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Estrogen and Insulin-like Growth Factor-I (IGF-I) Independently Down-regulate Critical Repressors of Breast Cancer Growth

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Abstract

Although estrogen receptor (ER) and insulin-like growth factor (IGF) signaling are important for normal mammary development and breast cancer, cross-talk between these pathways, particularly at the level of gene transcription, remains poorly understood. We performed microarray analysis on MCF-7 breast cancer cells treated with estradiol (E2) or IGF-I for 3hr or 24hr. IGF-I regulated mRNA of 5-10-fold more genes than E2, and many genes were co-regulated by both ligands. Importantly, expression of these co-regulated genes correlated with poor prognosis of human breast cancer patients. Closer examination revealed enrichment of repressed transcripts (compared to induced transcripts).. Interestingly, a number of potential tumor suppressors were down-regulated by IGF-I and estradiol, such as the B-cell linker BLNK. Analysis of three down-regulated genes showed that E2-mediated repression occurred independently of IGF-IR, and IGF-I-mediated repression occurred independently of ER. However, repression by IGF-I or estradiol required common downstream kinases, such as PI3K and MEK, suggesting downstream conversion of the two pathways. In conclusion, E2 and IGF-I co-regulate a set of genes that affect breast cancer outcome. There is enrichment of repressed transcripts, and the down-regulation by E2 and IGF-I is independent at the receptor level. This may be important clinically, as tumors with active ER and IGF-IR signaling may require co-targeting of both pathways.

INTRODUCTION

Breast cancer is a heterogeneous disease that is typically characterized by abnormal growth and survival of the epithelial cells of the breast. Both, steroid hormones, such as estrogen, and growth factors, such as insulin-like growth factor (IGF), can be major drivers of the disease, as both of these signaling pathways are highly mitogenic and anti-apoptotic.

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The effects of 17- β -estradiol (E2), the most potent estrogen, are mediated through the estrogen receptors (ER) α and β . Both receptors are modular in structure with several distinct domains, including an amino-terminally located ligand-independent transcriptional activation function (AF-1) domain, a DNA binding domain (DBD), a hinge region, and a ligand-dependent AF-2 domain. In the classical mode of action, ligand binding results in a conformational change of ER, which then allows it to directly interact with DNA at sequence-specific estrogen response elements (EREs). However, other modes of estrogen signaling have been described. These include indirect DNA binding through protein interactions with other transcription factors, such as Fos and Jun, ligand-independent activation of the receptor mediated by kinase cascades, and non-genomic, membrane-associated receptor signaling (12).

The IGF family consists of two ligands (IGF-I and IGF-II), two receptors (IGF-IR and IGF-IIR), and several high-affinity IGF binding proteins (3). Ligand-binding induces a conformational change in the receptor, resulting in activation of the intrinsic tyrosine kinase of the cytoplasmic domain of IGF-IR (34). Subsequent recruitment and phosphorylation of adaptor proteins, most notably the insulin receptor substrate (IRS) family of adaptors, transduces the intracellular signal. Activation of downstream kinases, such as MAPK and PI3K, are largely responsible for the proliferative and anti-apoptotic nature of active IGF signaling.

Interactions between estrogen and IGF action have been found in several tissues, including the uterus and mammary gland. In the uterus, studies have shown that estrogen treatment rapidly activates IGF-IR in an IGF-I-dependent manner (30, 31). Conversely, IGF-I stimulation can result in phosphorylation and activation of ER (18). Furthermore, this same study showed that IGF-I fails to stimulate proliferation in the uterus of the ER knockout mouse. A recent microarray study aimed at understanding the global transcriptional changes in the mouse uterus found that most of the gene regulation elicited by estradiol also occurred after growth factor treatment (13).

As previously stated, extensive cross-talk between estrogen and IGF action has also been documented in the mammary gland, a structure that critically relies on both of these signaling pathways for normal development. In fact, the ER knockout mouse (1) and the IGF-I knockout mouse (33) exhibit a similar defect in which elongation of the mammary ductal tree fails to occur. Furthermore, hypophysectomized and ovariectomized animals do not respond to estrogen treatment unless they also receive IGF-I (32). Additionally, IGF signaling components are hormonally regulated in the mouse mammary gland (19, 25). Not only do these data emphasize the mitogenic and pro-survival nature of these pathways, but they also highlight the importance of cooperation between the two.

In addition to their cooperative roles in normal mammary gland development, interaction between these two pathways has also been described in human breast cancer and in breast cancer cell line models. For example, there is a correlation between IGF-IR/IRS-1 and ER in breast cancer specimens (19), and this is likely due to the fact that components of the IGF system are estrogen-regulated. Thus, estrogen has the ability to sensitize cells to subsequent IGF-I stimulation. However, cross-talk occurs in both directions as active IGF signaling can phosphorylate ER and enhance its activity (7). Given the close interaction between these two pathways, it is not surprising that this cross-talk manifests itself at the clinical level as well. For example, treatment of breast cancer patients with anti-estrogens has been shown to reduce serum IGF-I levels (28). This is important since high serum IGF-I levels predict an increased risk of breast cancer (29). Furthermore, hyperactive IGF signaling has been associated with resistance to endocrine therapies (3).

Despite the extensive data showing the importance of interaction between these pathways for both normal mammary gland development and breast cancer pathogenesis, cross-talk between these pathways, particularly at the level of downstream gene transcriptional networks, remains poorly understood. In order to address this problem, we performed microarray analysis on MCF-7 breast cancer cells treated with E2 or IGF-I. We discovered that many genes are regulated by both ligands, and, interestingly, that there is enrichment of repressed targets within the co-regulated group. One of the most repressed target genes identified in our array was B-cell linker (BLNK). BLNK is a well-described tumor suppressor in lymphoma (10) and may also inhibit growth in breast cancer. We used BLNK as a candidate gene to gain a better understanding of how E2 and IGF-I repress transcriptional targets.

MATERIALS AND METHODS

Cell culture

MCF-7 human breast cancer cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Cellgro) supplemented with 5% characterized fetal bovine serum (HyClone), 100 IU/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B (antibiotics and antimycotic from Cellgro). Prior to ligand treatment for all experiments, cells were starved under serum-free conditions for at least one day. Serum-free medium (SFM) consists of phenol red-free improved minimal essential medium (IMEM; Invitrogen), 100 IU/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B, 10mM HEPES (Invitrogen), 1µg/mL transferrin (Sigma), and 1µg/mL fibronectin (BD Biosciences). For ligand stimulation, cells were treated with 10nM estradiol (E2; Sigma) and 100ng/mL insulin-like growth factor I (IGF-I; Novozymes), either alone or in combination. For untreated control samples, cells were simply maintained in SFM. In some experiments, cells were pre-treated for 30min with a chemical inhibitor prior to ligand stimulation. Inhibitors used include the IGF-IR antagonist BMS-536924 (1µM; Bristol-Myers Squibb), the pure anti-estrogen ICI 182780 (100nM; Sigma), the PI3K inhibitor LY-294002 (20µM; Calbiochem), the MEK inhibitor U0126 (10µM; Calbiochem), and the PKC antagonist Gö 6983 (0.25µM; Calbiochem).

