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ERE-independent ERα target genes differentially expressed in human breast tumors

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Abstract

The classical pathway for estrogen receptor (ER) signaling is mediated by ER binding to an estrogen response element (ERE) in DNA. ERα can also act via a nonclassical pathway by altering the activities of other transcription factors (e.g., Sp1, AP-1, or NF-κB) at their cognate sites on DNA. We previously generated a mutant form of ERα (E207A/G208A) that does not bind to EREs, and therefore lacks signaling via the classical pathway but retains signaling via the nonclassical pathway. In the current study, we introduce this mutant ERα into MDA-MB231 ERα-negative breast carcinoma cells to identify nonclassical pathway genes that respond to 17β-estradiol (E2), selective estrogen receptor modulators (SERMs) tamoxifen (TAM) or raloxifene (RAL), or the estrogen antagonist ICI 182,780 (ICI). Consistent with a role for nonclassical signaling in SERM action, microarray analyses identify 268 responsive nonclassical ERα pathway target genes. ICI elicits the largest number of nonclassical genes, followed by RAL, TAM, and E2. Custom microarrays containing identified nonclassical ERα responsive genes are used to compare gene expression in human breast tumor ($n = 34$) and normal mammary epithelial cell ($n = 9$) samples. A subset of nonclassical genes ($n = 32$) are differentially expressed in breast tumors. In summary, we show that nonclassical ERα pathway target genes exhibit a range of transcriptional responses to SERMs and identify targets of this pathway as potentially relevant to breast cancer. The identification of nonclassical ERα target genes offers new insight into estrogen receptor signaling and cross talk with pathways that mediate breast tumor response to SERM therapy.
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Keywords: Estrogen receptor alpha; SERM; Nonclassical; Microarray (human breast tumor)

1. Introduction

Estrogen receptor alpha (ERα), after binding its ligand, alters the transcription of target genes. This ‘genomic’ signaling can be further divided into classical and nonclassical pathways. In classical signaling, ERα binds to estrogen response elements (ERE) in target gene promoters and recruits cofactors to induce or repress transcription. In nonclassical signaling, ERα alters the transcription of genes without binding directly to an ERE. Rather, ERα alters the activities of other transcription factors such as AP-1, Sp1, or NF-κB. Examples of genes known to be regulated through this nonclassical ERα pathway include Hsp27 (Porter et al., 1997), RANTES (Kanda and Watanabe, 2003), IGF-1 (Umayahara et al., 1994), VCAM-1 (Simoncini et al., 2000), c-Myc (Dubik and Shiu, 1992), and EGFR (Salvatori et al., 2003). Mechanisms of nonclassical ERα regulation can vary for different interacting transcription factors. For example, ERα repression of IL-6 transcription depends on its ability to disrupt NF-κB and C/EBPβ interactions with DNA (Stein and Yang, 1995; Ray et al., 1997). In contrast, ERα has been shown to bind c-Jun and JunB, stabilize complex interactions with coactivator GRIP1 (Teyssier et al., 2001), and to alter AP-1 regulation of the collagenase promoter (Webb et al., 1995). Furthermore, regulation can be cell (Cerillo et al., 1998) and ligand specific (Webb et al., 1999, 2003; Kushner et al., 2000; Jakacka et al., 2001), perhaps suggesting a role for this pathway in conveying a tissue selective SERM response.

Patients with breast cancers that express estrogen receptor have a better prognosis and respond better to treatment with anti-estrogens, including Tamoxifen (O’Regan and Jordan, 2002).

