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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

We previously identified Uba3 as a nuclear hormone receptor coactivator that functions as part of the NEDD8 pathway to influence ER- and PR-mediated transcription. Although our previous model failed to demonstrate the connection between Uba3, the NEDD8 pathway, and transcription, we now have sufficient evidence to suggest the NEDD8 pathway is linked to ER- and PR-mediated transcription via the SCF E3 ligase complex. Here we show that Skp1 can associate with ERα by co-immunoprecipitation, and with the pS2 promoter by ChIP assay. We have investigated the mRNA expression of Uba3, PR, and ERα in several common tumor cell lines. We were not able to detect abnormal expression of hUba3 mRNA in the breast cancer cell lines. Furthermore, we were unable to correlate hUba3 mRNA expression with PR and ERα expression across all cell lines. We also investigated *in silico* the potential that Uba3 mRNA expression may be regulated by estrogen and progesterone. Although possible binding sites for ER and PR were localized within the Uba3 promoter, we were not able to detect induction or repression of Uba3 mRNA by estradiol, and SERMs tamoxifen, and raloxifene, or progesterone.

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TABLE OF CONTENTS

INTRODUCTION:

Estrogen and Progesterone regulate the growth and development of the mammary gland via a signaling cascade that is initiated through binding to their cognate nuclear hormone receptors (NHRs), the estrogen receptor α/β (ER) and progesterone receptor-A/-B (PR), members of the ligand-inducible transcription factor superfamily (1,2). ER and PR-target genes are further regulated by recruitment of coregulator proteins, which positively affect (coactivators) or negatively affect (corepressors) ligand-activated transcription (3).

We have previously identified human ubiquitin-activating enzyme 3 (hUba3) as a progesterone receptor (PR) interacting protein in a yeast 2-hybrid assay. Human Uba3 is a member of the NEDD8 (neural precursor cell-expressed developmentally down-regulated) ubiquitin-like protein modification pathway (4). The NEDD8 pathway functions through a pathway-specific E1 enzyme (a heterodimer composed of hUba3 and APP-BP1 (amyloid precursor protein binding-protein 1) and an E2 enzyme (Ubc12 (ubiquitin-conjugating enzyme 12)), but lacks a cognate E3 (and one may not exist) (4). We previously reported an intricate relationship between the NEDD8 pathway and NHR coactivation, although we were not able to demonstrate how this pathway influences ER- and PR-dependent transcription.

The Cullin proteins (Cul-1, -2, -3, -4a, -4b, and –5) have been the only identified target proteins of the NEDD8 modification pathway (5). The Cullin proteins are an integral component of the SCF ubiquitin-protein (E3) ligase (a complex of Skp1, Cullin-x, and an F-box protein). Modification of the Cullin proteins by NEDD8 appears to control the ubiquitin ligase activity of the SCF complex by recruiting Cdc34, a ubiquitin-conjuagting (E2) enzyme used by this complex (6). Since the E2-E3 components are juxtaposed to one another, it is thought that this proximity contributes to the enhancement of SCF complex function. Thus, the NEDD8 pathway may contribute to degradation of components involved in transcription via the SCF complex (7,8).

Several lines of evidence support the possibility that degradation is necessary for transcription to proceed (7,8). Using the ChIP assay as tool to understand how the NEDD8 pathway influences ER-mediated transcription we have been able to localize a component of the SCF complex to the pS2 promoter in an estradiol dependent manner. We have also shown ligand-dependent association between ERα and Skp1 by immunoprecipitation. In agreement with "Task 2" and "Task 3" in the original proposal, we investigated the expression of Uba3 in several tumor cell lines and compared its expression to that of ERα and PR. Finally we wished to investigate the potential for Uba3 mRNA expression to be inducible by estradiol, tamoxifen, raloxifene, and progesterone.

