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## Differential Estradiol and Selective Estrogen Receptor Modulator (SERM) Regulation of *Keratin 13* Gene Expression and its Underlying Mechanism in Breast Cancer Cells

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### Abstract

Expression of the *Keratin 13* (*KRT13*) gene, which encodes a cytoskeletal protein thought to play important roles in breast cancer growth and metastasis, is differentially regulated by estradiol (E2) and the selective estrogen receptor modulators (SERMs) tamoxifen and raloxifene. While stimulation of *KRT13* by tamoxifen is robust and prolonged, stimulation by E2 is more transient and raloxifene has virtually no effect. To investigate the mechanistic basis for the differential ligand regulation of *KRT13*, we have defined the regulatory regions of *KRT13*, compared gene expression by E2 and SERMs, and explored the magnitudes and time courses of estrogen receptor (ER) and cofactor recruitment patterns on these regions. Using a ChIP scanning approach and reporter transactivation assays, we identified a 2.5 kb upstream ER-binding regulatory region for *KRT13*. Directed composite mutations in this region revealed that three estrogen response elements and three Sp1 sites were involved in its ligand dependent regulation. Differential recruitment of ER $\alpha$  and cofactors to the *KRT13* regulatory sites paralleled the different time course and magnitude of regulation by these ligands: There was almost no ER $\alpha$  or cofactor recruitment with raloxifene, whereas there was strong, prolonged ER recruitment and histone acetylation with tamoxifen, and an early and more transient recruitment with E2. Taken together, our results suggest that the different ligand regulations of *KRT13* are due to ligand-differential recruitment of ER and coactivators, and they provide insight into the mechanisms responsible for the different agonistic activities and differential gene regulation by estradiol and the SERMs tamoxifen and raloxifene.

### Keywords

estrogen receptor; estradiol; tamoxifen; SERM; gene expression; breast cancer

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## Introduction

Estrogens are critical for the development and maintenance of reproductive tissues and have important roles in additional tissues such as bone and cardiovascular, and in nervous system function (Deroo and Korach, 2006). These actions are mediated through estrogen receptors alpha and beta ( $ER\alpha$  and  $ER\beta$ , respectively), which are members of the nuclear receptor superfamily. By binding to cognate ligands or pharmacologic agents such as the selective estrogen receptor modulators (SERMs) tamoxifen and raloxifene, the ER undergoes conformational changes and is able to display protein surfaces which allow the receptor to bind to DNA regulatory sequences in chromatin directly or indirectly through protein intermediaries. Direct ER binding sites include palindromic estrogen response elements (EREs), non-consensus EREs and ERE half sites. When binding to chromatin indirectly, ER regulates gene expression by tethering to other transcription factors, such as AP-1 and Sp1.

Depending on the properties of ligands, availability of coregulators in a given cell, and chromatin contexts, the ER is able to recruit different complexes of coregulators to either stimulate or repress the expression of target genes (Brzozowski et al., 1997, Kumar and Chambon, 1988, Lonard et al., 2007, Murdoch and Gorski, 1991). In the context of breast cancers, estrogens have long been known to promote tumor cell growth, presumably through regulating certain growth-promoting gene subsets such as stimulatory cell cycle-related genes (Frasor et al., 2003, Katzenellenbogen and Frasor, 2004). The SERM tamoxifen is widely utilized in breast cancer therapies (Smith and Good, 2003, Strassmer-Weippl and Goss, 2003), and exerts its effects by binding ER and differentially regulating target genes. By genome-wide microarray gene transcriptional profiling, Frasor *et al.* (Frasor et al., 2003, Frasor et al., 2004, Frasor et al., 2006) identified genes that were differentially regulated by E2 and SERMs. In most cases, genes were up-regulated or down-regulated by E2, with this regulation being reversed by the SERMs tamoxifen and raloxifene and the antiestrogen ICI 182, 780. However, some genes such as keratin 13 (KRT13) were highly stimulated by the SERM tamoxifen.

Keratins comprise the intermediate filament cytoplasmic cytoskeleton and are the major structural proteins of epithelial cells. Intermediate filaments are made up of heterodimeric polymers composed of both type I and type II keratins which are encoded by more than 50 genes in separate clusters on chromosomes 12 and 17 (Hesse et al., 2001). The specific structural or regulatory functions required for keratin filaments to act in a tissue-specific manner are likely to result from the directed transcriptional regulation of distinct keratin genes and the properties of the proteins they encode (Magin et al., 2007). Keratin 13, a type I keratin (KRT10-KRT-20), often pairs with KRT4, a type II keratin (KRT1-9), and both are expressed in suprabasal layers of non-cornified stratified squamous epithelium (e.g. mucosa). Keratin 13 expression is known to be regulated by calcium and nuclear receptor ligands such as retinoids and  $1\alpha,25$ -dihydroxyvitamin D3, but little is known about KRT13 regulation by estrogens or SERMs (Waseem et al., 1998). Olson et al. showed that keratin 13 was up-regulated in human luminal epithelial cells of secretory phase endometrium, suggesting that KRT13 might play a role in preparation for the implantation process (Olson et al., 2002).

In this study, we have characterized the differential regulation of *KRT13* gene expression by E<sub>2</sub> and the SERMs tamoxifen and raloxifene in breast cancer cells. We have identified the estrogen-responsive regulatory regions of *KRT13* by ChIP assay, and studied the recruitment of  $ER\alpha$  and the cofactors p300, SRC-2, and SRC-3 and the acetylation of histones on the *KRT13* regulatory regions in response to the different ER ligands over time. Also, to identify specific sites involved in *KRT13* gene regulation, we made an extensive series of deletions and mutations in the regulatory region of *KRT13* and tested their individual and combined responses to ER ligands. Our studies highlight marked differences between tamoxifen and raloxifene and

indicate that the differential regulation of this gene by different ER-ligand complexes is associated with differential recruitment of ER and coactivators and changes in histone acetylation.

## Materials and Methods

### Cell Culture and RNAi Studies

MCF-7 cells were maintained in Minimum Essential Medium (MEM) plus phenol red supplemented with 5% calf serum in a 5% CO<sub>2</sub> incubator. Cells were then cultured for 3 days in MEM (no phenol red) supplemented with 5% calf serum.  $2.5 \times 10^5$  cells/35-mm-well were plated in MEM plus 5% charcoal-dextran-treated calf serum (CDCS) 1 day before siRNA transfection. Transient transfections were performed using 100 pmol siRNA and 10  $\mu$ l Lipofectamine 2000 reagent (Invitrogen Corp.). The cells were incubated at 37 °C in a 5% CO<sub>2</sub>-containing incubator and were treated with ligands at 24 or 44 hrs after siRNA transfection and were harvested 48 hrs after transfection. siRNA duplexes targeting ER $\alpha$  (forward, UCAUCGCAUCCUUGCAAAdTdT; reverse, UUUGCAAGGAAUGCGAUGAdTdT, and control (GL3 luciferase, #D-001400-01) were obtained from Dharmacon (Lafayette, CO).