Abbreviations: aRNA, anti-sense RNA; E2, 17β-estradiol; ER, estrogen receptor; cRNA, cloned RNA; ERE, estrogen response element; ICI, ICI 182,780; IVT, In vitro transcription; MEC, mammary epithelial cell; NF-κB nuclear factor kappa B; RAL, raloxifene; SERM, selective estrogen receptor modulator; TAM, tamoxifen

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In addition, there is evidence that nonclassical ERα-interacting factors, such as AP-1 and NF-κB, play a role in the pathogenesis of breast cancer. Over-expression of the epidermal growth factor receptor (EGFR), known to signal through AP-1 to stimulate proliferation, is associated with aggressive tumor behavior and poor prognosis (Klijn et al., 1992). Additionally, levels of AP-1 transcription factor complex member fra-1 (Philips et al., 1998) correlate negatively with breast tumor ER status, response to treatment (Bamberger et al., 1999) and have been shown to alter tamoxifen action (Webb et al., 1995). Blockade of AP-1 with dominant negative c-Jun has been shown to inhibit growth of MCF-7 breast cancer cells in nude mice (Liu et al., 2002). Similarly, inhibition of NF-κB reduces metastatic potential in a murine mammary carcinoma model (Huber et al., 2004) and restores tamoxifen sensitivity to a cellular model of tamoxifen resistant, hormone-independent breast cancer (deGraffenried et al., 2004).

Studies pairing microarrays with cellular model systems have been used successfully to distinguish ERα from ERβ selective target genes and to determine temporal differences in gene expression in response to estrogen (Stossi et al., 2004). A recent array study using a dominant negative cJun identified 20 new AP-1 target genes regulated in response to estrogen (DeNardo et al., 2005). In the current study, we introduce a mutant ERα with ablated classical but intact nonclassical ERα signaling to selectively restore only nonclassical ERα signaling to an ERα negative breast epithelial cell line and screen for SERM responsive target genes. We further examine this nonclassical ERα target gene set for relevance to breast cancer by profiling their expression patterns in normal and tumor breast specimens.

2. Materials and methods

2.1. Construction of adenovirus, infection, and treatment of cells

The murine ERα receptor harboring two mutations (E207A/G208A, designated ER^{AA}) in the P-box of the first zinc finger of the DNA-binding domain was introduced into MDA-MB231 ERα-negative breast carcinoma cells using a recombinant adenovirus. The adenoviral vector (Ad-ER^{AA}) carries the ER^{AA} mutant driven by the cytomegalovirus (CMV) promoter. Ad-Empty carries no cDNA and was used as a negative control (Fig. 1). Ad-Gal (Lee et al., 1999) carrying β-galactosidase driven by the CMV promoter was used to test transduction efficiency. β-Galactosidase expression was detected in 95–100% of cells at 24–48 h after infection with Ad-Gal at a multiplicity of infection (MOI) of 7.5 plaque-forming units (PFU) per cell (data not shown). Therefore, subsequent experiments were performed using this amount (7.5 PFU/cell) of Ad-ER^{AA}.

MDA-MB231 ERα-negative breast carcinoma cells were cultured in charcoal-stripped serum for 3 days before being plated at 1.3×10^6 cells/10 cm plate. Cells were infected with either Ad ER^{AA} or Ad-empty vector and incubated for 8 h before changing the media. Four hours later, cells were treated with either E2 (1 nM), TAM (100 nM), ICI 182,780 (100 nM), RAL (1 μM) or ethanol vehicle control. Cells were incubated with ligand or vehicle for 12 h (40–60% confluent). RNA was extracted using Trizol reagent (Life Technologies), followed by DNase treatment, phenol/chloroform extraction and ethanol precipitation.

2.2. High density array screen

Sample RNA was labeled for hybridization to Affymetrix chips according to the manufacturer's "Eukaryotic Target Preparation" protocol. Briefly, double-stranded cDNA was synthesized from 10 μg of total RNA. In vitro transcription (IVT) was performed using T7 enzyme and biotin-labeled nucleotides. Twenty micrograms of purified cloned RNA (cRNA) was fragmented and then hybridized to Human Genome U95 Affymetrix GeneChip Arrays according to the manufacturer's protocol. Data were filtered to identify a SERM responsive gene set, defined as genes whose expression changed by at least 2.3-fold in response to any treatment versus vehicle control, but not with empty virus control in two independent replicate experiments. Available cDNA clones (Invitrogen) for 255 of the 268 identified genes were printed in triplicate on low density glass microarray slides along with GFP cDNA for positive and Arabidopsis cDNA for negative hybridization controls.

