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PRINCIPAL INVESTIGATOR: Harikrishna Nakshatri, B.V.Sc., PhD.
Kasi R. McCune, MD (fellow)

CONTRACTING ORGANIZATION: Indiana University
Indianapolis, IN 46202

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Harikrishna Nakshatri, B.V.Sc., PhD

Kasi R. McCune, MD

Email: hnakshat@iupui.edu

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14. ABSTRACT Estrogen receptor alpha (ERα)-positive breast cancers that co-express transcription factors GATA-3 and FOXA1 have a favorable prognosis. These transcription factors influence 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. We report that insulin increases the expression of T-bet in breast cancer cells, which correlates with reduced expression of GATA-3 and FOXA1. 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). MCF-7-T-bet cells were resistant to tamoxifen and displayed prolonged ERK and AKT activation in response to epidermal growth factor treatment. ERα-positive/T-bet-positive primary breast cancers express lower levels of FOXA1 (p=0.0137) and GATA-3 (p=0.0063) compared to ERα-positive/T-bet-negative breast cancers. Thus, T-bet expression and circulating insulin levels may serve as surrogate biomarkers to analyze progression of identify ERα-positive breast cancer.

15. SUBJECT TERMS
Diabetes, Intrinsic subtypes, Breast Cancer

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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 alpha (ERα), have one of the best prognosis with a 90% 5-year survival rate (3). This is partly attributed to their sensitivity to anti-estrogen 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, is associated with 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 coexpression 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 distal enhancer elements depending on the distribution of histone H3 lysine 4 mono and dimethylation; 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 estrogen's 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 three transcription factors are suggested to constitute a cell-lineage specific hormonal transcription factor network (6).

Most studies on anti-estrogen resistance have focused on the role of 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). While T-bet is essential for differentiation of T helper progenitors to 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, 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, since 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.

Body:

Objective: Investigate whether Luminal type A breast cancer cell line MCF-7 implanted into nude mice with experimentally induced type I diabetes (no insulin but hyperglycemia) or type II diabetes (elevated serum insulin and hyperglycemia) progress to become Luminal type B or any other intrinsic subtype.

Progress on the project and results obtained:

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 to ERα-positive breast cancers, a subset of ERα-positive breast cancers expressed higher levels of T-bet (Figure 1A) (29). 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 ($p=0.0001$) (Figure 1B). Using the same dataset, 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 ($p=0.0063$) (Figure 1C). T-bet expression was also associated with progesterone receptor (PR) negativity ($p<0.00005$) (30), a subgroup that is known to be associated with resistance to endocrine therapy (31) (Figure 1D). Analysis of T-bet expression among ERα/PR-positive breast cancer patients who received tamoxifen treatment in a different dataset revealed a trend of elevated T-bet expression in tumors of patients with recurrence (n=28) compared to patients who were disease-free (n=32) after five years of treatment, although this did not reach statistical significance ($p=0.066$) (32). Since microarray analysis was performed on laser capture micro-dissected tumor samples, this study demonstrates 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 factor I and II (IGF I and IGF II), epidermal growth factor (EGF), inflammatory cytokines, and estrogen on T-bet expression in MCF-7 cells. Only insulin and IGF I induced T-bet expression (Figure 2A).

We next examined the effects of insulin on GATA-3 and FOXA1 expression. If crosstalk 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 protein (Figure 2B). Results of multiple experiments on the effects of insulin on T-bet, GATA-3, FOXA1, and ERα proteins are shown in Figure 2C. Insulin-mediated reduction in GATA-3 correlated with 30% reduction in FOXA1 expression.

To determine 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 (33). Luminal B phenotype appears to be associated with reduced or loss of GATA-3 expression (Figure 2D). T-bet expression was markedly higher in BT-474 cells compared to other cell lines (Figure 2D). Insulin reduced FOXA1 expression in T47-D and ZR75-30 cells by ~30%. 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, Figure 3A). The expression of GATA-3, FOXA1, and ERα was lower in MCF-7-T-bet cells compared to parental (MCF-7p) cells, which is consistent with the effect of insulin on the expression of GATA-3 and FOXA1.

XBP-1 is a potential downstream target of ERα, FOXA1, and GATA-3 network based on a meta-analysis and contains binding sites for all three transcription factors (7, 14). While E2 readily induced XBP-1 expression in MCF-7p cells, it was markedly lower in T-bet overexpressing cells (Figure 3B, left panel). E2-inducible expression of GREB-1, which also contains both ERα and FOXA1 binding sites, was lower in MCF-7-T-bet cells compared to MCF-7p cells, although the magnitude of this effect was not as dramatic (Figure 3B, left panel).

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 (Figure 3C). E2-induced ERα binding to all three ERα binding sites of XBP-1 was substantially lower in MCF-7-T-bet cells compared to MCF-7p cells (Figure 3C). Like XBP-1, GREB-1 is associated with multiple ERα binding sites; a few of these sites are enriched for FOXA1 binding (Figure 3D). ERα binding to one of these binding sites that we examined was lower in T-bet overexpressing cells compared to 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-inducible expression of the above genes, we pretreated MCF-7 cells with insulin overnight and then exposed cells to ethanol or E2 for four hours. Insulin significantly reduced basal and E2-inducible expression of GREB-1 (Figure 3B, right panel).

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 demonstrated similar sensitivity to 1 μM 4-hydroxy tamoxifen in the absence of insulin (Figure 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, overall level of proliferation was significantly higher in MCF-7-T-bet cells compared to parental cells (under insulin plus tamoxifen or a combination of insulin, tamoxifen and E2). Similar results were obtained at variable insulin and tamoxifen concentrations (Figure 4B and C). Concentrations of insulin used in these experiments are similar to the levels seen in breast cancer patients with hyperinsulinemia (34). Note that at 0.1 μM tamoxifen, MCF-7-T-bet cells showed modest yet significant resistance to tamoxifen compared to MCF-7p cells and this resistance was further enhanced in the presence of insulin (Figure 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 upon insulin or E2 stimulation (Figure 4D).

Anti-estrogen 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) (35) to determine whether there is a correlation between anti-estrogen resistance and T-bet expression. Both MCF-7-T and MCF-7-F cells expressed higher levels of T-bet (Figure 5A). FOXA1 expression was significantly reduced in these resistant cells compared to 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 (Figure 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) anti-estrogen resistance of breast cancer cell lines.

We used short interfering RNA (siRNA) against T-bet to determine whether tamoxifen resistance of MCF-7-T cells can be partially reversed by reducing the levels of T-bet (Figure 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 ERα to genes that may be essential for E2-stimulated proliferation of cells.

T-bet overexpressing cells display elevated EGF stimulated MAP 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 anti-estrogen treatment (36). Conversely, elevated growth factor-dependent MAPK and/or AKT activation is associated with anti-estrogen resistance in breast cancer (37-39). To determine whether T-bet-mediated disruption of the ERα:FOXA1:GATA-3 transcriptional network leads to altered growth factor-dependent MAPK and AKT activation, we examined the levels of phospho-ERK and phospho-AKT in MCF-7p and MCF-7-T-bet cells upon treatment with EGF, heregulin, or insulin. pERK levels were higher and prolonged in EGF treated MCF-7-T-bet cells compared to MCF-7p cells (Figure 6A). Basal pAKT levels were consistently higher in MCF-7-T-bet cells (1.44 fold, $p=0.006$, $n=6$) compared to MCF-7p cells. Consequently, overall EGF stimulated pAKT level was also elevated in MCF-7-T-bet cells compared to MCF-7p cells. Similar to EGF, insulin stimulated ERK activation was prolonged in MCF-7-T-bet cells compared to MCF-7p cells (Figure 6B). Interestingly, heregulin-mediated ERK and AKT activation, which relies mostly on ERBB2:ERBB3 heterodimers, was similar in both cell types (Figure 6C). The effects of T-bet on EGF and insulin-mediated ERK and AKT is independent of growth factor receptor levels as the levels of EGFR, ERBB2/HER2, and ERBB3 were similar in both cell types.

In vivo analysis of effects of hyperinsulinemia on growth of ERα-cancer cell-derived tumors:

To study the effects of hyperinsulinemia on breast cancer cell growth in vivo, we established a xenograft models. 3T3L1 cells were implanted into the mesenteric region of six-week old female nude mice. Mice implanted with one million 3T3L1 cells develop type II diabetes and associated hyperinsulinemia (Shibasaki et al., Diabetologia 45:518-526). A week following injection of these cells, slow release estrogen pellets (0.72 mg per pellet, 60-day release) were implanted into animals. The control group received only estrogen pellets. Two million MCF-7 breast cancer cells were injected next day into the mammary fat pad of these animals. In first series of experiments neither control group nor 3T3L1 group developed tumors. This was most likely due to poor quality estrogen pellets. Unfortunately, several animals injected with 3T3 cells became sick within 2-3 weeks and died. We repeated these experiments two more time (35 animals each time) with variable doses of 3T3L1 cells. In both of these experiments, most animals in 3T3L1 group died prematurely. Nonetheless, two animals in 3T3L1 group survived beyond 10 weeks in the third set of experiment.

We analyzed tumors from control and 3T3L1 injected group by immunohistochemistry for the expression of ERα, FOXA1, and GATA-3. We did not observe any difference in the expression levels of ERα, FOXA1, and GATA-3 (Figure 7). Lack of differences in the expression of these markers in two groups could be related to the failure of 3T3L1 to induce hyperinsulinemia. That can also explain for the prolonged survival of these tumor-bearing animals compared to other animals. From these studies, we conclude that the current in vivo model is not suitable for investigating the effects of hyperinsulinemia on progression of ERα-positive breast cancer.