BODY

We have previously reported the characterization of hUba3 and APP-BP1 as nuclear receptor cofactors capable of functioning as coactivators in transient transfection assays in HeLa cells. Based on our experiements with hUba3 and Ubc12, we concluded that the NEDD8 pathway may play an important role in nuclear receptor-dependent transcription. Since we were not able to identify any physical interactions between hUba3 and PR and ER *in vitro* and *in vivo*, it was thought that hUba3 may interact with other protein components involved in transcription. We therefore developed a chromatin immunoprecipitation (ChIP) assay system to characterize the involvement of hUba3 during estrogen-dependent pS2 transcription in MCF-7 cells. Using an antibody we generated in rabbit, we attempted to localize hUba3 to the pS2 promoter upon administration of estradiol (Figure 1a). Unfortunately, we were not able to recruit hUba3 to the pS2 promoter whereas we were able to see recruitment of ERα (data not shown). We modified the ChIP assay detergents in the immunoprecipitation step to optimally precipitate hUba3 protein, but these alterations did not the change the results we observed in the ChIP assay. We

surmised that hUba3 and the NEDD8 pathway might be influencing ER-mediated transcription through the SCF E3 ligase complex. Using an antibody against one of the core proteins of the SCF complex, Skp1, we were able to see its recruitment to the pS2 promoter upon addition of estradiol (figure 1a). The recruitment was more profound when MG132, a proteasome inhibitor, was used in the experiment. We interpreted this result to mean that the interaction of the SCF E3 ligase complex with the pS2 promoter is likely transient and unstable due to the degradation-promoting (ubiquitylation) activity of this complex. To determine if ERα could interact with Skp1, we performed a co-immunoprecipitation experiment by immunoprecipitating Skp1 and detecting ERα (figure 1b). The ERα was fused to the N-terminus of luciferase, and therefore light units were used to quantify the interaction between Skp1 and ERα instead of a Western blot. The results show that ERα interacts with Skp1 in the presence of estradiol and this result agrees with the results we observed in the ChIP assay. Our results support a model where hUba3 and the NEDD8 pathway influence ER-dependent transcription through the SCF complex. Consistent with our results, hUba3 is not present at the promoter, but rather stimulates covalent attachment of NEDD8 to the Cullin protein within the SCF complex. This covalent modification activates the E3 ligase activity of the SCF complex, degrading components of the transcription apparatus. We are not able to conclude what the function of the ligase activity is, but the possibilities are intriguing. Ubiquitylation/degradation of transcription proteins may be necessary to permit promoter escape of RNA polymerase II. Ubiquitylation/degradation may also necessary to remove activated ligand-bound ERα from the promoter, thus attenuating estrogen signaling. Importantly, both possibilities may be correct; that is, activation of transcription and degradation are inherently coupled events. This way the cell has a mechanism to control the amount of transcription upon stimulation. Regardless of the correct downstream model, the data I have presented underscore the integration of the NEDD8 pathway in ER- and PR-dependent transcription.

Illumination of hUba3 and the NEDD8 pathway in ER- and PR-mediated transcription and the importance of ER and PR in breast cancer development support the possibility that disrupted expression of NEDD8 pathway proteins may play an important role in breast cancer. To this end we to wished to study the expression of hUba3, ERα, and PR in several breast cancer cell lines. We chose to study hUba3 expression in 7 cell lines: 4 breast cancer cell lines and 3 'non-breast' cancer cell lines. We included 3 'non-breast' cancer cell lines to infer expression of hUba3 in other cancer cell lines. (Details of the cell lines are described in figure 2.) Using real-time Taqman quantitative RT-PCR, we assessed the mRNA expression of hUba3, ERα, and PR (figure 2). The level of hUba3 mRNA was highest in HeLa cells and lowest in ZR-75 cells. Comparing hUba3 mRNA expression across all cancer cell lines, we were not able to observe significant differences between the breast cancer cell lines and the other cancer cell lines (Figure 2, compare MCF-7, T-47D, MDA, ZR-75 with that of DU145, Iahikawa, and Hela cell lines). Perhaps more importantly, hUba3 mRNA expression did not correlate with ER and PR mRNA expression in any of the cell lines tested (figure 2).

While these studies were ongoing, a group published a report that the overexpression of hUba3 promotes degradation of the ERα protein through the SCF E3 complex suggesting that regulation of ERα by the NEDD8 pathway occurs at the protein level (9). It is possible that hUba3 can be regulated by ER and/or PR, providing a negative feedback on ER and/ or PR signaling. To expedite this possibility we searched the hUba3 promoter (-5000bp to –1bp) for ER and PR binding sites *in silico* using MatInspector v2.2 software with the Transfac Database (transcfac.gbd.de). The results of the *in silico* search are shown in Table 1. We were able to find 5 ERE half sites and 1 PR half site, but we were not able to find any full palindromes. We limited our stringency to half sites because several ER target genes, for example PR and c-Myc, are known to be regulated by ER half-sites. We investigated the induction of hUba3 mRNA expression using real-time quantitative RT-PCR in the above mentioned cancer cell lines. We did not observe any induction of hUba3 mRNA by progesterone or estrogen, or co-stimulation

by both estrogen and progesterone (figure 3 and 4A). Rather, the expression of hUba3 mRNA is refractory to progesterone and estrogen in all cell lines tested. We also examined the repression of hUba3 mRNA expression by the SERMs, tamoxifen and raloxifene. Synonymous with the results observed with estradiol and progesterone, repression of hUba3 is unaffected by tamoxifen or raloxifene (figure 4B).

