Suppression of Estrogen Receptor-Mediated Transcription and Cell Growth by Interaction with TR2 Orphan Receptor

Yueh-Chiang Hu, Chih-Rong Shyr, Wenyi Che, Xiao-Min Mu, Eungsoek Kim, and Chawnshang Chang*

From the George Whipple Laboratory for Cancer Research, Departments of Pathology, Urology, and Radiation Oncology, University of Rochester Medical Center, Rochester, NY 14642, USA

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* To whom correspondence should be addressed: University of Rochester Medical Center, 601 Elmwood Ave., Box626, Rochester, NY 14642. Tel.: 585-275-9994; Fax: 585-756-4133; E-mail: chang@urmc.rochester.edu.
Summary

The transcriptional activity of estrogen receptor (ER) is known to be highly modulated by the character and amount of coregulator proteins present in the cells. TR2 orphan receptor (TR2), a member of the nuclear receptor superfamily without identified ligands, is found to be expressed in the breast cancer cell lines and function as a repressor to suppress ER-mediated transcriptional activity. Utilizing an interaction blocker, ER-#6 (amino acid 312-340), responsible for TR2 interaction, the suppression of ER by TR2 could be reversed, suggesting that this suppression is conferred by the direct protein-protein interaction. Administration of antisense TR2 resulting in an enhancement of ER transcriptional activity in MCF7 cells indicates that endogenous TR2 normally suppresses ER-mediated signaling. To gain insights into the molecular mechanism by which TR2 suppresses ER, we found that TR2 interrupted ER DNA binding by disrupting the ER homodimerization as a result of the formation of ER-TR2 heterodimers. The suppression of ER transcription by TR2, consequently, caused the inhibition of estrogen-induced cell growth and G1/S transition in estrogen-dependent breast cancer cells. Taken together, in addition to the potential roles in spermatogenesis and neurogenesis, our data provide a novel biological function of TR2 that may exert an important repressor in regulating ER activity in mammary glands.
Introduction

The human TR2 orphan receptor (TR2), a member of the nuclear hormone receptor superfamily, was cloned from human testis and prostate cDNA libraries and has no identified ligand(s) (1,2). TR2 is mapped to locate on chromosome 12q22 (3), known to be frequently deleted in various tumors, including testicular and ovarian germ cell tumors (4,5). Four RNA isoforms, TR2-5, -7, -9, and -11, have been identified. While TR2-11 encodes the full-length receptor, TR2-5, -7, and -9 encode truncated receptors with distinct deletions of ligand-binding domains (LBD) (1). TR2 has high homology with TR4, which places them in a unique subfamily within the nuclear hormone receptor superfamily (6). TR2 is evolutionarily conserved among species from primitive creatures to mammals, including sea urchin, rainbow trout, axolotl, xenopus, drosophila, mouse, and human (1,2,7-11). The facts that TR2 is broadly expressed in many tissues throughout development starting at as early as midgestation stage (12-15) and that drosophila with null mutations of DHR78 nuclear receptor, a homolog of human TR2, is lethal at the third-instar larval stage with severe defects in ecdysteroid-triggered metamorphosis (16) imply that the biological importance of TR2 is involved in the development process. With prominent expression throughout the active proliferating zones of the neural areas, the sensory nerve-targeted organs and the testes during development, TR2 has been proposed to play an important role in the early development of the nervous system and the male reproductive system.
(12-15). Also, it has been shown that TR2 is primarily expressed in the mouse testis, particularly in the developing germ cells, indicating a role of TR2 in spermatogenesis (12,17).

TR2 functions as a transcription factor that binds to its consensus response elements (AGGTCA) in a direct repeat (DR) orientation (AGGTCA(n)AGGTCA, x = 1-6) (15). Many TR2 target genes have been discovered, such as cellular retinol-binding protein II (CRBPII), retinoic acid receptor β (RARβ), SV40, erythropoietin, histamine H1 receptor, muscle-specific aldolase A, and ciliary neurotrophic factor receptor (CNTFR) (13-15,18-21), suggesting that TR2 has a broad range of biological functions. In terms of the regulation of TR2 expression, TR2 can be induced during neuronal differentiation in P19 embryonic carcinoma cells stimulated by ciliary neurotrophic factor (CNTF). In return, TR2 activates its target gene, CNTFR, expression which mediates CNTF signaling and is required for the motor neuron development (13,22). These may provide a linkage between TR2 and neurogenesis. The tumor suppressor genes, p53 and Rb, that induce cell cycle arrest can down-regulate TR2 expression in cells after ionizing radiation and in cells overexpressing p53 or Rb (23,24). TR2 can then go through a feed-back control mechanism to induce HPV-16 E6 and E7 target gene expression that are known to enhance the P53 protein degradation and inactivate the Rb function, respectively (23,25). TR2 is, therefore, thought to be involved in the cell cycle regulation.

Estrogen receptors (ERs), including ERα and ERβ, belong to nuclear hormone receptor superfamily and mediate estrogen actions in regulation of cell growth and differentiation,
particularly in mammary glands and uterus in females (see reviews in (26,27)). The proliferation of mammary glands is mainly dependent on estrogen stimulation; however, the proliferating epithelial cells detected in terminal end buds (TEBs) at the tip of elongating ducts in mammary glands are usually ER-negative (28-30). Despite the unclear role of ER in this process, in mice with a homozygous disruption of ER genes, the mammary glands remain undeveloped as demonstrated by the lack of TEBs and alveolar structures, even though the serum estrogen level is 10 times higher than those in wild-type mice (31,32). This indicates an indispensable role of ER in the growth of mammary glands. Also, the fact that more than two thirds of breast cancers from patients are ER-positive and benefit from antiestrogen or ovariectomy therapies strengthens the importance of ER in the stimulation of cell growth in mammary glands in response to estrogen (33). Therefore, understanding the mechanisms involving the suppression of the ER-mediated gene expression and cell proliferation may eventually help us to develop better drugs to battle the breast cancer.