RNA extraction and qRT-PCR

Total RNA was isolated using the RNeasy RNA isolation kit (Qiagen) as recommended by the supplier. Triplicate RNA samples were prepared for each treatment group. RNA was reverse transcribed using SuperScript II reverse transcriptase (Invitrogen) in accordance with manufacturer's instructions. The PCR reaction was then carried out on an ABI 7500 fast real-time thermocycler (Applied Biosystems) using the SYBR green master mix (Applied Biosystems) and 150nM each of both the forward and reverse primers. The cycling conditions were 50°C for 2min, 95°C for 10min, followed by 40 cycles at 95°C for 15s and 60°C for 30s. Primer Express 3.0 software (Applied Biosystems) was used to design all of the primers. The sequences of the primers used are listed in the supplementary material Table S1. The fold change for each gene was calculated using the cycle threshold ($\Delta\Delta C_T$) method as previously described (23), and data are represented as ligand-mediated fold change over SFM, unless otherwise stated. For each sample, real-time quantitative reverse transcription-PCRs (qRT-PCRs) were done in triplicate for both the genes of interest and the reference gene (β -actin) to normalize for input cDNA.

Microarray analysis

MCF-7 cells were plated in 10cm dishes and then switched to SFM on the next day. Following overnight starvation, cells were treated in triplicate with either E2 (10nM) or IGF-I (100ng/mL) for 3hr or 24hr. Control samples were maintained in SFM and left

untreated. Cells were lysed, and total RNA was isolated using the RNeasy kit (Qiagen) according to the manufacturer's instructions. cDNA synthesis, cRNA labeling, and array hybridization to Affymetrix HG U133A 2.0 gene chips were performed as previously described (14). Arrays were processed and normalized using the dChip software (<http://biosun1.harvard.edu/complab/dchip/>) (20). Data analysis was carried out by the Biostatistics and Bioinformatics Core of the Lester and Sue Smith Breast Center at Baylor College of Medicine. Briefly, all chips were modeled with the Array Analyzer GCRMA model, and ANOVA was performed separately for the 3hr and 24hr treatment groups.

Luciferase assays

For the BLNK promoter reporter assays, MCF-7 cells were plated in 6-well plates two days before transfection with 100ng/well pRL-Null (a *Renilla* luciferase construct for normalizing transfection efficiency) and 200ng/well pGL4 (firefly luciferase construct) containing a fragment of the BLNK promoter. Transfections were carried out using Lipofectamine 2000 (Invitrogen). After transfection, complete medium was aspirated and replaced with SFM. Cells were starved overnight and then treated with E2 (10nM) alone, IGF-I (100ng/mL) alone, or a combination of both ligands for 24hr. Luciferase assays using the dual-luciferase reporter assay system (Promega) were performed according to the manufacturer's instructions. Data were normalized to *Renilla* luciferase expression and represented as relative luciferase units (RLU).

Immunoblotting

Proteins were resolved on 8% SDS-PAGE and transferred to nitrocellulose. The membrane was blocked in 5% milk dissolved in phosphate-buffered saline + 0.1% Tween 20 (PBST) for 1hr at room temperature. Primary antibodies used include anti-BLNK (Santa Cruz Biotechnology; sc-8003) and anti- β -actin (Sigma; A5441). The BLNK antibody was diluted 1:500 in PBST + 5% milk and incubated overnight at 4°C. The β -actin antibody was diluted 1:5000 in PBST + 5% milk and incubated overnight at 4°C. After washing three times for 5min with PBST, the membranes were incubated with anti-mouse or anti-rabbit secondary antibodies conjugated to either IRDye700 or IRDye800 (Rockland) for 1hr at room temperature. Secondary antibodies were diluted 1:5000 in PBST + 5% milk. After incubation, membranes were washed three times for 5min with PBST, and the signal was visualized using the Odyssey imaging system (Li-Cor Biosciences).

ChIP assays

Chromatin immunoprecipitation (ChIP) assays were performed as previously described (35). Briefly, MCF-7 cells (5×10^6) were plated in DMEM. The next day, cells were washed twice with PBS and then switched to SFM. Following approximately 48hr starvation, cells were treated with E2 (10^{-8} M), IGF-I (100 ng/mL), or E2+IGF for 1hr. Control samples were left untreated in SFM. After DNA purification (QIAquick PCR purification kit; Qiagen), the BLNK promoter region was amplified by qRT-PCR using the following primer pair: forward 5' – TGCTCGGGCAAGTTTACTTC – 3'; reverse 5' – CGGCCACTCAAGTCTGATTTC – 3'. Data are represented as "% Input". The ChIP antibodies used were RNA polymerase II (Covance; MMS-126R) and IgG (Santa Cruz Biotechnology; sc-2027).

siRNA and MTS assay

MCF-7 cells were transfected with 50nM small interfering RNA (siRNA) using DharmaFECT1 (Thermo Fisher Scientific). The siRNAs used were as follows: siGENOME non-targeting siRNA #2 (Dharmacon) and BLNK (Sigma; 5' – GAUCCAAACAACCAUAUA – 3'). The next day cells were split and re-plated in a 96

well plate. Approximately 48hr after, cells were transfected with either BLNK siRNA or non-specific control siRNA. The next day, cells were split and re-plated in a 96-well plate. Cells were incubated in medium containing either 5% fetal bovine serum (FBS) or 5% charcoal-stripped FBS. Cell growth was assessed by MTS assay (Promega) on days 0, 1, 2, and 4. The experiment was performed with biological quintuplicates.

RESULTS

Microarray analysis reveals a number of genes that are regulated by both E2 and IGF-I in MCF-7 cells

We performed microarray analysis on MCF-7 breast cancer cells, which express high levels of both ER and IGF-IR, following treatment with either E2 (10nM) or IGF-I (100ng/mL) for 3hr or 24hr (Fig. 1A). RNA was isolated, and gene expression array profiles were analyzed. The data have been deposited in NCBI's Gene Expression Omnibus database and are accessible through GSE26834. Using a fold change > 1.5 and a p -value < 0.01 , approximately 3051 probesets were found to be significantly altered (up- or down-regulated) upon stimulation with IGF-I at the 3hr time point. Approximately 10-fold fewer probesets (297) were found to be either up- or down-regulated following treatment with E2 for 3hr. A similar pattern was observed after 24hr of treatment. IGF-I significantly altered about five times as many probesets (3659) as E2 (649). Representative gene lists can be found in Tables 1 and 2.