KEY RESEARCH ACCOMPLISHMENTS

- We have successfully determined how hUba3 and the NEDD8 pathway influences ER-mediated transcription through additional experiments not outlined in "Task 1", but necessary to complete "Task 1."
    - Through ChIP assay analysis, we have been able to show that Skp1 is localized to the pS2 promoter with ERα following administration of estradiol.
    - By co-immunoprecipitation, we observed an estrogen-dependent interaction with Skp1 and ERα.
- We have completed most of "Task 2" by examining the mRNA expression of hUba3, ERα, and PR in several breast cancer cell lines.
    - We have shown that hUba3 mRNA expression is widely expressed in breast cancer cell lines and cell lines derived from other tissues.
    - hUba3 mRNA expression does not correlate with ER/ PR mRNA levels in the cancer cell lines tested.
    - hUba3 mRNA expression is unaffected by ligands to ER and PR.

REPORTABLE OUTCOMES

None

CONCLUSIONS

We have made substantial progress in understanding how hUba3 and the NEDD8 pathway influence ER- and PR-mediated transcription. From the experiments we outlined in "Task 1" we were able to conclude that hUba3 and APP-BP1 are unique in their own right, but were not able to understand how this pathway is integrated with NHR function, an important aspect of "Task 1". Because of this unforeseen situation, we added several important experiments that provided insight into the true function of the NEDD8 pathway in ER- and PR-mediated transcription. We now conclude that the SCF E3 ligase complex is recruited with ERα upon addition of estradiol to the pS2 promoter. Therefore, hUba3 (at a site other than the promoter) functions as a coactivator by stimulating 'neddylation' (NEDD8 conjugation) of the SCF E3 ligase complex (at the promoter), leading to enhanced ubiquitylation of unknown proteins within the transcription apparatus. It is not quite clear yet, but ubiquitylation and/ or proteasome-mediated degradation somehow aides the process of ER-mediated transcription.

We have made significant progress in characterizing the expression of hUba3 mRNA in several cancer cell lines in agreement with "Task 2". Of the 7 cancer cell lines we investigated, we were able to observe differences in hUba3 mRNA expression, but were not able to conclude that mRNA expression of hUba3 contributes to carcinogenesis. Furthermore, we were not able to observe any significant correlation between hUba3 mRNA expression, and ERα and PR mRNA expression. Based on a report that the NEDD8 pathway controls degradation of ERα protein, we hypothesized that ER and PR signaling may regulate hUba3, providing a negative feedback mechanism that would attenuate this signaling. We observed that hUba3 mRNA expression is refractory to estrogen and progesterone signaling. Additionally, we were unable to alter hUba3 expression with the SERMs tamoxifen and raloxifene. We conclude that the mRNA expression of hUba3 is constant and unaffected by ER and PR signals. It is possible, and even likely that most differences among ER, PR, and hUba3 will be observed at the protein level. Comparison of hUba3 protein expression with ER and PR expression in tumor biopsy samples is the focus of "Task 3".

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APPENDIX:

