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Transfection of human prostate cancer CA-HPV-10 cells with cytosolic sulfotransferase SULT1E1 affects estrogen signaling and gene transcription

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SULT1E1 transfection and signal transduction

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ABBREVIATIONS

CA-HPV-10, a transformed cell line derived from a primary prostate adenocarcinoma; C_T, threshold cycle; ER-α, estrogen receptor alpha; ERE, estrogen response element; LNCaP, a transformed cell line derived from a metastatic prostate carcinoma; SEAP, secreted alkaline phosphatase; SULT1E1 (GenBank number: NM_005420), human estrogen sulfotransferase; TFRC (GenBank number: NM_003234), a gene encoding a transferrin receptor; TMEPAI (GenBank number: NM_020182), a gene encoding an androgen induced mRNA product.

ABSTRACT

Human cytosolic sulfotransferase SULT1E1 catalyzes the sulfation of estrogens and estrogenic drugs in human reproductive tissues. Logically, this estrogen-preferring sulfotransferase isoform could play a regulatory role in estrogen signaling activities in human reproductive cells including the prostate cells. This hypothesis was tested using DNA microarray and real-time RT-PCR methods in the present work. Potential changes in the transcriptional expression of selected signal transduction-related genes in human prostate cancer CA-HPV-10 cell line following SULT1E1 transfection were examined by DNA microarray methods. Notable changes were observed in the mRNA expression levels of TFRC, a cell membrane transferrin receptor gene, and TMEPAI, a gene encoding a steroid-dependent mRNA product. Expression of TFRC was down-regulated while expression of TMEPAI was up-regulated by SULT1E1 transfection in CA-HPV-10 cells. Data from the current studies also showed that the estrogen induced estrogen response element activation in CA-HPV-10 cells was repressed after the cells were transfected with SULT1E1. These results indicate that SULT1E1 may function as a transcriptional mediator in human prostate cancer CA-HPV-10 cells.

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INTRODUCTION

Human sulfotransferase SULT1E1 catalyzes the sulfation of endogenous and exogenous estrogenic chemicals (Duffel et al., 2001; Nishiyama et al., 2002; Schrag et al., 2004; Song, 2001; Strott, 1996). This reaction involves the transfer of a sulfuryl group from 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to an estrogen molecule such as β-estradiol producing adenosine 3', 5'-diphosphate (PAP) and an estrogen sulfuric acid ester. Addition of a sulfuryl group changes the chemical nature of estrogenic substrates making them more water-soluble. The expression of human sulfotransferase SULT1E1 can be detected in selected normal and cancerous reproductive tissues and the tissue distribution of SULT1E1 varies considerably with gender and age (Demyan et al., 1992; Duanmu et al., 2006; Dunn and Klaassen, 1998; Song et al., 1997; Whitnall et al., 1993).

Estrogens are a group of steroid compounds that play important regulatory roles in the development and propagation of human reproductive malignancies such as breast and prostate cancers (Bosland, 2000). In the estrogen receptor (ER)-positive cells, estrogens and estrogenic drugs mainly exert their biological effects through the ER-coupled signal transduction pathways. In addition, estrogens may also interact with other signal transduction pathways involving conventional second messengers such as G-protein, free intracellular calcium, and mitogen-activated protein kinase (Cato et al., 2002; Falkenstein et al., 2000; Nabekura et al., 1986; Zakon, 1998).

The regulatory mechanisms for the estrogen-initiated or estrogen-mediated signal transduction processes in human reproductive cancer cells are intrinsic. One of the potential mechanisms is that the availability of free estrogen molecules in the cell is modulated by the presence of estrogen biotransformation enzymes such as SULT1E1. Logically, human estrogen sulfotransferase SULT1E1 could serve as a regulator of the estrogen-related signal transduction processes by controlling the number of active estrogenic molecules in the cell. This hypothesis has been supported by the results

from previous studies suggesting that SULT1E1 is involved in the regulation of cell growth response to estrogen signaling (Falany et al., 2002; Qian et al., 1998).

The present work is aimed at identifying the potential specific intracellular signaling processes regulated by cytosolic sulfotransferases. We seek to gain new insights into the molecular basis for the regulatory rules of SULT1E1 in intracellular signal transduction and gene transcription. In this work, the transcriptional changes of a group of genes related to various intracellular signaling processes in human prostate cancer CA-HPV-10 cells by SULT1E1 transfection were investigated using DNA microarray and real-time RT-PCR methods. Results from the current studies showed that SULT1E1 transfection altered the mRNA expression levels of TFRC, a human gene locus encoding a transferrin receptor, and TMEPAI, a gene encoding a steroid-dependent protein product. In addition, the molecular mechanisms responsible for the regulatory effects of SULT1E1 on gene transcription were also studied.