In addition to functioning as a transcription regulator, TR2 can modulate other signaling via different mechanisms. For example, TR2 suppresses RXR- and RXR/RAR-mediated transcription by binding to the same DNA response element (DRE) with a higher binding affinity (15) and represses thyroid receptor α (TRα)/RXR signaling by competing for limited amounts of DREs (20). TR2 can also exert its suppressive effects via the recruitment of class I and class II histone deacetylases (HDAC) (34). Here, we demonstrate a new role of TR2 in the breast cancer
cells where TR2 suppresses ER-mediated transcription and cell growth by direct protein-protein interaction, thus representing a novel signaling pathway within nuclear hormone receptor superfamily.
MATERIALS AND METHODS

Antibodies- ER rabbit polyclonal (H-184), ER mouse monoclonal (C-314), progesterone receptor (PR) rabbit polyclonal (H-190), and actin goat polyclonal (C-11) were obtained from Santa Cruz Biotechnology. Mouse monoclonal anti-TR2 IgM antibody (G204) were described previously (14). Monoclonal anti-FLAG antibody (M2) was purchased from Sigma. AP-conjugated secondary antibodies (goat anti-rabbit IgG, donkey anti-goat IgG and goat anti-mouse IgM) were from Santa Cruz Biotechnology.

Constructs- The pCMV-TR2, pGEX-3x-TR2, and pCMX-VP16-TR2 were constructed by insertion of full-length TR2 cDNA (1,2) into individual vectors. The doxycycline-inducible expression vector pBIG2i bearing hygromycin B resistance gene was a gift from Dr. Jay Reeder (University of Rochester, NY) (35). pBIG2i and pBIG2i-FLAG-TR2 were used for generating MCF7-pBIG and MCF7-TR2 stable clones, respectively. The GAL4-ER (aa 282-595) and pCMV-mERβ were gifts from Dr. Hinrich Gronemeyer (Strasbourg, France) and Vincent Giguère (McGill University, Québec, Canada), respectively. To construct GST-ER fragments, ER cDNA fragments were released from pSG5-ER (36) using adequate restriction enzymes and inserted into the pGEX vector series (Amersham Pharmacia) to produce pGEX-3X-ER-#1 (aa 1 to 165), pGEX-2T-ER-#2 (aa 123-340), pGEX-2T-ER-#3 (aa 312-595), pGEX-3X-ER-#4 (aa 552-595), pGEX-2T-ER-#5 (aa 123-312), and pGEX-2T-ER-#6 (aa 312-340). The pGEX-KG-
TR2-#1, #2, and #3 plasmids were constructed by insertion of PCR-generated cDNA fragments corresponding to aa 1-112, aa 88-196, and aa 179-603, respectively, into pGEX-KG vector (a gift from Dr. Frank B. Furnari, University California-San Diego, CA). pCDNA3-TR2-fl AS and pIRES-TR2-N AS were constructed by insertion of opposite orientation of cDNAs encoding full length and N terminal (aa 1-112) into pCDNA3 (Invitrogen) and pIRES (Clontech), respectively.

**Transient Transfections**- Transfections and chloramphenicol acetyltransferase (CAT) assays were performed using the calcium phosphate precipitation method, as described previously (37). CAT reporter plasmids containing one copy of estrogen response element (ERE-CAT), or mouse mammary tumor virus (MMTV-CAT) were used as indicated. Also, a β-galactosidase expression plasmid, pCMV-β-gal, was used for transfection efficiency. For luciferase assay, luciferase reporters (ERE-luc and MMTV-luc) were transfected into cells using calcium phosphate precipitation method or SuperFect Transcription Reagent (Qiagen) as indicated. pRL-TK vector (Promega) encoding renilla luciferase was used for internal control and luciferase activity was analyzed using the Dual-Luciferase Reporter Assay System (Promega) following manufacturer’s instructions.

**Co-immunoprecipitation**- MCF7 cells plated on 100-mm dishes were solubilized in 1 ml RIPA buffer containing 0.5% NP-40 and protease inhibitors. Immunoprecipitation was performed
using rabbit anti-ER antibody (1:100) (H-184) and then analyzed by Western blotting with anti-ER (1:1000) (H-184) or anti-TR2 (1:1000) (G204) antibodies, followed by incubation with AP-conjugated goat anti-rabbit IgG or rabbit anti-mouse IgM antibodies, and visualized with Alkaline Phosphatase Conjugate Substrate Kit (Bio-Rad).

**GST Pull-down Assay**- GST alone and GST fusion proteins were purified by Glutathione-Sepharose 4B beads as instructed by manufacturer (Amersham Pharmacia). The pull-down assay was performed with 5 µl of *in vitro*-translated [35S]-labeled proteins as described previously (37).