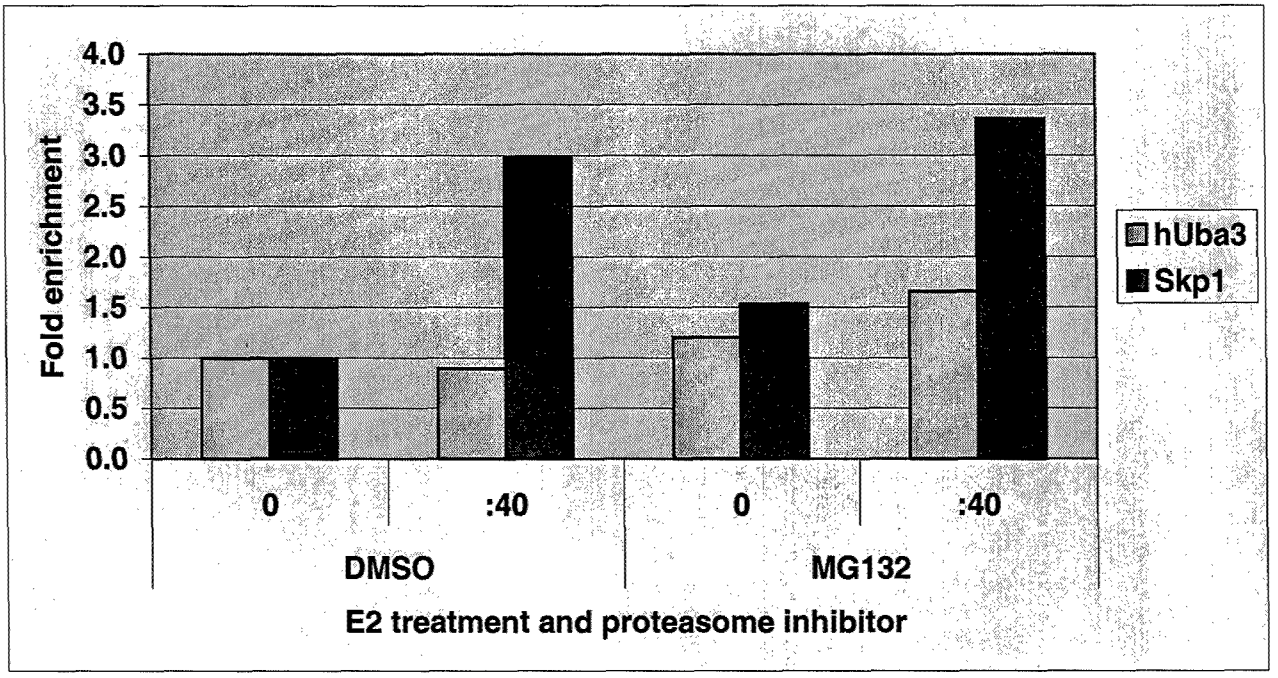

Figure 1A. Skp1, but not hUba3 is recruited to the pS2 promoter following admistration of estradiol in MCF-7 cells. Cells were treated with DMSO or MG132 with or without estradiol for 40 minutes. The data were quantified using real-time PCR. The DMSO 0 zero time point was made to equal 1 and 'fold enrichment' was calculated from the ΔCt values. The data were normalized to input DNA and a non-specific internal control (not seen).