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MATERIALS AND METHODS

Chemicals-β-estradiol, β-estradiol sulfate and tamoxifen were obtained from Sigma-Aldrich (St.Louis, MO). 3-*(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide* (MTT) was obtained from Calbiochem (San Diego, CA).

Cell culture-Human prostate cancer CA-HPV-10 cell line was purchased from American Type Culture Collection (ATCC) and routinely cultured in Keratinocyte Serum Free Medium (Invitrogen, Carlsbad, CA) with 0.1 ng/mL epidermal growth factor and 25 μg/mL bovine pituitary extract according to the ATCC guidelines. All cell culture experiments were performed in strict aseptic conditions in a class II biological hood.

Construction of the SULT1E1 mammalian expression vectors-The full-length cDNA for the coding region of SULT1E1 (Genbank number: NM_005420) (Aksoy et al., 1994) was isolated from human liver total RNA (Agilent Technologies, Santa Clara , CA) by RT-PCR using the gene specific primers listed in Table 1. The isolated SULT1E1 cDNA was then inserted into a pTargeT mammalian expression vector (Promega, Madison, WI). Final vector construct was verified by DNA sequencing.

Generation of the SULT1E1-transfected CA-HPV-10 cell line- The wild-type CA-HPV-10 cells were transfected with the SULT1E1-pTargeT vector using SuperFect™ transfection reagent from Qiagen, Valencia, CA according to the manufacturer's instructions. The transfected cells were selected and maintained with G418 sulfate (1 mg/mL; Promega) added to the growth medium.

DNA microarray analysis-Total RNA samples were isolated from the harvested cell samples using an RNeasy Mini kit (Qiagen), amplified and biotin-labeled with a TrueLabeling-AMP™ Linear RNA Amplification kit (SuperArray, Frederick, MD) according to the manufacturer's instructions. The resulting cRNA probes from the SULT1E1-transfected and empty pTargeT vector-transfected CA-HPV-10 cell samples were hybridized in parallel with the Oligo GEArray Human Signal Transduction PathwayFinder Microarray Blot (SuperArray) that encodes 113 genes related to various steroid nuclear

receptor- and cell membrane receptor-coupled signal transduction pathways. The biotin-labeled cRNA probes were hybridized to the microarray blot membranes at 60 °C overnight. CDP-*Star* (SuperArray), a chemiluminescent alkaline phosphatase substrate, was used to detect the hybridized biotinylated probes on the membranes. Chemiluminescence was measured using a ChemImager 5500 imaging system (Alpha Innotech, San Leandro, CA). The data were quantitatively analyzed using the GEArray Expression Analysis Suite software (SuperArray).

MTT colorimetric assay-The empty pTargeT vector transfected CA-HPV-10 cells were seeded in 96-cell plates in the subculture medium (100 μL), and treated with tamoxifen at various concentrations. After 24 hours, 10 μL of MTT (5 mg/mL) was added to each well, and the cells were further incubated for five more hours before the medium was discarded. The crystals in each well were then dissolved in 200 μL of DMSO. The optical density at 570 nm was then determined using an MRX microplate reader (Dynex Technologies, Chantilly, VA). The proliferation rates were calculated from the OD readings with various drug treatments using the OD reading from the DMSO-treated cells as 100%.

Quantitative real-time RT-PCR-Total RNA samples were prepared using the PureLink Total RNA Purification Kit (Invitrogen). Measurement of gene expression by quantitative real-time RT-PCR was carried out using the gene-specific primers for TFRC and β-actin (Table 1) and the ABsolute™ MAX QRT-PCR SYBR Green Mix from ABgene, Rochester, NY according to the manufacturer's instructions. Samples were simultaneously cycled and monitored by an MX3000P quantitative real-time PCR instrument (Agilent Technologies, Santa Clara, CA).

To study the molecular mechanism underlying the regulatory effect of SULT1E1 on TFRC expression, the SULT1E1-pTargeT- and empty pTargeT-transfected cell lines were treated with 1 nM tamoxifen for 24 hours and subjected to real-time RT-PCR analysis using gene specific primers (Table 1). Tamoxifen was first dissolved in DMSO to form a stock solution and then added to the growth

medium. The final concentration of DMSO in culture medium was always less than 1% (v/v) and the control cultures received the same value of DMSO as the tamoxifen-treated cultures.