Key Research Accomplishments:

- **Insulin confers resistance to tamoxifen**
- **Insulin represses GATA-3 expression and thus disrupt ERα:FOXA1:GATA-3 axis in Luminal type A breast cancer cells**
- **Insulin induces the transcription factor T-bet, which also downregulates GATA-3.**
- **T-bet enhances growth factor receptor signaling, which may contribute to tamoxifen resistance.**

Reportable outcome:

1) Abstract presented in San Antonio Breast Cancer Symposium-2008: Kasi R. McCune, Poornima Bhat-Nakshatri, Mangesh Thorat, Sunil Badve, Harikrishna Nakshatri. Control of Luminal Type A Intrinsic Subtype Enriched Transcription Factor Network by Insulin: Implications of Diabetes on Breast Cancer Classification. This abstract received 31st Annual CTRC-AACR San Antonio Breast cancer symposium AstraZeneca Clinical Scholar Award.

2) Harikrishna Nakshatri, Kasi R. McCune, Poornima Bhat-Nakshatri, Mangesh Thorat, and Sunil Badve. Control of Luminal Type A Intrinsic Subtype Enriched Transcription Factor

Network by Insulin: Implications of Diabetes on Breast Cancer Subtypes. Era of Hope meeting, Baltimore, June 2008.

3) Kasi McCune, Poornima Bhat-Nakshatri, Mangesh A Thorat, Kenneth P Nephew, Sunil Badve, and Harikrishna Nakshatri. Disruption of Estrogen Receptor, GATA-3, and FOXA1 Transcription Factor Network by Insulin-Inducible T-bet: Implications in Hormone-Dependent Breast Cancer. Manuscript under review in Cancer Research.

Conclusion: Figure 8 illustrates how insulin may promote progression of Luminal type A breast cancers to anti-estrogen resistant phenotype. Insulin through T-bet disrupts ERα:FOXA1:GATA-3 axis, which leads to loss of estrogen dependence and upregulation of growth factor signaling.

Key personnel who received salary support involved in the project:

Harikrishna Nakshatri (PI)

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Appendices:

P28-1: CONTROL OF LUMINAL TYPE A INTRINSIC SUBTYPE ENRICHED TRANSCRIPTION FACTOR NETWORK BY INSULIN: IMPLICATIONS OF DIABETES ON BREAST CANCER SUBTYPES

Harikrishna Nakshatri,[1] Kasi R. McCune,[1] Poornima Bhat-Nakshatri,[1] Mangesh Thorat,[1] and Sunil Badve[2]
[1]Indiana University-Purdue University, Indianapolis, and [2]Indiana University, Indianapolis

Background: Breast cancer is classified into five intrinsic subtypes based on gene expression pattern; luminal type A, luminal type B, ErbB2/Her-2-positive, basal, and normal-like. Luminal type A and type B represent estrogen receptor α (ERα)-positive breast cancers with luminal type A expressing higher levels of ERα and is associated with better prognosis. Recent studies have identified a specific functional transcription factor network comprising GATA-3, FOXA1, and ERα in normal luminal cells as well as in luminal type A breast cancer that dictates their hormone dependence. Signaling molecules that may disrupt this network and force these cells to acquire hormone-independence are not known. T-bet (Tbx21) has been described as a major negative regulator of GATA-3 activity. As the expression and/or activity of some of the above factors are controlled by insulin, the objective of this study was to investigate whether elevated level of insulin, as evidenced in type II diabetes, alters gene expression pattern in luminal type A breast cancers by interrupting GATA-3:FOXA1:ERα network and thus forcing these cancers to acquire nonluminal phenotype and/or hormone-independence.

Methodologies: The effect of insulin on the expression of ERα, FOXA1, GATA-3, and T-bet was measured in ERα-positive MCF-7 cells by western blot analysis. The effect of T-bet on estrogen-regulated gene expression was measured by transient transfection assays and stable overexpression of T-bet in MCF-7 cells. Publicly available Oncomine database was used to determine the expression pattern of T-bet and its relation to ERα status in primary breast cancers. Immunohistochemistry was used to determine T-bet expression in normal breast and breast cancer.

Results: Insulin induced the expression of T-bet, which was partially reversed by estrogen. In transient transfection assays, T-bet reduced estrogen response element-driven reporter gene expression. ERα and GATA-3 levels were reduced in MCF-7 cells stably overexpressing T-bet suggesting that T-bet reduces GATA-3-dependent ERα expression. Estrogen-inducible expression of estrogen target gene GREB-1 was lower in T-bet overexpressing cells compared to parental cells. Importantly, ERα-negative breast cancers showed higher T-bet expression suggesting mutual antagonism between ERα and T-bet.

Conclusions: Insulin may change the gene expression pattern through T-bet-mediated disruption of master cell-type-specific transcriptional network including GATA-3, ERα, and FOXA1 that dictates the phenotype of hormone-dependent luminal type A breast cancer. T-bet may serve as a marker to differentiate FOXA1+/GATA-3+ breast cancers that may have progressed to hormone-independence.

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P28-2: FUNCTIONAL RELATIONSHIPS BETWEEN HER2 AND THE LEPTIN SYSTEM IN BREAST CANCER

Eva Surmacz
Temple University

Background: The obesity hormone leptin (Ob) has been implicated in tumorigenesis, especially in the development of breast cancer (BC). The mitogenic, angiogenic, and antiapoptotic activities of Ob are mediated through the leptin receptor (ObR). Data obtained in HEK293T cells engineered to coexpress ObR and the oncoreceptor HER2 suggested that Ob can transactivate HER2 via ObR. To address this putative interaction, we studied whether simultaneous expression of Ob, ObR, and HER2 can occur in human BC. In cellular models, we studied if ObR and HER2 can physically interact and if activation of Ob/ObR is able to transactivate HER2.

Material and Methods: The expression of Ob and ObR was evaluated by immunohistochemistry in 59 BCs (31 HER2-positive, 28 HER2-negative). Ob and ObR were classified as positive (at least +) or negative (below +). The relationships among Ob and ObR and the clinicopathological features, that is, grading (G1, G2, and G3), tumor size (diameter in mm), node involvement (positive or negative), vascular invasion (positive or negative), and ER and PgR expression (positive or negative) were analyzed using the Chi square test. The mechanistic relationships between ObR and HER2 were studied by western blotting, co-immunoprecipitation, and immunofluorescence/deconvoluted microscopy in MCF-7 cells that are sensitive to Ob and coexpress both receptors.

Results: Ob and ObR were coexpressed in 78% of BC and were correlated in all BCs (p10 mm) node-positive tumors (trends p=0.06 and p=0.08). The simultaneous expression of Ob/ObR and HER2 was found in 39% of BCs, but the Ob/ObR system was also frequent in HER2-negative BCs. Ob, ObR, and combined Ob/ObR did not correlate with HER2, grading, VI, and ER/PgR. Using MCF-7 cells, we found that a fraction of ObR and HER2 are colocalized and can be found in one immunocomplex. We also demonstrated that 100 ng/mL Ob can activate HER2 tyrosine phosphorylation upon 15 min–1 h treatment.

Conclusions: Ob and ObR are often coexpressed, and a subset of Ob/ObR-positive tumors exhibits concomitant expression of HER2. In BC cells, HER2 and ObR may physically interact and Ob can cross-activate HER2. Thus, high levels of Ob found in obese patients might lead to the activation of the Ob/ObR/HER2 signaling in BC contributing to the resistance of BC to anti-HER2 treatments.

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P28-3: PROLACTIN PROMOTES MAMMARY TUMORIGENESIS SECONDARY TO LOSS OF p53

Kathleen Oleary and Linda Schuler
University of Wisconsin, Madison

Multiple factors and pathways contribute to mammary tumorigenesis. Two factors that have separately been implicated in breast cancer are the hormone prolactin (PRL) and the tumor suppressor p53. This study has begun to examine the interaction between these factors using two mouse models: $p53^{-/-}$ mice made congenic on the FVB/N background and the NRL-PRL transgenic mouse model which employs a non-hormonally regulated, mammary selective promoter to drive expression of PRL in mammary epithelium. Mammary gland transplants were performed on the following genotypes: wildtype, $p53^{-/-}$, NRL-PRL/ $p53^{+/+}$, and NRL-PRL/$p53^{-/-}$ to circumvent the problem that $p53^{-/-}$ mice are prone to multiple tumors. This study demonstrates that increased PRL combined with loss of p53 cooperatively affect mammary tumorigenesis in multiple ways. Tumor latency is decreased. Mean survival of the NRL-PRL/$p53^{-/-}$ recipients was 209 days as compared to 247 days for the $p53^{-/-}$ recipients. In addition, the tumors appeared to be more aggressive. For the NRL-PRL/$p53^{-/-}$ tumors, 5/15 (33%) invaded into the peritoneal cavity. In contrast, this was not observed for the $p53^{-/-}$ tumors 0/9 (0%); all were confined to the mammary fat pad. However, NRL-PRL/$p53^{-/-}$ and $p53^{-/-}$ tumors were similar histologically. Both were highly anaplastic and were identified as either carcinosarcomas, spindle cell tumors or adenosarcomas, indicating that loss of p53 appears to dictate the tumor histology. Thus, it appears that PRL and p53 pathways interact to promote breast cancer by decreasing latency and increasing invasiveness. Further studies are being done to examine the contribution of genomic instability to this process.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0647.