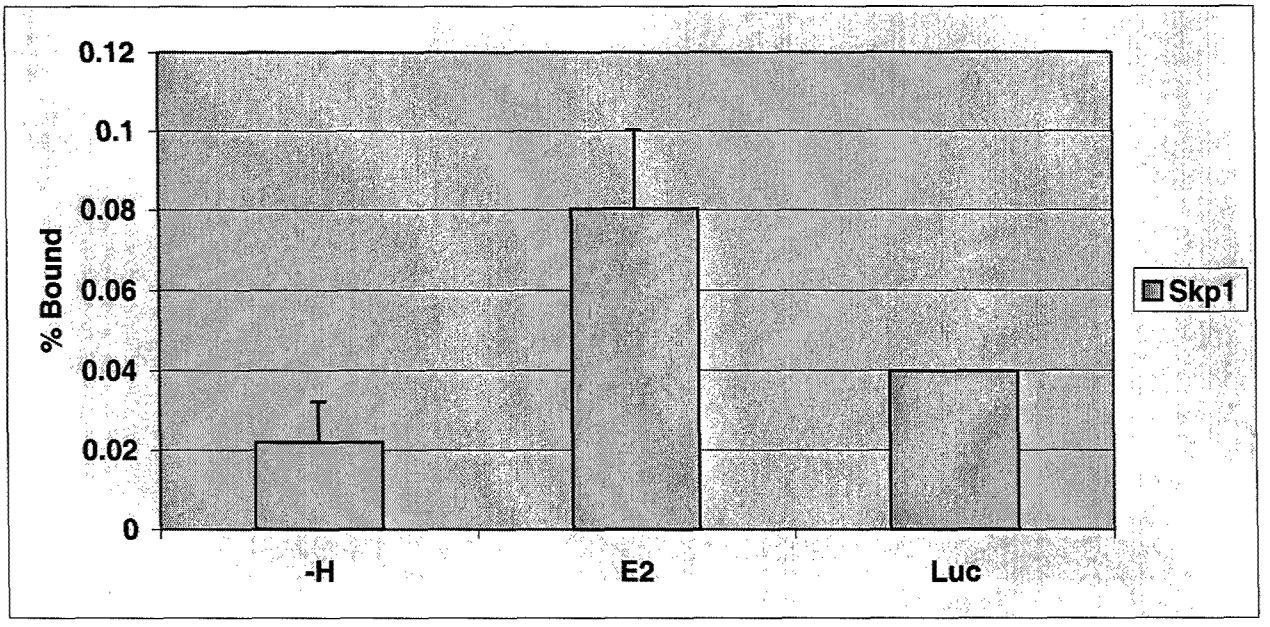

Figure 1B. Skp1 interacts with ERα in the presence of estradiol. ERα-luciferase fusion protein was transcfected into HeLa cells and immunoprecipitated with the Skp1 antibody. Skp1 interacts with ERα approximately 4-fold in the presence of estradiol relative to absence of hormone. Luciferase alone was used as a negative control. Data are presented as a percent of input from each condition. –H, no hormone. E2, addition of estradiol. Luc, luciferase negative control.

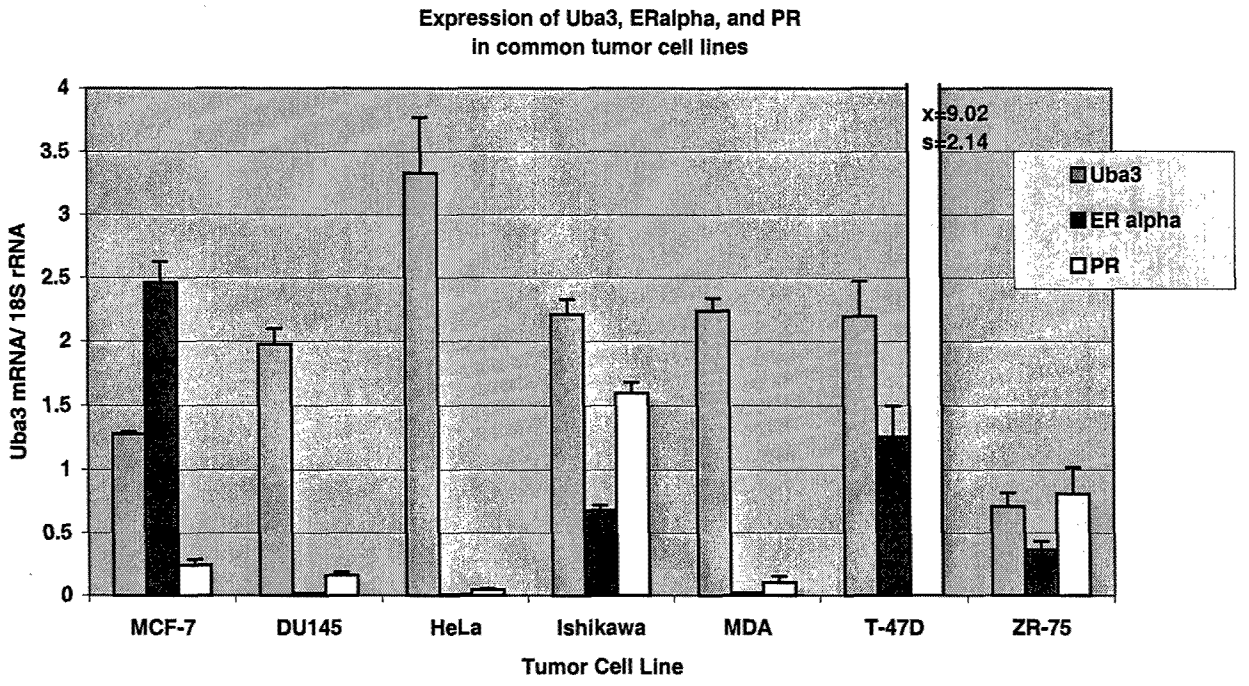

Figure 2. mRNA expression analysis of hUb3, ERα, and PR is common cancer cell lines. **MCF-7** (breast cancer cell line), ERα$^+$, PR inducible; **DU145** (prostate cancer cell line), ERα$^-$, PR non-inducible; **HeLa** (cervical carcinoma cell line), ERα$^-$, PR non-inducible; **Ishikawa** (uterine cancer cell line), ER$^+$, PR inducible; **MDA-MB-231** (breast cancer cell line), ERα$^-$, PR non-inducible; **T-47D** (breast cancer cell line), ER$^+$, PR$^+$; **ZR-75-1** (breast cancer cell line), ERα$^+$, PR inducible. Using real-time quantitative RT-PCR, the cell lines were screened for mRNA expression of hUba3, ERα, and PR. The results were normalized to 18S rRNA and compared across each cell line. Expression of PR in T-47D cells is known to be high and was modified for clarity in the figure. The expression is 9.02 and the standard deviation is 2.14.