### Chromatin Immunoprecipitation (ChIP)

MCF-7 cells maintained in MEM (plus phenol red) supplemented with 5% calf serum, were then cultured in MEM plus 5% CDCS at 37 °C in a 5% CO<sub>2</sub>-containing incubator for 3 days prior to treatment with vehicle,  $10^{-8}$  M estradiol or  $10^{-7}$  M trans-hydroxytamoxifen (Tam) or  $10^{-7}$  raloxifene (Ral) for the times indicated. Chromatin was crosslinked using 1% formaldehyde at 37 °C for 10 min and cells were then washed twice with PBS and harvested in ice-cold PBS plus protease inhibitor cocktail (Roche) plus 1 mM DTT. Cell pellets were first resuspended in nuclei isolation buffer (50 mM Tris, pH 8.0, 60 mM KCl, 0.5% NP40, protease inhibitor and 10 mM DTT), centrifuged at 1000 g for 3 min and then resuspended in lysis buffer (0.5% SDS, 10 mM EDTA, 0.5 mM EGTA, 50 mM Tris-HCl, pH 8.0, protease inhibitor and 10 mM DTT) and sonicated (Fisher Scientific, Sonic Dismembrator Model 100) at 80% of maximum power three times for 10 seconds. The cell lysate was centrifuged at 14,000 g. The supernatant was diluted 1:4 with dilution buffer (1% Triton X-100, 2mMEDTA, 150mM NaCl, 20 mM Tris-HCl, pH8.0, protease inhibitor and 10 mM DTT ) and precleared with 15  $\mu$ l preimmune IgG (Santa Cruz Inc.), 2 $\mu$ g salmon sperm DNA, 50  $\mu$ l 25% protein A agarose slurry (Santa Cruz Inc.). Complexes were incubated at 4°C overnight with 2–5  $\mu$ g antibody, then pulled down at 4°C for 1 h with 60  $\mu$ l of 25% protein A-agarose slurry containing 2  $\mu$ g salmon sperm DNA. Precipitates were sequentially washed with 1ml washing buffer I (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.0), 150 mM NaCl), 1 ml washing bufferII (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.0), 500 mM NaCl), 1ml washing bufferIII (0.25 mM LiCl, 1% NP40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl (pH 8.0)) and twice with 1 ml TE buffer (1 mM EDTA, 10 mM Tris-HCl (pH 8.0)). Chromatin complexes were incubated at room temperature for 20 min with 100–300  $\mu$ l elution buffer (1% SDS, 0.1 M NaHCO<sub>3</sub>). The crosslinking was reversed by incubating at 65°C overnight with 200 mM NaCl and 200  $\mu$ g/ml proteinase K (Invitrogen Corp.). DNA was purified with QIAquick columns (Qiagen) and quantified by real-time PCR.

### Real-time PCR

Real-time PCR was performed to quantify gene expression levels and ChIP samples. Each real-time PCR reaction contained 4  $\mu$ l of diluted cDNA or ChIP sample, 5  $\mu$ l of 2X SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 0.5  $\mu$ l of 1.25  $\mu$ M forward and reverse primers and 0.5  $\mu$ l of ddH<sub>2</sub>O. The real time PCR was carried out in an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems) for 40 cycles (95°C for 15 s and 60°C for 1 min) after an initial 10 min incubation at 95°C. The primers used for real-time PCR are listed

in Table 1. The fold change in expression of each gene was calculated as described previously, with 36B4 cDNA as an internal control (Frasor et al., 2003). The CHIP results were calculated as percent of input chromatin.

### Genomic Cloning, Mutagenesis, and Luciferase Reporter Assays

The *KRT13* promoter region was amplified from human genomic DNA (Roche Corp.) and then cloned into the pGL3-promoter reporter plasmid using primers as indicated in Table 1B. Mutagenesis of putative response elements was performed using the QuickChange Site-Directed Mutagenesis protocol. The original sequences and mutagenesis primers are listed in Table 2, with the mutated sequences highlighted. All reporter constructs were sequenced for verification. Briefly, 1000 ng pGL3 reporter vector and 25 ng pRL-SV40 were co-transfected into MCF-7 cells in 24-well plates using Lipofectamine 2000 in OptiMEM as per manufacturer's instructions (Invitrogen, Carlsbad, CA). Cells were transfected for 6 h, washed, and treated with indicated ligands prior to cell lysis in 1x passive lysis buffer (Promega, Madison, WI) and measurement of luciferase activity in an MLX Microtiter Plate Luminometer (Dynex Technologies, Chantilly, VA).

## Results

### Differential up-regulation of *KRT13* mRNA level by E<sub>2</sub>, Tamoxifen and Raloxifene

Our previous work identified *KRT13* as being differentially regulated by estrogen and SERMs and suggested that *KRT13* might serve as a marker of breast tumor responsiveness to tamoxifen therapy (Frasor et al., 2006). To characterize the regulation of *KRT13*, ligand treatment time course experiments were performed in MCF-7 breast cancer cells treated with estradiol (E<sub>2</sub>), tamoxifen or raloxifene and *KRT13* mRNA levels were quantified by real-time PCR (Fig. 1). *KRT13* was differentially regulated by E<sub>2</sub>, tamoxifen and raloxifene in a time-dependent manner. *KRT13* mRNA increased upon 1 h E<sub>2</sub> treatment, peaked at 4 h and returned to almost the control basal level at 24 h. Compared to the E<sub>2</sub> treatment curve, the tamoxifen treatment curve was shifted right, and showed a slower increase in *KRT13* mRNA with a maximum response at 8h that remained high through 24 h. By contrast, raloxifene had only a very mild effect on *KRT13* expression at all times. To investigate whether the gene expression stimulations were primary responses to the ligands, cells were treated with DMSO vehicle or the translation inhibitor cycloheximide together with vehicle, E<sub>2</sub>, tamoxifen or raloxifene for 6 h. Fig. 2 shows that cycloheximide treatment still allowed up-regulation of *KRT13* mRNA expression by E<sub>2</sub> and SERMs, indicating that *KRT13* is likely a primary, direct gene target of the ER. We cannot, however, rule out possible involvement of secondary effects in the tamoxifen stimulations since the fold change with tamoxifen was reduced relative to basal in the cycloheximide vs. no cycloheximide treatment groups.

### Involvement of ER $\alpha$ in regulation of *KRT13* gene expression

To verify the involvement of ER $\alpha$  in *KRT13* regulation, ER $\alpha$  was knocked down by siRNA transfection in MCF-7 cells and *KRT13* mRNA levels were then determined after ligand treatments. As shown in Fig. 3 panels A and B, siER $\alpha$  treatment (which reduced ER $\alpha$  protein > 90%) eliminated *KRT13* mRNA induction by either E<sub>2</sub> or tamoxifen monitored at 4 h and 24 h of treatment. These results imply that ER $\alpha$  is required for the *KRT13* mRNA induction by E<sub>2</sub> and SERMs. We also obtained no stimulation of *KRT13* mRNA by E<sub>2</sub> or the SERMs in ER-negative 231 human breast cancer cells (data not shown), further suggesting the importance of ER in the *KRT13* response to ligands.

### Ligand-dependent ER $\alpha$ recruitment on the *KRT13* promoter

Utilizing a bioinformatic sequence analysis of the *KRT13* promoter (–5000 to +200 bp of the transcription start site), we found four putative ERE sites, four AP-1 sites, three Sp1 sites and nine half-ERE sites (Fig. 4A). In order to identify the ER binding regions in the *KRT13* promoter, ChIP scanning (Wang et al., 2004) was carried out (Fig. 4B). First, cells were treated with vehicle, E<sub>2</sub> or tamoxifen for 45 min and a standard ChIP assay was performed using sonicated chromatin with a median size of 500 bp, and the chromatin fragments were immunoprecipitated by antibody against ER $\alpha$ . Second, the precipitated DNA was analyzed by qPCR with 7 primer sets, each targeting a putative regulatory element in the *KRT13* promoter (Fig. 4A). As shown in Fig. 4B, regions 3.5, 4 and 5 showed strong ER $\alpha$  recruitment when cells were treated with E<sub>2</sub> or tamoxifen, and recruitment at this 45 min time was always greater with E<sub>2</sub> vs. Tam treatment. Region 2.5 recruited ER $\alpha$  following E<sub>2</sub> treatment but not tamoxifen treatment, suggesting that region 2.5 might be a differential ligand-dependent binding site for ER $\alpha$ .

### ER $\alpha$ and factor recruitment on the *KRT13* promoter proximal region

From the findings in Fig. 4, it is clear that estradiol and tamoxifen bound ER $\alpha$  was recruited to specific regions within the *KRT13* proximal promoter. To investigate whether other potential regulatory factors are recruited to the same regions as well, cells were treated with estradiol, tamoxifen or raloxifene for times up to 24 h, and factor recruitment on the *KRT13* promoter region P2.5, region P4, and the transcription start site (TSS) was analyzed with ChIP and qPCR. In all cases, recruitment was greatest at region P4 (Fig. 5).