2.3. Breast tumor sample preparation and hybridization

All studies involving human samples were approved by the Northwestern University Institutional Review Board. High quality sample RNA was isolated from normal and tumor breast tissues as previously described (Ariazi et al., 2002). Briefly, frozen pulverized tumor specimens were obtained from the National Cancer Tissue Resource SPORE at Baylor College of Medicine (Houston, TX). Normal mammary epithelial cells (MECs) were obtained from reduction mammoplasties and processed to enrich for epithelial cells (Ethier, 1996). To avoid altering their transcriptional profiles, MECs were not expanded

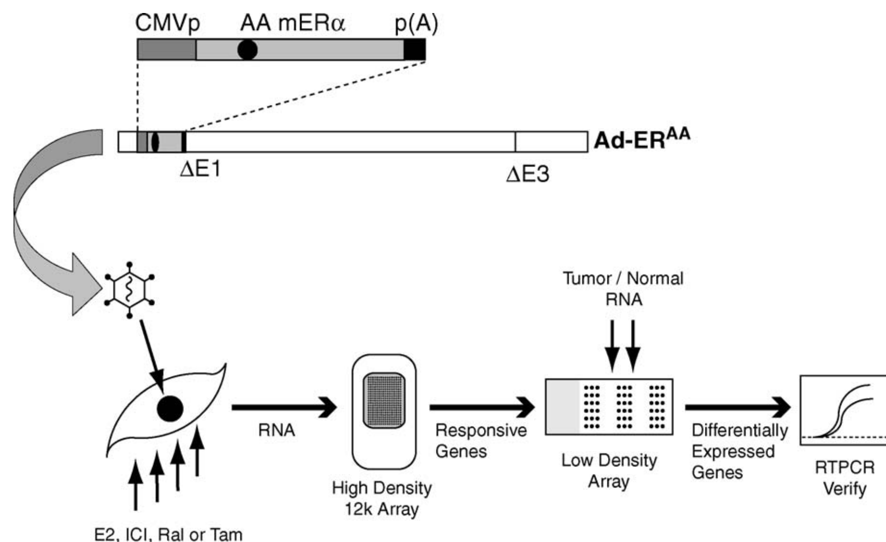

Fig. 1. Adenoviral constructs and experimental approach.

in culture. Since RNA from these human samples was limited, and microarrays required more than was available, 1 μg of total RNA from each normal and tumor breast sample was subjected to one round of T7 enzyme-based linear amplification using the MessengerAmp aRNA kit (Ambion). Appropriate size distribution of cRNA was confirmed using the RNA 6000 Nano Labchip Kit (Agilent Technologies). A normal reference cRNA pool was prepared by mixing equal parts of each normal MEC cRNA. GFP antisense RNA (aRNA) was prepared by IVT (Megascript kit, Ambion) using RNA Polymerase SP6 and linearized pGEM-3Z vector containing GFP sequence. Hybridization probes were prepared using aminoallyl dUTP (Ambion) incorporation during cDNA synthesis and subsequent coupling to Cy dyes (Amersham Biosciences) of 8 μg of individual experimental (normal or tumor) cRNA, or an equal amount of the normal reference pool, spiked with 100 ng of GFP aRNA internal control. Labeled probes (Cy3 experimental and Cy5 reference) were column purified, mixed, precipitated, and resuspended in hybridization buffer (25% formamide, 5× SSC, 0.1% SDS, 0.5 μg/μl Poly dA, 0.2 μg/μl BSA, 1 μg/μl denatured salmon sperm DNA). Probes were boiled 4 min, then applied to denatured, prehybridized glass microarrays under lifter cover slips (Erie Scientific Company) and incubated 16 h at 42 °C in hybridization chambers with controlled humidity. Arrays were washed 1 × 4 min in 2× SSC, 0.1% SDS at 50 °C, followed by 1 × 4 min in 2× SSC, 0.1% SDS, and 2 × 4 min in 0.2× SSC at room temperature with gentle shaking.