**Electrophoretic Mobility Shift Assay (EMSA)**- EMSA was carried out as described previously (38) with some modifications. Human complement C3 ERE (containing one imperfect palindromic inverted repeat: 5'-AGGTGGCCCTGACCC-3') end-labeled with γ-[32P]-ATP was used as probe. ER and TR2 were *in vitro* translated by TNT system (Promega) as instructed by manufacturer. Reactions were performed in 20 µl of EMSA binding buffer (10 mM HEPES/pH 7.9, 100 mM KCl, 1 mM Dithiothreitol, 0.5 mM EDTA, 2.5 mM MgCl₂, and 6% glycerol). For the antibody supershift analysis, 1 µl of the monoclonal anti-ERα antibody (C-314) was used. The protein-DNA complexes were analyzed on a 5% polyacrylamide native gel containing 2.5% glycerol in 1X TBE.
RESULTS

TR2 mRNA Is Expressed in the Breast Cancer Cell Lines- The studies of TR2 tissue distribution have demonstrated that TR2 is expressed in many tissues with higher expression in brain and male reproductive organs (1,2,13,14). To explore TR2 roles in female tissues, the expression of TR2 mRNA was examined in several breast cancer cells using Northern blot analysis. As shown in Fig. 1, TR2 transcripts around 2.5 kb were detected in three ER-positive breast cancer cell lines (MCF7, T47D, and ZR-75-1) at different expression levels. Also, TR2 transcripts could be detected in LNCaP and PC-3 prostate cancer cells as a control (Fig. 1).

TR2 Selectively Suppresses ER-mediated Transcription- ER is known to be an important regulator for breast cancer development as two thirds of breast tumors contain a functional ER that mediates estrogen actions to stimulate cell growth. Since the TR2 was detected in the breast cancer cells, we wondered if TR2 could affect ER function. Using ERE-CAT reporter system, TR2 consistently suppressed either exogenous (in PC-3 and H1299 cells) or endogenous (in MCF7 and T47D cells) ER transactivation in a dose-dependent manner (Fig. 2A). To determine whether TR2 affects the expression of ER proteins, we used a stable clone of MCF7-TR2 cells, where the expression of FLAG-tagged TR2 driven by pBIG2i vector could be induced by doxycycline treatment (2 μg/ml) and detected by Western blotting with anti-FLAG antibody (Fig. 2B). The expression of doxycycline-induced FLAG-TR2 was not influenced by 17β-
estradiol (E2) treatment. After treating with doxycycline and/or E2, MCF7-TR2 cell lysates were subjected to co-immunoprecipitation followed by Western blotting using anti-ER antibody (H-184). As shown in Fig. 2B, although ER expression was reduced by E2 treatment, the expression level of ER was not suppressed by overexpressed FLAG-TR2 induced by doxycycline, indicating that the suppression of ER by TR2 does not result from the down-regulation of ER expression. We also found that, in transient transfection assay, both of endogenous and exogenous ER levels were not affected by transiently overexpressed TR2 in MCF7 and COS-1 cells (data not shown). Consistent with the phenomenon in Fig. 2A, ER was suppressed by doxycycline-induced TR2 in MCF7-TR2 cells (Fig. 2C). By contrast, the doxycycline treatment did not affect ER transcriptional activity in the MCF7-pBIG cells, which were stably transfected with the pBIG2i parent vector (Fig. 2C). To rule out the artificial effects linked to foreign reporters as demonstrated in Fig. 2A & C, PR expression, an endogenous target gene of ER, was examined. As shown in Fig. 2D, TR2 could repress E2-induced PR expression at mRNA and protein levels in T47D cells as well as in MCF7 cells (data not shown). Interestingly, TR2 could also suppress the basal level of ER transcription in the absence of E2 (data not shown). For examining the specificity, we also tested the effect of TR2 on other classical steroid receptors. As shown in Fig. 2E, while TR2 could also suppress ERβ- and AR-mediated transcription in HEK293 (no detectable ER) and PC-3 cells, respectively, TR2 has little effect on the PR- or glucocorticoid receptor (GR)-mediated transcription in T47D cells. Taken together, results from
Fig. 2 demonstrate that TR2 can suppress ERα-mediated transcription and these suppressive effects are rather receptor-specific.

**TR2 Physically Associates with ER** - To investigate whether TR2 and ER are physically associated, co-immunoprecipitation and GST pull-down assays were carried out for examination of *in vivo* and *in vitro* interaction. Cell extracts from MCF7 cells treated with ethanol, E2, and tamoxifen were co-immunoprecipitated with anti-ER antibody (H-184). Immunocomplexes were then Western blotted with anti-TR2 antibody (G204). As shown in Fig. 3A, TR2 was in the ER-immunocomplexes in the presence of ethanol, E2, or tamoxifen. Using GST pull-down assay, GST-TR2 fusion protein could directly interact with *in vitro* translated [35S]-labeled ER and AR, but not RXRα (Fig. 3B). For testing ligand effects on the ER-TR2 binding, no much difference was found among different treatments (Fig. 3C). Collectively, these results suggest that ER and TR2 are directly associated with each other in a ligand-independent manner.

To dissect the TR2 interaction domain on ER, six ER peptides fused with GST were tested in GST pull-down assays. As shown in Fig. 4A, GST-ER-#2 (aa 123-340) and GST-ER-#3 (aa 312-595), but not GST-ER-#1 (aa 1-165) and GST-ER-#4 (aa 552-595) can interact with TR2 in the presence or absence of E2. Furthermore, GST-ER-#6 (aa 312-340), the overlapping region between GST-ER-#2 and -#3, but not GST-ER-#5 (aa 123-312), showed positive interaction with TR2, indicating that ER-#6 domain is responsible for this interaction. On the other hand, three
GST-fused TR2 fragments, GST-TR2-#1, -#2, and -#3, corresponding to N-terminus (aa 1-112), DBD (aa 88-196), and LBD (aa 179-603), respectively, were also examined to locate the ER-binding region. As shown in Fig. 4B, GST-TR2-#2, but not GST-TR2-#1 or -#3, was responsible for binding to ER.