P28-4: INHIBITION OF ESTROGEN-INDUCED GROWTH OF BREAST CANCER CELLS BY MODULATING IN SITU OXIDANT LEVELS

Deodutta Roy, Quentin Felty, Victor Okoh, and Nana-Aisha Garba
Florida International University

The purpose of the our BCRP-funded proposal (BC051097) was to examine an untested and highly innovative concept in estrogen-induced carcinogenesis research, i.e., estrogen-induced mitochondrial oxidants are involved in the promotion/progression of breast cancer through modulating signaling that controls the early G1 stage of the cell cycle. We previously reported that 17-β-estradiol (E2)-induced mitochondrial (mt) reactive oxygen species (ROS) act as signaling molecules. Here we have examined whether antioxidants, N-acetylcysteine and ebselen, overexpression of ROS lowering genes, such as catalase and silencing of mtTFA are able to induce cell growth arrest in the presence of estrogen by analysis of the expression of early cell cycle biomarkers, cyclin D1 and PCNA, by real-time RT-PCR, the rate of DNA synthesis by BrDu incorporation, and different phases of cell cycle by flow cytometry. We also determined the morphology and behaviors of cells that overexpress mtSOD, catalase or silenced with mtTFA siRNA compared to those that do express these genes normally exposed to estrogen. Our data revealed that E2-induced cell growth was reduced by antioxidants N-acetyl-L-cysteine (NAC), catalase, and the glutathione peroxidase mimic ebselen. mtTFA siRNA transfection inhibited estrogen-induced proliferation of MCF-7 cells that is evident from the lower incorporation of BrDU in SiRNA treated cells compared to wild-type cells in the presence of E2. We observed similar results by flow cytometery. In E2 treated MCF7 cells, the percentage of DNA content in S phase was 18% while this decreased to 6.8% in mtTFA silenced MCF7 after 24 h. The FACS data not only confirms the results shown by the BrdUrd assay, it also shows that impairment of mitochondrial biogenesis prevents E2-induced entry of MCF7 cells into the S phase by arresting them in the G0/G1 phase. Both antoxidant treatment and dextoxification of ROS prevented E2-induced expression of cyclin D1 and pcna, markers of cell proliferation detected by Real time PCR. In cells overexpressed with adenovirus construct containing catalase that lowers oxidant production as well as in mtTFA silenced cells using their SIRNA, E2 was not able to produce any colony. Both antioxidants ebselen and N-acetylcysteine produced similar effects. It appears that E2-dependent colony formation rate of MCF-7 cells is dependent on ROS or mitochondrial signaling. Since neither antioxidants nor mito-

HTF9C, CEACAM5, NDRG1, SLC7A5 recorded as previously described. Each case was assigned a Mammostrat score and RFS and OS analysed by marker positivity and Mammostrat score.
Results: Staining for all 5 antibodies was successful in 1174/1390 (84%) of cases. In the primary analysis of 531 N0/ER+ve Tamoxifen only treated patients Mammostrat was significantly associated with relapse free survival (RFS) in univariate (p=0.025) & multivariate proportional hazards analysis (p=0.01, HR=1.3, 95%C.I. 1.08-1.74). PgR, multifocality and menopausal status were significant co-variates (p<0.05, HR 0.89, 2.0 & 0.6 respectively). The Nottingham prognostic index was non-significant. Of the 5 antibodies, only p53 (p=0.04) was independently predictive of survival.
In a secondary univariate analysis of 781 patients (including N+ve and chemo/tam treated patients) Mammostrat was predictive of RFS & OS (p<0.01) with NDRG1/CEACAM5/p53 also predictive of RFS(p<0.05). However Mammostrat was not independent of nodal status, pathological size, grade or multifocality in a proportional hazards analysis.
Discussion: In the Edinburgh BCS population Mammostrat was predictive of RFS (both local and distant relapses) in N-ve/ER+ve patients treated with tamoxifen alone irrespective of menopausal status. There was a strong correlation between Mammostrat scores and grade, however, in a multivariate analysis Mammostrat contributed significantly to prognostication along with PgR, multifocality and menopausal status.

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Overexpression of CD44 in acquired endocrine resistant breast cancer modulates erbB activity and promotes an invasive phenotype.

Baruah BP, Smith C, Jordan N, Nicholson RI, Robertson J, Barrett-Lee P, Hiscox S. Velindre Cancer Centre, Cardiff, United Kingdom; Tenovus Centre for Cancer Research, Cardiff, United Kingdom; Cardiff University, Cardiff, United Kingdom; Nottingham City Hospital, Nottingham, United Kingdom

Background: Acquired endocrine resistance in breast cancer cells is accompanied by altered growth factor receptor signalling and a highly migratory cell phenotype. In tamoxifen-resistant ('TamR') MCF7 cells, our microarray analysis has demonstrated elevated levels of CD44, a transmembrane glycoprotein, known to interact with and modulate the function of a number of growth factor receptors and plays a major role in tumour metastasis. Here, we have explored the role of CD44 as a modulator of erbB family activity in TamR cells and evaluated the expression of CD44 in a series of breast cancer patients on endocrine therapy.
Methods: RT-PCR was performed to confirm overexpression of CD44 at the gene level in TamR cells. Western blotting and immunocytochemistry were used to investigate expression of CD44 at the protein level together with the activity of erbB signalling pathways following stimulation of CD44 by its ligand, hyaluronan (HA), or following stimulation with erbB ligands (TGFα, heregulin β1) after knockdown of CD44 expression using siRNA. Immunofluorescence microscopy was used to determine association between CD44 and erbB family members. Cell migration was assessed by seeding cells onto fibronectin-coated microporous inserts for 24 hours. CD44 expression levels were evaluated using paraffin-embedded tissue sections in a series of primary breast cancer tissues (n=103) from patients receiving endocrine therapy by immunohistochemical staining.
Results: CD44 was overexpressed at the gene and protein level in TamR cells versus their endocrine sensitive counterparts. siRNA-mediated suppression of CD44 expression reduced basal EGFR/Her2 activity in TamR cells together with suppressing their migratory phenotype. siRNA-mediated inhibition of CD44 expression greatly attenuated the ability of erbB ligands to activate erbB receptors and inhibited TGFα and heregulin β1-induced migration. Conversely, stimulation of TamR, but not MCF7 cells, with HA promoted activation of erbB family members together with their downstream signalling intermediates ERK1/2, Src and AKT. Analysis of CD44 expression in clinical tissue revealed an association between elevated CD44 expression and a shortened response to endocrine therapy.
Conclusions: These data demonstrate that overexpression of CD44 accompanies endocrine resistance where it enhances the sensitivity of these cells to factors (erbB ligands, HA) found within the tumour microenvironment. This may implicate CD44 in loss of endocrine response clinically and, therefore, provide a novel therapeutic target in endocrine resistant breast cancer.

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Overexpression of L1CAM accompanies acquired endocrine resistance and is associated with the development of an aggressive cell phenotype.

Hiscox S, Jordan N, Crandon-Lewis A, Jiang W, Nicholson RI, Gee J. Cardiff University, Cardiff, United Kingdom; Astrazeneca, Macclesfield, United Kingdom

Background: Acquired resistance to endocrine therapies is associated with a gain in aggressive features in vitro which may impact on tumour spread in vivo. To identify potential candidate molecules which may promote this adverse cell behaviour, we performed microarray analysis on MCF-7 cell models of fulvestrant resistance ('FasR' cells) applying a commercial algorithm for signalling network discovery. We have subsequently investigated the significance of overexpression of L1CAM, a cell-surface adhesion molecule implicated in a MAPK signalling network and recently associated with tumour metastasis, in FasR cells and in clinical tissue.
Materials and Methods: A network analysis algorithm was applied to t-test significant Affymetrix gene lists derived from FasR vs. MCF-7 cells (Almac Diagnostics). Subsequent to revealing a prominent L1CAM network, L1CAM gene and protein expression were assessed using RT-PCR, Western blotting and immunofluorescence. Inhibition of L1CAM was achieved using siRNA transfection. Cell invasion and migration were measured by seeding onto Matrigel- or fibronectin-coated microporous membranes respectively. After 48 hours, invasive/migratory cells were fixed, stained and counted. Analysis of cell signalling molecules was determined using Western blotting with phospho-specific antibodies. Clinical significance of L1CAM expression was determined by analysis of L1CAM expression in tumour (n=101) vs. normal (n=23) breast tissue using Q-PCR.
Results: Development of fulvestrant resistance in MCF7 cells was associated with ER loss and a gain in migratory and invasive capacity in vitro. L1CAM was overexpressed at both gene (p<0.05) and protein (p<0.01) level in FasR cells whereas little or no L1CAM was detectable in MCF7 cells confirming microarray findings. Network and ontological analysis predicted L1CAM lies upstream of an ERK2 network (26 genes; p<0.0001) impinging on transcription factors/regulators which may promote aggressive cell behaviour. In agreement, siRNA-mediated inhibition of L1CAM suppressed the activity of nuclear MAP kinase and reduced the intrinsic migratory and invasive nature of FasR cells in vitro. Preliminary analysis indicated L1CAM expression was inversely associated with ER status (p<0.01) and reduced overall survival (p=0.01).
Conclusions: These data suggest that L1CAM overexpression associates with emergence of ER-negative fulvestrant resistance in breast cancer, where it may confer a highly motile and invasive phenotype. Additionally, our clinical studies suggest L1CAM may also contribute to inherently aggressive, ER-negative breast cancers. L1CAM signalling may thus provide a new therapeutic target to subvert such aggressive states *in vivo*.

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Control of luminal type A intrinsic subtype enriched transcription factor network by insulin: implications of diabetes on breast cancer classification.