2.4. Data collection and analysis

Due to intrinsic differences between Cy5 and Cy3 signal strengths, data were normalized by altering the detection laser power to adjust the ratio of GFP internal hybridization control Cy5/Cy3 to 1.0 (Scan Array Express Microarray Analysis System, Perkin-Elmer Life Science). Quality assessment of array spot morphology and template alignment was performed. Data were further normalized such that the median of all GFP spots equaled 1.0. Ratio data for triplicate spots were averaged, log transformed and analyzed by unsupervised hierarchical clustering using the Cluster and Tree software programs (http://www.microarrays.org/software.html) with Spearman Rank correlation (Eisen et al., 1998). Expression data were tested against previously determined sample characteristics (Ariazi et al., 2002) for correlation using Multivariate analysis by Spearman Rank.

2.5. Data confirmation

Correlation of expression data nodes with tumor status obtained by unsupervised hierarchical clustering was confirmed using multivariate analysis by Spearman Rank correlation in the SPSS statistical analysis package. Microarray expression data were confirmed using semi-quantitative RT-PCR (IQ SYBR Green Super Mix, iCycler Instrument, BioRad) on normal and tumor pools.

3. Results

3.1. Identification of estrogen receptor α nonclassical pathway target genes

Using the nonclassical ERα signaling cellular model system (Fig. 1) and gene expression profiling, we identified 268 nonclassical ERα target genes (Supplementary Table 1). In response to E2 or ICI, a larger number of genes were stimulated than suppressed (ratio of approximately 60:40) (Fig. 2A). In contrast, genes that responded to tamoxifen or raloxifene were overwhelmingly suppressed. Treatment of cells with estradiol elicited the smallest group of responsive genes ($n = 29$), whereas ICI elicited the largest group of responsive genes ($n = 195$). Raloxifene and tamoxifen elicited responses from intermediate numbers of genes ($n = 100$ and 46, respectively). Note that the sum ($n = 370$) exceeds the total of 268 identified target genes as many genes respond to more than one treatment.

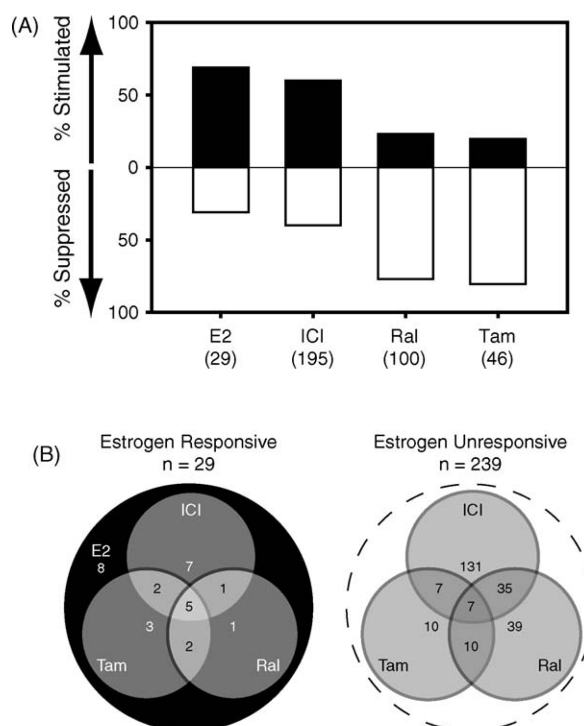

Fig. 2. Identification of nonclassical ERα target genes. (A) Distribution and directionality of responsive genes identified in the Affymetrix high density screen. Numbers of responsive genes for each treatment are indicated in parentheses. (B) Overlapping ligand responses of nonclassical ERα regulated genes. Genes are segregated into estrogen-responsive (left panel, $n = 29$) and estrogen-unresponsive (right panel, $n = 239$). Overlapping areas represent genes that respond to more than one ligand.

