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TR2 Orphan Receptor Functions as Negative Modulator for Androgen Receptor in Prostate Cancer Cells PC-3

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BACKGROUND. Both androgen receptor (AR) and orphan receptor TR2 (TR2) belong to the steroid nuclear receptor superfamily and are expressed in prostate cancer tissue and cell lines. AR has been known to be involved in prostate proliferation and prostate cancer progression. AR binds to androgen response elements and regulates target gene expression via a mechanism involving coregulators. However, the function of TR2 in prostate and prostate cancer and the relationship between TR2 and AR in the prostate cancer is unclear.
METHODS. Transient transfection and CAT reporter gene assays were employed to assess AR-mediated transactivation. The expression level of prostate specific antigen (PSA) was measured by Northern blot analysis. The interaction between AR and TR2 was assessed by glutathione-S-transferase (GST) pull-down assay and mammalian two-hybrid system assay.
RESULTS. Orphan nuclear receptor TR2 suppressed androgen-mediated transactivation in prostate cancer PC-3 cells, and over-expression of TR2 suppressed PSA expression. The suppression of AR mediated transactivation by TR2 is not due to competition for the limited coregulator availability by these two receptors, but possibly through the interaction between TR2 and AR nuclear receptors.
CONCLUSIONS. TR2 may function as a negative modulator to suppress AR function in prostate cancer. Further studies on how to control TR2 function may result in the ability to modulate AR function in prostate cancer. *Prostate 57: 129–133, 2003.* © 2003 Wiley-Liss, Inc.

KEY WORDS: **orphan receptor; androgen receptor; transactivation; suppression; interaction; PC-3 cell**

INTRODUCTION

The human orphan receptor TR2 (TR2), a member of the nuclear receptor superfamily, was isolated from testis and prostate cDNA libraries and its cDNA encodes a protein of 603 amino acids with a calculated molecular mass of 67 kDa [1,2]. The expression of TR2 has been detected widely in the male reproductive system including testis, prostate, and seminal vesicle [1–3]. TR2 is also relatively highly expressed in prostate cancer tissue and cell lines [1–4]. TR2 was originally found as a transcription factor that binds to its consensus response element (AGGTCANxAGGTCA, x = 1–6) to regulate its target gene expression. The possible TR2 target genes identified so far include cellular retinol binding protein II (CRBP II) [5,6], retinoic acid receptor (RAR) [5,6], simian virus 40 (SV40) [7], erythropoietin [8], histamine H1 receptor [9], muscle-specific aldolase A [10], and ciliary neurotrophic factor (CNTF) receptor [11]. TR2 can be down-regulated by the tumor suppressor genes *p53* and Rb genes in cells after ionizing radiation and in cells over-expressing p53 and Rb. TR2 could also control the

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expression of p53 and Rb through the regulation of human papillomavirus 16 E6/E7 genes [12,13]. Therefore, TR2 is thought to be involved in cell cycle regulation and tumorigenesis.

The androgen receptor (AR), another member of the steroid receptor superfamily, is composed of a variable amino-terminal domain, a highly conserved DNA-binding domain, and a ligand-binding domain [14]. Ligand-dependent transcriptional activation of AR is mediated by the COOH-terminal domain that includes the ligand binding domain and an activation function domain (AF-2) [15]. Crystallographic studies show that ligand-bound steroid receptors undergo a conformational change in the AF-2 core motif. This ligand-induced conformational change presumably recruits coregulators such as SRC-1, TIF-2, CBP/P300, ARA70, ARA54, ARA55, ARA24, ARA160, p/CIP/ACTR/AIB1, Rb, and NCoA-1 [16].

Androgen and AR play essential roles in prostate proliferation and prostate cancer progression. Here we demonstrated that TR2 could modulate AR-mediated transactivation and target gene expression possibly through TR2 and AR interaction in human prostate cancer PC-3 cells.

MATERIALS AND METHODS

Plasmids

The plasmids MMTV-CAT, PSA-CAT, pCMV-AR, pCMV-TR2, pSG5-PR, pSG5-GR, pSG5-SRC-1, pSG5-TIFII, pSG5-ARA55, GST-TR2, pCMX-VP16-TR2, pCMX-GAL4-AR, pCMX-VP16-RXR, and pCMV-β-Gel were reported previously [7–13,16].