Estrogen response element (ERE) reporter assay-To examine the effect of SULT1E1 transfection on the estrogen-induced ERE activation, human CA-HPV-10 cells were co-transfected with the SULT1E1-pTarget vector and the pERE-TA-SEAP vector from BD Biosciences, Mountain View, CA. The pERE-TA-SEAP vector encodes an ERE region followed by a TA promoter. A secreted alkaline phosphatase (SEAP) reporter gene is also located downstream of the TA promoter. pTA-SEAP (BD Biosciences), a vector without the ERE sequence, was used as a control vector. The experimental cells were treated with 100 nM β-estradiol or 100 nM β-estradiol sulfate. The reporter gene SEAP product was quantitatively assayed using a Great EscAPe SEAP Fluorescence Detection kit (BD Biosciences). The fluorescence signal (excitation/emission wavelengths, 360/449 nm) was read by a Spectramax M5 fluorescence plate reader (Molecular Devices; Sunnyvale, CA).

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RESULTS

SULT1E1 transfection down-regulated the mRNA expression of TFRC in human prostate cancer CA-HPV-10 cells.

A SULT1E1-pTargeT vector transfected CA-HPV-10 cell line was developed and characterized by RT-PCR analysis in the current studies (Figure 1). An empty pTargeT vector-transfected CA-HPV-10 cell line was also developed as a control. DNA microarray experiments with a focused gene microarray blot (Figure 2) were carried out as a first approach to test whether SULT1E1 transfection affects any known cytosolic signal transduction pathway in human CA-HPV-10 prostate cancer cells. The oligo DNA microarray blot (SuperArray) used in the current work was designed based on current knowledge on cellular signal transduction pathways.

As shown in Figure 2, data from the DNA microarray analysis indicated that SULT1E1 transfection down-regulated the transcriptional level of the TFRC gene (GenBank number: NM_003234), which encodes a transferrin receptor. The DNA microarray results were confirmed by real-time RT-PCR experiments (Figure 3). The difference between the TFRC mRNA levels of the SULT1E1-pTargeT and empty pTargeT transfected cell lines was statistically significant ($p<0.05$).

Effect of tamoxifen on the mediation of TFRC expression by SULT1E1 transfection in human CA-HPV-10 cell line

Tamoxifen is a selected estrogen signaling mediator that competitively binds to ERs. In the present studies, 1 nM tamoxifen was used to test the role of ERs in the SULT1E1 effect on TFRC transfection. At 1 nM, tamoxifen does not significantly change the cell proliferation rate of human CA-HPV-10 cells (Figure 4). The modification of the SULT1E1 effect on TFRC transfection by tamoxifen was determined by real-time RT-PCR methods. TFRC expression was relatively quantified by the ΔC_T (C_T of TFRC – C_T of β-actin) value, which is inversely proportional to the number of target mRNA copies

present in the cell sample. After the treatment of 1 nM tamoxifen, the TFRC mRNA expression level in the empty pTargeT transfected CA-HPV-10 cells was not down-regulated but instead it was slightly up-regulated ($p<0.05$). The down-regulation of TFRC expression by SULT1E1 transfection was also reversed after tamoxifen treatment (Figure 3).

Effect of SULT1E1 transfection on β-estradiol-activated ERE reporter gene expression in human CA-HPV-10 cells

The expression of human estrogen receptor alpha (ER-α) in human CA-HPV-10 cells has been reported previously (Cheung et al., 2005). The ER-α mRNA expression was also verified in the present work with RT-PCR methods and gene specific primers (Lau et al., 1999) (Table 1; Figure 1). The ERE activation by 100 nM β-estradiol was detected after CA-HPV-10 cells were transfected with the pERE-TA-SEAP vector. The ERE was also activated when the cells were treated with 100 nM β-estradiol sulfate. The reporter-gene responses were reversed when the cells were co-transfected with the SULT1E1-pTargeT vector (Figure 5).

TMEPAI expression was up-regulated by SULT1E1 transfection in human CA-HPV-10 cell line.

Additionally, the mRNA expression level of TMEPAI (GenBank number: NM_020182) was also significantly altered by SULT1E1 transfection (Figure 2). The TMEPAI gene locus is located on chromosome 20q13 and encodes an androgen-induced transcriptional product (Xu et al., 2003).

