

Roles of Testicular Orphan Nuclear Receptors 2 and 4 in Early Embryonic Development and Embryonic Stem Cells

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The testicular orphan nuclear receptors (TRs) 2 and 4 act as either transcriptional activators or regulatory proteins of other nuclear receptor superfamily members. With no identified cognate ligands, their physiological roles remain unclear. Here we showed the phenotypes of $TR2^{-/-}$: $TR4^{-/-}$ mutant embryos, which reveal that the loss of TR2 and TR4 causes early embryonic lethality and increased cell death. We also found that TR2 and TR4 are expressed in blastocysts and embryonic stem (ES) cells, and can act as transcriptional activators in ES cells. The results on further investigating the roles of TR2 and TR4 in ES cells showed that TR2 and TR4 were differentially expressed when ES cells were induced into different specialized cell types, and their expression is regulated by retinoic acid. Knocking down TR2 and TR4 mRNAs decreased the expression of Oct-3/4 and Nanog genes. Mechanism dissection suggests that TR2 and TR4 may affect the Oct-3/4 gene by binding to a direct repeat-1 element located in its promoter region, which is influenced by retinoic acid. Together, our findings highlight possible roles for TR2 and TR4 in early embryonic development by regulating key genes involved in stem cell self-renewal, commitment, and differentiation. (*Endocrinology* 150: 2454–2462, 2009)

The orphan nuclear receptors, testicular receptors (TRs) 2 and 4, are closely related in many aspects such as amino acid sequence homology, tissue distributions, and biochemical functions (1, 2). Orphan nuclear receptors belong to the nuclear receptor superfamily, which comprises ligand-activated transcriptional factors that control gene expression in response to developmental, physiological, and environmental cues (2, 3). Orphan nuclear receptors are named because their ligands remain unknown, resulting in difficulties in searching for their physiological functions.

A number of orphan nuclear receptors have found their ligands in recent years. These receptors function in regulating steroid, lipid, or xenobiotic metabolism and homeostasis (2, 4).

Consequently, there are increased interests in finding ligands for remaining orphan nuclear receptors such as TR2 and TR4 to expand our understanding of nuclear receptor signaling. Because orphan nuclear receptors are ancient components of the nuclear receptor family (3, 5), they possible act in early embryo development and differentiation in primitive cells.

Early embryonic development includes cleavage of the fertilized egg to form a blastula, gastrulation, and organogenesis. These events involving stem cell proliferation, differentiation, and apoptosis are controlled by a complex network of transcriptional factors that up-regulate or down-regulate essential genes for development. The embryonic stem (ES) cells, first isolated from the inner cell masses (ICMs) of mouse blastocysts, are plu-

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Abbreviations: CHIP, Chromatin immunoprecipitation; Coup-TF, chicken ovalbumin upstream promoter-transcription factor; DAPI, 4',6-diamidino-2-phenylindole; DR1, direct repeat-1; EB, embryoid body; E7.5, embryonic d 7.5; ES, embryonic stem; GCNF, germ cell nuclear factor; ICM, inner cell mass; PEPCK, phosphoenolpyruvate carboxylase; qRT-PCR, quantitative RT-PCR; RA, retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor; shRNA, short hairpin RNA; SUMO, small ubiquitin-related modifier; TR, testicular receptor; tRA, all-trans-retinoic acid; TR2/4-RE, testicular receptor 2/4 responsive element; TUNEL, terminal deoxynucleotidyl transferase-mediated uridine 5'-triphosphate end labeling.

ripotent cells with the ability to self-renew and differentiate into cell types of all three germ layers (6, 7). With a defined culture condition containing required inducing factors, ES cells are able to differentiate and form embryo bodies that then can undergo different transformations including, neuronal (8), hematopoietic (9), osteogenic (10), and adipogenic (11) differentiation.

Maintaining ES cells in an undifferentiated state requires leukemia inhibitory factor to activate signal transducer and activator of transcription 3 (Stat3) that serves as a key transcriptional determinant of ES cell self-renewal (12, 13). Beside signal transducer and activator of transcription 3 (Stat3), other transcriptional factors such as Oct-3/4 (14) and Nanog (15, 16) also control early ES cell potency, self-renewal, and embryonic cell fate specification.

As for inducing ES cells into differentiation, many signaling molecules play a role in influencing lineage differentiation (17). Among them, retinoic acid (RA) was shown to be a master regulator in the induction and lineage selection of ES cells into neuronal cells (8, 18), adipocytes (11), osteoblasts (19), or germ cells (20). RA, an active metabolite of vitamin A, functions through two subfamilies of nuclear receptors, retinoic acid receptor (RAR) and retinoid X receptor (RXR). Activated by both all-trans RA (tRA) and 9-cis RA, RARs have three subtypes, RAR α , RAR β , and RAR γ , which are products of three individual genes. However, the RXR subfamily is only activated by 9-cis RA (2, 21). RA was shown to repress Oct-3/4 expression and then induce transcription of specific target genes linked to the ability of ES cells to differentiate into different cell types (22, 23). Therefore, RA signaling may play a critical role in embryogenesis and cellular differentiation, but the exact molecular events of RA signaling that lead a pluripotent stem cell to a fully differentiated cell are yet to be determined.