Transient Transfection and CAT Assay

Human prostate cancer PC-3 cells were maintained in DMEM containing penicillin (25 U/ml), streptomycin (25 g/ml), and 5% fetal calf serum (FCS). Human prostate cancer LNCaP cells were maintained in RPMI-1640 containing penicillin (25 U/ml), streptomycin (25 g/ml), and 10% FCS. PC-3 cell were transfected using the calcium phosphate precipitation method as described previously [17], and LNCaP cells were transfected using Superfect™ according to the manufacturer's procedures (Qiagen, Chatsworth, CA). CAT assay was performed as described previously [13,17].

Glutathione-S-Transferase (GST) Pull-Down Assay

GST control protein and GST-TR2 fusion proteins were purified by glutathione-sepharose 4B beads as described by the manufacturer (Amersham Biosciences). Five microliters of in vitro-translated ^{35}S-methionine-labeled proteins was used to perform the pull-down assay as described previously [4].

Northern Blot

Total RNA from transfected LNCaP cells was prepared using TRIZOL reagents (Life Technology) as instructed by the manufacturer. The probe was obtained from PSA gene by PCR and labeled with ^{32}P dCTP. Northern blot hybridization was performed as described previously [13].

RESULTS

TR2 Suppresses AR Mediated Transactivation in Prostate Cancer PC-3 Cells

AR is known to be highly involved in prostate cancer progression, and both TR2 and AR are expressed in prostate cancer, therefore we studied the TR2 effect on AR transactivation activity. As shown in Figure 1, in the presence of 10 nM DHT, addition of AR in prostate cancer PC-3 induced the activity of both MMTV-CAT (A) and PSA-CAT (B), two common AR target gene reporters. Addition of TR2 expression plasmid strongly suppresses MMTV-CAT (Fig. 1A) and PSA-CAT (Fig. 1B) activity (lanes 3–5 vs. 2) in a dosage-dependent manner. Glucocorticoid receptor (GR) can also

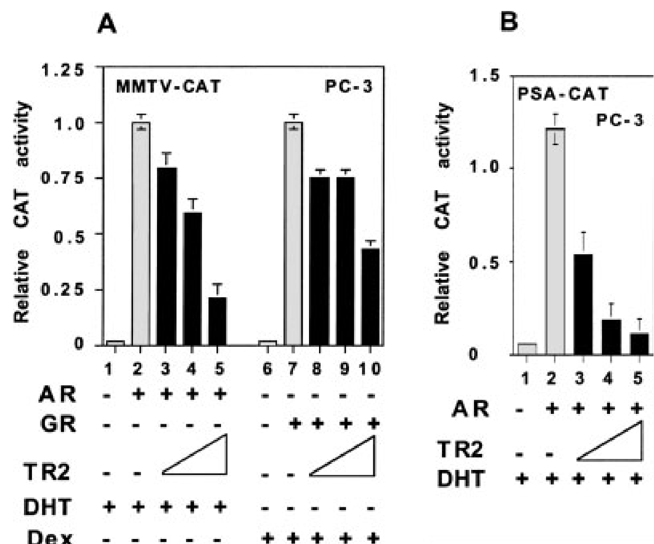

Fig. 1. TR2 suppresses AR-mediated transactivation. Two micrograms of AR transactivation reporter gene MMTV-CAT (**A**) and PSA-CAT (**B**), 0.5 μg AR, and GR expression plasmid pCMV-AR (A, **lanes 2–5**), pSG5-GR (A, **lanes 7–10**) were transfected into PC-3 cells. Increasing amounts (2, 4, and 7 μg) of TR2 expression plasmid pCMV-TR2 as indicated were also transfected into PC-3 cells. After 18 hr transfection, the cells were treated with either 10 nM DHT (A and B, **lanes 1–5**), or 10 nM dexamethasone (Dex) (A, **lanes 6–10**). CAT assay was performed as described in Materials and Methods.

induce MMTV CAT activity in the presence of 10 nM dexamethasone (A, lane 7 vs. 6). As a control, addition of TR2 has much less suppression effect on the GR-mediated transactivation (A, lanes 8–10 vs. 7).