We found significant overlap between genes increased by exogenously administered IGF-I in our study and genes that were found to be elevated in MCF-7 cells overexpressing IGF-I. Specifically, of the 24 genes reported as up-regulated by IGF-I (27), 15 genes were likewise increased ($p < 0.05$) by IGF-I at 24hr in our data set ($p < 0.000001$, one-sided Fisher exact). Furthermore, comparison of the estrogen-regulated transcripts to the numerous previously published microarray studies showed extensive overlap as expected (data not shown).

While E2 and IGF-I independently regulate a number of genes, we also found significant and extensive overlap between target genes regulated by both ligands. For example, of the 297 estrogen-regulated probesets at the 3hr time point, 183 overlapped with the IGF-I-regulated probesets as represented in a Venn diagram (Fig. 1B). The overlap between estrogen- and IGF-I-regulated genes is highly statistically significant ($p < 0.001$) (X^2 , with continuity correction in SPLUS). Furthermore, the overlap is even more striking after 24hr of ligand stimulation with 454 of the 649 estrogen-regulated genes also being regulated by IGF-I. We performed quantitative real-time RT-PCR to verify genes within each section of the Venn diagrams, and representative target genes are shown (Fig. 1C).

Additionally, we performed gene ontology (GO) analysis on genes that were found to be either induced by both E2 and IGF-I or repressed by both ligands at the 3hr and 24hr time points. Genes induced at both time points were enriched for DNA metabolism and replication. For example, at the 3hr time point, 16 genes fell into one of these two GO categories. At the 24hr time point, 29 genes were associated with these two groups. Taking into account the total number of genes within each population, these represent two of the most statistically significant categories that we identified ($p < 0.00001$). In contrast, GO terms enriched for repressed genes at the 3hr and 24hr time points were more distinct. At the 3hr time point, genes repressed by both E2 and IGF-I were generally enriched for development and embryonic specification ($p < 0.005$), while at 24hr there was enrichment of chromosome organization and nucleosome assembly ($p < 0.0001$). A more detailed description of the GO analysis can be found in the supplemental material (Fig. S1).

Expression of E2 and IGF-I co-regulated genes is correlated with poor prognosis of human breast cancer

Given the close interaction between the ER and IGF signaling pathways, not only in normal mammary gland development, but also in breast cancer cells and in xenograft models, several researchers have suggested a need for co-targeting these pathways as part of breast cancer therapy. In order to determine if the E2 and IGF-I co-regulated genes were associated with any clinical significance, we examined whether or not they had any prognostic value in human breast cancer. To address this question, we defined an “E2-IGF co-regulated gene activation score” for a given tumor as the Pearson correlation between the E2-IGF co-regulated genes at 24hr (by using 1 for up and -1 for down) and the gene expression values of the 295 tumors from the publicly available and well-described van de Vijver dataset (37). Due to the fact that we were looking at estrogen-regulated genes (and also due to the fact that ER-negative tumors have a poor prognosis), we focused solely on the 226 ER-positive tumors. Tumors that showed a positive Pearson's correlation ($p < 0.01$) were then classified as having an “activated” E2-IGF co-regulated signature (i.e. significant correlation, $p < 0.01$, to the pattern of up- and down-regulation by E2 and IGF-I observed in vitro). Tumors that had a significant anti-correlation ($p < 0.01$) had a “deactivated” signature. Finally, those that showed no significant association ($p > 0.01$) were termed “intermediate”. Using this classification, Kaplan-Meier curves showed significant differences in time to metastasis among the three groups (log rank $p < 1E-6$), with patients in the activated group having a shorter time to metastasis compared to those patients in either the deactivated or intermediate groups (Fig. 2A). Thus, genes that are co-regulated by both estrogen and IGF-I in MCF-7 breast cancer cells confer poor prognosis when similarly regulated (either induced or repressed) in human breast cancers. Importantly, the effect on prognosis was not simply due to the fact that estrogen and IGF-I induce proliferative genes (proliferation is a known poor prognostic marker in breast cancer), as removal of genes that were either annotated as “cell cycle” by gene ontology (GO) analysis or were experimentally found to be correlated with cell cycle progression (39) had very little effect on the Kaplan-Meier analysis (log-rank $p = 1.7E-06$; Fig. S2 in supplemental material).

Additionally, we sought to determine if the E2-IGF-I co-regulated gene signature correlated with a particular subtype of ER-positive tumors. To address this question, we once again turned to the publicly available and well-described van de Vijver dataset (37). Within the 226 ER-positive human breast tumors, we found a range of gene expression patterns. Approximately 25% of the tumors examined had an activated E2-IGF-I co-regulated gene expression signature (Fig. 2B; denoted by black box). That is, genes induced by both E2 and IGF-I in MCF-7 cells were similarly expressed at high levels in these tumors. Likewise, genes found to be repressed by both ligands in vitro were similarly repressed in these tumors. Tumors with this activated co-regulated gene signature correlated with the luminal B subtype of ER-positive breast cancers, and this luminal B subtype is known to have a worse prognosis than its luminal A counterpart (36). Interestingly, tumors with a deactivated signature (i.e. tumors that had low expression of the genes found to be induced in MCF-7 cells and high expression of the repressed genes) were found to correlate with the luminal A subtype of ER-positive breast cancer.

Genes that are co-regulated by E2 and IGF-I are enriched for repressed targets

While estrogen receptor has been largely studied as an activator of transcription, recent gene expression profiling studies have revealed that ER can repress at least as many targets as it activates (2, 6, 11). Although not as much is known about how IGF-I regulates transcriptional targets, in our microarray study, we found that both estrogen and IGF-I repress and induce an approximately equal number of genes. We identified genes that were either induced by E2 at both 3hr and 24hr or repressed by E2 at both time points. When we

used the microarray data to examine the expression level of these genes in response to IGF-I treatment, we found that the overlap of genes within the repressed group was more pronounced (Fig. 3A).