Direct Association Is Required for TR2-mediated Suppression on ER- Small proteins (< 20~30 kD) are presumably capable of quickly crossing nuclear pore complexes through passive diffusion (39). Ideally, introducing small peptides containing interaction sequences may mask the binding sites for interaction proteins and prevent them from binding to the target proteins in either cytoplasm or nucleus. The small peptide ER-#6 was, therefore, tested to determine whether it can serve as an interaction blocker to interfere with ER-TR2 binding, using GST pull-down assay and mammalian two-hybrid system where in vitro translated HA-tagged ER-#6 and pCDNA3-HA-ER-#6 plasmid were introduced, respectively. Firstly, the interaction of GST-TR2 with $^{35}$S-labeled ER was inhibited by increasing amounts of HA-ER-#6 peptide (Fig. 5A). Secondly, GAL4-ER can interact with VP16-TR2 in the presence of E2, according to the induction of CAT activity, and this ER-TR2 interaction was suppressed when co-transfecting with pCDNA3-HA-ER-#6 (Fig. 5B). Thus, based on these results, ER-#6 is able to be an interaction blocker. Next, to determine whether direct association is required for TR2 to suppress ER, pCDNA3-HA-ER-#6 was applied in an ERE-CAT reporter gene assay. As shown in Fig. 5C,
the E2-induced ER transcription was significantly repressed by the doxycycline-induced TR2 in a dose-dependent fashion in MCF7-TR2 cells. Addition of ER-#6 was then capable of reversing this suppression, suggesting that TR2 suppresses ER through direct interaction.

*The Biological Significance of TR2 on ER Activity* - Antisense TR2 expression plasmids, pCDNA3-TR2-fl AS and pIRES-TR2-N AS, were assessed in ERE-luciferase assay to determine whether blocking endogenous TR2 expression might significantly enhance ER activity in MCF7 cells. First, using Western blotting with anti-TR2 antibody (G204), those two antisense constructs were proven to be able to reduce the expression of endogenous TR2 as well as overexpressed TR2 (Fig. 6A). This reduction of endogenous TR2 by antisense plasmids resulted in an increase in ER transcription in a dose-dependent manner (Fig. 6B), indicating that endogenous TR2 normally suppresses ER activity in MCF7 cells.

*ER DNA-binding and Homodimeric Formation Are Disrupted by Associating with TR2* - To elucidate the molecular mechanisms by which ER was suppressed by interacting with TR2, we tested ER expression, stability, nuclear translocation, DNA binding, and interaction with coregulators. We found that overexpression of TR2 did not influence ER expression (Fig. 2B), stability, or nuclear translocation (data not shown). Using GST pull-down assay and mammalian two-hybrid assay, TR2 did not affect the binding between ER and some coregulators such as
SRC-1, TIF-II, and ARA70 (data not shown). After ruling out these mechanisms, we suspect that TR2 may mainly influence ER on DNA binding. Using the EMSA assay as shown in Fig. 7A, two specific ER-ERE bands could be detected (lane 3 and 4) and were supershifted by ER antibody (C-314) (lanes 5 and 6, indicated as an arrowhead). Two ER-ERE bands presumably consist of the monomer and dimer of ER bound to ERE, respectively. Since the sequences of ERE used in this assay contain one imperfect palindromic inverted repeat, the ER was bound to the half side of ERE as monomer. However, the monomer of ER bound to ERE does not occur {	extit{in vivo}} due to the instability (40). Then, Addition of 100-fold molar excess of unlabeled ERE could effectively eliminate these specific bands (lanes 7 and 8). Interestingly, the intensity of these ER-ERE complexes were decreased upon addition of increasing amounts of TR2 in either the absence (lanes 9-11) or the presence of 10 nM E2 (lanes 12-14). Since no ERE-TR2 specific band (lane 2) and no extra supershifted band formed as TR2-ER-ERE complexes (lanes 3-4 vs 9-14) were found, we may conclude that TR2 interacts with ER resulting in ER dissociating from binding to DNA. Accordingly, our competition assay (Fig. 7B) showed that the ER homodimer formation, as illustrated by the interaction between GST-ER-LBD and [35S]-ER, was decreased by presence of TR2 and, conversely, heterodimeric formation of ER-TR2 was increased along with the increasing amounts of TR2. It indicates that TR2 forms a TR2-ER heterodimer but not a TR2-ER-ER complex to interfere with ER homodimeric formation. Furthermore, the reduction of ER homodimerization by TR2 could be rescued when the ER-#6 peptide which blocked TR2
from interacting with ER, was added (Fig. 7B). Taken together, Fig. 7 suggests that TR2 may suppress ER-mediated transactivation via the formation of TR2-ER heterodimers that reduce the formation of the ER homodimer and cause ER to associate from ERE.