On the *KRT13* P4 region (Fig. 5 middle panels), the recruitment of ER $\alpha$  was markedly increased with estradiol or tamoxifen treatment, but not with raloxifene treatment. With estradiol or tamoxifen, acetylation of histone H4 (ACh4) and recruitment of p300, SRC-2 and SRC-3 were increased and showed different patterns. In estradiol treated cells, recruitment of ER $\alpha$ , p300, SRC-2, SRC-3 and histone acetylation was highest at 45 min, then decreased by 4 h, and further decreased to the basal levels at 24 h (Fig. 5 middle panels). Following tamoxifen treatment, ER $\alpha$  and p300 recruitment and histone H4 acetylation were low at 45 min, rose to high levels at 4 h and remained high at 24 h, whereas SRC-2 and SRC-3 recruitment kept increasing and reached the highest level at 24 h (Fig. 5 middle panels). Of interest, the level of ER $\alpha$  recruitment with tamoxifen was two fold higher than that with E<sub>2</sub> at any time monitored and the ER $\alpha$  level was high over a prolonged period of time. By contrast, with raloxifene treatment, recruitment of ER $\alpha$ , cofactors and acetylation of histone H4 changed very little over time, consistent with our observations that this ligand had almost no ability to increase *KRT13* gene expression. Thus, of most note was the very different time course of ER $\alpha$  and cofactor recruitment and acetylation of histone H4 at the P4 region by the three ligands, being transiently increased very early after E<sub>2</sub> treatment and then declining, whereas the increases with tamoxifen were slower and usually higher and more sustained over time, and no increases were observed with raloxifene.

On the *KRT13* P2.5 region (Fig. 5 left panels), and on the *KRT13* transcription start site (Fig. 5 right panels, TSS), ER $\alpha$  was not recruited with any ligands, and with estradiol treatment, only weak increases in SRC-2, p300 and ACh4 were seen. In tamoxifen and raloxifene treated cells, there was no increase in recruitment of any cofactors. Hence, the greatest changes in ER $\alpha$  and cofactor recruitments were seen at region P4 (Fig. 5 middle panels).

### Analysis of regulatory elements in the *KRT13* promoter

Bioinformatic analysis of the *KRT13* promoter region revealed multiple potential EREs, Sp1, AP-1 and half ERE sites. The ChIP assay results in Figs. 4 and 5 showed binding of ER and cofactors to this promoter region. To explore the regulatory activity of the *KRT13* promoter in

greater detail, the -2500 to +200 bp region flanking the transcription start site of *KRT13* was cloned into the pGL3-luciferase reporter plasmid and its transcriptional activity was monitored in MCF-7 cells (Fig. 6A). This full length region showed a 9-fold stimulation with estradiol treatment and a 7-fold stimulation with tamoxifen treatment relative to the vehicle treatment (Fig. 6B).

In order to evaluate the cis-activation function of different regions in the *KRT13* promoter, serial truncated constructs were generated and cloned and their transcriptional activities were analyzed (Fig. 6A). Relative to the full length *KRT13* promoter the first truncated construct, denoted KRT13T1 (-1329 - +200 bp), which deleted the most distal ERE (named ERE1, see Fig. 4) and several Sp1 and AP1 sites and a half-ERE, retained approximately 40% of the E<sub>2</sub>-stimulated activity and 35% of the tamoxifen-stimulated activity seen with the full length promoter. Further truncation to remove also ERE 2, KRT13T2 (-784 - +200 bp), or to remove ERE 3, KRT13T3 (-525 - +200 bp), or to remove all four EREs, KRT13T4 (-204 - +200 bp), yielded constructs that had very low transcription activity with either E<sub>2</sub> or tamoxifen (Fig. 6B). These results suggest that the majority of the E<sub>2</sub> and tamoxifen inducible activity within the *KRT13* promoter is derived from the regulatory elements in the -2500 to -784 region.

To evaluate further the contribution of the ERE and Sp1 sites to the transcription activity of the *KRT13* promoter, each site was mutated individually or in various combinations by site directed mutagenesis and the E<sub>2</sub> or Tam inducible activities were assessed (Fig. 6). As shown in Fig. 6A and B, individual mutation of ERE 1, 2, or 3 (mut 1, mut2 or mut 3) diminished, but did not abolish, E<sub>2</sub> or Tam responses. Mutation of ERE 4 had no effect, consistent with our findings from the promoter truncations also. It is interesting that mutation of ERE 1 reduced only the E<sub>2</sub> response, but not the Tam response. Among all four individual ERE mutations, mutation of ERE 2 (mut2) had the greatest impact on both E<sub>2</sub> and Tam transcriptional activity. Double ERE mutations of EREs 1-2 and 2-3 and the triple mutation of EREs 1-2-3 further reduced E<sub>2</sub>/Tam responses almost to the basal level, whereas with the double ERE mutations at sites 1-2 or 2-3 about half of the E<sub>2</sub>/Tam responses of the wild type promoter were retained. The findings with the ERE mutations demonstrate that EREs 2 and 3 are involved in both E<sub>2</sub> and Tam regulation of *KRT13*, that ERE 1 may be mainly involved in the E<sub>2</sub> response, and that ERE 4 does not appear to be involved in regulation of *KRT13*.

Because truncation T1 more greatly reduced ligand stimulation than was observed with just mutation of ERE 1 (mut1), we examined the possible roles of the two Sp1 sites present near ERE 1 as well as the possible involvement of the Sp1 site nearest to the promoter since even truncation T4 showed some low ligand regulation. In examining the involvement of Sp1 sites, we found that all single, double and triple mutations of the three Sp1 sites gave similarly reduced E<sub>2</sub> and Tam responses compared to the wild type *KRT13* construct, suggesting that the Sp1 sites are involved in the full activity of the *KRT13* promoter. The Sp1 triple mutation still evoked about half of the full E<sub>2</sub>/Tam responses, this remaining transcriptional activity probably being due to the EREs. These deletion and mutation analyses implicate ERE 2, along with EREs 1 and 3, as being most central in the regulation of *KRT13*.

## Discussion

Our findings reveal differential time dependent up-regulation of keratin 13 gene expression by estradiol and two SERMs. *KRT13* was up-regulated by estradiol at early time points, and was up-regulated by tamoxifen at later time points. Thus, for the regulation of *KRT13*, estradiol was a short-term agonist and tamoxifen was a more long-term agonist, being considerably more effective than E<sub>2</sub> in sustaining up-regulation of the mRNA for *KRT13* over time. The SERM, raloxifene, by contrast, was quite ineffective in up-regulating *KRT13* gene expression.

To understand the basis for the differential ligand regulation of this gene, we used a ChIP scanning approach to identify the estrogen receptor binding and potential ligand regulated region of *KRT13*, and we cloned and further characterized this region by site directed mutagenesis and serial truncations. By ChIP scanning, we identified a regulatory region (−2500 bp to +200 bp) that has ligand-dependent ER $\alpha$  binding. The region we identified overlaps with the −2572 bp to −1035 bp region identified by ChIP-on-chip studies of Carroll *et al.* (Carroll *et al.*, 2006) as an ER binding region close to the *KRT13* gene. Within the 2.7-kb region we studied, we found four putative ERE sites and three Sp1 sites using sequence analysis. This 2.7-kb region was further dissected by mutagenesis, and we found that three EREs and three Sp1 sites are involved in the *KRT13* hormonal regulation we have examined.

Upon estrogen treatment, ER and other cofactors are known to be recruited to gene regulatory regions and to modulate gene expression (Metivier *et al.*, 2003, Shang *et al.*, 2000). Depending on the promoter context and cellular background, tamoxifen and raloxifene can regulate gene expression in a manner similar to or different from that of estradiol (Frasor *et al.*, 2004, Rivera *et al.*, 2005, Shang and Brown, 2002). In our study, tamoxifen, raloxifene and estradiol regulated *KRT13* differentially. Our ChIP time course studies revealed that the time-dependent differential regulation effects of these ligands on *KRT13* gene stimulation reflected a differential pattern of recruitment of ER and coregulators to these regulatory sites. Beyond the differential recruitment of coactivators such as SRC-2, SRC-3 and p300, and changes in histone acetylation that we examined, recent work has also highlighted the involvement of FoxA1 and related factors that affect chromatin remodeling that accompanies the changes in gene expression by different ligand-receptor complexes (Carroll *et al.*, 2005, Carroll *et al.*, 2006, Green and Carroll, 2007, Leu *et al.*, 2004, Lupien *et al.*, 2008).