We expanded on this initial observation by looking at patterns of induction and repression specifically within the co-regulated group of genes ($p < 0.05$; fold > 1.1 for induction, fold < 0.9 for repression). Interestingly, and in accordance with our previous findings, when we performed this analysis, we noticed enrichment for repressed targets. For example, at the 3hr time point, 51% of the 183 co-regulated probesets were repressed by both E2 and IGF-I (Fig. 3B). This is in contrast to the only 30% of probesets that were induced by both E2 and IGF-I. The least abundant patterns of regulation were those patterns in which E2 and IGF-I had opposite effects on target genes; only 17% of the probesets at the 3hr time point were induced by E2 but repressed by IGF-I, and only 2% were repressed by E2 but induced by IGF-I at this time. A similar pattern was observed after 24hr of ligand treatment. Of the 454 co-regulated probesets at the 24hr time point, 62% were repressed by both E2 and IGF-I. This is in stark contrast to the 35% of probesets that were induced by both ligands. Again, E2-induced/IGF-I-repressed (2.3%) and E2-repressed/IGF-I-induced (0.7%) represented the least prevalent patterns of target gene regulation. Thus, these data show a highly significant percentage of genes that are co-regulated by E2 and IGF-I, that these ligands act predominantly in a concordant fashion, and that the genes co-regulated by both ligands are enriched for repressed transcripts.

Many of the co-regulated genes identified in our study may be key targets that estrogen and IGF-I use to mediate cancer progression. This hypothesis is supported by the fact that genes that are co-regulated by these ligands in MCF-7 cells confer poor prognosis when similarly regulated (either induced or repressed) in human breast cancers (Fig. 2). Given that the list of estrogen and IGF-I co-regulated genes is enriched for repressed targets, it is not surprising that there are a number of potential tumor suppressor genes that appear on the list. For example, the top 20% of genes repressed by estrogen and IGF-I at either the 3hr or 24hr time point contains several candidates that have been described as tumor suppressors in previously published literature (Fig. S3 in the supplemental material). We validated a number of these genes by quantitative real-time RT-PCR in MCF-7 cells that were either maintained in serum-free medium (SFM) or treated with E2 (10nM) or IGF-I (100ng/mL) for 3hr or 24hr (Fig. 3C). BLNK, SOCS2, and NR3C1 were all strongly repressed by both ligands at 3hr and 24hr. ING4 was strongly repressed by IGF-I at both time points, but the E2-mediated repression was prominent only at the 24hr time point. BIK mRNA levels were reduced by approximately 40% by E2 and IGF-I following 3hr of treatment. At 24hr, the E2-mediated repression was much prominent than the IGF-I-mediated repression of BIK.

E2-mediated repression of tumor suppressor genes is independent of IGF-IR and IGF-I-mediated repression is independent of ER, but both require common downstream kinases

Since estrogen signals through ER, and IGF-I elicits its biological effects by binding to IGF-IR, we wanted to determine the importance of both receptors in the estrogen- and IGF-I-mediated repression of several co-regulated target genes. To address this question, we pre-treated MCF-7 cells with or without the anti-estrogen ICI 182780 (100nM) or with a small molecule inhibitor of IGF-IR (BMS-536924, 1uM). Following 30min of pre-treatment, cells were stimulated with estradiol (10nM) or IGF-I (100ng/mL) for 3hr or 24hr. Blockade of ER by ICI 182780 (ICI) dramatically reversed estrogen-mediated repression of BLNK, SOCS2, and CCNG2 at all time points examined (Figs. 4A and 4B). Surprisingly, ICI had little or no effect on the IGF-I-mediated repression of these same genes. Similarly, the anti-IGF-IR inhibitor BMS-536924 (BMS) was able to completely reverse IGF-I-mediated repression of all genes, but it had essentially no effect on estrogen-mediated repression. These results were somewhat surprising given the previous data that we and others have generated

indicating positive interaction between these two signaling pathways (ref). Further investigation is needed to determine if this independence is specific to the set of genes that we have chosen for study (e.g. maximal repression) or whether there are other genes that may rely more upon ER-IGF-IR cross-talk. Taken together, these data show, for the target genes we have selected, that repression mediated by each ligand is dependent upon that ligand's receptor. Furthermore, and quite interestingly, the data also show that the IGF-I-mediated repression of target genes is independent of ER, and the estrogen-mediated repression is independent of IGF-IR.

To build upon this initial observation of receptor independence, we decided to examine the effect of dual ER/IGF-IR blockade on target gene repression. We pre-treated MCF-7 cells with either BMS alone (IGF-IR inhibitor; 1 μ M), ICI alone (ER antagonist; 100nM), or the combination of both inhibitors. This was followed by simultaneous stimulation with estrogen (10nM) and IGF-I (100ng/mL) for 3hr (Fig. 4C). In the absence of any inhibitor, combined treatment of E2+IGF-I strongly repressed BLNK expression. This repression could only be partially relieved when ER alone or IGF-IR alone was blocked. In order to fully restore BLNK levels back to basal and fully abrogate the E2- and IGF-I-mediated repression, combined blockade of ER and IGF-IR had to be administered.

We next sought to establish whether or not this independence existed downstream of the receptor level as well. Given that both estrogen and IGF-I have been shown to activate multiple kinases, including PI3K, MAPK, and PKC, we decided to explore the role of these enzymes in the estrogen- and IGF-I-mediated repression of BLNK using various chemical inhibitors. MCF-7 cells were maintained in serum-free medium overnight and then pre-treated with inhibitor for 30min. Inhibitors used include the PI3K inhibitor LY-294002 (20 μ M), the MEK inhibitor U0126 (10 μ M), and the PKC antagonist Gö 6983 (0.25 μ M). Following this 30min pre-treatment, cells were administered E2 (10nM), IGF-I (100ng/mL), or E2+IGF-I for 24hr in the continued presence of the inhibitor. As control, cells were treated with estradiol and IGF-I in the absence of any chemical inhibitor as well. In the absence of inhibitor blockade, both E2 and IGF-I strongly repressed BLNK mRNA levels (Fig. 4D). Blockade of PI3K signaling with the LY compound almost completely relieved the E2-mediated repression and significantly impeded the ability of both IGF-I alone and E2+IGF-I to repress BLNK. Blockade of MEK with U0126 partially abrogated BLNK repression under all treatment conditions but was unable to restore BLNK levels to those seen under basal conditions. Inhibition of the PKC signaling pathway had no effect on either the E2- or IGF-I-mediated BLNK repression, suggesting that PKC does not lie downstream of estrogen or IGF signaling in repression of this particular target gene. Clearly, these experiments do not rule out the possibility that other kinases may be involved in the estrogen- and IGF-I-mediated repression of BLNK. They do, however, provide a more general understanding of how these pathways may cooperate to regulate target genes. Although independent at the level of ER and IGF-IR, mechanisms of estrogen- and IGF-I-mediated repression of targets, such as BLNK, may converge further downstream on various kinases, such as PI3K and MEK.