**TR2 Suppresses E2/ER-induced Cell growth and G_{1}/S Transition** - E2, through ER, is known to enhance G_{1}/S transition and stimulate cell proliferation in estrogen-dependent breast cancer cells (41-45). To investigate whether the suppression of ER by TR2 can affect breast cancer cell growth in response to estrogen, MTT (thioazyl blue) assays were carried out for examining the cell viability. The data from MTT assays (Fig. 8A) showed that addition of E2 for 5 days apparently stimulated cell growth in both MCF7-pBIG (MTT_{OD570} 0.82 ± 0.008) and MCF7-TR2 cells (MTT_{OD570} 0.74 ± 0.019), as compared to both cells treated with ethanol for 5 days (MTT_{OD570} 0.58 ± 0.007 and 0.48 ± 0.032, respectively). Although doxycycline had mild toxic effect on cell growth as demonstrated by causing the slight growth inhibition in MCF7-pBIG cells, the presence of doxycycline obviously arrested cell growth of the MCF7-TR2 cells (MTT_{OD570} 0.30 ± 0.014 in ethanol treatment and 0.36 ± 0.054 in the presence of E2), indicating that TR2 expression abrogate the E2-induced cell proliferation in breast cancer cells. To determine whether TR2 can interrupt E2/ER-induced G_{1}/S transition, the cell cycle profile was obtained from flow cytometric analysis using MCF7-TR2 cells, which were treated with ethanol, E2 and doxycycline for 12 hours. As shown in Fig. 8B, addition of E2 to MCF7-TR2 cells
cultured in RPMI medium with 10% of charcoal-dextran-treated fetal bovine serum (CD-FBS) can induce the G₁/S transition (G₁: from 42.6 to 32.9%; S: from 27.5 to 37.1%). In contrast, TR2 expression inhibited the E2-induced G₁/S transition, leading to the G₁ arrest (G₁: from 32.9 to 55.3%; S: from 37.1 to 21.4%). In the absence of E2, doxycycline treatment also resulted in an accumulation of G₁ cells (G₁: from 42.6 to 54.6%), which is consistent with the data that overexpression of TR2 could also suppress the basal level of ER transactivation in the absence of E2 in the ERE-luciferase reporter assay (data not shown). Meanwhile, we also observed that the cell size of MCF7-TR2, but not that of MCF7-pBIG cells, became larger after 3 days of doxycycline induction. Whether the changes of the cell size might be due to cell cycle arrest as occurred in many other cases (46,47) remains to be further investigated.
DISCUSSION

The discovery that TR2 is expressed in breast cancer cells (Fig. 1) and suppresses ER-mediated signaling (Fig. 2) may demonstrate a novel biological function of TR2 in the estrogen-responsive organs, in addition to other potential physiological roles of TR2 in neurogenesis and spermatogenesis (12-15). TR2 was originally identified as a transcriptional factor that can modulate target genes’ expression via binding to the TR2 response elements (1,2,15) and also can influence the activity of other transcription factors, such as RXR, RAR, and TRα, through competing the same DNA response elements (15,20). In this study, we found that TR2 is capable of interacting and regulating ER-mediated transcription (Fig. 2-4). Furthermore, The interaction blocker, ER-#6, an ER fragment (aa 312-340) responsible for TR2 binding (Fig. 4A), was able to rescue ER from suppression by TR2 (Fig. 5), and administration of antisense TR2 could enhance ER transcription in MCF7 cells (Fig. 6). Thus, these data provide a new molecular function of TR2 in modulating other nuclear receptor activity via the mechanism of direct protein-protein interactions, implying that TR2 may function as one of the repressors of ER-mediated signaling.

To further understand the molecular mechanisms by which TR2 suppresses ER, TR2 may possibly influence ER by changing the expression, protein stability, nuclear translocation, DNA binding, interacting with coregulators, and/or post-translational modifications such as phosphorylation and acetylation. From our preliminary studies, we found that TR2 neither affects ER expression levels (Fig. 2B) and the nuclear translocation nor the interaction with some
coactivators, such as SRC-1a, TIF-II, and ARA70 (data not shown). Despite not ruling out other possible mechanisms, such as post-translational modifications, data from EMSA (Fig. 7A) clearly demonstrate that addition of TR2 may interrupt ER binding to DNA and this dissociation may be due to the disruption of ER homodimerization as a result of the formation of non-functional TR2-ER heterodimers (Fig. 7B). It may consequently result in the suppression of ER transcription. Similarly, the disruption of ER homodimers through the interaction with truncated estrogen receptor product-1 (TERP-1) also shows an interruption of ER-ERE binding, resulting in the suppression of ER-mediated transcription (48). By contrast, a tumor suppressor, P53, suppresses ER via interfering with the DNA binding without affecting the dimerization (49). However, the mechanism by which p53 suppresses ER remains unclear.