Coordinate regulation of responsive genes is illustrated in Fig. 2B. For clarity, responsive genes are separated into two groups: estrogen responsive (Fig. 2B, left) and estrogen unresponsive (Fig. 2B, right). The sum of genes regulated by multiple treatments is displayed in the overlapping areas. Only 11% ($n = 29$) of identified genes regulated through the nonclassical ERα pathway were responsive to E2. Most genes identified were not responsive to E2 ($n = 239$) and either responded to ICI ($n = 131$) or raloxifene ($n = 39$), or similarly to both ICI and raloxifene ($n = 35$).

3.2. Nonclassical ERα target genes with altered expression in breast tumors

Identification of these 268 gene targets provides an opportunity to examine the potential relevance of nonclassical ERα signaling in breast cancer pathogenesis and treatment. A custom cDNA microarray was created using available clones (255/268) for the identified nonclassical ERα pathway genes and used to determine expression profiles in a previously characterized set of 34 breast tumors and 9 samples of purified normal mammary epithelial cells (Ariazi et al., 2002). Unsupervised hierarchical clustering revealed a clear partitioning of the normal and tumor samples (Fig. 3, *x*-axis) and identified statistically correlated gene clusters, including genes up and down regulated in tumors (Fig. 3, *y*-axis). Correlation of expression data for genes up-

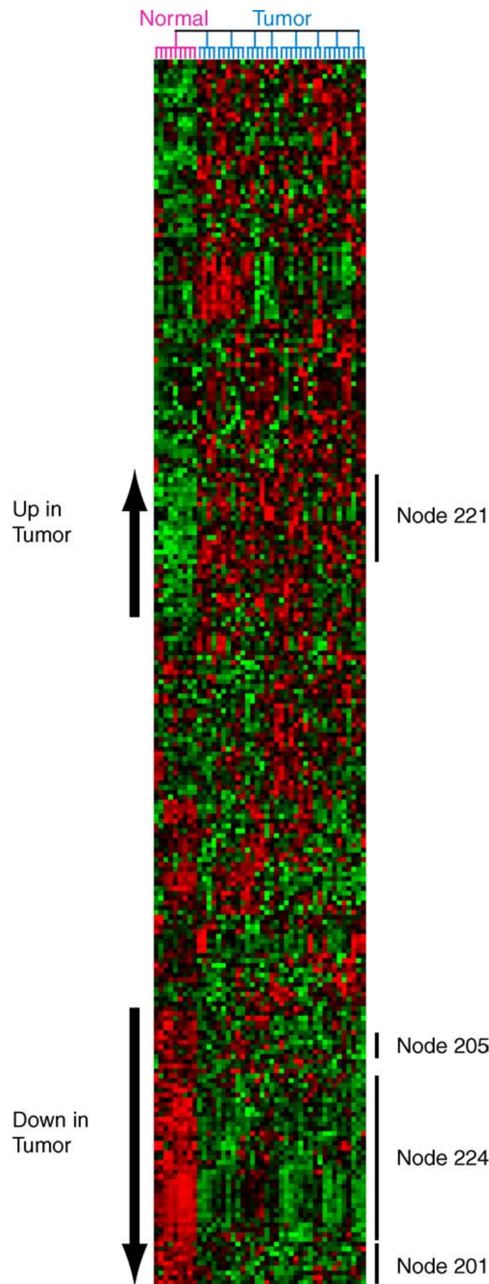

Fig. 3. Clustering of nonclassical ERα target genes. Expression profiles of nonclassical ERα target genes were determined in normal and tumor breast tissue samples. Data were subjected to unsupervised hierarchical clustering. In this figure, red denotes genes up-regulated and green denotes genes down-regulated as compared to the median expression level across all samples against normal reference pool. Note the clear partitioning of normal from tumor samples (top) based on specific clusters of genes (nodes). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