BLNK inhibits MCF7 cell growth

BLNK was one of the top genes identified in our microarray study to be strongly repressed by both E2 and IGF-I. Although not much is known about the role of BLNK in breast cancer, previous reports have shown that the BLNK chromosomal locus undergoes frequent loss of heterozygosity in prostate cancer (15) and that *BLNK* knockout mice spontaneously develop lymphomas (10). If BLNK regulation by estrogen and IGF-I is important for breast cancer cell biology, then its protein level, in addition to its mRNA level, should be regulated by both ligands. To determine if estradiol and IGF-I could lower BLNK protein levels, we

treated MCF-7 cells with estradiol (10nM), IGF-I (100ng/mL), or the combination of both ligands for 48hr (Fig. 5A). Control cells, maintained in serum-free medium, had the highest level of BLNK expression. Treatment of cells with either E2 or IGF-I resulted in a decrease in BLNK protein. Interestingly, the simultaneous administration of E2+IGF-I resulted in a complete loss of BLNK protein, which was undetectable by immunoblot. These data support the idea that both estrogen and IGF signaling repress BLNK protein levels via distinct mechanisms that can cooperate in an additive manner. Thus, the strong repression of BLNK mRNA by E2 and IGF-I corresponds to a loss at the protein level as well.

Given its potential role as a tumor suppressor, we wanted to investigate the function of BLNK and its effect on MCF-7 cell biology. We decided to mimic the E2- and IGF-I-mediated repression of BLNK by knocking down BLNK expression using transient siRNA transfection in MCF-7 cells. Cells cultured in charcoal-stripped FBS and transfected with non-specific siRNA (Fig. 5B, top panel, solid line) failed to proliferate, emphasizing the dependence of these cells on estrogen for growth. However, knockdown of BLNK enhanced MCF-7 cell growth when cultured in charcoal-stripped serum (Fig. 5B, top panel, dashed line). In contrast, depletion of BLNK levels in MCF-7 cells cultured in full serum (FBS; Fig. 5B, bottom panel, dashed line) did not enhance growth compared to cells transfected with the non-specific siRNA control (Fig. 5B, bottom panel, solid line). This may be due to the fact that steroid hormones, such as estradiol, and growth factors, such as IGF-I, are present in full serum. BLNK levels may already be extremely low due to repression by these ligands, and siRNA may be unable to further reduce BLNK levels. As proof of principle of this concept, we cultured MCF-7 cells either in 5% complete fetal bovine serum (FBS) or 5% charcoal-stripped FBS (CSS) for 72hr (Fig. 5B). While BLNK protein was undetectable in cells cultured in FBS, cells cultured in CSS had appreciable BLNK protein expression. Furthermore, and in line with this train of thought, cells cultured in medium devoid of any serum had the highest BLNK expression levels (data not shown). Thus, our data show that BLNK is dynamically regulated by E2 and IGF-I and that loss of BLNK may confer a growth advantage.

DISCUSSION

Here we report a novel examination of estrogen and IGF-I action in MCF-7 breast cancer cells. A majority of earlier work has focused on interaction between these two pathways at the signal transduction level. For example, estrogen can induce components of the IGF system, activated IGF-IR can phosphorylate ER and enhance its activity, and non-nuclear ER has been shown to physically interact with IGF-IR at the membrane. Overall, a theme of bi-directional cross-talk has emerged. Despite this work, interaction between these pathways at the level of downstream gene transcriptional networks remains poorly understood. Estrogen and IGF-I are major drivers of breast cancer growth and survival, and it is through changes in target gene expression that these ligands elicit their biological effects. Thus, it is critical to determine the transcriptional targets of the estrogen and IGF signaling pathways and the mechanisms by which these genes are regulated.

We performed microarray analysis of MCF-7 cells treated with E2 or IGF-I for either 3hr or 24hr. We found that, under serum-free conditions, IGF-I is a much more potent regulator of transcription than is E2. In fact, at the 3hr time point, IGF-I significantly regulated about 10 times as many probe sets as did E2. This is likely due to the extensive and pleiotropic downstream effects of IGF-I, whereby activation of the receptor can lead to multiple phosphorylation cascades. Previous microarray studies examining effects of IGF-I have been published (9, 26, 27), and, importantly, we found significant overlap between genes regulated by exogenously administered IGF-I in our study and genes that were found to be regulated in other studies as well. In contrast to IGF-I microarray studies, there have been

many more previously published studies examining the effect of estrogen treatment on target gene expression (5, 8, 11, 21, 38). Interestingly, data have shown that ER, classically described as an activator, can repress at least as many genes as it induces (24). This is exactly what we found in our microarray study as well. Additionally, comparison of the estrogen-regulated transcripts from our array to the numerous previously published microarray studies showed extensive overlap as expected. Despite this fact, a number of genes shown to be regulated by E2 in other microarray studies were not significantly regulated by E2 in our array. This may be the result of starving our cells in serum-free medium, in contrast to the more common starvation of cells in medium containing charcoal/dextran-treated fetal bovine serum. This does not come as a complete surprise given the previous work in the field. As an example, Katzenellenbogen et al. found that estrogen-mediated induction of progesterone receptor (PR), a classical ER target gene, does not occur when cells are in medium containing serum concentrations below 1% (17). However, estrogen-mediated activation of PR could be restored by either increasing serum concentrations or supplementing the medium with insulin or IGF-I. Our data presented here lends support to and builds upon this idea that a large fraction of ER transcriptional activity is dependent on ER interaction with other cellular pathways.

We found that estrogen and IGF-I co-regulate a large number of target genes, and that these genes are biologically important. That is, human breast tumors showing the same pattern of gene expression to the pattern we observed in MCF-7 cells following stimulation with E2 or IGF-I had a significantly worse prognosis. This is important since some of the genes we identified in our array may be key targets that E2 and IGF-I use to elicit their biological response. Taking this into consideration, and given the fact that there is enrichment for repressed genes within our data set, it is not surprising that several of the genes have been previously described as tumor suppressors and growth inhibitors. Many studies focus on how E2 and IGF-I “turn on” genes to promote tumorigenesis. However, the data we provide here highlight the fact that what these ligands deactivate may be just as important, if not more important, than what they activate in terms of promoting carcinogenesis.