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DISCUSSION

Growth and maintenance of human reproductive cells are largely controlled by the actions of sex steroid hormones including estrogens. The molecular mechanisms responsible for the mediation of estrogen actions in human reproductive tissues are intrinsic. The presence of SULT1E1 in the reproductive tissues such as the prostate has been reported in the literature (Nakamura et al., 2006; Takase et al., 2007). In the ER-positive cells, SULT1E1 could serve as a mediator of estrogen-activated signal transduction processes due to its involvement in estrogen biotransformation. Previous studies have shown that SULT1E1 was able to mediate cell growth response of human reproductive cells to estrogen stimulation (Falany et al., 2002; Qian et al., 1998). In the current studies, new evidence that supports the hypothesis that SULT1E1 can function as an estrogen signaling mediator in human ER-positive prostate cancer cells was obtained.

The hypothesis that SULT1E1 functions as an estrogen signaling and transcriptional mediator was first tested via knowledge-based DNA microarray methods in human prostate cancer CA-HPV-10 cell line. Human prostate cancer CA-HPV-10 cell line is an ER-positive cell line derived from a primary human prostate adenocarcinoma (Cheung et al., 2005; Weijerman et al., 1994). The microarray blot used in this experiment covers 113 genes associated with various steroid nuclear receptor- and cell membrane receptor-coupled signal transduction pathways. These genes are either signal transduction target genes such as TMEPAI or genes encoding cytosolic second messengers such as protein kinase C. The reason that both steroid nuclear receptor- and cell membrane receptor-coupled signaling pathways were studied by the DNA microarray experiment is that, although the biological effects of estrogens are primarily attributed to the regulation of the target gene expression by activating estrogen nuclear receptors, they have also been linked to other signal transduction systems involving cell membrane receptors and conventional second messengers (Cato et al., 2002; Falkenstein et al., 2000; Nabekura et al., 1986; Zakon, 1998).

The mRNA levels of two genes, TFRC and TMEPAI, among the genes tested by the DNA microarray analysis were significantly altered after SULT1E1 transfection (Figure 2). The mRNA expression of TFRC was significantly down-regulated by SULT1E1 transfection (Figure 2). TFRC encodes a transferrin receptor, which is responsible for the regulation of cellular uptake of iron from transferrin, a plasma protein (Schneider et al., 1984). Transferrin receptors are also involved in the transport of drugs including chemotherapeutic agents across the cell membrane (Qian et al., 2002). The expression of transferrin receptors on the cytoplasm membrane is estrogen-inducible (Poola and Kiang, 1994; Poola and Lucas, 1988). Data from the present studies support the hypothesis that this estrogen-inducible TFRC expression can be mediated by SULT1E1.

A possible signaling pathway responsible for the SULT1E1 effect on TFRC expression would be the ER/ERE coupled signaling pathway. In ER-positive cells, estrogenic molecules, such as β-estradiol, mainly exert their genomic effects by binding to ERs and the resulting estrogen-bound ERs are able to activate EREs which control target gene transcription in the cell nucleus. Based on previous studies, human CA-HPV-10 cell line is an ER-positive cell line (Cheung et al., 2005). The expression of ER-α, which is responsible for ERE activation (Klinge, 2001), was also confirmed by RT-PCR and gene specific primers in the current studies (Figure 1). With a pair of ER-α gene specific primers designed by Lau and co-workers, the wild-type ER-α transcript along with an ER-α variant mRNA was detected (Figure 1) (Lau et al., 1999). Thus, it is possible that the modification of TFRC expression by SULT1E1 transfection in CA-HPV-10 cells was related to the ER/ERE coupled signaling processes. This hypothesis has been further tested with ERE reporter gene assay in the current studies (Figure 5). The data showed that the ERE activation by β-estradiol, as indicated by the reporter gene SEAP product, was significantly repressed after SULT1E1 transfection (Figure 5). This supports the hypothesis that SULT1E1 is able to regulate the ER/ERE coupled signaling activities. The data also

showed that the ERE reporter gene expression level was increased after the cells were treated with 100 nM β-estradiol sulfate (Figure 5). Currently, it is not clear that the β-estradiol sulfate induced ERE activation is caused by the direct action of β-estradiol sulfate on ERs or the effect of β-estradiol produced by the hydrolysis of the β-estradiol sulfate molecule in the cell.

If SULT1E1 regulates TFRC expression through an ER-coupled signaling pathway, the effect of SULT1E1 would be similar to those produced by ER antagonists. In the current studies tamoxifen was initially used as an ER antagonist to test this assumption. However, the experimental results (Figure 3) showed that, at 1 nM, tamoxifen did not significantly down-regulate TFRC expression in the empty-pTargeT vector transfected CA-HPV-10 cells. Instead, tamoxifen treatment resulted in a slight increase of TFRC expression in the empty-pTargeT vector transfected control CA-HPV-10 cells. The down-regulating effect of SULT1E1 transfection on TFRC transfection was also eased after tamoxifen treatment (Figure 3). The unexpected effect of tamoxifen on TFRC expression could be explained by the fact that tamoxifen is a competitive antagonist as well as a weak agonist of ERs (Lerner and Jordan, 1990). In CA-HPV-10 cells, tamoxifen may act as a weak ER agonist and oppose the down-regulating effect of SULT1E1 on TFRC expression.