Although it is still unknown whether the region spanning from aa 341 to 551 on ER provides the binding sites for TR2, the ER-#6 (aa 312-340) is sufficient to bind TR2 and functions as a interaction blocker (Fig. 4 & 5). The ER-#6 covers the region spanning from helix 1 to part of helix 3 within the N-terminal of the ER ligand binding domain (LBD), which is located outside the ER LBD pocket and has no critical amino acids responsible for hormone binding and dimerization (50). This binding region for TR2 is different from the region, known as the AF-2 domain, for most other ER coactivators, such as SRC-1 and the related p160 family, which contain the signature motif of the NR box (LXXLL) responsible for interacting with ER in the presence of ligands (51). The AF-2 interaction surface is comprised of the specific amino
acids in helix 3, 4, 5, and 12, and, upon ligand binding, forms a hydrophobic cleft where helix 12 is positioned over the ligand-binding pocket providing a surface for those coregulators binding (52,53). The different binding sites for TR2 and ligand-dependent coactivators on ER may provide an explanation for our results showing that the ER-TR2 interaction was ligand-independent (Fig. 3 & 4), and that TR2 did not interfere with ER interacting with those coactivators (data not shown). Consistent with this phenomena, an antiestrogen, tamoxifen, did not affect their interaction as shown in Fig. 3 A & C. It is also consistent with the finding that a signature motif, LXXLL, located on the TR2 LBD (aa 547-551) is not required for ER interaction since the ER binding region is located on the TR2 DBD (Fig. 4B). The similar phenomenon has also been demonstrated by Tanenbaum, et al (54). They demonstrated that REA, a repressor of estrogen receptor activity, interacts with ER through a ligand-independent fashion, where the LXXLL motif of REA and the helix 12 of ER are not involved in the binding. However, they showed that the integrity of the LXXLL motif is still important for REA to perform its suppressive effect on ER, although it is not required for interaction. Therefore, it will be interesting to determine whether the LXXLL motif within TR2 is also necessary for suppression of the ER.

It has long been known that the beneficial effects of antiestrogens on the ER-positive breast tumors is probably due to blockage of E2/ER-mediated cell growth (33). Therefore, to find the repressors of ER and understanding those molecular mechanisms may provide information
towards the development of therapeutic drugs to battle the E2/ER-dependent tumors. However, few of ER suppressors have been identified and characterized (55) and the detailed suppression mechanisms also remain largely unknown. Early reports suggested several possible mechanisms including: 1) the interference of the binding capacity of ER homodimers to ERE, such as P53 (49) and TERP-1 (48); 2) competition with coactivators for binding to ER, such as short heterodimer partner (SHP) (56), DAX-1 (57), TERP-1 (48), and REA (54); 3) recruitment of HDACs to ER, such as metastatic-associated protein 1 (58), and SMRT (59). Here we provide another repressor, TR2, functioning through the formation of non-functional ER-TR2 heterodimers that result in ER dissociating from ERE.

ER is also known to function as a modulator to regulate the function of other nuclear receptors, such as TR, RAR, and RXR, through protein-protein interaction (60). ER also interacts with and suppresses proapoptotic forkhead transcription factor transcription activity in the presence of estrogen (61). Unexpectedly, we also found that ER could suppress TR2-mediated transcription in a ligand-independent manner (data not shown). This suppression was not mediated via interruption of TR2 DNA binding although the ER interaction site is located on TR2 DBD (data not shown). The mechanism by which ER suppresses TR2 remains unclear at this moment. Nevertheless, these findings represent a mutual regulation between ER and TR2 within the nuclear hormone receptor superfamily.
Fig. 8 demonstrates that TR2 can suppress E2/ER-induced G₁/S transition and cause cell growth inhibition in MCF7-TR2 cells where TR2 could be induced by treatment with doxycycline. This growth suppression is suggested to mainly go through suppression of ER signal since TR2 lost this suppressive effect on cell growth in the presence of tamoxifen (data not shown). However, we can not rule out the possibility that TR2 mediates growth inhibition through the pathways independent of ER. Earlier studies have shown that TR2 induction is involved in neuronal differentiation in mouse P19 stem cells stimulated by either retinoic acid or CNTF (13,62), suggesting that TR2 may have a role in cell differentiation and negatively regulating cell proliferation. As TR2 is located in chromosome 12q22, a known region frequently deleted in various tumors including testicular and ovarian germ cell tumors (4,5), it will be interesting to link TR2 as one of the tumor suppressor candidates that can negatively regulate cell growth.

Taken together, we suggest that TR2 may function not only a transcription factor, but also an important repressor in regulating ER-mediated transcription in mammary glands. Therefore, our future study may expand to determine the physiological roles of TR2 in TR2 knockout mice, especially in the development of mammary glands as well as brain, nervous, and reproductive systems where ER is known to exert an essential role.
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Abbreviations

aa, amino acid; CAT, chloramphenicol acetyltransferase; DBD, DNA-binding domain; E2, 17β-estradiol; ER, estrogen receptor; ERE, estrogen response element; CD-FBS, charcoal-dextran-treated fetal bovine serum; GST, glutathione S-transferase; LBD, ligand-binding domain; PR, progesterone receptor; TR2, TR2 orphan receptor.
REFERENCES


Figure legends

**Fig. 1. Expression of TR2 mRNA in the breast cancer cell lines.** 20 µg of total RNA was extracted from cancer cells. A TR2 cDNA encoding LBD (aa 179-603) was random-primed labeled with α-[32P]-dCTP and used as probe. Northern hybridization was performed with Rapid-hyb buffer (Amersham Pharmacia) according to manufacturer’s instructions. 28S ribosome RNA was stained with 0.04% methylene blue in sodium acetate (pH 5.0) for RNA integrity and quantity control.