There are several possible explanations for our observations. First, the structures of the ER-tamoxifen and ER-raloxifene complexes are not identical and show some important differences (Brzozowski *et al.*, 1997, Shiau *et al.*, 1998, Wijayaratne *et al.*, 1999) that may contribute to differences in their patterns of gene regulation, and to our observations that tamoxifen was more stimulatory than raloxifene on the *KRT13* gene. Second, estradiol-bound ER is reported to be degraded through a ubiquitin proteasome pathway, whereas SERM-bound ER is relatively more stable (Fan *et al.*, 2004, Lonard *et al.*, 2000, Wijayaratne and McDonnell, 2001). Through gene expression profiling, we have observed that tamoxifen and raloxifene can have very different agonistic (stimulatory) activities on different genes, and although the mechanistic basis for these differences have not been elucidated, differences in ligand-dependent degradation of ER may, in part, account for the differential regulation of gene expression in our study. Since the tamoxifen-bound ER appears to be more stable and less susceptible to proteasome-mediated degradation and down-regulation than is the E2-ER complex (Shao *et al.*, 2004, Wijayaratne and McDonnell, 2001), the up-regulation of *KRT13* might remain sustained for a longer time by the ER-tamoxifen complex. Finally, it has been shown that 2–24 hour treatments with tamoxifen and raloxifene increased p160 coactivator levels in HeLa and MCF-7 cells (Frasor *et al.*, 2003, Lonard *et al.*, 2004). The elevated levels of coactivators may boost the agonistic effects of tamoxifen on *KRT13*, especially when the coactivator levels are high, a situation similar to the late time point in our experiments.

Several studies have highlighted marked cell-specific differences in the hormonal regulation of specific genes. For example, whereas GREB1 has been shown to be up-regulated by estrogen in several estrogen receptor-positive breast cancer cell lines, other genes were regulated by estrogen in MCF-7 but not in other estrogen receptor-positive breast cancer cells (Rae *et al.*, 2005, Stender *et al.*, 2007). In this regard, it is of interest that we also observed cell-specific differences in hormonal regulation of *KRT13*. Although *KRT13* was up-regulated by tamoxifen and by estradiol but not by raloxifene in MCF-7 cells, we observed no up-regulation of *KRT13* by either of the three ligands over a 48 h time period in 231 breast cancer cells stably

expressing ER (at 30% the level of MCF-7 cells), and we observed only weak (2-fold) up-regulation by estradiol at 1 and 4 h and negligible regulation monitored over 48 h by tamoxifen or raloxifene in ZR75 cells, which contained 8% the ER level of the MCF-7 cells. What accounts for these cell-specific differences is unclear, but might include alterations in chromatin modifications in or near the *KRT13* gene, differences in ER levels, presence or absence of different coregulators, and differences in additional transcription factors such as FOXA1 in the different ER-positive cells (Eeckhoutte et al., 2006, Katzenellenbogen and Katzenellenbogen, 2002, Lupien et al., 2008, Shang and Brown, 2002).

Our results provide insight into the mechanisms responsible for differential gene regulation by estrogen and different SERMs. Although we have reported that another keratin, keratin 19, is regulated by estradiol in breast cancer cells (Choi et al., 2000) and others have shown keratin involvement in breast cancer migration (Chu et al., 1993, Chu et al., 1996, Hendrix et al., 1996, Hendrix et al., 1997), this is the first report on the hormonal regulation of *KRT13*. Our findings indicate important differences in the regulation of *KRT13* by estradiol and different SERMs that might impact on the activity of *KRT13* in breast cancer cell migration and metastasis.

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## Abbreviations

<b>ChIP</b>	chromatin immunoprecipitation
<b>E2</b>	17 $\beta$ -estradiol
<b>ER</b>	estrogen receptor
<b>ERE</b>	estrogen response element
<b>KRT13</b>	keratin 13
<b>Ral</b>	raloxifene
<b>SERM</b>	selective estrogen receptor modulator
<b>Tam</b>	tamoxifen

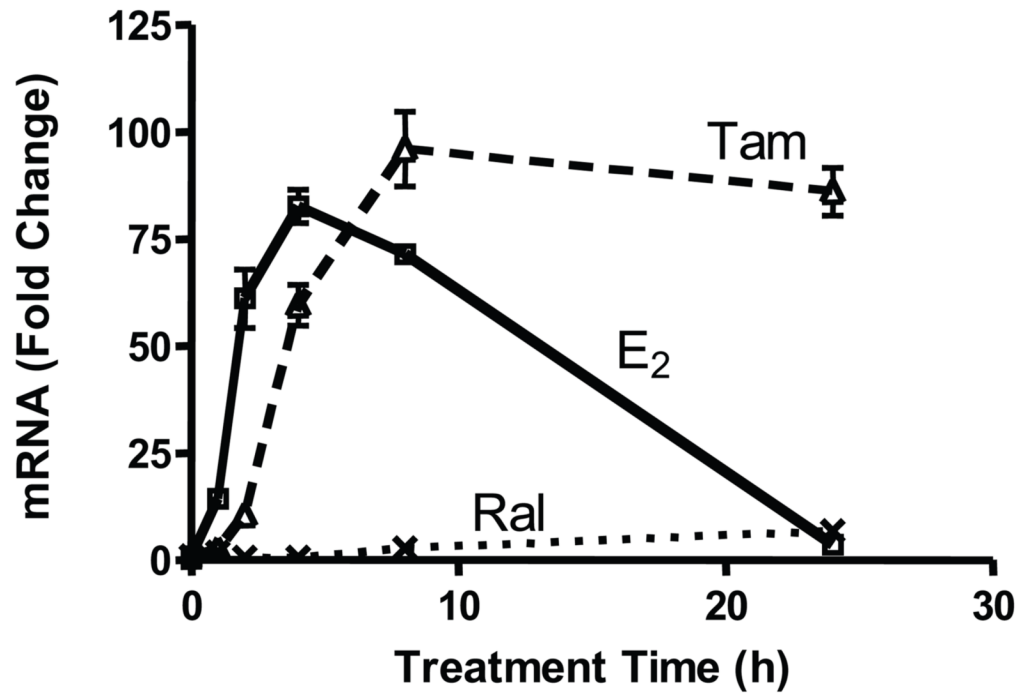
## References

- Brzozowski AM, Pike ACW, Dauter Z, Hubbard RE, Bonn T, Engstrom O, Ohman L, Greene GL, Gustafsson JA, Carlquist M. Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature* 1997;389:753–758. [PubMed: 9338790]
- Carroll JS, Liu XS, Brodsky AS, Li W, Meyer CA, Szary AJ, Eeckhoutte J, Shao W, Hestermann EV, Geistlinger TR, Fox EA, Silver PA, Brown M. Chromosome-wide mapping of estrogen receptor