Alternatively, non-ER/ERE-coupled signaling mechanisms in human CA-HPV-10 cells could also be responsible for the regulatory effect of SULT1E1 on TFRC transfection. Firstly, estrogens are not the only steroid substrates of SULT1E1 in human prostate tissues. Besides estrogens, other endogenous steroid compounds such as pregnenolone and dehydroepiandrosterone (DHEA) are also the substrates of SULT1E1 (Falany et al., 1995; Petrotchenko et al., 1999). These steroids often work together with estrogens to maintain a balance of biological activities in the cell. Secondly, as mentioned above, the biological effects of estrogens have also been linked to cell membrane receptor coupled signal transduction systems involving conventional second messengers (Cato et al., 2002; Falkenstein et al.,

2000; Nabekura et al., 1986; Zakon, 1998). Some of these second messenger systems such as the protein kinase A and mitogen activated signal transduction pathways were also reportedly responsible for the regulation of transferrin receptor expression (Lok et al., 1995; Ouyang et al., 1993).

Results from the DNA microarray experiments also showed an up-regulated expression level of TMEPAI by SULT1E1 transfection (Figure 2). The TMEPAI gene locus encodes an androgen-induced transcriptional product that has been considered as an androgen signaling biomarker in human prostate cells (Xu et al., 2003). It has exhibited a high expression level in the prostate. We speculate that the molecular mechanism for the regulatory effect of SULT1E1 on TMEPAI expression might be related to both androgen and estrogen signaling pathways because, although SULT1E1 is an estrogen-preferring isoform, its substrates also include other hydroxysteroid molecules (Falany et al., 1995; Petrotchenko et al., 1999). In addition, previous studies also showed that estrogens were capable of interacting with a mutated androgen receptor in human prostate cancer LNCaP cells (Veldscholte et al., 1990). Currently, it is not clear whether or not this mutated androgen receptor is also expressed in CA-HPV-10 cells.

In summary, results from the current work provide new evidence indicating that SULT1E1 may function as an estrogen signaling and gene transcription mediator in human ER positive prostate cells such as human prostate cancer CA-HPV-10 cells. The transcriptional response of estrogen-inducible genes such as TFRC to estrogen stimulation could be controlled by the presence of human sulfotransferase SULT1E1. As the transferrin/transferrin receptor system is involved in the transport of irons as well as drugs and other xenobiotics across the cell membrane (Qian et al., 2002), the influence of SULT1E1 on transferrin receptor expression may thus have important pharmaceutical implications.

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LEGENDS FOR FIGURES

Figure 1. RT-PCR analysis. The total RNA samples, extracted from the SULT1E1-pTargeT- and empty-pTargeT-transfected CA-HPV-10 cells, were analyzed respectively with RT-PCR methods using the primers derived from the ER-α and SULT1E1 genes (Table 1). *Lanes M*: DNA molecular marker (GeneRuler DNA ladder mix, Fermentas, Burlington, Ontario, Canada); *lane 1:* Expression of the wild-type ER-α transcript along with an ER-α transcript variant (the shorter RT-PCR product) was detected in the empty pTargeT vector-transfected CA-HPV-10 cells; *lane 2*: the control mRNA expression of human β-actin in the empty pTargeT vector-transfected CA-HPV-10 cell line was determined by RT-PCR and gene specific primers (Table 1); *lane 3*: the mRNA expression of human SULT1E1 in the empty pTargeT vector transfected CA-HPV-10 cell line was not detectable by RT-PCR and gene specific primers (Table 1); *lane 4*: the control mRNA expression of human β-actin in the SULT1E1-pTargeT-trasfected CA-HPV-10 cell line was determined; *lane 5*: the SULT1E1 mRNA expression became detectable by RT-PCR and gene specific primers in human CA-HPV-10 cells after transfected with the SULT1E1-pTargeT vector.

Figure 2. SULT1E1 transfection alters the mRNA expression levels of TFRC and TMEPAI in human CA-HPV-10 cells. The total RNA samples from the SULT1E1-transfected and control cell lines were hybridized in parallel with the oligo GEArray Human Signal Transduction PathwayFinder Microarray blot that covers 113 genes related to various signal transduction pathways (Superarray). Hybridization patterns were determined using a FluroChem 5500 imaging system (Alpha Innotech) and the integrated density values (IDVs) were analyzed using the GEArray Expression Analysis Suite software (SuperArray). The expression levels of TFRC and TMEPAI were significantly altered (Boundary = 3.0). Data represent the average of three experiments.