**Fig. 2. The effects of TR2 on ERα-, ERβ-, AR-, PR-, and GR-mediated transactivation.** A, Cells in 60-mm dishes were transfected with 2 µg of ERE-CAT and expression plasmids, as indicated, by calcium phosphate precipitation methods. 1 µg of β-galactosidase expression plasmid, pCMV-β-gal, was used as an internal control for transfection efficiency. Sixteen hours after transfection, cells were treated with ethanol or 10 nM E2 for another 16 h and were then harvested for CAT assay. B, MCF7-TR2 cells were treated with 10 nM E2 and/or 2 µg/ml doxycycline for 24 h. Cells lysates were subjected to Western blotting using anti-FLAG antibody (M2) to monitor the induction of FLAG-tagged TR2. The ER expression level was determined by co-immunoprecipitation followed by Western blotting with anti-ER antibody (H-184) using cell lysates containing 400 µg of total proteins. C, MCF7-pBIG and MCF7-TR2 cells were transfected with 2 µg of ERE-CAT and 1 µg of pCMV-β-gal and, after 16 hr, were treated with...
or without 2 μg/ml doxycycline. Cells were then harvested for CAT assay. D, T-47D cells seeded in 60-mm dishes were transfected with 10 μg of pCMV or pCMV-TR2 for 16 h, followed by treatment with 10 nM E2 for another 16 h. Cell extracts (80 μg) and total RNA (15 μg) were used for Western blotting with anti-PR antibody (H-190) and Northern blotting, respectively. Relative mRNA expression amounts were normalized by 28S expression and quantitated by ImageQuant V.1.2 (Molecular Dynamics). E, Methods used are the same as described in (A). ERE- and MMTV-luciferase reporter genes were used for examination of ERβ and AR transactivation, respectively. MMTV-CAT reporter was used for PR and GR transactivation. Luciferase activity was analyzed following manufacturer’s instructions (Promega). 10 nM of E2, DHT, progesterone (P), and dexamethasone (Dex), were used as indicated. CAT and luciferase activity are presented relative to the response to ethanol, which is set as one. Values are the means ± SD of three independent experiments.

**Fig. 3. The physical association analysis between TR2 and ER.** A, 500 μg of total proteins from MCF7 cells treated with ethanol, 10 nM E2, or 1 μM tamoxifen for 24 h were immunoprecipitated with normal rabbit IgG or rabbit anti-ER antibody (H-184) as indicated. The immunoprecipitates were subjected to Western blotting with anti-ER (1:1000, H-184) or anti-TR2 (1:1000, G204) antibodies. B, The GST and GST-TR2 fusion proteins were purified as instructed by the manufacturer (Amersham Pharmacia). 5 μl of *in vitro*-translated [35S]-labeled
AR, ER, and RXR were incubated with the GST or GST-TR2 fusion proteins bound to glutathione-Sepharose beads in a pull-down assay. After extensive washing, bead-bound protein complexes were loaded onto 8% SDS-PAGE and analyzed by PhosphorImager (Molecular Dynamics). The input represents 20% amount of [35S]-labeled proteins used in each pull-down assay. C, Ligand effects on the interaction between ER and TR2. Three kinds of treatments (ethanol, 10 nM E2, 1 µM Tamoxifen) were added, individually, in each GST pull-down reaction as indicated. The input represents 10% amount of [35S]-labeled ER used in each pull-down assay.

**Fig. 4. Mapping the interaction domains on ER and TR2.** A, The construction of GST-ER fragments is illustrated schematically on the upper panel. GST alone and GST fusion proteins were purified as described by the manufacturer instructions. 5 µl of [35S]-TR2 was incubated with GST or GST-ER fusion proteins bound to glutathione-Sepharose beads in the absence or presence of 1 µM E2. After extensive washing, bead-bound protein complexes were loaded onto 8% SDS-PAGE and analyzed by PhosphorImager. B, Schematic representation of GST-TR2 constructs is illustrated on the upper panel. GST or three GST-TR2 fusion proteins were purified and incubated with 5 µl of [35S]-ER in the pull-down assay. The input represents 10% amount of [35S]-labeled proteins used in each pull-down assay. DBD, DNA binding domain; LBD, ligand binding domain.
Fig. 5. ER-#6 serves as ER-TR2 interaction blocker capable of reversing the suppression of ER by TR2. A, ER-#6 blocks ER-TR2 interaction in GST pull-down assay. GST and GST-TR2 fusion proteins were purified as described by the manufacturer (Amersham Pharmacia). Glutathione-Sepharose beads bound GST-TR2 were then incubated with 5 µl of [35S]-ER with increasing amounts of HA-ER-#6, which was in vitro translated from a pCDNA3-HA-ER-#6 plasmid, for 2 h at 4 °C in the absence of E2. After extensive washing, bead-bound protein complexes were loaded onto 8% SDS-PAGE and analyzed by PhosphorImager (Molecular Dynamics). The input represents 10% amount of [35S]-ER used in each pull-down assay. B, ER-#6 inhibits ER-TR2 interaction in mammalian two-hybrid system. PC-3 cells plated on 60-mm dishes were co-transfected with 2 µg of pG5-CAT reporter with expression plasmids as indicated. 1 µg of pCMV-β-gal was also used as an internal control for transfection efficiency. CAT activity was analyzed in the presence of 10^{-8} M E2. C, ER-#6 reverses TR2-mediated suppression of ER transactivation. MCF7-TR2 cells were co-transfected with 2 µg of ERE-CAT, 1 µg of pCMV-β-gal, and 7 µg of pCDNA3 or pCDNA3-HA-ER-#6. Sixteen hours after transfection, cells were treated with ethanol, 10 nM E2, and/or increasing amount of doxycycline, as indicated, for another 16 h. CAT activity is presented relative to the response to ethanol, set as one. Values are the means ± SD of three independent experiments.
Fig. 6. Enhancement of ER transcriptional activity by administration of antisense TR2 in MCF7 cells. A, MCF7 cells cultured in 35-mm dished were transfected with 1 µg of pCDNA3-TR2-lf AS, pIRES-TR2-N AS, and pCMV-TR2 plasmids using SuperFect Transfection Reagent (Qiagen). The total amount of plasmids in each dish was made up to 2 µg by adding the parent vectors. After 32 h, cells were harvested and 80 µg of cell lysates were subjected to Western blotting with anti-TR2 antibody (G204) and anti-actin antibody (C-11). B, MCF7 cells cultured in 35-mm dishes were transfected with 0.125 µg of ERE-Luc and increasing amounts (0.5 ~ 1.875 µg) of pCDNA3-TR2-lf AS and pIRES-TR2-N AS plasmids, as indicated. 10 ng of pRL-TK (Promega) was used as internal control. The total amount of plasmids in each dish was made up to 2 µg by adding the pCDNA3 parent vector. Sixteen hours after transfection, cell were treated with or without 10 nM E2 for another 16 h. Luciferase activity was analyzed according to manufacturer’s instructions (Promega). Luciferase activity is presented relative to response to ethanol, which is set as 1. Values are the means ± SD of three independent experiments.