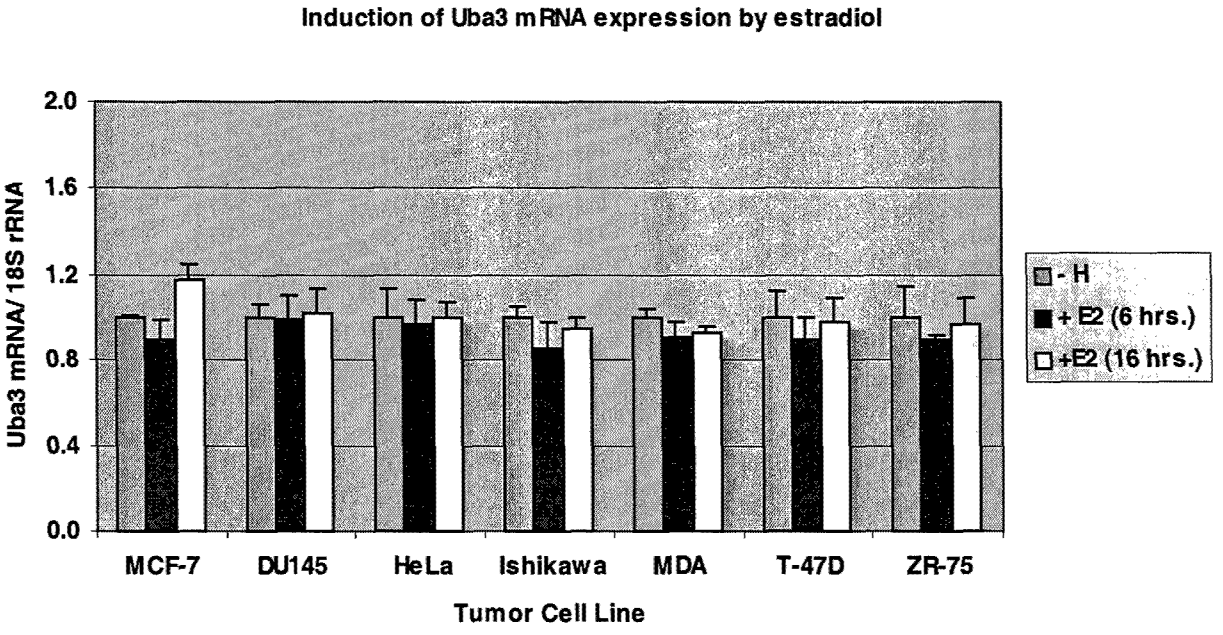

Figure 3A. Induction of hUba3 mRNA expression by estradiol in the same 7 cell lines as in figure 2. Expression was assayed at 6 and 16 hours following addition of estradiol. The zero time point (-H) was made equal to 1 and the other time points were made relative to -H. Analysis performed by real-time quantitative RT-PCR. Values were normalized to 18S rRNA. -H, no hormone. +E2, addition of estradiol.