Suppression of AR-Mediated Transactivation is not Due to the Competition of Limited Coregulator Availability Between AR and TR2

It has been known that AR transactivation activity is highly regulated by coregulators, and the amount of coregulator in a specific cell could be relatively limited. This limited coregulator amount may lead to competition with each other for their maximal transactivation activity. As shown in Figure 2, to demonstrate whether the suppression of AR transactivation by TR2 is due to competition for the limited coregulator amount, we transfected MMTV-CAT, pCMV-AR, and increasing amounts of pCMV-TR2 into human prostate PC-3 cell, as well as AR coregulators SRC-1 (lanes 6–10), ARA70 (lanes 11–15), TIF II (lanes 16–20), and ARA55 (lanes 21–25) at AR:AR coregulator plasmid ratio of 0.5:1. Co-transfection of these limited amounts of co-regulators did not affect the suppression of AR mediated-transactivation by TR2. These data suggested that suppression of AR-mediated transactivation was not due to the competition of limited coregulator availability between AR and TR2 and the suppression of AR-mediated transactivation by TR2 could be a specific event in PC-3 cells.

TR2 Suppresses PSA Expression

PSA is an androgen target gene, which is widely used as a marker for clinical prostate cancer progression. We transfected prostate cancer LNCaP cells with either vector control or TR2 expression plasmid pCMV-TR2, and then treated cells with DHT or vehicle. Total RNA was extracted and PSA expression was measured by Northern blot analysis. As shown in Figure 3, addition of 10 nM DHT induces PSA mRNA expression (lane 2 vs. 1). Addition of TR2 expression plasmid suppresses PSA expression level in LNCaP cells (lane 4 vs. 2). These in vivo TR2-mediated suppressive effects

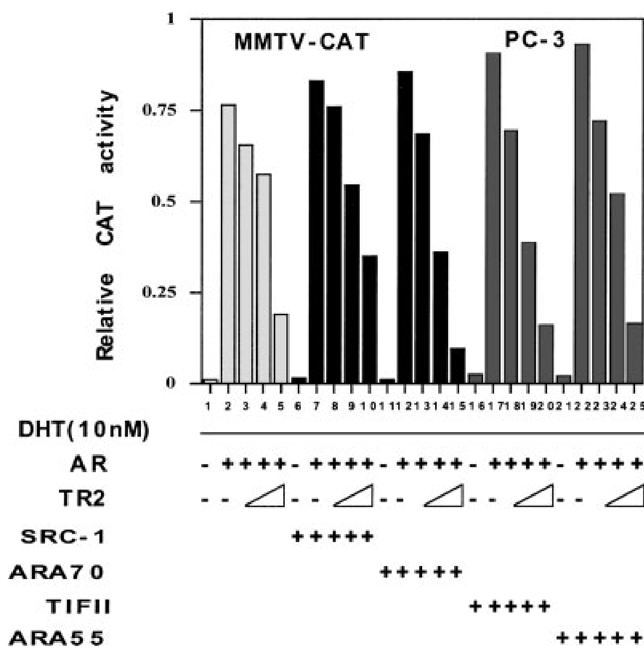

Fig. 2. Suppression of AR-mediated transactivation by TR2 is not due to competition for the limited coregulator availability by these two receptors. Two micrograms of AR transactivation reporter gene MMTV-CAT, 0.5 μg AR expression plasmid pCMV-AR, and increasing amounts of (2, 4, and 7 μg) TR2 expression plasmid pCMV-TR2 were transfected into PC-3 cells. One micrograms of each of pSG5-SRC-1 (**lanes 6–10**), pSG5-ARA70 (**lanes 11–15**), pSG5-TIF II (**lanes 16–20**), and pSG5-ARA55 (**lanes 21–25**) were also transfected. CAT assay was performed as described in Materials and Methods.