Figure 3. Real-time RT-PCR analysis of TFRC expression. The SULT1E1-pTargeT transfected CA-HPV-10 cells and the empty pTargeT transfected cells were treated with 1 nM tamoxifen respectively. After 24 hours, the TFRC mRNA expression was determined by real-time RT-PCR analysis using gene specific primers (Table 1) for TFRC and β-actin. The data represent the average of eight replicates in each group and displayed as a percentage of the control values from the empty pTargeT-vector transfected cells treated with DMSO. * $P < 0.05$ as compared to the empty pTargeT-vector transfected cells treated with DMSO.

Figure 4. Cell proliferation assay. The dose-dependent effect of tamoxifen on the cell proliferation of the empty pTargeT vector transfected CA-HPV-10 cell line was determined by MTT analysis. Cells were treated with various concentrations of tamoxifen. The resulting purple color from the reduced MTT was analyzed colorimetrically at 570 nm. The proliferation rates were calculated from the average OD readings with various tamoxifen concentrations using the average OD reading from the DMSO-treated cells as 100%. The data shown in the current figure are the mean ± S.E of eight replicates in each case. * $P < 0.05$ as compared to the DMSO treated cells.

Figure 5. Effect of SULT1E1 transfection on the β-estradiol-induced ERE activation measured by SEAP reporter gene assay. The SEAP reporter gene assay was performed to determine the effect of SULT1E1 transfection on the β-estradiol-induced ERE activation in CA-HPV-10 cells. The data represents an average of eight replicates in each group and is displayed as a percentage of the fluorescence units exhibited by the pTargeT/pTA-SEAP co-transfected control cells treated with 100 nM β-estradiol. * $P < 0.05$ as compared to the pTargeT/pTA-SEAP co-transfected cells treated with 100 nM β-estradiol.

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Table 1. Primers used for RT-PCR experiments

Target gene	Primer sequence	GenBank number
SULT1E1(for cDNA cloning)		
Forward primer	5'-ATGAATTCTGAACTTGACTA-3'	NM_005420
Reverse primer	5'-TTAGATCTCAGTTCGAAACTTC-3'	
TFRC (for quantitative RT-PCR analysis)		
Forward primer	5'-GTGTGAGAGACTGGCAGGAA-3'	NM_003234
Reverse primer	5'-GCTGGTGAAGTCTGTGCTGT-3'	
ER-alpha(for RT-PCR analysis)		
Forward primer	5'-GTGGGAATGATGAAAGGTGG-3'	NM_000125
Reverse primer	5'-TCCAGAGACTTCAGGGTGCT-3'	
β-actin (for quantitative RT-PCR analysis)		
Forward primer	5'-ATGGATGATGATATCGCCGC-3'	NM_001101
Reverse primer	5'-ACCATCACGCCCTGGTGCCTG-3'	