Fig. 7. Interference with ER binding to ERE by ER-TR2 heterodimer formation. A, Interruption of ER binding to ERE by TR2 in EMSA. [32P]-end-labeled ERE probe (4 x 10^8 dpm/µg) was incubated with in vitro translated TR2 and ER proteins (ratios from 1:1 to 1:4) in EMSA binding buffer and analyzed on a 5% acrylamide native gel containing 2.5% glycerol. 1 µl of anti-ERα monoclonal antibody (C-314) was added for antibody supershifts (lane 5 and 6).
A 100-fold excess molar of unlabeled ERE probe was added as a cold competitor (lane 7 and 8). Ethanol or 10 μM E2 was added as indicated. The migration positions of the supershifted band formed by Ab-ER-ERE are indicated as an *arrowhead*. *ns*, non-specific binding. *B*, ER homodimeric formation is disrupted by TR2, but rescued by ER-#6. GST-ER-#3 (LBD) and GST proteins were purified as described by the manufacturer (Amersham Pharmacia). *In vitro* translated [35S]-ER with increasing amounts of [35S]-TR2 were co-incubated with GST-ER-#3 or GST alone which were bound to glutathione-Sepharose beads. ER-#6 peptide was obtained using the thrombin protease cleavage method (ROCHE) to release ER-#6 peptide from bead-bound GST-ER-#6. Equal amounts of GST-ER-#3 and GST-ER-#6 were used as determined by a coomassie-staining gel. After extensive washing, bead-bound protein complexes were loaded onto 8% SDS-PAGE and analyzed by PhosphorImager (Molecular Dynamics). The input represents 0.5 μl of [35S]-labeled ER and TR2 as used in each reaction.

**Fig. 8. The TR2 suppresses E2-induced breast cancer cell growth and G<sub>1</sub>/S transition.** *A*, Growth assays were performed by MTT method as instructed by manufacturer (Sigma). 5 X 10<sup>3</sup> MCF7-pBIG and MCF7-TR2 cells were seeded in 24-well plates and incubated in RPMI with 10% CD-FBS for 48 h. Cells were then treated with ethanol, 10 nM E2, and/or 2 μg/ml doxycycline as indicated. After 1-, 3- and 5-day of treatments, cells were harvested for MTT assay. Values are the means ± SD of O.D.<sub>570</sub> from three independent wells of cells. *B*, The
inhibition of E2-induced G_/S transition by TR2 in MCF7-TR2 cells. Cells were incubated in RPMI with 10% CD-FBS for 48 h and then treated with ethanol, 10 nM E2 and 2 µg/ml doxycycline, as indicated, for 12 h. Cells were then trypsinized and fixed overnight in 70% Ethanol. After cells were incubated with 1 µg/ml RNase A (Sigma) and propidium iodide (Roche Molecular Biochemicals), the DNA contents of cells were measured by a flow cytometry.
Fig. 1, Hu et al.
Fig. 2, Hu et al.
Fig. 3, Hu et al.
Fig. 4, Hu et al.
Fig. 5, Hu et al.
Fig. 6. Hu et al.

A

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<td></td>
<td>-</td>
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B

- **ERE-LUC**
  - TR2-N AS: -
  - TR2-fl AS: -
  - E2 (10 nM): +

- **MCF7**
  - TR2-N AS: -
  - TR2-fl AS: -
  - E2 (10 nM): +

**Relative Luciferase Activity (fold)**

- ERE-LUC:
  - TR2-N AS: -
  - TR2-fl AS: -
  - E2 (10 nM): +

- MCF7:
  - TR2-N AS: -
  - TR2-fl AS: -
  - E2 (10 nM): +
**Fig. 7, Hu *et al.***

### A

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### B

**Input GST GST-ER-#3 (LBD)**

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**ER-ERE complexes**

**BS**
Fig. 8, Hu et al.

A  

MCF7-pBIG

MTT (O.D. 570) Values

Percentage of Cells

B  

MCF7-TR2

Percentage of Cells

E2 (10 nM)  -  -  +  +  
Doxycycline  -  +  -  +
Suppression of estrogen receptor-mediated transcription and cell growth by interaction with TR2 Orphan Receptor
Yueh-Chiang Hu, Chih-Rong Shyr, Wenyi Che, Xiao-Min Mu, Eungseok Kim and Chawnshang Chang

J. Biol. Chem. published online July 1, 2002

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