McCune KR, Bhat-Nakshatri P, Thorat M, Badve S, Nakshatri H. Indiana University School of Medicine, Indianapolis, IN

Background: Luminal type A and type B represent estrogen receptor alpha (ERalpha)-positive breast cancers with luminal type A expressing higher levels of ERalpha and is associated with better prognosis. Recent studies have identified a specific functional transcription factor network comprising GATA-3, FOXA1 and ERalpha in normal luminal cells as well as in luminal type A breast cancer that dictates their hormone dependence. Signaling molecules that may disrupt this network and force these cells to acquire hormone-independence are not known. T-bet (Tbx21) has been described as a major negative regulator of GATA-3 activity. As the expression and/or activity of some of the above factors are controlled by insulin, the objective of this study was to investigate whether elevated level of insulin, as evidenced in type II diabetes, alters gene expression pattern in luminal type A breast cancers by interrupting GATA-3:FOXA1:ERalpha network and thus forcing these cancers to acquire non-luminal phenotype and/or hormone-independence.
Methodologies: The effect of insulin on the expression of ERalpha, FOXA1, GATA-3 and T-bet was measured in ERalpha-positive MCF-7 cells by Western blot analysis. The effect of T-bet on estrogen-regulated gene expression was measured by stable overexpression of T-bet in MCF-7 cells and subsequent qRT-PCR analysis. Publicly available Oncomine database was used to determine the expression pattern of T-bet and its relation to ERalpha status

in primary breast cancers. A proliferation assay was used to determine sensitivity of T-bet overexpressing cells to tamoxifen in the presence and absence of insulin.
Results: Insulin induced the expression of T-bet, which was partially reversed by estrogen. ERalpha and GATA-3 levels were reduced in MCF-7 cells stably overexpressing T-bet suggesting that T-bet reduces GATA-3-dependent ERalpha expression. Estrogen-inducible expression of estrogen target genes GREB-1 and Myb was lower in T-bet overexpressing cells compared to parental cells, although basal expression was elevated in T-bet overexpressing cells. Treatment of T-bet overexpressing cells with insulin decreased tamoxifen sensitivity. Although T-bet expression was generally higher in ERalpha-negative breast cancers compared to ERalpha-positive breast cancers, a subpopulation of ER-positive breast cancers express elevated levels of T-bet.
Conclusions: Insulin may change the gene expression pattern through T-bet-mediated disruption of master cell-type-specific transcriptional network including GATA-3, ERalpha and FOXA1 that dictates the phenotype of hormone-dependent luminal type A breast cancer. T-bet may serve as a marker to identify ERalpha-positive breast cancers that express low levels of GATA-3 and have progressed to hormone-independence.

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Proteomics analysis of tissue samples obtained before and during letrozole treatment in postmenopausal patients with locally advanced breast cancer.

Hans V, Evans DB, Lonning PE, Geisler J. University of Oslo, Oslo, Norway; Novartis Institutes for BioMedical Research, Basel, Switzerland; University of Bergen, Bergen, Norway

Introduction
We report on the analysis of protein expression in the tumor tissue of postmenopausal patients before and after 4 months of letrozole treatment in the neoadjuvant setting. All patients had ER and/or PGR positive breast cancer and were enrolled in the "FAST-study". Letrozole was given orally as 2.5 mg once daily. Tumor tissue was obtained by large, open biopsies prior to treatment and during final surgery (in general: mastectomy).
Methods
We used paired samples from 13 postmenopausal breast cancer patients. Breast cancer tissue and plasma samples were obtained prior to treatment and after 4 months of therapy with letrozole in the neoadjuvant setting. Thus, all in all 26 tumor samples were used from 13 individual patients. Proteins were extracted from 20-50 mg of breast cancer tissue, labeled with fluorescent dyes and separated using 2-dimensional difference gel electrophoresis (2-D DIGE). After image analysis, differentially expressed proteins ($p<0.01$, paired T- and Wilcoxon tests) were identified using MALDI tandem mass spectrometry.
Results and discussion
The 2-D DIGE based method allowed monitoring of 2000-2500 protein species in each sample. After alignment of all these patterns, around 25 cellular proteins were found to be differentially expressed in the pairwise pre-/post-treatment comparison, along with several plasma proteins. Among these cellular proteins were several reported tumor markers such as stathmin and NDPKA, chaperones such as HSP-60, and members of the annexin family. We are currently investigating the correlation between these proteins and the clinical parameters.
To our knowledge this is the first report on proteomics analysis comparing samples obtained before and during treatment with a third-generation aromatase inhibitor. The study demonstrates the feasibility of performing a high-resolution proteomics analysis on tumor material derived from needle biopsies and yielded a number of potential markers, which will require further confirmation in a larger population. The results might be useful to elucidate adaptive mechanisms to estrogen suppression in vivo.

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Lapatinib can activate or supress estrogen receptor (ER) signaling in cell models of endocrine resistant breast cancer.

Leary AF, Martin L-A, Lykkesfeldt AE, Dowsett M, Johnston SRD. Royal Marsden Hospital, London, United Kingdom; Breakthrough Breast Cancer Research Center, London, United Kingdom; Institute of Cancer Biology, Copenhagen, Denmark

Background
Growth factor (GF) signaling is frequently upregulated in hormone resistant breast cancer (BC) and provides the rationale for using the dual EGFR/HER2 inhibitor, lapatinib (LAP) in an effort to overcome endocrine resistance. Hormone resistant BC can also vary in its degree of continued ER dependence. We previously demonstrated potent synergy between LAP and TAM in cell models of acquired endocrine resistance to tamoxifen (TAMR cells), and to estrogen deprivation (LTED cells)[1]. This study aimed to characterize the phenotype of these two models with a focus on the ER pathway, and to determine the biological basis for the observed synergy between LAP and TAM.
Methods
LTED cells and TAMR cells were both derived from hormone-sensitive MCF7 parental cells as previously described[1]. The differential effects of LAP, TAM, estrogen deprivation (ED) or the combination were assessed by proliferation assays, ERE reporter assays, quantitative PCR and western blot analyses.
Results
In LTEDs: Increased ER expression and ERE transactivation were seen in LTEDs compared to MCF7 cells, as well as continued sensitivity to the ER downregulator, fulvestrant (FUL), indicating that in LTEDs, ER activity is not only enhanced but is also a significant driver of growth. In LTEDs, LAP alone suppressed ER activity as measured by both ERE transactivation and ER phosphorylation at Ser118; the most effective suppression of ER activity was achieved with a combination of LAP+TAM/ED where the effects were additive.
In TAMRs: In contrast, TAMRs demonstrated decreased ER activity (low basal ERE transactivation and loss of PgR) and ER independent proliferation (FUL had minimal growth inhibitory effects despite ER downregulation). In TAMRs, neither LAP nor TAM had significant antiproliferative effects on their own: LAP alone decreased pMAPK/pAkt but increased basal ERE transactivation and transcription of the ER-responsive gene pS2. TAM alone inhibited ERE activation but increased HER2 gene and protein expression. Inhibiting both HER2 and ER pathways with LAP+TAM resulted in synergistic growth inhibition.
Conclusions
In LTED cells with enhanced ER function attributable in part to GF receptor activation, the synergistic antiproliferative effects from LAP and TAM or ED may be largely due to enhanced inhibition of ER signaling. In contrast in TAMR cells with suppressed ER activity, LAP alone appears to enhance ER signaling, while TAM alone inhibits ER activity but increases HER2 expression. In TAMRs, the combination of TAM+LAP is required to interrupt both ER and GF signaling. These results suggest that the basis for synergy between LAP and endocrine therapy in hormone resistant BC may vary depending on the nature of the cross-talk between the ER and GF pathways.
[1]SABCS, 2006 Abs 303.

3032

Coassociation of ERα and p160 proteins predicts resistance to endocrine treatment; SRC-1 is an independent predictor of breast cancer recurrence.

Redmond AM, Bane FT, Stafford AT, McIlroy, Dillon MF, Hill ADK, Young LS. Royal College of Surgeons in Ireland, Dublin, Ireland

The p160 coactivators AIB1 and SRC-1 are known to play a critical role in modulating transcription in breast cancer cells in conjunction with ligand-bound estrogen receptor. The interactions of the p160 proteins with this nuclear receptor are also important in the development of resistance to endocrine treatments. Using quantitative coassociation immunofluorescent microscopy, the colocalisation of the p160s and ERα was increased in the LY2 endocrine resistant cell line following treatment with the anti-estrogen tamoxifen in comparison to the endocrine sensitive MCF-7 cell line. In cell cultures derived from patient tumours at the time of primary surgery prior to treatment, there was an increase in association of the coactivators with ERα following treatment with estrogen but dissociation was evident in the presence of tamoxifen. Immunohistochemical staining of a tissue microarray, constructed from 560 breast cancer patients, revealed that SRC-1 was a strong predictor of reduced disease-free survival, both in patients receiving adjuvant tamoxifen treatment and untreated patients ($p<0.0001$ and $p=0.0111$ respectively). AIB1 was not a significant independent predictor of disease recurrence. SRC-1 was assigned a hazard ratio of 2.12 when survival analysis using a Cox proportional hazards model was applied. Quantitative coassociation analysis of the p160 coactivators with ERα in the patient TMA revealed significantly stronger colocalisation of SRC-1 and ERα in patients who are known to have relapsed than those patients who did not recur ($p=0.00001$). This data suggests SRC-1 is pivotal in tumour aggressiveness and is a powerful predictor of progression of disease in breast cancer patients.

Figure legends:

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 (29) were extracted from Oncomine (www.oncomine.org). Difference in expression between 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 the expression levels. C). Expression levels of FOXA1 and GATA-3 in ERα+/T-bet- and ERα+/T-bet+ subgroups. D). T-bet expression negatively correlates with PR negativity. As in A, data was extracted from a published study (30).

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 nM) and/or E2 (0.1 nM) for indicated time and T-bet expression was measured by western blotting. B) The effect of insulin on GATA-3, FOXA1, and ERα expression. Cells were treated with insulin and/or E2 for 24 hours and the expression levels of different proteins were measured by western blotting. C) Densitometric scanning data of 3 or more experiments showing insulin-mediated significant increase in T-bet expression and reduction of GATA-3 and FOXA1 expression in MCF-7 cells. Mean and standard error of the mean are presented. 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) showed significantly lower levels of GATA-3 compared to the luminal A cell line T47-D. Note that insulin reduced FOXA1 expression in T47-D and ZR75-30 cell lines.