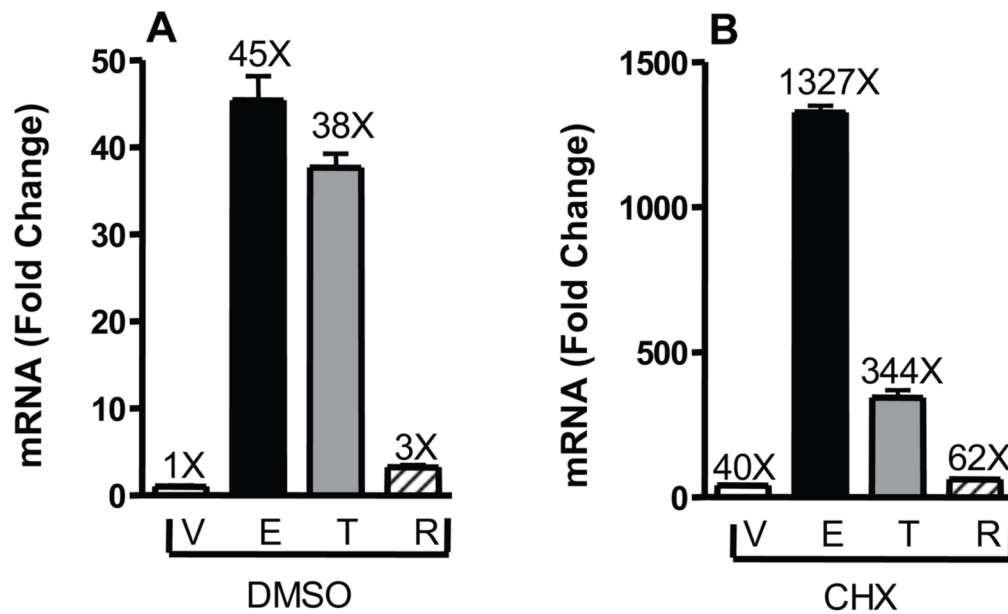


- binding reveals long-range regulation requiring the forkhead protein FoxA1. *Cell* 2005;122:33–43. [PubMed: 16009131]
- Carroll JS, Meyer CA, Song J, Li W, Geistlinger TR, Eeckhoutte J, Brodsky AS, Keeton EK, Fertuck KC, Hall GF, Wang Q, Bekiranov S, Sementchenko V, Fox EA, Silver PA, Gingeras TR, Liu XS, Brown M. Genome-wide analysis of estrogen receptor binding sites. *Nat Genet* 2006;38:1289–1297. [PubMed: 17013392]
- Choi I, Gudas LJ, Katzenellenbogen BS. Regulation of keratin 19 gene expression by estrogen in human breast cancer cells and identification of the estrogen responsive gene region. *Mol Cell Endocrinol* 2000;164:225–37. [PubMed: 11026574]
- Chu YW, Runyan RB, Oshima RG, Hendrix MJ. Expression of complete keratin filaments in mouse L cells augments cell migration and invasion. *Proc Natl Acad Sci U S A* 1993;90:4261–5. [PubMed: 7683431]
- Chu YW, Seftor EA, Romer LH, Hendrix MJ. Experimental coexpression of vimentin and keratin intermediate filaments in human melanoma cells augments motility. *Am J Pathol* 1996;148:63–9. [PubMed: 8546227]
- Deroo BJ, Korach KS. Estrogen receptors and human disease. *J Clin Invest* 2006;116:561–70. [PubMed: 16511588]
- Eeckhoutte J, Carroll JS, Geistlinger TR, Torres-Arzayus MI, Brown M. A cell-type-specific transcriptional network required for estrogen regulation of cyclin D1 and cell cycle progression in breast cancer. *Genes Dev* 2006;20:2513–26. [PubMed: 16980581]
- Fan M, Nakshatri H, Nephew KP. Inhibiting proteasomal proteolysis sustains estrogen receptor-alpha activation. *Mol Endocrinol* 2004;18:2603–15. [PubMed: 15284335]
- Frasor J, Chang EC, Komm B, Lin CY, Vega VB, Liu ET, Miller LD, Smeds J, Bergh J, Katzenellenbogen BS. Gene expression preferentially regulated by tamoxifen in breast cancer cells and correlations with clinical outcome. *Cancer Res* 2006;66:7334–40. [PubMed: 16849584]
- Frasor J, Danes JM, Komm B, Chang KC, Lyttle CR, Katzenellenbogen BS. Profiling of estrogen up- and down-regulated gene expression in human breast cancer cells: insights into gene networks and pathways underlying estrogenic control of proliferation and cell phenotype. *Endocrinology* 2003;144:4562–4574. [PubMed: 12959972]
- Frasor J, Stossi F, Danes JM, Komm B, Lyttle CR, Katzenellenbogen BS. Selective estrogen receptor modulators: discrimination of agonistic versus antagonistic activities by gene expression profiling in breast cancer cells. *Cancer Res* 2004;64:1522–33. [PubMed: 14973112]
- Green KA, Carroll JS. Oestrogen-receptor-mediated transcription and the influence of co-factors and chromatin state. *Nat Rev Cancer* 2007;7:713–722. [PubMed: 17721435]
- Hendrix MJ, Seftor EA, Chu YW, Trevor KT, Seftor RE. Role of intermediate filaments in migration, invasion and metastasis. *Cancer Metastasis Rev* 1996;15:507–25. [PubMed: 9034607]
- Hendrix MJ, Seftor EA, Seftor RE, Trevor KT. Experimental co-expression of vimentin and keratin intermediate filaments in human breast cancer cells results in phenotypic interconversion and increased invasive behavior. *Am J Pathol* 1997;150:483–95. [PubMed: 9033265]
- Hesse M, Magin TM, Weber K. Genes for intermediate filament proteins and the draft sequence of the human genome: novel keratin genes and a surprisingly high number of pseudogenes related to keratin genes 8 and 18. *J Cell Sci* 2001;114:2569–2575. [PubMed: 11683385]
- Katzenellenbogen BS, Frasor J. Therapeutic targeting in the estrogen receptor hormonal pathway. *Semin Oncol* 2004;31:28–38. [PubMed: 15052541]
- Katzenellenbogen BS, Katzenellenbogen JA. Biomedicine. Defining the “S” in SERMs. *Science* 2002;295:2380–1. [PubMed: 11923515]
- Kumar V, Chambon P. The estrogen receptor binds tightly to its responsive element as a ligand-induced homodimer. *Cell* 1988;55:145–56. [PubMed: 3167974]
- Leu YW, Yan PS, Fan M, Jin VX, Liu JC, Curran EM, Welshons WV, Wei SH, Davuluri RV, Plass C, Nephew KP, Huang THM. Loss of estrogen receptor signaling triggers epigenetic silencing of downstream targets in breast cancer. *Cancer Res* 2004;64:8184–8192. [PubMed: 15548683]
- Lonard DM, Lanz RB, O’malley BW. Nuclear receptor coregulators and human disease. *Endocr Rev* 2007;28:575–87. [PubMed: 17609497]