regulated in tumors (node 221) with normal or tumor status did not achieve statistical significance and was not explored further. Expression data of genes down regulated in tumors (nodes 205, 224, and 201) was more consistent and significantly correlated with normal or tumor status. Genes that showed a minimum 2.5-fold response against the normal reference pool at a frequency of at least 50% of tumors were selected for final analysis. Among all known sample parameters tested, down-regulated genes show negative correlations to tumor ERα, and ERRγ indicating genes are down regulated when ERα or ERRγ levels are high. Conversely, down-regulated genes show positive correlation with EGFR and MDM2 copy numbers indicating genes are down when EGFR or MDM2 are suppressed (Supplementary Table 2). Expression data for these 32 genes, mainly derived from node 224, are summarized in Table 1. As an example, the median expression of interleukin 8 is −44.8-fold with a decrease of more than 2.5-fold reported in all 35 tumor specimens when compared to the normal reference pool.

3.3. Ligand response patterns for nonclassical ERα target genes differentially expressed in breast tumors

To gain insight into how SERMs signal through the nonclassical ERα pathway in breast cancer, target genes identified as differentially expressed in breast tumors were compared using ligand response data from the original high density screen. E2 responsive genes were selected for further analysis. These 14 genes were sorted by E2 fold response and then grouped by their SERM response, with cut off 1.7-fold (Table 2). The first group was coordinately regulated by both E2 and tamoxifen. The second group was stimulated by estrogen but suppressed by both tamoxifen and raloxifene. These findings underscore the differential regulation of nonclassical pathway target genes by different ER ligands.

4. Discussion

In this study, we identified 268 genes regulated by the nonclassical ERα pathway in response to E2, ICI, or the SERMs TAM and RAL. In addition, we find that a nonclassical SERM response can occur in the absence of estradiol, supporting the hypothesis that SERMs affect gene transcription through the nonclassical ERα pathway by mechanisms distinct from competitive ligand binding. It is notable that a large number (131/268) of genes regulated through the nonclassical ERα pathway in our high density screen are stimulated by ICI but unresponsive to E2, TAM, or RAL (Fig. 2B). The larger number of ICI-responsive genes, as compared to E2, was unexpected and suggests a global mechanism such as the AF-independent model. In this model, an ERα-ICI complex is proposed to sequester transcriptional repressors away from AP-1 elements to allow for unopposed transcription (Kushner et al., 2000; Uht et al., 2004). Alternatively, ICI can lead to accumulation of ER in the cytoplasm and accelerated receptor degradation (Hermenegildo and Cano, 2000). One caveat to the interpretation of our results is that the ERα negative MDA-MB231 breast carcinoma cell line expresses low levels of ERβ (Vladusic et al., 2000). Therefore, genes identified in our screen may be regulated by nonclassical ERα and ERβ receptor heterodimers.

Expression analysis of breast tumor samples identified 32 nonclassical ERα target genes. We have previously shown that the tumors in this set of samples have higher ERα, ERRγ, and lower EGFR than normal specimens (Ariazi et al., 2002). Since the expression of ERα and EGFR biomarkers have been asso-