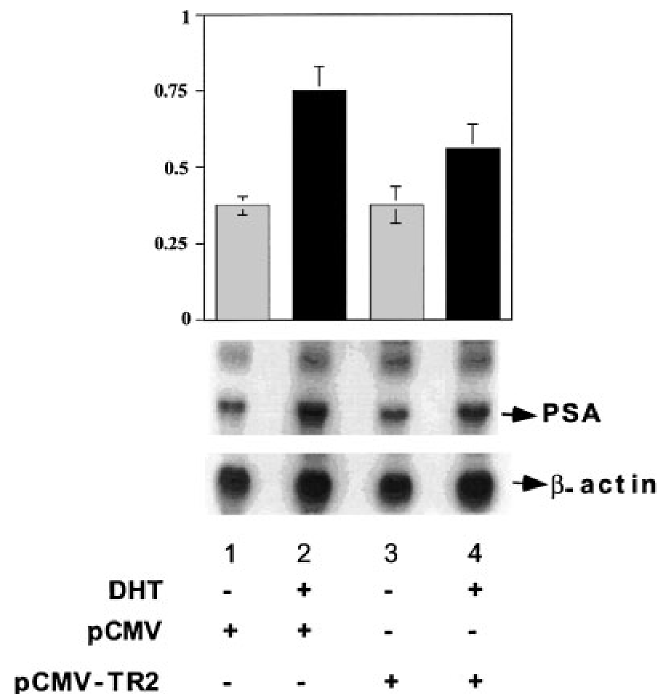

Fig. 3. TR2 suppresses PSA expression. Vector control (**lanes 1** and **2**) and TR2 expression plasmid pCMV-TR2 (**lanes 3** and **4**) were transfected into prostate cancer LNCaP cells without (lanes 1 and 3), or with (lanes 2 and 4) the treatment of 10 nM DHT. Northern blot (**lower panel**) was performed as described in Materials and Methods. The relative mRNA levels are presented as the ratio of PSA mRNA:β-actin mRNA, and values of bars represent the mean ± SE of triplicate determination in three similar experiments (**upper panel**).

strongly support our data from reporter gene assays in Figure 1 and demonstrate that TR2 may function as a repressor to negatively regulate PSA expression.

Interaction Between TR2 and AR

We applied GST pull-down assay to further dissect the mechanism of how TR2 can suppress AR-mediated transactivation. As shown in Figure 4A, ^{35}S methionine-labeled AR was able to interact with GST-TR2 fusion protein (lanes 4 and 6), but not GST alone (lanes 3 and 8). This interaction was relatively specific for AR, as TR2 was not able to interact with retinoid X receptor α (RXRα) (lane 5 and 7), a common nuclear receptor that binds to many other nuclear receptors.

The interaction between TR2 and AR was further evaluated by the mammalian two-hybrid system assay. A full-length TR2 was fused to the transcriptional activator VP16 (VP16-TR2) and then cotransfected with GAL4-DBD fused with AR ligand binding domain (GAL4-AR) and a GAL4-responsive CAT reporter (pG5-CAT) in PC-3 cells. The cells were treated with 10 nM DHT, 18 hr after transfection. As shown in Figure 4B, both VP16-TR2 and GAL4-AR alone showed a low background (lanes 1 and 2), and cotransfection of VP16-TR2 and GAL4-AR showed a significant induction of CAT reporter gene activity (lane 3). As a control, cotransfection of VP-16-RXR and GAL4-AR only showed the background level of CAT activity (lane 4). The data from mammalian two-hybrid system assay in Figure 4B is consistent with the data from GST pull-down assay in Figure 4A, both indicating the interaction between TR2 and AR.