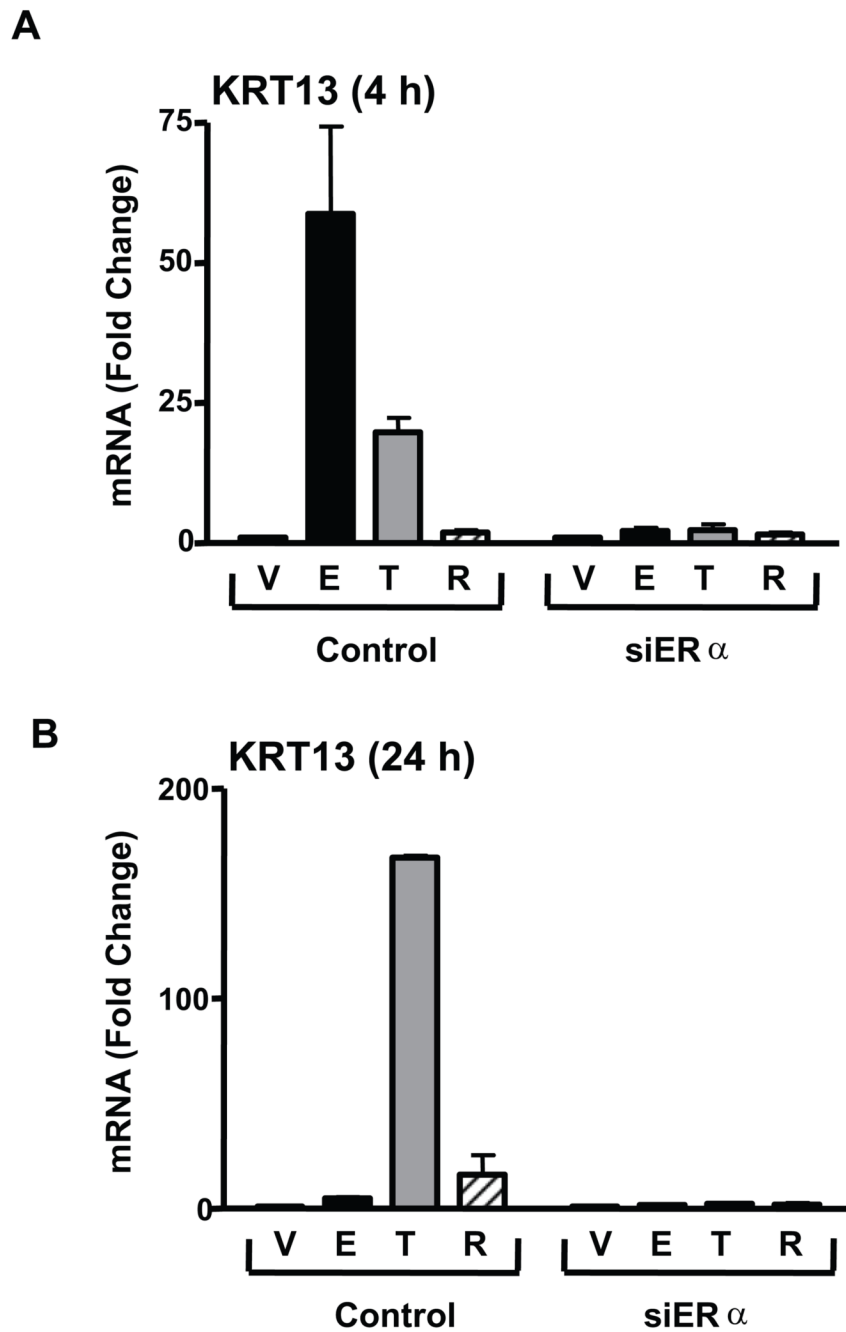
- Lonard DM, Nawaz Z, Smith CL, O'malley BW. The 26S proteasome is required for estrogen receptor-alpha and coactivator turnover and for efficient estrogen receptor-alpha transactivation. *Mol Cell* 2000;5:939–48. [PubMed: 10911988]
- Lonard DM, Tsai SY, O'malley BW. Selective estrogen receptor modulators 4-hydroxytamoxifen and raloxifene impact the stability and function of SRC-1 and SRC-3 coactivator proteins. *Mol Cell Biol* 2004;24:14–24. [PubMed: 14673139]
- Lupien M, Eeckhoutte J, Meyer CA, Wang Q, Zhang Y, Li W, Carroll JS, Liu XS, Brown M. FoxA1 translates epigenetic signatures into enhancer-driven lineage-specific transcription. *Cell* 2008;132:958–970. [PubMed: 18358809]
- Magin TM, Vijayaraj P, Leube RE. Structural and regulatory functions of keratins. *Exp Cell Res* 2007;313:2021–32. [PubMed: 17434482]
- Metivier R, Penot G, Hubner MR, Reid G, Brand H, Kos M, Gannon F. Estrogen receptor-alpha directs ordered, cyclical, and combinatorial recruitment of cofactors on a natural target promoter. *Cell* 2003;115:751–63. [PubMed: 14675539]
- Murdoch FE, Gorski J. The role of ligand in estrogen receptor regulation of gene expression. *Mol Cell Endocrinol* 1991;78:C103–8. [PubMed: 1778298]
- Olson GE, Winfrey VP, Blaeuer GL, Palisano JR, Nagdas SK. Stage-specific expression of the intermediate filament protein cytokeratin 13 in luminal epithelial cells of secretory phase human endometrium and peri-implantation stage rabbit endometrium. *Biol Reprod* 2002;66:1006–15. [PubMed: 11906920]
- Rae JM, Johnson MD, Scheys JO, Cordero KE, Larios JM, Lippman ME. GREB 1 is a critical regulator of hormone dependent breast cancer growth. *Breast Cancer Res Treat* 2005;92:141–9. [PubMed: 15986123]
- Rivera JA, Alturaihi H, Kumar U. Differential regulation of somatostatin receptors 1 and 2 mRNA and protein expression by tamoxifen and estradiol in breast cancer cells. *J Carcinog* 2005;4:10. [PubMed: 16018813]
- Shang Y, Brown M. Molecular determinants for the tissue specificity of SERMs. *Science* 2002;295:2465–8. [PubMed: 11923541]
- Shang Y, Hu X, Drenzo J, Lazar MA, Brown M. Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription. *Cell* 2000;103:843–52. [PubMed: 11136970]
- Shao W, Keeton EK, McDonnell DP, Brown M. Coactivator AIB1 links estrogen receptor transcriptional activity and stability. *Proc Natl Acad Sci U S A* 2004;101:11599–604. [PubMed: 15289619]
- Shiau AK, Barstad D, Loria PM, Cheng L, Kushner PJ, Agard DA, Greene GL. The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell* 1998;95:927–37. [PubMed: 9875847]
- Smith RE, Good BC. Chemoprevention of breast cancer and the trials of the National Surgical Adjuvant Breast and Bowel Project and others. *Endocr Relat Cancer* 2003;10:347–57. [PubMed: 14503912]
- Stender JD, Frasier J, Komm B, Chang KC, Kraus WL, Katzenellenbogen BS. Estrogen-regulated gene networks in human breast cancer cells: involvement of E2F1 in the regulation of cell proliferation. *Mol Endocrinol* 2007;21:2112–23. [PubMed: 17550982]
- Strassmer-Weippl K, Goss PE. Prevention of breast cancer using SERMs and aromatase inhibitors. *J Mammary Gland Biol Neoplasia* 2003;8:5–18. [PubMed: 14587860]
- Wang JC, Derynck MK, Nonaka DF, Khodabakhsh DB, Haqq C, Yamamoto KR. Chromatin immunoprecipitation (ChIP) scanning identifies primary glucocorticoid receptor target genes. *Proc Natl Acad Sci U S A* 2004;101:15603–8. [PubMed: 15501915]
- Waseem A, Alam Y, Dogan B, White KN, Leigh IM, Waseem NH. Isolation, sequence and expression of the gene encoding human keratin 13. *Gene* 1998;215:269–79. [PubMed: 9714826]
- Wijayaratne AL, McDonnell DP. The human estrogen receptor-alpha is a ubiquitinated protein whose stability is affected differentially by agonists, antagonists, and selective estrogen receptor modulators. *J Biol Chem* 2001;276:35684–92. [PubMed: 11473106]
- Wijayaratne AL, Nagel SC, Paige LA, Christensen DJ, Norris JD, Fowlkes DM, McDonnell DP. Comparative analyses of mechanistic differences among antiestrogens. *Endocrinology* 1999;140:5828–40. [PubMed: 10579349]



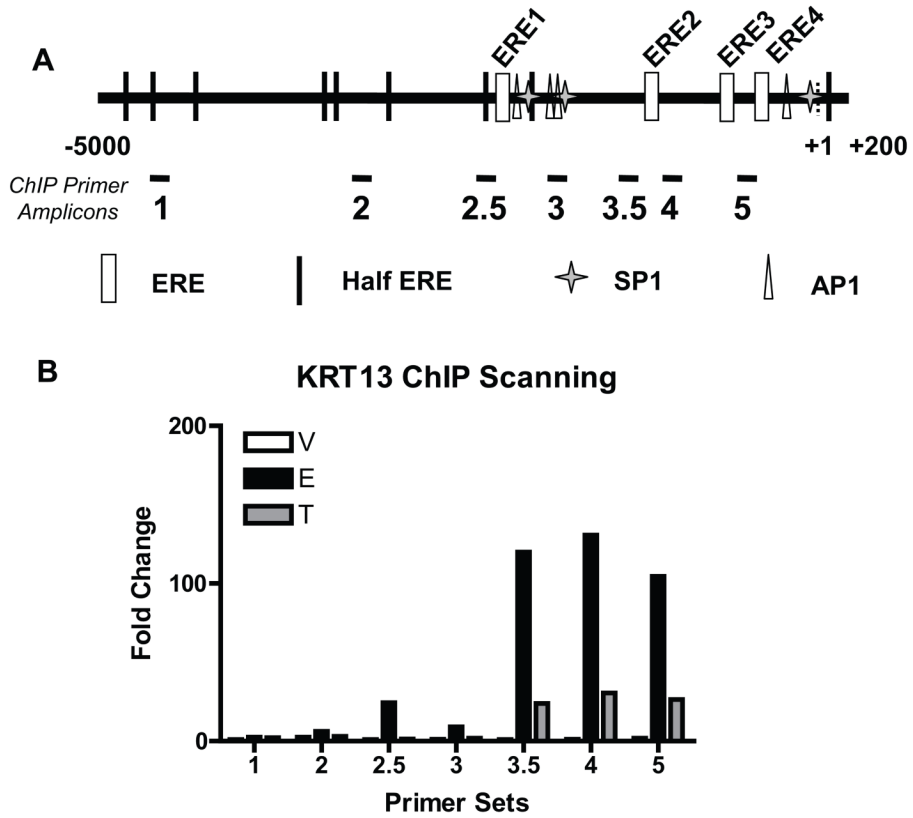
**Figure 1.** Differential up-regulation over time of *KRT13* mRNA levels by E<sub>2</sub>, Tam (trans-hydroxytamoxifen) and Ral. MCF-7 cells were treated with 10 nM E<sub>2</sub>, 100 nM Tam or 100 nM Ral for 0, 1, 2, 4, 8, and 24 h. RNA was extracted and reverse transcribed, and real time PCR was carried out. Each value represents the mean  $\pm$  SEM from three separate experiments.