Figure 1

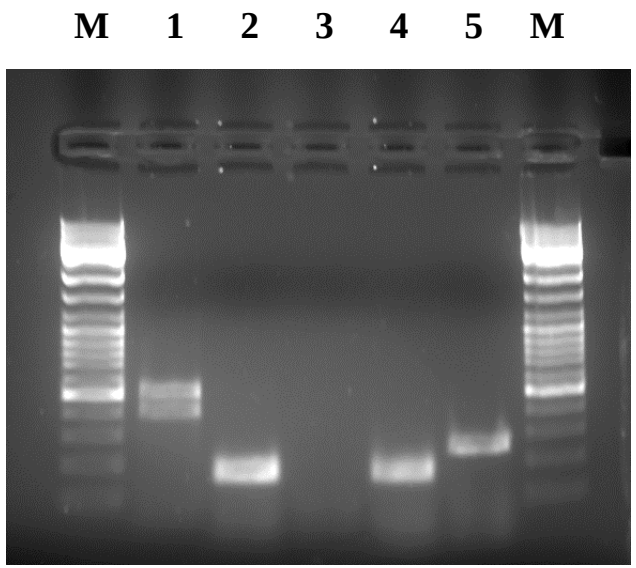

Figure 2

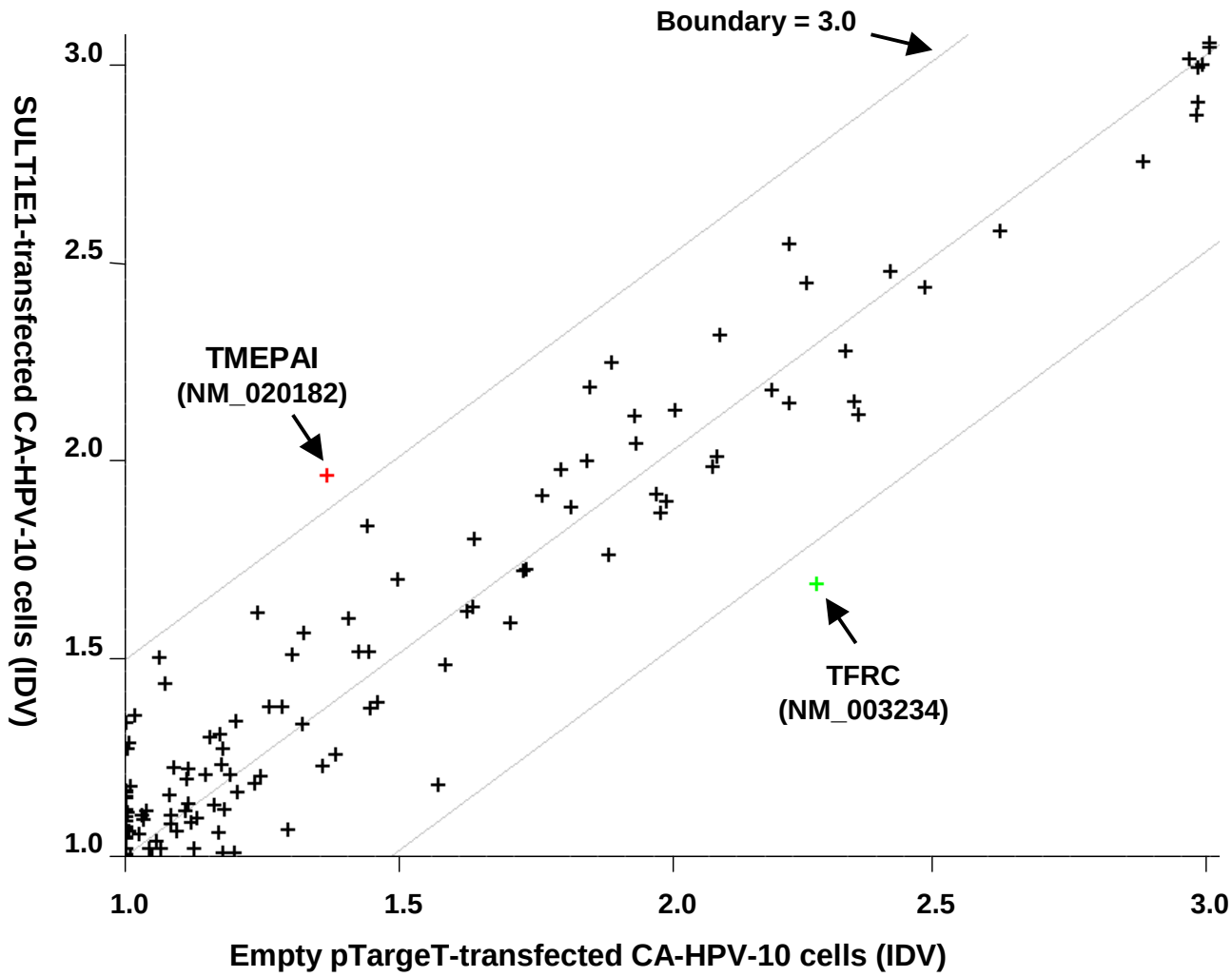