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 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 vs MCF-7-T-bet. B) E2-inducible expression of XBP-1 and GREB-1 in MCF-7p and MCF-7-T-bet cells (left panel). Results of three or more experiments are presented (mean ±SEM). *$p=0.01$. The effect of insulin (INS) on basal and E2-inducible expression of GREB-1 is shown in the right panel. Cells were pre-treated with insulin overnight and then exposed to ethanol or E2 for four hours. *$p=0.01$ control versus insulin treatment; **$p=0.02$, E2 vs E2+ insulin (n=5). C) ERα binding to enhancer regions of XBP-1 was markedly lower in MCF-7-T-bet cells compared to MCF-7p cells. ERα and FOXA1 binding sites associated with XBP-1 from previous ChIP-on-Chip (13, 17) are shown on the top (black bars) along 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 q-PCR. Asterisks denote statistically significant differences in ERα binding under identical treatment conditions between two cell types. D) ChIP assay was used to measure ERα binding to one of the ERα binding regions (black bars) associated with GREB-1 gene (indicated by inverted arrow on the top).

Figure 4: Insulin confers resistance to tamoxifen. A) The effect of insulin on proliferation and tamoxifen sensitivity of MCF-7p and MCF-7-T-bet cells. Cells were plated on 96 well plates and treated with E2 (0.1 nM), 4-hydroxy-tamoxifen (1 μM) and/or insulin (50 nM) as described in

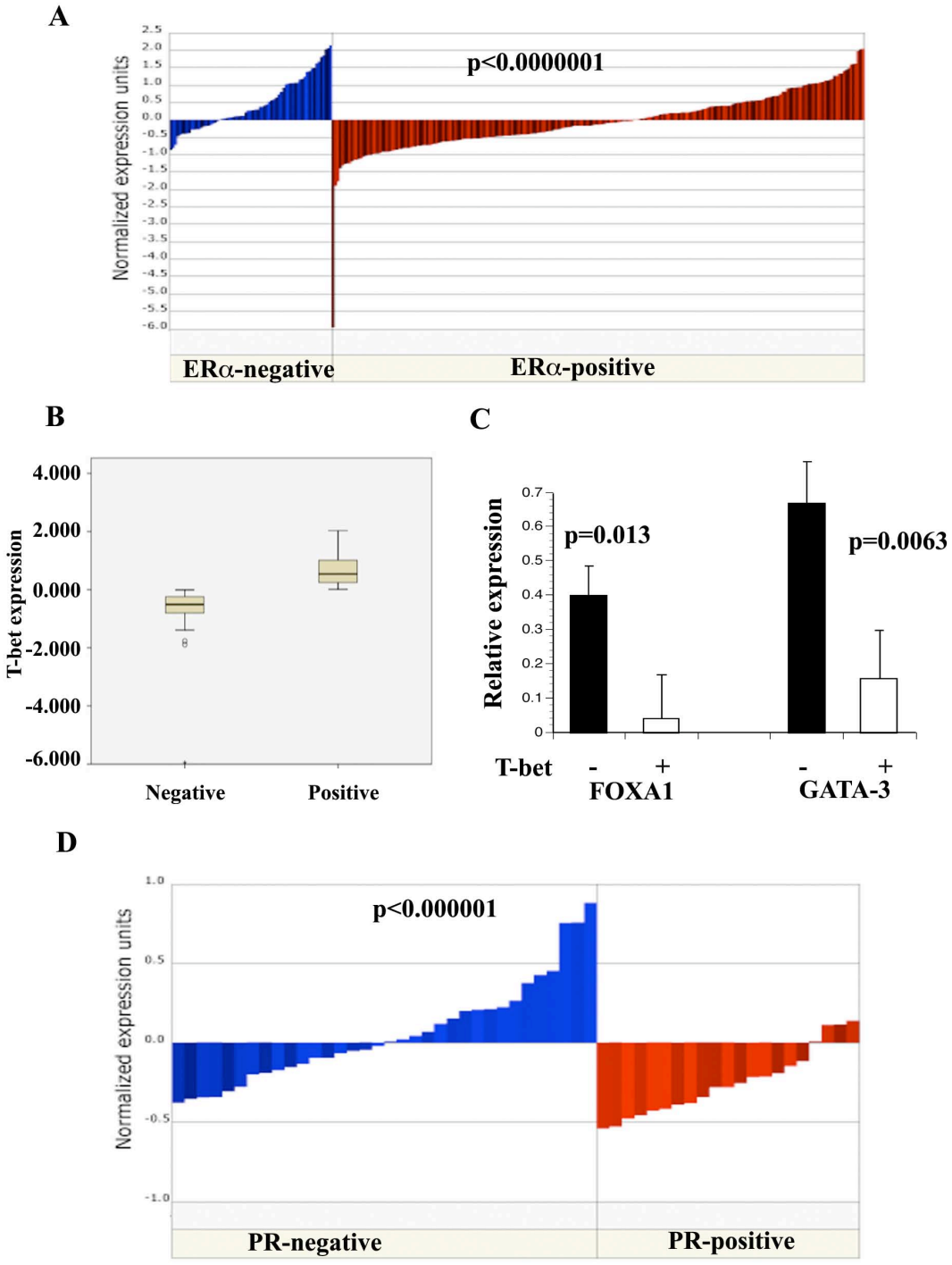
materials and methods. *p= 0.0001, MCF-7p *vs* MCF-7-T-bet cells under identical treatment condition. B) The effect of different concentrations of insulin on proliferation and tamoxifen sensitivity (1 μM) of MCF-7p and MCF-7-T-bet cells. *p<0.01 between MCF-7p and MCF-7-T-bet cells under identical treatment condition. C) The effect of insulin on proliferation under variable concentration of tamoxifen. Experiments are done as in B. D) T-bet is required for E2- and insulin-dependent proliferation of T47-D cells. T47-D cells were treated with siRNA against T-bet or control non-specific siRNA targeting luciferase gene for four days. T-bet siRNA reduced T-bet protein levels by 30% (left panel). Cells were treated with E2, insulin or both for six days. *p<0.01 control *vs* T-bet siRNA.

Figure 5: Changes in ERα:FOXA1:GATA-3 axis in MCF-7 cells that acquired resistance to tamoxifen (MCF-7-T) or fulvestrant (MCF-7-F). A). Basal and insulin-regulated expression pattern of T-bet, ERα, FOXA1, and GATA-3 in MCF-7, MCF-7-T and MCF-7-F cells. Right panel displays 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, reduction in FOXA1 expression in MCF-7-T and MCF-7-F cells compared to parental cells is significant (p<0.05). B) E2 fails to induce ERα:FOXA1:GATA-3 target gene GREB-1 in MCF-7-T and MCF-7-F cells. GREB-1 expression was measured by qRT-PCR (n=3). C) T-bet siRNA inhibits growth of MCF-7-T cells. Cells were treated with siRNA as in Figure 4D and cell proliferation was measured by BrDU-ELISA. As in T47-D cells, T-bet siRNA reduced T-bet protein levels by 30% (left panel) and transcript levels by 50% (middle panel).

Figure 6: MCF-7p and MCF-7-T-bet cells show differential ERK and AKT activation in response to growth factor signaling. A) EGF-inducible ERK activation in MCF-7p and MCF-7-T-bet cells. Cells were treated with 20 ng/ml EGF for indicated time and ERK and AKT activation was measured using phospho-specific antibodies. Ratio between phosphorylated ERK and the loading control β-Actin is presented. B) Insulin-inducible (50 ng/ml) ERK and AKT activation in MCF-7p and MCF-7-T-bet cells. C) Heregulin-inducible (50 ng/ml) ERK and AKT activation in MCF-7p and MCF-7-T-bet cells.

Figure 7: Expression of ERα, FOXA1, and GATA-3 in MCF-7 cell derived mammary fat pad tumors of animals with or without 3T3L1 cell injection. 3T3L1 cells were injected into mescenteric region of nude mice before injecting MCF-7 cells into the mammary fat pad. Tumors were isolated after ~12 weeks and fixed in formalin. Sections were stained for antibodies against ERα, FOXA1, and GATA-3 as described previously (8).

Figure 8: Model depicting the effects of insulin on progression of luminal type A breast cancer. LRIG is a repressor of growth factor signaling and its expression is dependent on ERα, FOXA1 and GATA-3. TLE3 is a transcription repressor, which represses ERBB3 expression. The expression of TLE3 requires ERα, FOXA1, and GATA-3. Insulin mediated reduction in GATA-3 expression could result in reduced expression of LRIG1 but increased expression of ERBB3. Consequently, cancer cells acquire estrogen-independence and activated growth factor receptor signaling.