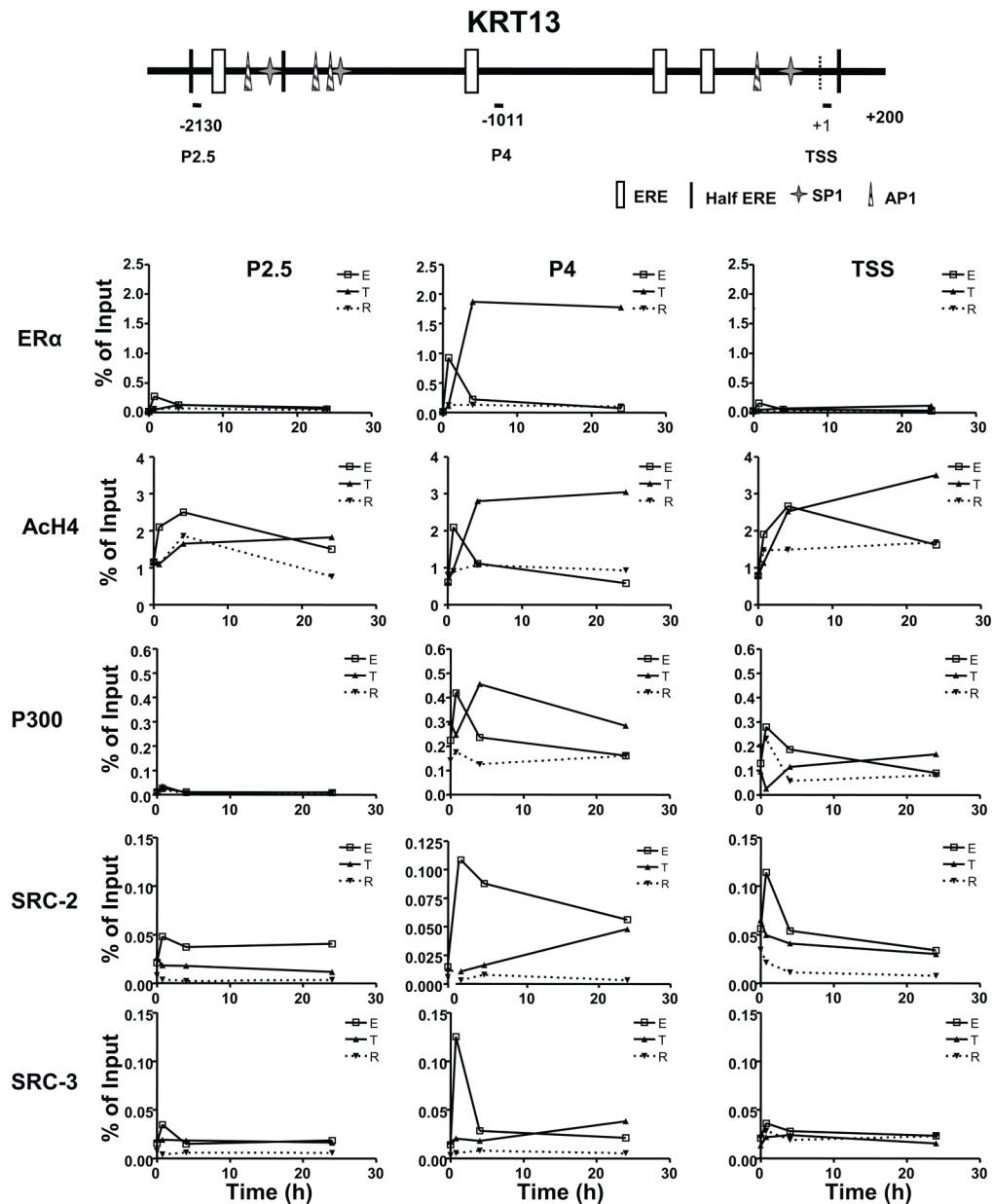
**Figure 2.** Examination of the effect of cycloheximide on the up-regulation of *KRT13* gene expression by E<sub>2</sub>, Tam and Ral. MCF-7 cells were pre-treated with DMSO vehicle (A) or 10  $\mu$ g/mL cycloheximide (B) for 30 min and then treated with vehicle, 10 nM E<sub>2</sub>, 100 nM Tam (trans-hydroxytamoxifen) or 100 nM Ral with or without 10  $\mu$ g/mL cycloheximide for 6h. RNA was extracted and reverse transcribed, and real time PCR was carried out. Each value represents the mean  $\pm$  SEM from three separate experiments.



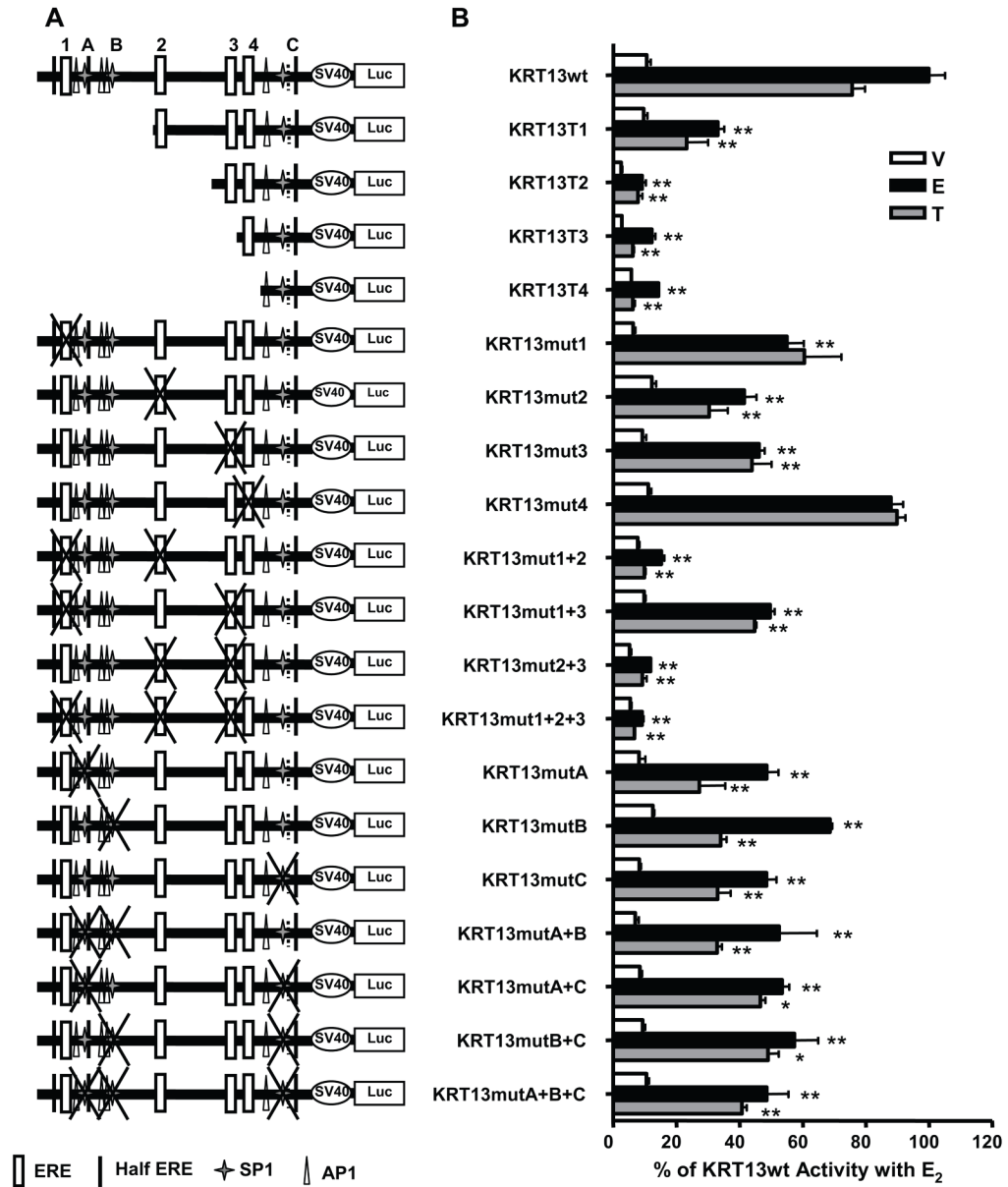
**Figure 3.** ER $\alpha$  mediates ligand-induced *KRT13* gene expression. MCF-7 cells were transfected with siRNA for control GL3 luciferase, or with siRNA for ER $\alpha$  and were treated with vehicle, 10nM E<sub>2</sub>, 100nM trans-hydroxytamoxifen or 100 nM Ral for 4 h (panel A) or 24 h (panel B). RNA was extracted and reverse transcribed, and real time PCR was carried out. Each value represents the mean  $\pm$  SEM from three separate experiments.