For the target genes we examined, we found that E2 and IGF-I use some common downstream kinases, including MEK and PI3K, to elicit repression. This is distinct from what occurs upstream, as we found that repression at the level of ER and IGF-IR occurs independently. That is, estrogen-mediated repression of targets does not rely on IGF-IR, and IGF-I-mediated repression of target genes is independent of ER. Initially, this may be somewhat surprising given the previous data that we and others have generated indicating positive interaction between these two signaling pathways. For example, IGF-I-mediated induction of cyclin D1 was shown to be dependent on ER expression (40). Additionally, E2-mediated induction of the classical ER target gene pS2 was significantly enhanced by the addition of IGF-I (4). The authors of this study concluded that this augmentation was due to the fact that E2 and IGF-I synergize in the recruitment of multiple ER-associated proteins, including coregulators and RNA polymerase II, to the pS2 ERE site. Despite these findings, our data argue that there may be multiple ways in which E2 and IGF-I can cooperate to repress target genes and that it does not have to occur in a strictly linear pathway. In total, we examined 10 genes (Figs. 4A, 4B and data not shown) that were either repressed or induced by E2 and IGF-I in the presence or absence of either BMS or ICI. For these 10 genes, we found that the estrogen-mediated regulation of transcripts did not rely on IGF-IR, and the IGF-I-mediated regulation occurred independently of ER. Further investigation is needed to determine the nature of this independence, but it should be noted that this is not the first time that such results have been reported. A previous study using microarray-based evaluation of ER/growth factor cross-talk *in vivo* in the mouse uterus revealed that a number of genes are regulated by IGF-I in an ER-independent manner (13).

One of the top targets found in our array to be repressed by both E2 and IGF-I was *BLNK*, a well-documented tumor suppressor in cancer of the immune system. *BLNK* knockout mice spontaneously develop lymphoma (10). Additionally, 50% of childhood pre-B cell acute lymphoblastic leukemias display a decrease in *BLNK* expression (16). Although not much is known about *BLNK*'s role in breast cancer, our preliminary data suggest that *BLNK* regulates growth of MCF-7 cells as well. One interesting observation we made concerns the dynamic regulation of *BLNK* protein levels in medium devoid of serum or containing either full serum or charcoal-stripped serum. *BLNK* protein was highest in serum-free conditions, somewhat lower in charcoal-stripped serum, and barely detectable in full serum. It is likely that the differential response of MCF-7 cell growth that we observed in response to siRNA-mediated knockdown in full serum compared to charcoal-stripped serum was due to the aforementioned dynamic regulation of *BLNK* protein in different culture conditions. Although the experiment was not performed, one could speculate that *BLNK* knockdown in MCF-7 cells cultured in serum-free medium would have the most dramatic effect on cell growth. The additive repression of *BLNK* protein seen with full serum compared to charcoal-stripped serum correlates with the total loss of *BLNK* protein following simultaneous administration of E2+IGF. Importantly, some *BLNK* protein remained detectable after treatment with either E2 or IGF-I alone. These data, along with the luciferase reporter and ChIP data, emphasize the independent nature of *BLNK* repression elicited by both E2 and IGF-I.

In summary, our study provides evidence that E2 and IGF-I repress a large number of common target genes. For many of these gene transcripts, the E2-mediated repression occurs independently of IGF-IR, and the IGF-I-mediated repression does not rely on ER, emphasizing the independent and distinct nature of target gene repression. This fact may be clinically relevant. For example, if *BLNK* or other target genes are key suppressors of tumorigenesis, then one would reason that maintaining *BLNK* levels would be effective in limiting disease progression. Given that estrogen and IGF signaling can independently repress *BLNK*, it stands to argue that tumors with active estrogen and IGF signaling, for example, luminal B breast tumors, would have extremely low *BLNK* expression. Furthermore, in order to completely restore *BLNK* levels, one would have to inhibit both the ER and IGF signaling pathways. This lends support to the idea that there is likely a subset of breast tumors that would benefit from co-targeted therapy of both the ER and IGF signaling pathways. Not only has this idea been tested pre-clinically—breast cancer cell lines treated with a combination of the aromatase inhibitor letrozole and an IGF-IR inhibitor synergistically induced apoptosis (22)—but there are also several clinical trials aimed at testing this hypothesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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FIG. 1.

Global gene transcription patterns initiated by E2 and IGF-I treatment in vitro. (A) Affymetrix profiles were taken of MCF-7 cells in serum-free medium (SFM) with or without the presence of either E2 (10nM) or IGF-I (100ng/mL) for 3hr or 24hr. Shown are genes found to be significantly ($p < 0.01$) induced (yellow) or repressed (blue) following ligand stimulation (fold change > 1.5). (B) Venn diagrams showing the extensive and significant ($p < 0.001$) overlap of target genes regulated by both E2 and IGF-I at 3hr and 24hr. (C) Quantitative real-time RT-PCR (qRT-PCR) was used to calculate relative mRNA expression as ligand-mediated fold change compared to the untreated SFM control. MCF-7 cells were starved overnight in SFM and then treated with either E2 or IGF-I for 3hr or 24hr.

RNA of select genes from each section of the Venn diagrams was measured by qRT-PCR. The data are an average of three replicates $\pm$ standard error of the mean (SEM).

FIG. 2.

An activated E2/IGF-I co-regulated gene signature in clinical breast tumors is associated with poor prognosis. (A) Kaplan-Meier analysis of the van de Vijver tumor profile data set comparing the differences in risk among three groups of patients when only tumors classified as ER-positive were considered. Green line, tumors having an “activated” co-regulated gene signature (i.e. significant correlation, $p < 0.01$, to the pattern of up- and down-regulation by E2 and IGF-I observed in vitro); pink line, “deactivated” tumors that have low similarity (i.e. anti-correlation, $p < 0.01$) to the E2/IGF-I pattern; and yellow line, tumors that were neither similar nor dissimilar to the co-regulated gene expression pattern ($p > 0.01$). The log-rank test evaluated whether there are significant differences among any of the three groups. (B) An E2/IGF-I co-regulated gene expression signature was defined and the 226 ER-positive tumors from the van de Vijver data set were analyzed. Tumors having high expression of induced genes (yellow) and low expression of repressed genes (blue) were classified as “activated” (black box outline). These tumors were found to correlate with the

luminal B subtype (red) of ER-positive breast cancers. In contrast, tumors classified as “deactivated” correlated with the luminal A subtype (blue).

FIG. 3.

Enrichment of repressed targets within the E2/IGF-I co-regulated gene set. (A) Genes found to be induced (yellow) by E2 at both 3hr and 24hr or repressed (blue) by E2 at both time points were identified. Microarray data was then used to examine expression of these genes in response to IGF-I treatment followed by examination of overlap between E2 and IGF-I regulation of targets within the induced and repressed groups. (B) Probe sets within the co-regulated group of genes ($p < 0.05$; fold > 1.1 for induction, fold < 0.9 for repression) were analyzed at both 3hr and 24hr and divided into one of four groups—induced by both E2 and IGF-I, repressed by both ligands, induced by E2 but repressed by IGF-I, and repressed by E2 but induced by IGF-I. $\uparrow$ = induced, $\downarrow$ = repressed. (C) Quantitative real-time RT-PCR (qRT-

PCR) was used to calculate relative mRNA expression as ligand-mediated fold change compared to the untreated SFM control. MCF-7 cells were starved overnight in SFM and then treated with either E2 (10nM) or IGF-I (100ng/mL) for 3hr or 24hr. The data are an average of three replicates $\pm$ standard error of the mean (SEM).