Table 1
Breast tumor associated nonclassical ERα target genes

Gene name	Accession no.	Tumor		Normal	
		Fold	Frequency	Fold	Frequency
Beta-thromboglobulin-like protein	M17017	−53.0	35/35	1.0	0/9
Interleukin 8 (IL8)	M28130	−44.8	35/35	1.0	1/9
Chemokine exodus-1	U64197	−43.5	35/35	−1.0	2/9
IFN-beta 2a	X04430	−41.6	35/35	−1.0	1/9
Cytokine (GRO-beta)	M36820	−39.7	35/35	1.0	0/9
Cytokine (GRO-gamma)	M36821	−31.3	35/35	1.0	2/9
Arg-Serpin (plasminogen activator-inhibitor 2, PAI-2)	Y00630	−24.1	35/35	1.0	2/9
Stromelysin	X05232	−21.5	35/35	−1.2	1/9
Prointerleukin 1 beta	X04500	−13.4	35/35	−1.2	2/9
Granulocyte colony-stimulating factor (G-CSF)	X03656	−9.2	35/35	−2.4	3/9
MAD-3 mRNA encoding IkB-like activity	M69043	−8.4	35/35	−1.0	0/9
Tumor necrosis factor (TNF-alpha)	X02910	−8.0	35/35	−1.5	1/9
MDA-7 (mda-7)	U16261	−7.7	35/35	−2.7	5/9
Gravin	U81607	−6.4	34/35	−1.9	3/9
Bone morphogenetic protein 2A (BMP-2A)	M22489	−5.8	34/35	−1.8	2/9
Heme oxygenase 1 (HO-1)	Z82244	−5.8	33/35	−1.3	0/9
Erythroblastosis virus oncogene homolog 2 (ets-2)	J04102	−5.7	35/35	−1.1	0/9
Dioxin-inducible cytochrome P450 (CYP1B1)	U03688	−5.4	34/35	−1.4	2/9
Placental protein 5 (PP5)	D29992	−5.1	33/35	−1.7	3/9
Cellular oncogene c-fos	V01512	−4.7	29/35	−1.1	0/9
Bcl-2 related (Bfl-1)	U27467	−4.0	30/35	−1.5	1/9
HIV-1, Nef-associated factor 1 beta (Naf1 beta)	AJ011896	−3.7	34/35	−1.2	0/9
Tumour suppressor protein, HUGL[a]	X86371	−3.6	34/35	−1.5	2/9
Helix–loop–helix basic phosphoprotein (G0S8)	L13463	−3.6	25/35	−1.3	1/9
Decidual protein induced by progesterone, DEPP	AB022718	−3.6	29/35	−1.5	1/9
Pim-1 oncogene	M16750	−3.4	28/35	−1.5	0/9
Follistatin	M19481	−3.3	28/35	−1.8	2/9
c-jun proto oncogene (JUN), clone hCJ-1	J04111	−3.3	25/35	−1.2	0/9
Synaptojanin 2B	AF039945	−3.1	24/35	−1.4	0/9
Lysophosphatidic acid receptor homolog	U80811	−2.6	21/35	−1.3	0/9
Platelet-derived growth factor PDGF-A	X06374	−2.6	23/35	−1.4	0/9
TRAF-interacting protein I-TRAF mRNA	U59863	−2.6	18/35	1.0	0/9

Table includes genes whose expression data statistically confirmed to associate with tumor or normal status and are differentially expressed by at least 2.5-fold in at least 50% of tumors. Genes appear sorted by median fold tumor against normal reference pool. Frequency denotes the number of individual tumor or normal samples found differentially expressed against the normal reference pool.

[a] HUGL data were not confirmed because RT-PCR was unsuccessful with multiple different primers.

ciated with clinical outcome (Klijn et al., 1992), it is possible that these newly identified nonclassical pathway genes may provide additional markers for the identification of high risk ERα positive or ERα negative breast tumors (Zhou et al., 2005a).

Few (11%) of the identified nonclassical ERα target genes are responsive to E2 but these E2-responsive genes are somewhat enriched (22%) among those differentially expressed in breast tumors. Among these 14 genes, we observe that tamoxifen regulates one group similarly to estrogen, whereas it regulates another group discordantly relative to estrogen. Thus, mechanism of SERM action varies in a gene-specific manner.

Many of the identified nonclassical pathway genes encode proteins with known roles in inflammation, such as interleukin 8 and pro-interleukin 1 beta. Further, many either affect NF-κB activation or are themselves NF-κB target genes. For example, MDA-7 is known to potentiate TNF stimulation of NF-κB transcription and inhibit TNF-mediated apoptosis (Aggarwal et al., 2004). G-CSF is induced in response to NF-κB activation (Bar-Yehuda et al., 2002). Heme-oxygenase 1 (Lavrovsky et al., 1994) and IL-8 (Bobrovnikova-Marjon et al., 2004) induction are regulated by NF-κB and AP-1 sites in their proximal promoters, and Pim-1 kinase is induced by NF-κB activation through CD40 receptor signaling (Zhu et al., 2002). The overlap between nonclassical ERα and NF-κB target genes is perhaps not surprising given the growing evidence of ERα/NF-κB reciprocal antagonism or cross talk (Evans et al., 2001). Described mechanisms of ERα repression of NF-κB transcription include a competition for cofactors, disruption of NF-κB binding to DNA, and a stabilization of IκB and sequestration of NF-κB in the cytoplasm (McKay and Cidlowski, 1999). We observed estrogen dependent induction of the MAD-3 gene encoding IκB activity. It is possible that the transcriptional activation of this inhibitor constitutes an additional mechanism of the nonclassical ERα mediated suppression of NF-κB transcription.