**Figure 4.** Mapping ER $\alpha$ -binding regions in the *KRT13* promoter by ChIP scanning. (A) Schematic diagram of the -5000 to +200 region of the *KRT13* gene. The putative elements and PCR-amplified fragments indicated are: ERE1 (-2129 to -2117), ERE2 (-1289 to -1277), ERE3 (-587 to -575), ERE4 (-394 to -382), amplicon 1 (-4613 to -4511), amplicon 2 (-3314 to -3214), amplicon 2.5 (-2296 to -2171), amplicon 3 (-1888 to -1762), amplicon 3.5 (-1448 to -1334), amplicon 4 (-1157 to -1010), amplicon 5 (-567 to -428). (B) ChIP analysis of E<sub>2</sub>-induced and tamoxifen-induced ER $\alpha$ -binding to the *KRT13* promoter region. MCF-7 cells were treated with vehicle, 10nM E<sub>2</sub> or 100nM Tam (trans-hydroxytamoxifen) for 45 min. ChIP assays were performed with antibody against ER $\alpha$  and the ChIP samples were analyzed by real time PCR. Each value represents the mean from two separate experiments.

**Figure 5.**

Recruitment of ER $\alpha$ , changes in histone H4 acetylation (AcH4), and recruitment of p300, SRC-2, and SRC-3 on the *KRT13* promoter over time in response to E<sub>2</sub>, Tam or Ral. MCF-7 cells were treated with vehicle, 10nM E<sub>2</sub>, 100nM Tam or 100nM Ral for 0, 45 min, 4 h and 24 h. ChIP assays were performed with antibody against ER $\alpha$ , AcH4, p300, SRC-2 or SRC-3 and the ChIP samples were analyzed by real time PCR. Recruitments on the *KRT13* promoter regions P2.5, P4 and the TSS (transcription start site) are shown. Each value represents the mean from three to five separate experiments.



**Figure 6.** Analysis of the E<sub>2</sub> and tamoxifen-induced transcriptional activity of the wild type (wt) *KRT13* promoter or the *KRT13* promoter containing the indicated truncations or mutations. ERE positions are: ERE1 (-2129 to -2117), ERE2 (-1289 to -1277), ERE3 (-587 to -575), ERE4 (-394 to -382). (A) Schematic diagrams of the wild type and mutant constructs. (B) E<sub>2</sub> and tamoxifen-induced transcriptional activities of the wild type and *KRT13* promoter mutants were evaluated in a luciferase reporter system in MCF-7 cells. Values are the mean ± SEM from three to six separate experiments. Dunnett’s test was performed to compare each mutant with the wild type promoter for each ligand treatment. \* p < 0.05, and \*\* p < 0.01.



**Table 1**

<b>Table 1A. Primers used for real-time PCR</b>		
	<b>Forward primer</b>	<b>Reverse primer</b>
36B4	5'-GTGTTTCGACAATGGCAGCAT	5'-GACACCCTCCAGGAAGCGA
KRT13	5'-GGCAGAGATGAGGGAGCAGTA	5'-TCTTGGCGTGGAACCATTCT
SRC-1	5'-CTCATGGTGTGGCTCGTTCAT	5'-TGCTGGCGGTTTATCTGGT
SRC-2	5'-AGGACAGCCACCTCCATG	5'-TGACTGGGTGGGATTCGAG
SRC-3	5'-CCATGGGAAGACCAGGAGG	5'-CGAAGAGGCAATGTGGGAA
KRT13-P1	5'-CCCTTGCTGAGTTCAGTGGG	5'-AGCCTCAATGCCTTAGGCTCTT
KRT13-P2	5'-GAACCCAGGAAAACAGTGCAC	5'-ACCTCGCCTGTGTATCTCTTC
KRT13-P2.5	5'-GGCATTACAAGTCCCCGCTAA	5'-CAATGCTGCAGATCCTCCAA
KRT13-P3	5'-CTGCTGATACCCCTGCGG	5'-GGTTCCTGGAACCCCT
KRT13-P3.5	5'-TGGGTCTGTGTACATCCTC	5'-TCACATCCAGGAAGACGGC
KRT13-P4	5'-CCCAAGTTGTGACAACCAAA	5'-CAATTACCTGACTCCACACCCC
KRT13-TSS	5'-AAGAGCCACAGTCTCGGC	5'-TGGCAGAGGAGCTCTGCAG

<b>Table 1B. Primers used for Cloning</b>		
	<b>Forward primer</b>	<b>Reverse primer</b>
KRT13wt	5'-ATGGTACCGTTGTTGTTGTTTGGCCA	5'-TATGATCTCCATAGCCCCCAGCTGATCCC
KRT13T1	5'-ATTGGTACCGCCCTAAAGCAGTCAGCGTAAT	5'-TATGATCTCCATAGCCCCCAGCTGATCCC
KRT13T2	5'-ATTGGTACCGCAATAGTAGGACCCTCCTCC	5'-TATGATCTCCATAGCCCCCAGCTGATCCC
KRT13T3	5'-ATTGGTACCGGCAGGTGATTCTATCGAACCA	5'-TATGATCTCCATAGCCCCCAGCTGATCCC
KRT13T4	5'-ATTGGTACCTGTCCCTCTGAATCATCTTTTT	5'-TATGATCTCCATAGCCCCCAGCTGATCCC

Table 2

Primers used for Site-Directed Mutagenesis

	Original Sequence	Forward primer	Reverse primer
KRT13mut1	GGGGCA <b>T</b> GT <b>T</b> A <b>C</b> CC	5'-GGTTTGGGGCATTGTTTAGCCAGGCATGGCAGGTT <b>C</b>	5'-GAACCTGCCATGCCCTGGCTAAACAATGCCCCAAACC
KRT13mut2	GGCCAGACTG <b>A</b> CC <b>C</b>	5'-CAACCCGAGGGCCAGACTGGAA <b>C</b> AGGCATCTCAACCTCAAC	5'-GTTGAGGTTGAGATGCCCTGTTCCAGTCTGGCCCTCGGGTTG
KRT13mut3	CTGTACTGTG <b>A</b> CT <b>T</b>	5'-GGTCTCTGTCACTGTGGAA <b>T</b> TTTCTGATGGCCCTCGG	5'-CCGAGGCCATCAGAAAA <b>T</b> TCCACAGTGA <b>C</b> AGAGACC
KRT13mut4	GGGCTACGGT <b>G</b> AC <b>C</b> T	5'-GTCGAGGGCTACGGTGGAA <b>T</b> TGCAAAAGCACAGAG	5'-CTCTGTGCTTTGGCA <b>A</b> TTCCACCGTAGCCCTCGAC
KRT13mutA	CGGGCAGGG <b>T</b>	5'-GGGCAGGAGGGCG <b>A</b> TAA <b>G</b> GGTCAGGGGGTTAG	5'-CTAACCCCTGACCCT <b>T</b> ATCGGGCCCTCC <b>T</b> GGCC <b>C</b>
KRT13mutB	ACCCAG <b>C</b> CC <b>C</b>	5'-GTGCCACCCCA <b>C</b> CC <b>A</b> TATCCTGGCCTG <b>G</b> TATC	5'-GATACCCAGGGCCAGG <b>A</b> TATGGGTGGGGTGG <b>C</b> AC
KRT13mutC	AAC <b>C</b> AG <b>C</b> CC <b>C</b>	5'-GGGAGGAGAGAGATA <b>A</b> CC <b>A</b> TATCCTATGGAGGTGTATA <b>A</b> AA <b>G</b>	5'-CTTTTATACACCTCCATAGG <b>A</b> TATGGTTATCTTCTCTC <b>C</b> CT <b>C</b> CC

The mutation sequences are bolded.