FIG. 4.

Repression of target genes by E2 and IGF-I is independent at the receptor level but relies on common downstream kinases. (A and B) MCF-7 cells were starved overnight in SFM and then pre-treated for 30min with either the IGF-IR inhibitor BMS (1 μ M) or the anti-estrogen ICI (100nM). Following pre-treatment, cells were stimulated with E2 (10nM) or IGF-I (100ng/mL) for 3hr (A) or 24hr (B). RNA was isolated and qRT-PCR was performed. The data are an average of three replicates $\pm$ standard error of the mean (SEM). (C) MCF-7 cells were starved overnight in SFM and then pre-treated for 30min with either BMS (1 μ M), ICI (100nM), or the combination of BMS+ICI. Cells were then simultaneously treated with E2+IGF-I for 3hr. RNA was isolated and qRT-PCR was performed. The data are an average of three replicates $\pm$ standard error of the mean (SEM). (D) MCF-7 cells were maintained in SFM overnight and then pre-treated for 30min with either LY-294002 (20 μ M), U0126 (10 μ M), or Gö6983 (0.25 μ M). Following pre-treatment, cells were administered E2 (10nM), IGF-I (100ng/mL), or the combination of E2+IGF-I for 24hr. RNA was isolated and qRT-PCR was performed. The data are an average of three replicates $\pm$ standard error of the mean (SEM).

FIG. 5.

Estradiol and IGF-I independently repress BLNK, a regulator of MCF-7 cell growth. (A) MCF-7 cells were starved overnight in SFM, and then treated with E2 (10nM), IGF-I (100ng/mL), or the combination of E2+IGF-I for 48hr. Cells were harvested and lysed in 5% SDS. Western blot was performed using antibodies directed against BLNK and β -actin, which was used as a loading control. (B) MCF-7 cells were transiently transfected with BLNK siRNA (dashed lines) or non-specific control siRNA (solid lines), and then incubated in medium containing 5% charcoal-stripped serum (top graph) or 5% fetal bovine serum (FBS; bottom graph). Cell growth was assessed by MTS assay on days 0, 1, 2, and 4. The assay was performed with biological quintuplicates, and each point represents the average

value $\pm$ SEM. The very bottom panel represents a Western blot showing BLNK protein levels from MCF-7 cells that were cultured for 72hr in medium containing 5% FBS or 5% charcoal-stripped serum (CSS). (C) MCF-7 cells were transfected with the *BLNK* -0.8kb promoter and maintained in SFM or treated with E2 (10nM), IGF-I (100ng/mL), or E2+IGF-I for 24hr. Luciferase reporter assays were performed. The data represent relative luciferase units (RLU) and are an average of three replicates $\pm$ SEM. (D) ChIP assays for RNA polymerase II and IgG (negative control) in MCF-7 cells maintained in SFM or after treatment with E2 (10nM), IGF-I (100ng/mL), or E2+IGF-I for 1hr. Quantitative real-time PCR was performed with primers designed around the *BLNK* transcriptional start site. The data is represented as % input and is an average of three independent experiments $\pm$ SEM.

Table 1

Genes identified in microarray analysis, 3hr time point.

	E2-Regulated				IGF-I-Regulated				Co-Regulated			
	Gene		Fold		p-value		Gene		Fold		p-value	
	Gene		Fold		p-value		Gene		Fold		p-value	
Induced	PLAC1		7.873		0.000004		LRP8		5.484		0.002173	
	MYBL1		5.463		0.000034		HMGCS1		4.708		0.035979	
	PDLIM3		4.416		0.000046		CORO1A		4.588		0.000002	
	RET		4.374		0.005893		MVD		4.499		0.000000	
	IL17RB		3.956		0.000001		TUBB		4.392		0.000001	
	SGK		3.751		0.000043		SLC7A11		4.369		0.009523	
	UGT2B15		3.495		0.000019		MCM10		4.299		0.000000	
	ABHD2		3.364		0.000009		SCD		4.268		0.001216	
	PTGER3		3.237		0.009288		CDC6		4.208		0.000000	
	CXorf6		3.217		0.000086		HMGCS1		4.158		0.000131	
	CYP26B1		3.184		0.000448		PDLIM7		4.157		0.001252	
	CELSR2		3.132		0.000772		LRP8		3.923		0.000008	
	CALCR		3.083		0.000043		LDLR		3.883		0.000052	
	ABHD2		3.037		0.000690		SLC7A11		3.880		0.036210	
	SYBU		2.939		0.000215		SFN		3.760		0.000052	
	PGR		2.932		0.000068		PHLDA2		3.712		0.000011	
	CXCL12		2.904		0.002804		BCMSUNL		3.598		0.000000	
	CXCL12		2.892		0.025237		INSIG1		3.564		0.000231	
	RAB31		2.891		0.000000		EGR3		3.561		0.000007	
	RAPGEF1		2.882		0.000358		MVK		3.487		0.000031	
Repressed	TENS1		0.267		0.011009		STON1		0.068		0.000000	
	FAM110B		0.329		0.000000		KLHL24		0.094		0.000001	
	DRAM1		0.348		0.000772		N4BP2L1		0.109		0.000000	
	ANXA3		0.354		0.007054		BLNK		0.156		0.000000	
	TNFRSF11B		0.362		0.002349		HBP1		0.180		0.000000	