Cross talk between ERα and NF-κB has been proposed to mediate some of the preventative effects of estrogen against cardiovascular disease (Evans et al., 2001) and osteoporosis (Galien and Garcia, 1997). A similar role for nonclassical ERα suppres-

Table 2
SERM response patterns for a group of breast tumor associated nonclassical pathway target genes

	E2	RAL	TAM	ICI
Genes regulated coordinately by E2 and TAM				
IFN-beta 2a	-2.8	-2.1	-4.3	3.1
Interleukin 8	-3.8	-1.3	-3.8	4.0
Prointerleukin 1 beta	-2.2	-1.6	-3.8	1.9
MDA-7 (mda-7)	-2.7	1.2	-2.7	11.5
Beta-thromboglobulin-like protein	-2.1	-0.1	-2.2	3.8
Chemokine exodus-1	-1.8	0.0	-2.1	4.5
Plasminogen activator-inhibitor 2	-2.9	2.3	-1.8	5.5
Follistatin	-1.8	-1.3	-1.6	2.4
Cytokine GRO-gamma	-1.7	1.2	-1.6	9.0
Pim-1 oncogene	1.9	1.4	1.4	2.7
Genes regulated conversely by E2 and TAM				
Granulocyte colony-stimulating factor	2.8	-5.3	-3.6	-1.9
Decidual protein induced by progesterone	3.5	-4.7	-3.1	-4.8
MAD-3 mRNA encoding IkB-like activity	2.2	-2.5	-2.0	-1.6
Heme Oxygenase 1	4.4	-4.4	-1.7	-3.3

Data from the high density array screen was used to identify estrogen responsive genes among the identified tumor associated nonclassical ERα target genes (1.7-fold cut off). These genes are grouped based on whether estrogen (E2) and tamoxifen (TAM) regulate them coordinately or discordantly.

sion of NF-κB transcription in SERM signaling may be relevant for the treatment of breast cancer. Activation NF-κB is known to play a role in the promotion of epithelial–mesenchymal transition (Huber et al., 2004), metastasis (Huber et al., 2004), progression to hormone-independent growth (Nakshatri et al., 1997) and hormone resistance, (Zhou et al., 2005b; deGraffenried et al., 2004) in breast tumor cells. SERM ligands, such as WAY-169916, that specifically target the NF-κB pathway will likely modulate some of the nonclassical ERα pathway genes (Chadwick et al., 2005). The use of such ligands may avoid the uterotropic effects associated with the tamoxifen treatment of breast cancer.

In conclusion, we report 268 genes regulated by the nonclassical ERα pathway in response to treatment with E2, ICI, and the SERMs tamoxifen and raloxifene. We demonstrate that SERMs can signal through the nonclassical ERα pathway in the absence of estradiol and that a subset of target genes ($n = 32$) are differentially expressed in breast cancers. These genes are enriched for estrogen responsive genes which exhibit distinct patterns of transcriptional regulation in response to estradiol, raloxifene, tamoxifen, or the estrogen antagonist ICI. Furthermore, many of these genes are known NF-κB or AP-1 targets, implicating nonclassical ERα cross talk with these transcription factors as important to breast cancer. Identification of the nonclassical ERα pathway adds a new dimension to the study of estrogen and SERM action and identifies potential target genes that may be involved in the pathogenesis and treatment of breast cancer.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mce.2005.10.003.

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