A
p<0.0000001
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ERα-negative
ERα-positive
B
T-bet expression
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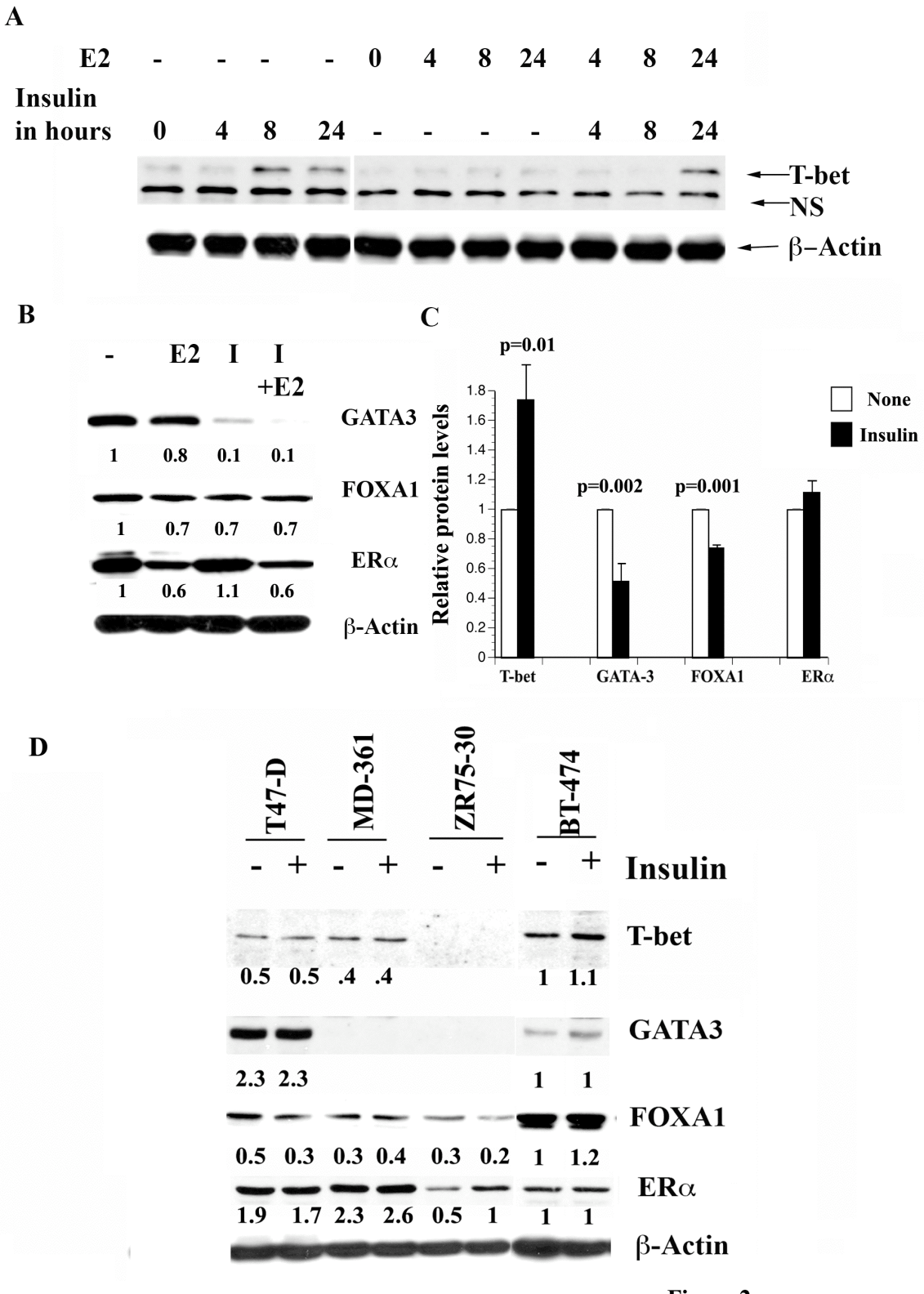
2.000
0.000
-2.000
-4.000
-6.000
Negative
Positive
C
Relative expression
p=0.013
p=0.0063
T-bet
FOXA1
GATA-3
D
p<0.000001
Normalized expression units
PR-negative
PR-positive

Figure 1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ERα
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β-Actin
C
p=0.01
p=0.002
p=0.001
Relative protein levels
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Insulin
T-bet
GATA-3
FOXA1
ERα
D
T47-D
MD-361
ZR75-30
BT-474
- + - + - + - + Insulin
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ERα
1.9 1.7 2.3 2.6 0.5 1 1 1
β-Actin

Figure 2

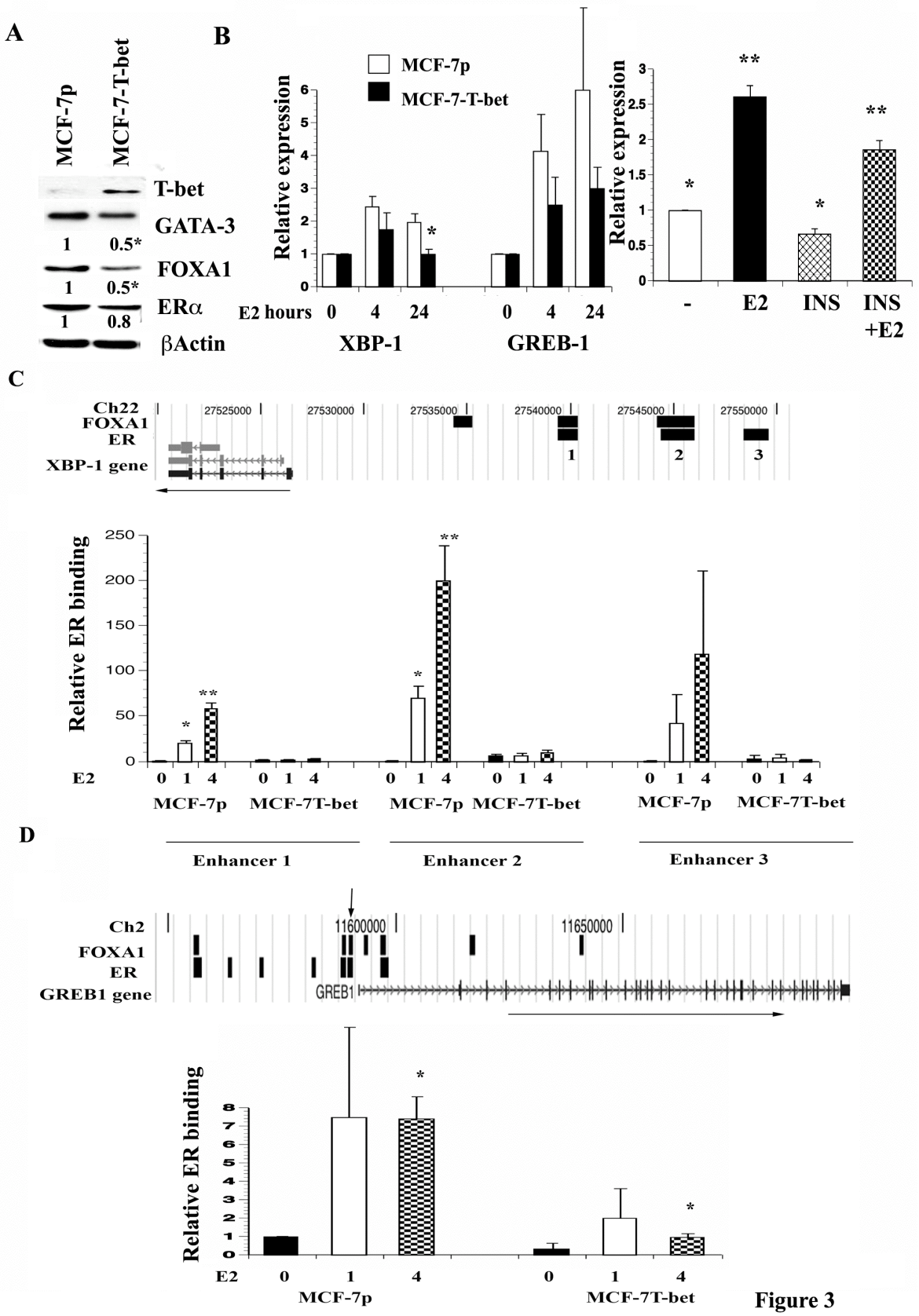
A
MCF-7p
MCF-7-T-bet
T-bet
GATA-3
1
0.5*
FOXA1
1
0.5*
ERα
1
0.8
βActin
B
MCF-7p
MCF-7-T-bet
Relative expression
E2 hours 0 4 24 0 4 24
XBP-1
GREB-1
Relative expression
- E2 INS INS +E2
C
Ch22
FOXA1
ER
XBP-1 gene
27525000
27530000
27535000
27540000
27545000
27550000
1
2
3
Relative ER binding
E2
MCF-7p
MCF-7T-bet
MCF-7p
MCF-7T-bet
MCF-7p
MCF-7T-bet
D
Enhancer 1
Enhancer 2
Enhancer 3
Ch2
FOXA1
ER
GREB1 gene
11600000
11650000
GREB1
Relative ER binding
E2
MCF-7p
MCF-7T-bet