	E2-Regulated			IGF-I-Regulated			Co-Regulated			
	Gene	Fold	p-value	Gene	Fold	p-value	E2		IGF-I	
							Fold	p-value	Fold	p-value
	CMKOR1	0.365	0.009288	NR3C1	0.182	0.000009	0.433	0.030916	0.340	0.000627
	BLNK	0.374	0.002725	CITED2	0.184	0.000288	0.576	0.042359	0.235	0.000001
	ADFP	0.384	0.000026	CITED2	0.193	0.000001	0.384	0.000026	0.436	0.000016
	EFEMP1	0.385	0.005874	RIN2	0.204	0.000000	0.329	0.000000	0.502	0.000016
	NR3C1	0.390	0.012622	SEPP1	0.207	0.013234	0.472	0.001539	0.360	0.000006
	BMP7	0.392	0.000726	CALCOCO1	0.211	0.000910	0.447	0.000002	0.386	0.000000
	GALNT10	0.399	0.017414	NR3C1	0.214	0.000014	0.348	0.000772	0.489	0.002956
	NR3C1	0.402	0.010481	NR3C1	0.223	0.000010	0.420	0.040057	0.417	0.006073
	IER3	0.406	0.004042	HIST1H2BG	0.227	0.000016	0.365	0.009288	0.480	0.012503
	TCF7L1	0.408	0.000772	NR1D2	0.230	0.000043	0.429	0.003558	0.428	0.000391
	PLK2	0.413	0.000030	C6orf80	0.231	0.000000	0.438	0.000559	0.445	0.000074
	NR3C1	0.413	0.027953	M17S2	0.234	0.000012	0.466	0.001005	0.435	0.000048
	NBEA	0.420	0.040057	PIK3IP1	0.234	0.000000	0.504	0.000716	0.410	0.000005
	RPRM	0.420	0.029515	C5orf4	0.235	0.000104	0.462	0.030356	0.457	0.004319
	ELF5	0.429	0.003558	N4BP2L1	0.235	0.000001	0.696	0.025280	0.234	0.000000

Table 2

Genes identified in microarray analysis, 24hr time point.

	E2-Regulated			IGF-I-Regulated			Gene	Co-Regulated		
	Gene	Fold	p-value	Gene	Fold	p-value		E2	IGF-I	p-value
Induced	PLAC1	10.917	0.000001	SLC7A11	11.237	0.000633	PLAC1	10.917	0.000001	3.11
	SEMA3B	5.143	0.011892	SLC7A11	9.423	0.000329	SEMA3B	5.143	0.011892	3.613
	MYBL1	4.87	0.000073	CHAC1	7.612	0.000287	MYBL1	4.87	0.000073	3.183
	WISP2	4.293	0.01755	SLC7A1	5.862	0.009763	SLC7A5	2.432	0.001582	4.824
	IL17RB	3.99	0.000001	ATF3	5.642	0.001791	TNIK	2.356	0.037222	4.547
	PDLIM3	3.953	0.000096	CA4	5.518	0.000123	DHRS2	2.259	0.037534	3.811
	OLFML3	3.899	0.011892	ASNS	5.244	0.000712	CORO1A	2.2	0.007286	3.539
	CYP26B1	3.608	0.000111	C1orf24	5.227	0.009847	TNIK	2.29	0.009596	3.239
	MGP	3.419	0.000395	PDLIM7	5.209	0.000329	LAD1	2.37	0.044638	3.152
	SYBU	3.383	0.000047	C1orf24	5.017	0.018335	NRGN	2.239	0.021445	3.148
	HOXC4	3.34	0.006405	CEBPG	5.014	0.000008	HOMER2	2.085	0.002703	3.158
	UGT2B15	3.283	0.000035	SLC7A5	4.824	0.000001	AREG	2.35	0.000455	2.868
	ABHD2	3.208	0.000258	CLGN	4.788	0.002793	MYBL2	2.026	0.015641	3.153
	ABHD2	3.192	0.000017	TP73L	4.665	0.001329	EGR3	2.931	0.000243	2.174
	COL21A1	3.129	0.000086	DDIT3	4.607	0.001838	BOP1	1.792	0.049265	3.227
	SERPINA1	3.095	0.037747	PCK2	4.602	0.003066	MCM10	1.907	0.008851	3.105
	CXorf6	2.989	0.000143	VEGF	4.578	0.000033	C1orf135	1.634	0.019928	3.297
Repressed	ARSJ	2.981	0.028393	RASSF1	4.573	0.000005	FHL2	2.553	0.040267	2.258
	SGK	2.935	0.000286	TNIK	4.547	0.000092	SLC7A1	1.86	0.030323	2.879
	EGR3	2.931	0.000243	FILIP1L	4.4	0.000302	CDC6	2.001	0.00255	2.587
	CXCR4	0.224	0.015076	STON1	0.119	0.000001	CXCR4	0.224	0.015076	0.207
	NELL2	0.231	0.000334	SEPP1	0.121	0.001436	STON1	0.324	0.004824	0.119
	CXCR4	0.257	0.035768	N4BP2L1	0.13	0.000001	CXCR4	0.257	0.035768	0.258
	BLNK	0.273	0.000111	PTGER4	0.152	0.000006	CXCR4	0.281	0.020681	0.239
	CXCR4	0.281	0.020681	CAV1	0.168	0.000004	BLNK	0.273	0.000111	0.305

	E2-Regulated				IGF-I-Regulated				Gene	Co-Regulated			
	Gene	Fold	p-value	p-value	Gene	Fold	p-value	p-value		E2		IGF-I	
										Fold	p-value	Fold	p-value
	NR3C1	0.289	0.000433		FMO5	0.173	0.000005		HIST1H2AE	0.42	0.022263	0.23	0.000057
	FAM110B	0.29	0		KLHL24	0.194	0.000067		C18orf1	0.357	0.005056	0.312	0.00031
	NR3C1	0.298	0.001767		ELOVL2	0.196	0.002268		MATN2	0.437	0.012476	0.248	0.000021
	NR3C1	0.302	0.001184		N4BP2L1	0.204	0		ADFP	0.371	0.000017	0.332	0.000001
	SASH1	0.315	0.015225		CXCR4	0.207	0.001976		PLK2	0.317	0.000001	0.416	0.000005
	PLK2	0.317	0.000001		CAV1	0.211	0.000005		HBPI	0.514	0.001339	0.222	0
	CTNND2	0.318	0.019756		HBPI	0.222	0		RHOBTB1	0.366	0.001257	0.37	0.000234
	STON1	0.324	0.004824		HIST1H2AE	0.23	0.000057		NELL2	0.231	0.000334	0.516	0.027157
	KIAA0922	0.33	0.000181		MAFF	0.234	0.000147		HIST1H2AM	0.409	0.009016	0.347	0.000396
	TNFRSF11B	0.334	0.000679		CXCR4	0.239	0.001851		PMP22	0.352	0	0.418	0
	EFNB2	0.337	0.002577		RLN2	0.244	0		N4BP2L1	0.58	0.028945	0.204	0
	NR3C1	0.34	0.004697		MATN2	0.248	0.000021		NR3C1	0.298	0.001767	0.49	0.015744
	SASH1	0.34	0.013216		GALC	0.248	0.003528		HIST1H2BG	0.42	0.012101	0.392	0.001261
	PMP22	0.352	0		CXCR4	0.258	0.00864		KIAA0922	0.33	0.000181	0.505	0.002264
	LIMA1	0.354	0.002316		NPY5R	0.273	0		NR3C1	0.34	0.004697	0.501	0.01804