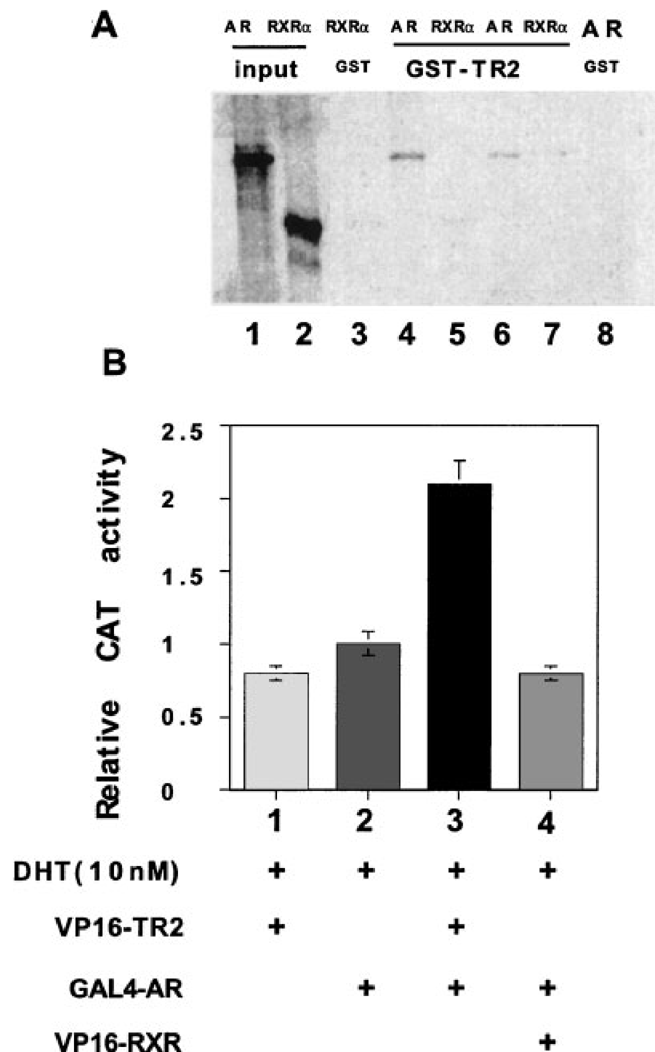

Fig. 4. The interaction between AR and TR2 in GST pull-down assay (**A**) and mammalian two-hybrid system assay. A: The GST-TR2 fusion protein and GST control were purified according to manufacturer's protocol. Five microliters of in vitro-translated ^{35}S methionine-labeled AR (**lanes 4**, **6**, and **8**) and RXRα (**lanes 3**, **5**, and **7**) were incubated with the GST-TR2 (lanes 4–**7**) and GST (lanes 3 and 8) bound glutathione-sepharose beads. The pull-down complex was loaded on 10% SDS–PAGE and visualized by autoradiography. **B**: PC-3 cells were transfected with VP16-TR2 (**lane 1**), GAL4-AR (**lane 2**) alone, or VP16-TR2 and GAL4-AR together (lane 3), or VP16-RXR and GAL4-AR together (lane 4). A GAL4 responsive CAT reporter gene has also been cotransfected. The PC-3 cells were treated with 10nM DHT 18 hr after transfection. The cells were harvested 24 hr after treatment, and CAT assay was performed.

DISCUSSION

It has been known that TR2 suppresses many genes through three molecular mechanisms. The first mechanism is that TR2 directly binds to the consensus response element (TR2-RE) in the promoter region of its target genes and thus regulates the expression of these genes. The first DNA response element for TR2 was identified in the SV40 major late promoter +55 region. TR2 can bind with this response element with high affinity [7]. Human erythropoiein gene [8], Histamine H1 receptor gene [9], aldolase A gene [10], CNTF receptor gene [11], and human papillomavirus type 16 long control region [18] have been demonstrated to be regulated through this mechanism by TR2.

The second mechanism by which TR2 modulates other genes is through competition with other nuclear receptors to bind to the same response element and thus influence the functional activity of these genes. Human TR2 binds to synthetic TR2-REs consisting of two AGGTCA half sites with various nucleotides of spacing in the following order, from greatest to least affinity: DR1 > DR2 > DR5 = DR4 = DR6 > DR3 (5). Through this mechanism, TR2 has been shown to be able to modulate RAR/RXR-mediated transactivation [5,6].

The third molecular mechanism through which TR2 suppresses other genes, is via protein–protein direct

interaction. Dimerization is essential for most nuclear receptor functions. RAR, thyroid hormone receptor, vitamin D receptor, and orphan receptor LXR and FXR all form heterodimers with the common partner RXR [19]. Recently the interaction and mutual suppression between AR and orphan receptor TR4, an orphan receptor closely related to TR2, have been reported [20]. Suppression of estrogen receptor (ER)-mediated transactivation by TR2 and TR4 through protein–protein interaction has also been reported [4,21]. Homodimerization of TR2 and TR4 and heterodimerization between TR2 and TR4 have been discovered [22]. Thus the complicated homodimer and heterodimer complexes may play roles in the modulation of AR- or ER-mediated transactivation. The interaction of AR and TR2 may be only one step of these complicated dimer complex formations in prostate or prostate cancer. In this study we demonstrated that TR2 could also function as a negative modulator for AR transactivation in prostate cancer cells. Both AR and TR2 are expressed in prostate and prostate cancer. The cross-talk between these two nuclear receptors may represent a new regulatory mechanism in prostate and prostate cancer.

In conclusion, the finding that AR mediated transactivation could be suppressed by TR2 through the interaction between these two receptors represents a unique TR2 function. Further studies may expand the role of TR2 on the androgen/AR function.