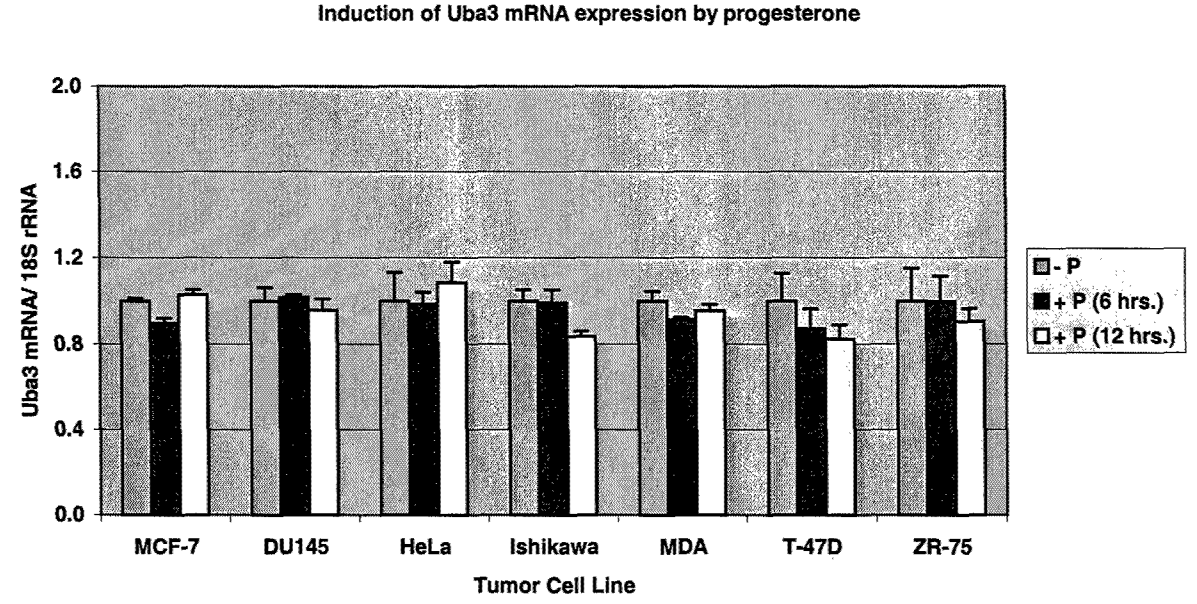

Figure 3B. Induction of hUba3 mRNA expression by progesterone in the same 7 cell lines as in figure 2. Expression was assayed at 6 and 16 hours following addition of progesterone. The zero time point (-H) was made equal to 1 and the other time points were made relative to -H. Analysis performed by real-time quantitative RT-PCR. Values were normalized to 18S rRNA. -P, no hormone. +P, addition of progesterone.

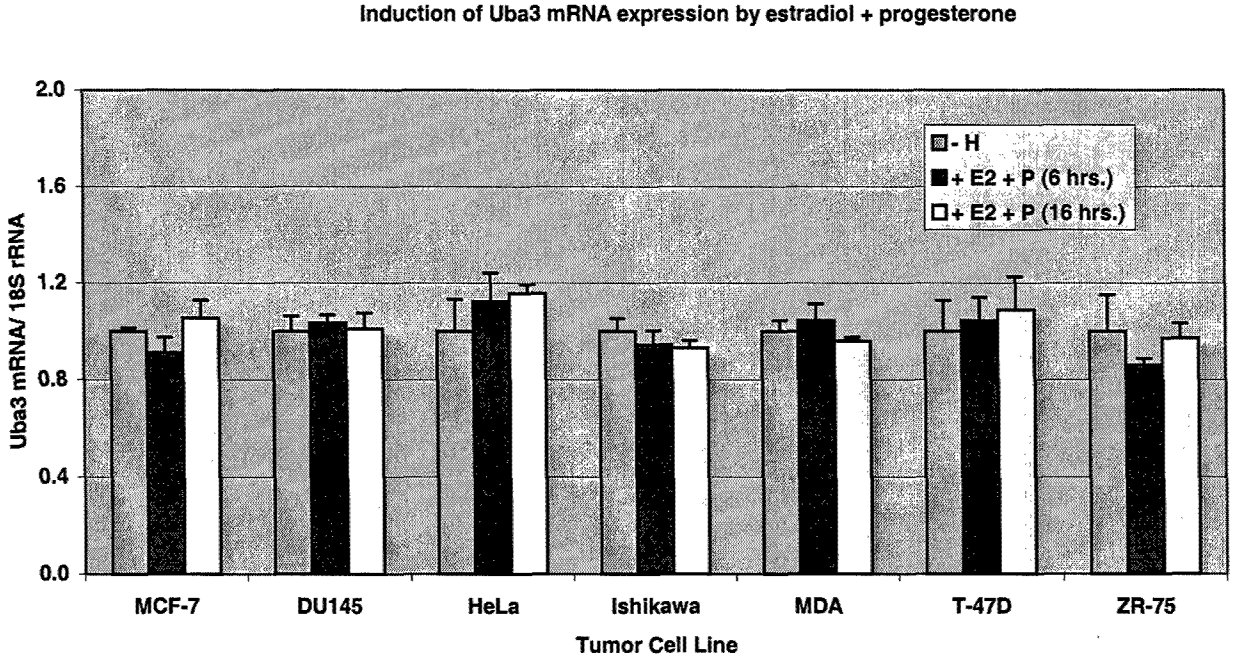

Figure 4A. Induction of hUba3 mRNA expression by progesterone and estrogen in the same 7 cell lines as in figure 2. Expression was assayed at 6 and 16 hours following addition of progesterone and estrogen. The zero time point (-H) was made equal to 1 and the other time points were made relative to -H. Analysis performed by real-time quantitative RT-PCR. Values were normalized to 18S rRNA. –H, no hormone. +E2 +P, addition of estrogen and progesterone.