Figure 3

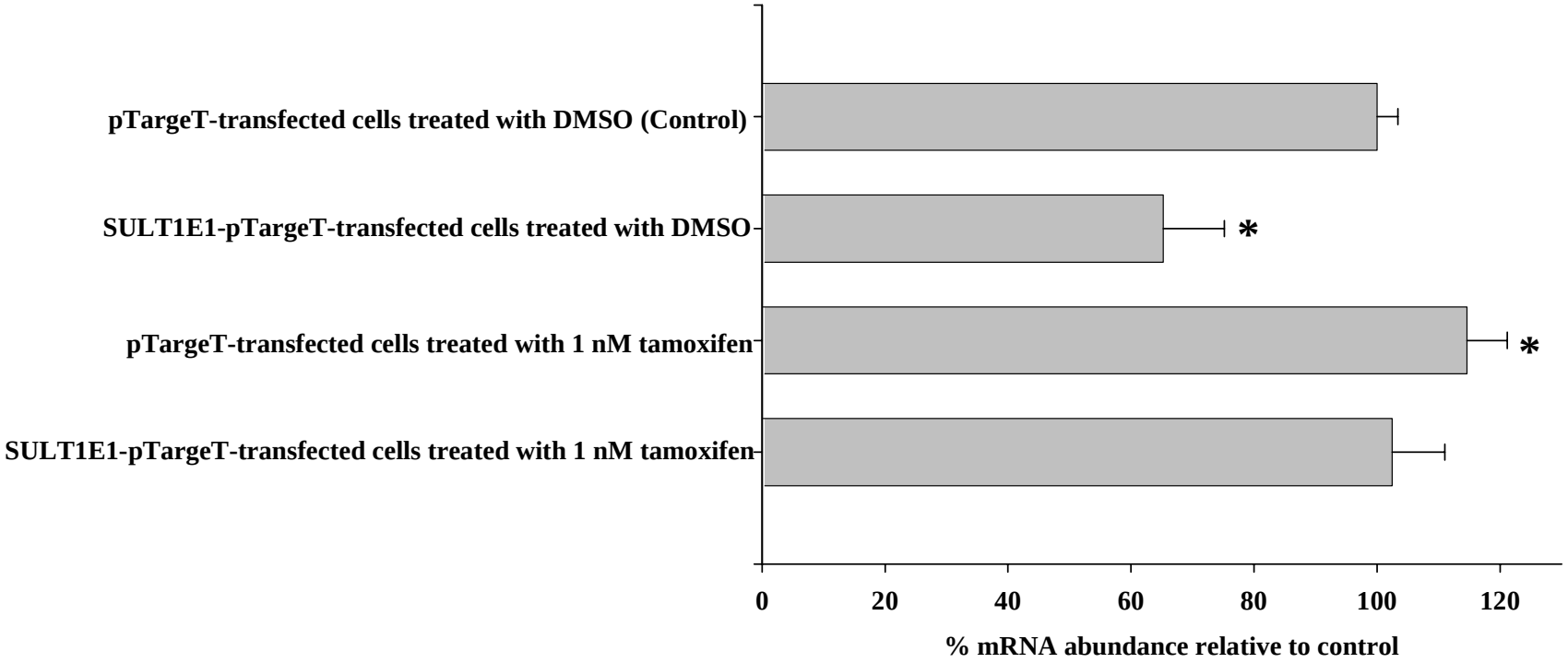

Figure 4

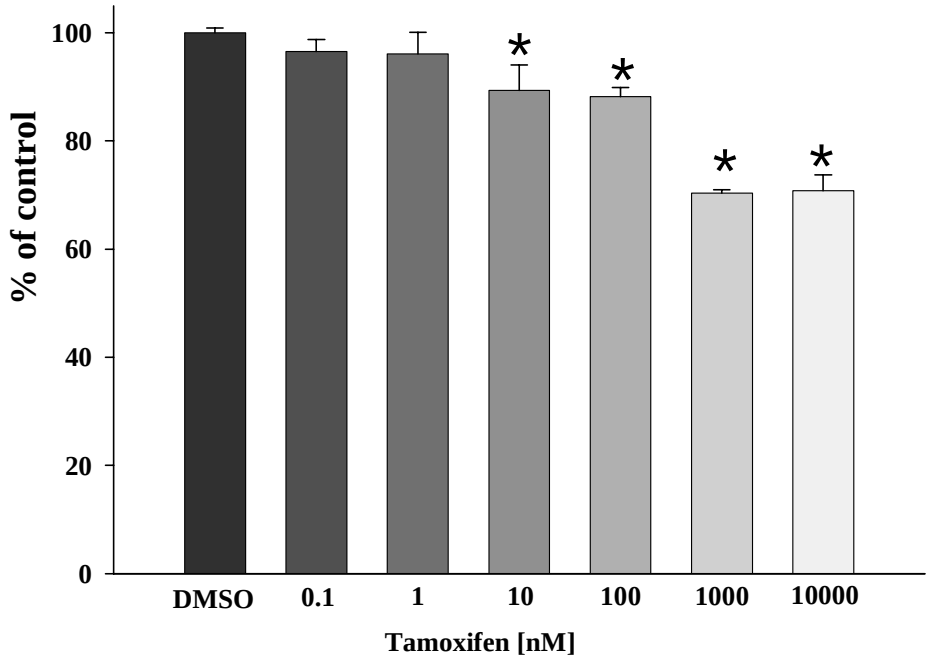

Figure 5