Figure 3

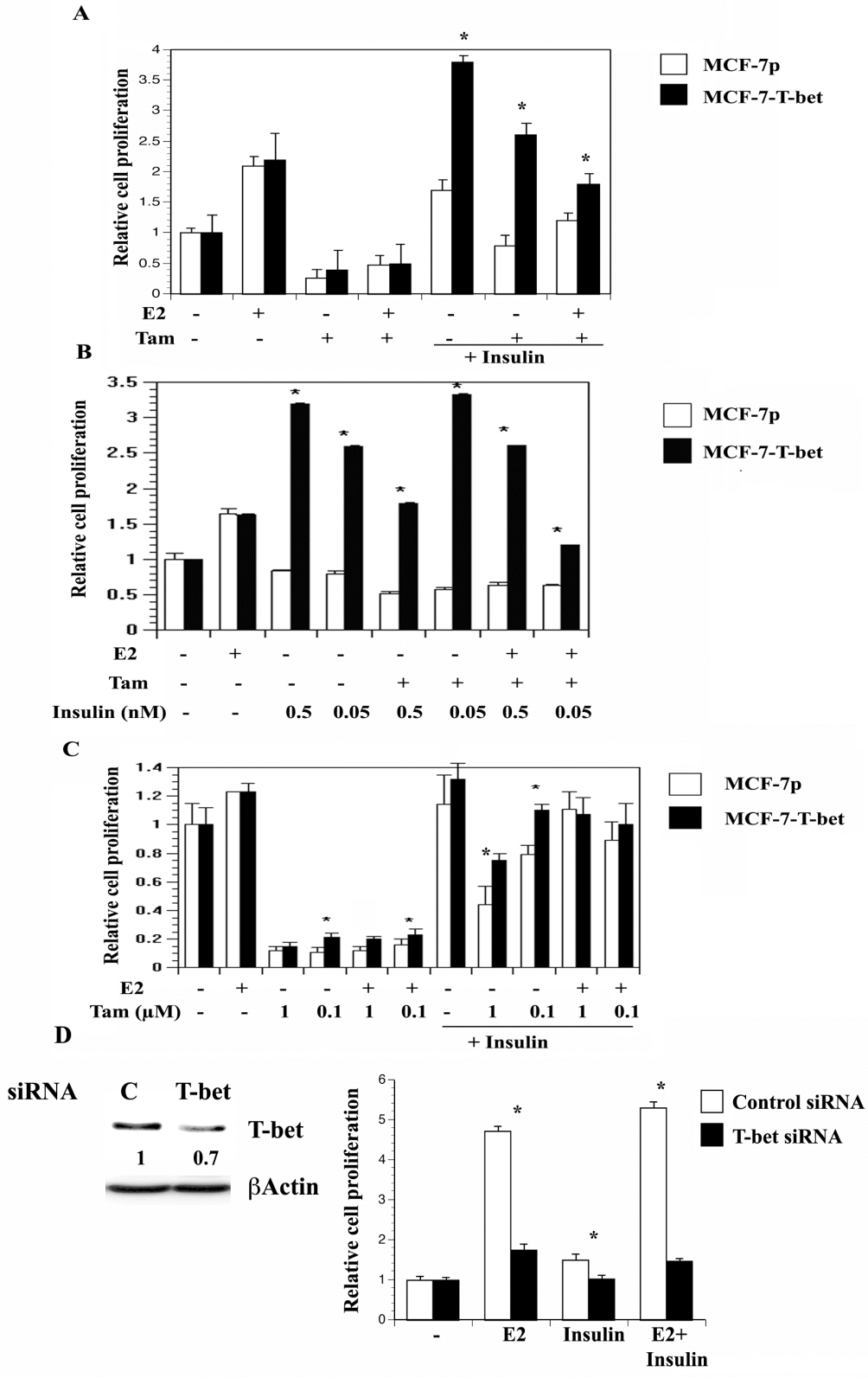
A
Relative cell proliferation
MCF-7p
MCF-7-T-bet
E2
Tam
+ Insulin
B
Relative cell proliferation
MCF-7p
MCF-7-T-bet
E2
Tam
Insulin (nM)
C
Relative cell proliferation
MCF-7p
MCF-7-T-bet
E2
Tam (μM)
+ Insulin
D
siRNA
C
T-bet
T-bet
1
0.7
βActin
Relative cell proliferation
Control siRNA
T-bet siRNA
-
E2
Insulin
E2+ Insulin

Figure 4

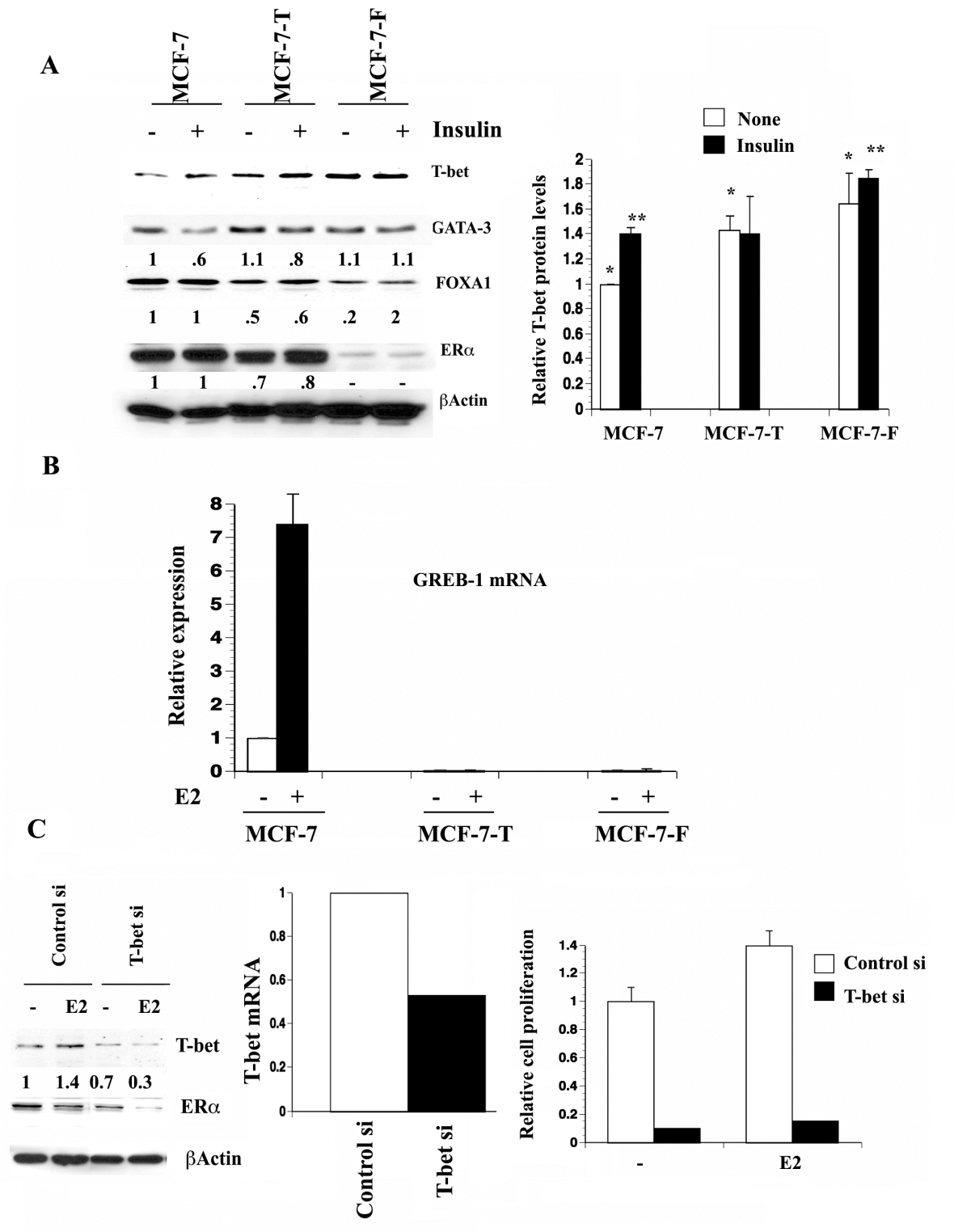
A
MCF-7
MCF-7-T
MCF-7-F
- + - + - +
Insulin
T-bet
GATA-3
1 .6 1.1 .8 1.1 1.1
FOXA1
1 1 .5 .6 .2 2
ERα
1 1 .7 .8 - -
βActin
None
Insulin
Relative T-bet protein levels
MCF-7
MCF-7-T
MCF-7-F
B
Relative expression
GREB-1 mRNA
E2
- + - + - +
MCF-7
MCF-7-T
MCF-7-F
C
Control si
T-bet si
- E2 - E2
T-bet
1 1.4 0.7 0.3
ERα
βActin
T-bet mRNA
Control si
T-bet si
Relative cell proliferation
Control si
T-bet si
-
E2