REFERENCES

1. Chang C, Kokontis J. Identification of a new member of the steroid receptor superfamily by cloning and sequence analysis. Biochem Biophys Res Commun 1988;155:971–977.
2. Chang C, Kokontis J, Acakpo-satihivi L, liao S, Takeda H, Chang Y. Molecular cloning of new human TR2 receptor: A class of steroid receptor with multiple ligand-binding domain. Biochem Biophys Res Commun 1988;165:735–741.
3. Lee C-H, Chang L-M, Wei L-N. Molecular cloning and characterization of a mouse nuclear orphan receptor expressed in embryos and testis. Mol Reprod Dev 1996;44:305–314.
4. Hu Y-C, Shyr C-R, Che W-Y, Mu X-M, Kim E, Chang C. Suppression of estrogen receptor-mediated transactivation and cell growth by interaction with TR2 orphan receptor. 2002; J Biol Chem 277:33571–33579.
5. Lin T-M, Young W-J, Chang C. Multiple function of the TR2-11 orphan receptor in modulating activation of two cis-acting elements involved in the retinoic acid signal transduction system. J Biol Chem 1995;270:30121–30128.
6. Wei L-N, Hu X, Chinpaisal . Constitutive activation of retinoic acid receptors beta 2 promoter by orphan receptor TR2. J Biol Chem 2000; 11907–11914.
7. Lee HJ, Chang C. Identification of human TR2 orphan receptor response element in the transcriptional initiation site of the simian virus 40 major late promoter. J Biol Chem 1995;270:5434–5440.
8. Lee H-J, Young W-J, Shih CC-Y, Chang C. Suppression of the human erythropoietin expression gene by the TR2 orphan receptor: A member of steroid receptor superfamily. J Biol Chem 1996;271, 10405–10412.
9. Lee H-J, Lee Y-F, Chang C. Identification of the histamine H1 receptor gene as the differentially repressed target for the human TR2 orphan receptor. Mol Cell Biochem 1999;194:199–207.
10. Chang C, Lee H-J, Lee Y-F. Identification of the human adolase A gene as the first induced target for the TR2 orphan receptor, a member of the steroid hormone receptor superfamily. Biochem Biophys Res Commun 1997;235:205–211.
11. Young WJ, Lee YF, Smith SM, Chang C. A bidirectional regulation between the TR2/TR4 orphan receptors (TR2/TR4) and the ciliary neurotrophic factor (CNTF) signal pathway. J Biol Chem 1998;273:20877–20885.
12. Lin D-L, Chang C. P53 is a mediate for radiation-repressed human TR2 orphan receptor expression in MCF-7 cells, a new pathway from tumor suppressor to member of steroid receptor superfamily. J Biol Chem 1996;271:14649–14652.
13. Mu XM, Liu YX, Collins LL, Kim E, Chang C. The p53/Retinoblastoma-mediated repression of testicular orphan receptor-2 in the rhesus monkey with cryptorchidism. J Biol Chem 2000;275:23877–23883.
14. Chang C, Kokontis J, Liao S. Molecular cloning of human and rat complementary DNA encoding androgen receptor. Science 1988;240:324–326.
15. O'Malley B. The steroid receptor superfamily: More excitement predicted for the future. Mol Endocrinol 1990;4:363–369.
16. Heinlein CA, Chang C. Androgen receptor (AR) coregulators: An overview. Endorcrinol Rev 2002;23:175–200.
17. Mu XM, Young WJ, Uemura H, Chang C. Induction of an intronic enhancer of the human ciliary neurotrophic factor receptor (CNTFR alpha) gene by the TR3 orphan receptor. 1998; Endocrine 9:27–32.
18. Collins LL, Lin D-L, Mu X-M, Chang C. Feedback regulation between orphan nuclear receptor TR2 and human papilloma virus type 16. J Biol Chem 2001;276:27316–27321.
19. Mangelsdorf DJ, Evans RM. The RXR heterodimer and orphan receptors. Cell 1995;83:841–850.
20. Lee Y-F, Shyr C-R, Thin TH, Lin W-J, Chang C. Convergence of two repressors through heterodimer formation of androgen receptor and testicular orphan receptor-4: A unique signaling pathway in the steroid receptor superfamily. Proc Natl Acad Sci USA 1999;96:14724–14729.
21. Shyr C-R, Hu Y-C, Kim E, Chang C. Modulation of estrogen receptor-mediated transactivation by orphan receptor TR4 in MCF cells. J Biol Chem 2002;277:14622–14628.
22. Lee C-H, Chinpaisal C, Wei L-N. A novel nuclear receptor heterodimerization pathway mediated by orphan receptor TR2 and TR4. J Biol Chem 1998;273:25209–25215.