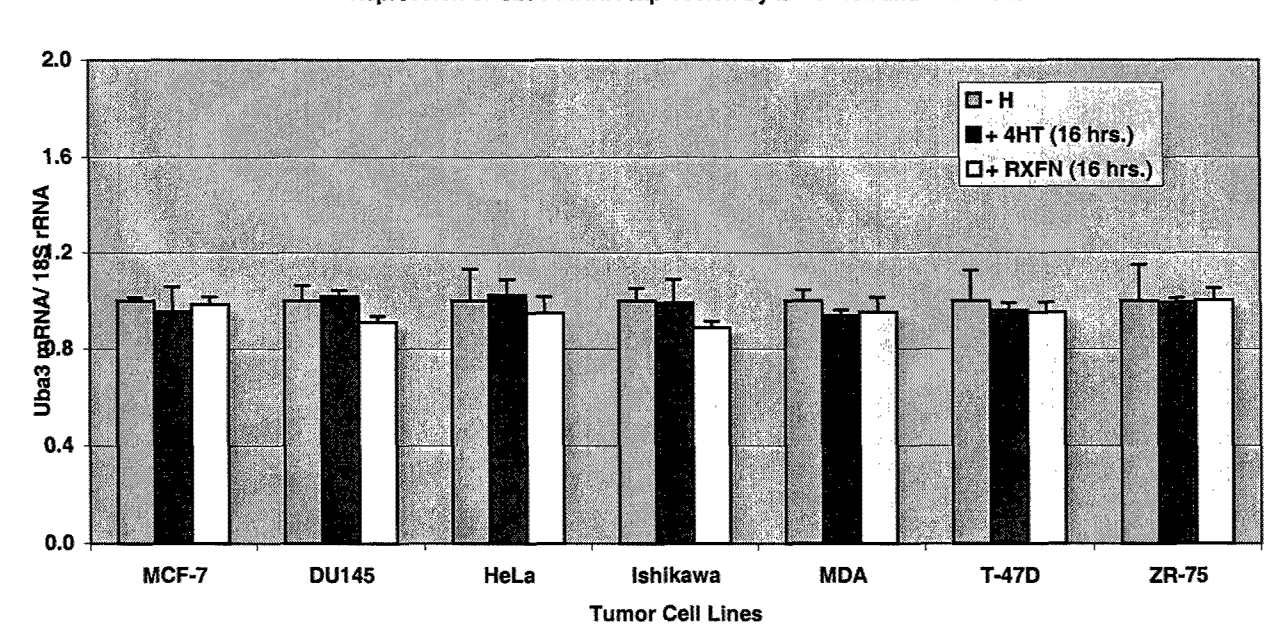

Figure 4B. Repression of hUba3 mRNA expression by tamoxifen and raloxifene in the same 7 cell lines as in figure 2. Expression was assayed at 6 and 16 hours following addition of SERM. The zero time point (-H) was made equal to 1 and the other time points were made relative to -H. Analysis performed by real-time quantitative RT-PCR. Values were normalized to 18S rRNA. –H, no hormone. 4HT, 4 hydroxytamoxifen. RXFN, raloxifene.

Table 1: Results of *in silico* search for ER and PR binding sites within the Uba3 promoter.

Binding Site	Binding site location relative to Tx start site (strand of DNA)	Core Similarity	Matrix Similarity	Sequence
ER	- 2149 (+)	1.000	0.891	tacagtgaatcTGACccct
ER	- 2510 (-)	1.000	0.900	tcagaactgcaTGACcaaa
ER	- 2610 (+)	1.000	0.872	cactctcatctTGACcaag
PRE	- 3925 (-)	1.000	0.875	tgaactagtTGTTctg
ER	- 4105 (+)	1.000	0.891	tctgttctacaTGACcttc
ER	- 5500 (-)	1.000	0.865	gacagagaaagTGACcacg