Figure 5

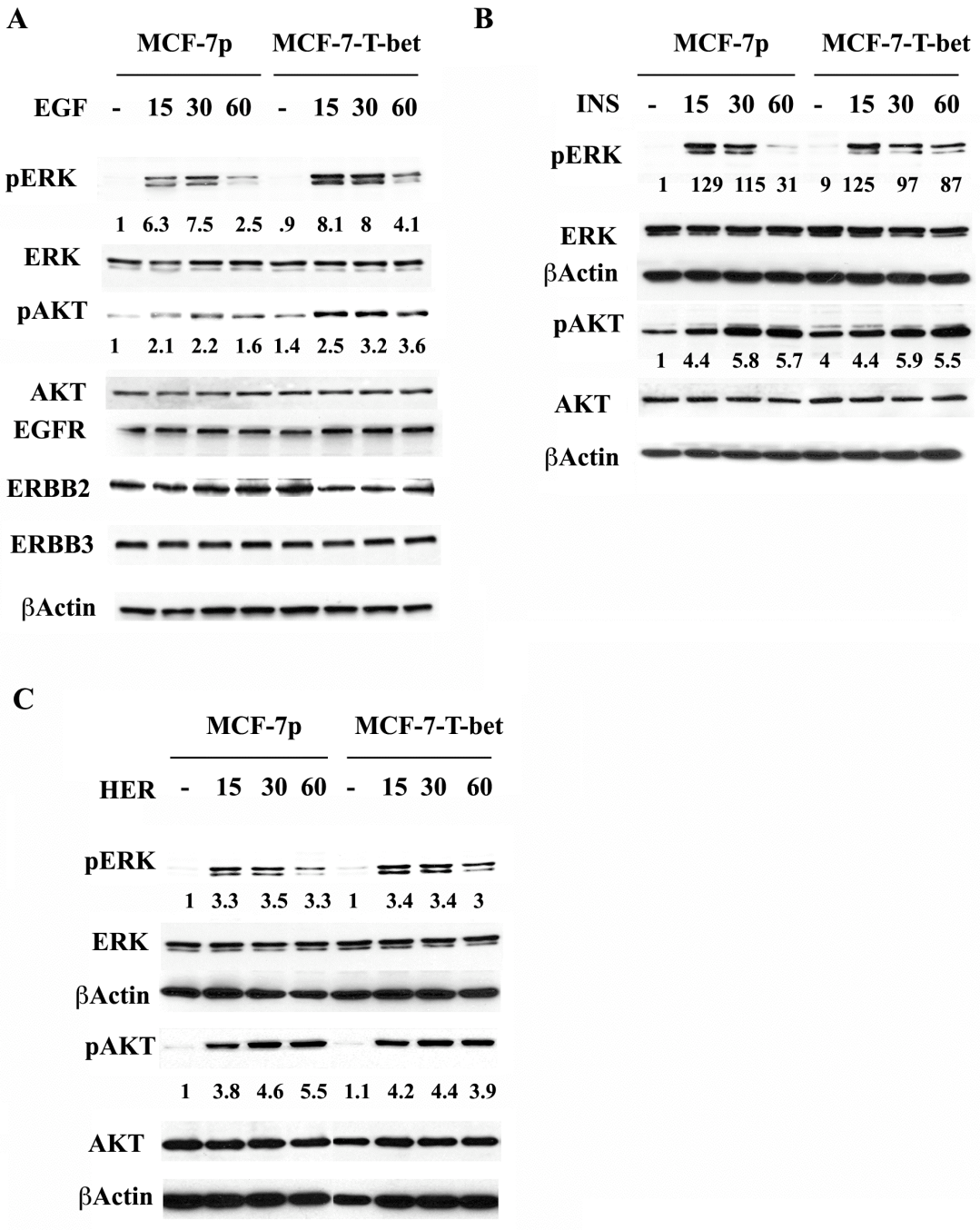
A
MCF-7p
MCF-7-T-bet
EGF - 15 30 60 - 15 30 60
pERK
1 6.3 7.5 2.5 .9 8.1 8 4.1
ERK
pAKT
1 2.1 2.2 1.6 1.4 2.5 3.2 3.6
AKT
EGFR
ERBB2
ERBB3
βActin
B
MCF-7p
MCF-7-T-bet
INS - 15 30 60 - 15 30 60
pERK
1 129 115 31 9 125 97 87
ERK
βActin
pAKT
1 4.4 5.8 5.7 4 4.4 5.9 5.5
AKT
βActin
C
MCF-7p
MCF-7-T-bet
HER - 15 30 60 - 15 30 60
pERK
1 3.3 3.5 3.3 1 3.4 3.4 3
ERK
βActin
pAKT
1 3.8 4.6 5.5 1.1 4.2 4.4 3.9
AKT
βActin

Figure 6

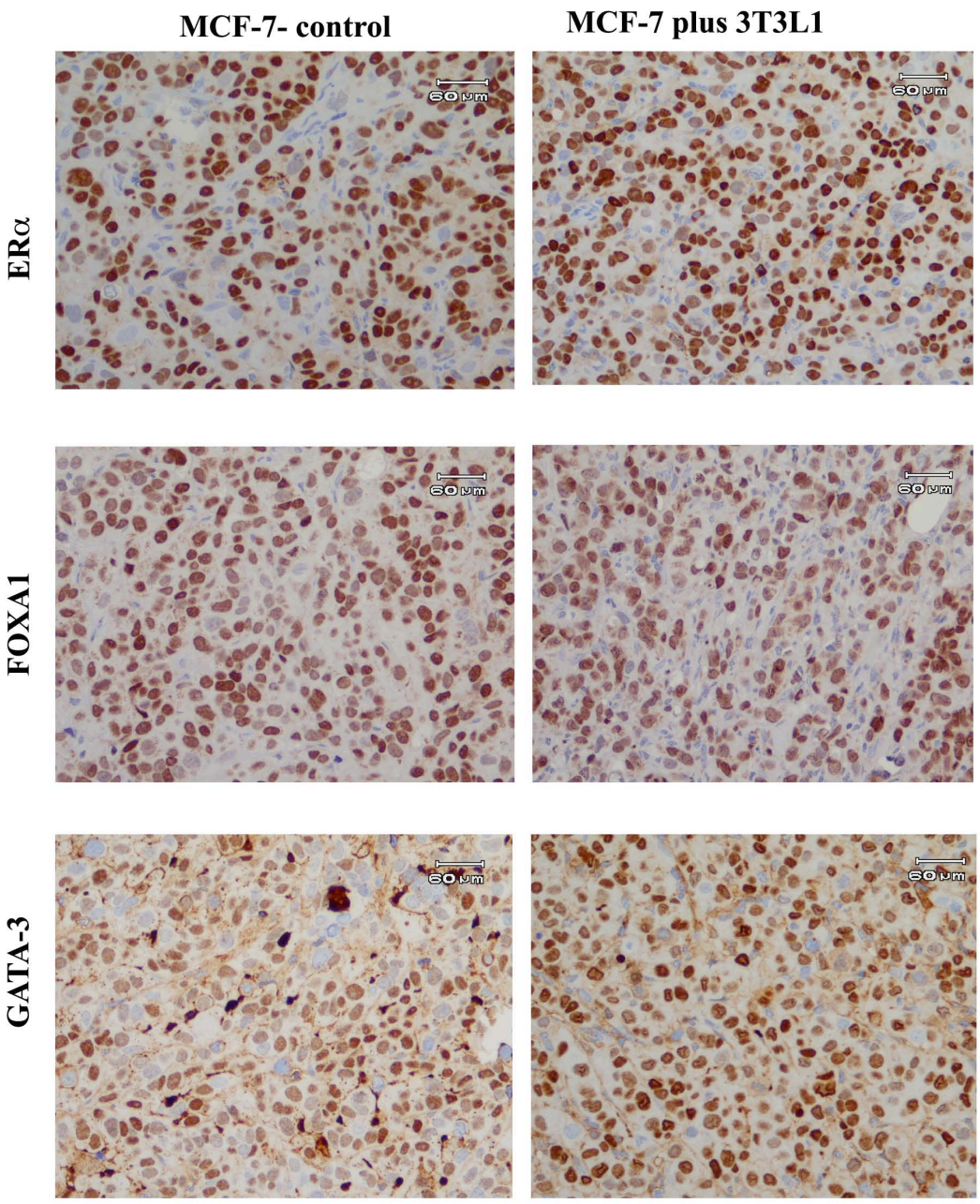

Figure 7

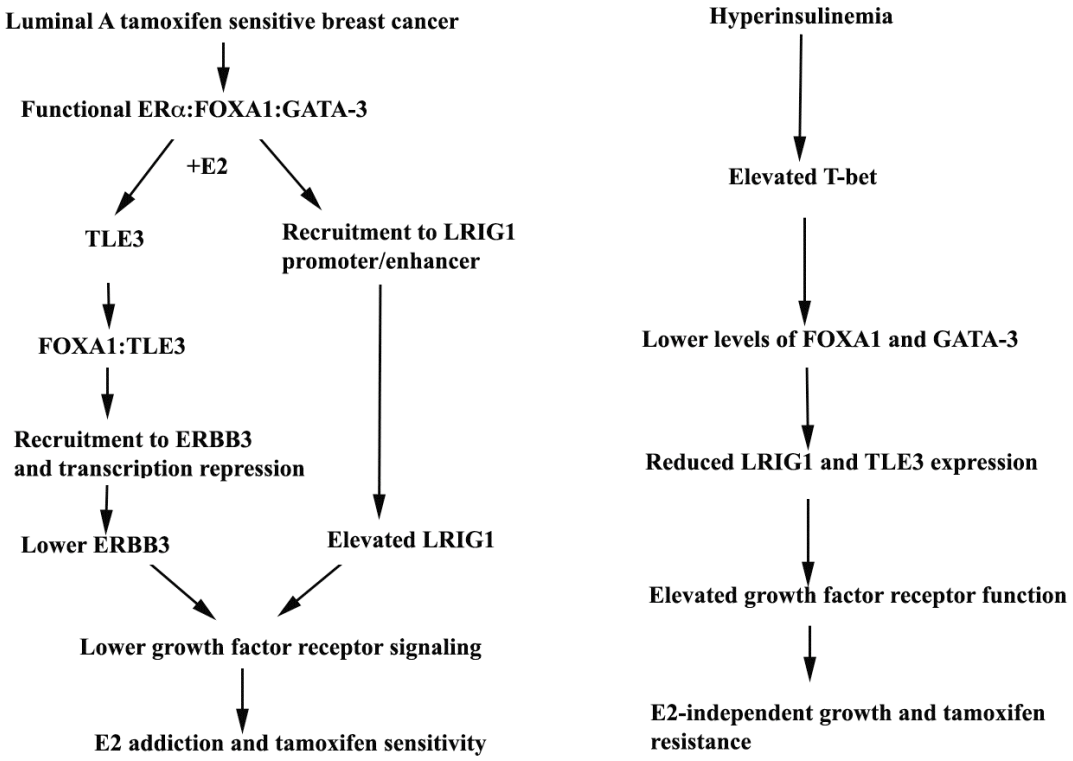
Luminal A tamoxifen sensitive breast cancer
Functional ERα:FOXA1:GATA-3
+E2
TLE3
Recruitment to LRIG1 promoter/enhancer
FOXA1:TLE3
Recruitment to ERBB3 and transcription repression
Lower ERBB3
Elevated LRIG1
Lower growth factor receptor signaling
E2 addiction and tamoxifen sensitivity
Hyperinsulinemia
Elevated T-bet
Lower levels of FOXA1 and GATA-3
Reduced LRIG1 and TLE3 expression
Elevated growth factor receptor function
E2-independent growth and tamoxifen resistance

Figure 8