In vitro studies showed that TR2 and TR4 act similarly as regulatory proteins for other nuclear receptors, including estrogen receptors (24, 25), and RARs/RXR (8, 26). In addition to interfering with nuclear receptor signaling, they can function either as transcriptional activators via their DNA binding elements (27–30), or transcriptional repressors (31, 32). The studies on the physiological functions of TR2 or TR4 by using the gene knockout approach demonstrated that the TR2 knockout (TR2^{-/-}) mice appear normal with no overt defects (33), but the TR4 knockout (TR4^{-/-}) mice exhibit growth retardation and defects in reproduction and maternal behavior in females (34), as well as deficits in spermatogenesis and motor coordination with aberrant cerebellar development (35, 36). Furthermore, the observation that the number of TR4^{-/-} pups generated by the mating of TR4 heterozygous mice is well under that predicted by the normal mendelian ratio (34) indicates that TR4 plays an essential role in embryonic development.

In addition, TR2 and TR4 were shown to be expressed in embryonic carcinoma cells that are capable of being derived into three germ cell types, and modulate RA-RAR/RXR signaling in embryonic carcinoma cells (8, 26). TR4 was reported to affect the RA-mediated apoptosis or differentiation in embryonic carcinoma cells (37). A recent study demonstrated the function of TR2 in fine-tuning Oct-3/4 expression and regulating stem cell proliferation (38). These findings, as well as their tissue and cell

expression patterns, suggest that TR2 and TR4 may work very closely and have common functions in living organisms, but may also have their unique roles. Therefore, the roles of TR2 and TR4 in embryonic development need further investigation.

Based on the findings of the action of TR2 and TR4 on RA signaling in embryonic carcinoma cells (27, 30, 37–39), we suspected that TR2 and TR4 may affect RA target genes that are critical in early embryonic events. Here, we investigated how TR2 and TR4 act in early embryonic development by exploring the phenotypes of TR2^{-/-}:TR4^{-/-} compound mutant mice. To delineate further the molecular mechanism by TR2 and TR4 to regulate development, we used ES cells for simulating early embryonic developmental events, and find how TR2 and TR4 function in ES cells. Our reports show that TR2^{-/-}:TR4^{-/-} mutant mice die in early embryonic stages and exhibit increased apoptosis. TR2 and TR4 are expressed in blastocysts, ES cells, and embryoid bodies (EBs), and they could act as a transcriptional factor in ES cells. In addition, TR2 and TR4 are expressed differentially during the ES cells undergoing neuronal, adipogenic, or osteogenic differentiation, and their expression is up-regulated by RA in ES cells. TR2 and TR4 bind a direct repeat-1 (DR1) sequence that locates within the Oct-3/4 promoter (40), and the binding is repressed in the presence of RA. The Oct-3/4 expression is influenced by the expression level of TR2 or TR4. Together, our results suggest that TR2 and TR4 play a fundamental role in early embryogenesis, and regulate the ES cell differential and developmental events, affecting RA-induced differentiation through the regulation of pluripotency genes such as Oct-3/4 and Nanog.

Materials and Methods

Animals

Mice were housed in our animal care facility with a controlled 12-h light, 12-h dark cycle and a controlled temperature range of approximately 25°C. Interbreedings between TR2^{-/-} and TR4^{+/-} mice were done to generate compound heterozygotes (TR2^{+/-}:TR4^{+/-}). Double knockout mutants (TR2^{-/-}:TR4^{-/-}) were obtained by intercrossing TR2^{-/-}:TR4^{+/-} mice from breeding of compound heterozygotes. All animals received humane animal care as outlined in *A Guidebook for the Care and Use of Experimental Animals* (Chinese-Taipei Society of Laboratory Animal Sciences, Taiwan, 2005).

For analysis of mouse genotypes, tail snips from 3-wk-old mice or embryos dissected at designated times were used for extracting genomic DNA to perform PCRs to determine their genotypes as described previously, including the information for the primer sets used in genotyping (33, 34).

ES cell culture, EB formation, and differentiation

The mouse blastocyst-derived ES cell line D3 was obtained from the American Type Culture Collection (Manassas, VA). Mouse ES cells (ES-D3) were grown as described in Ref. 41.

To induce the formation of EBs, ES cells were treated with trypsin-EDTA (Sigma-Aldrich Corp., St. Louis, MO) to obtain suspended cells, and 2×10^4 cells were added into a conical tube to allow aggregation for 5 d by following the method as described in Ref. 42. Five-day EBs were transferred to 35-mm tissue culture dishes coated with 0.1% gelatin and processed for differentiation. For adipogenic differentiation, EBs were treated with 1 μ M tRA for 3 d, and then kept in the medium containing 0.5 μ g/ml insulin and 2 nM T₃ for 6 d. For neural differentiation, EBs were

induced by 1 μM tRA (Sigma-Aldrich) for 10 d. To induce osteogenic differentiation, EBs were cultured in the medium supplemented with 1 μM dexamethasone, 50 $\mu\text{g}/\text{ml}$ ascorbic acid, and 50 mM β -glycerophosphate for 21 d.

Preparation of blastocysts and embryos

The blastocysts were obtained as described in Ref. 41. Blastocysts were flushed out from the uterus on d 3.5 pregnancy with PBS solution and incubated in a four-well chamber slide (Nalge Nunc Intl., Thermo Fisher Scientific, Tokyo, Japan) of DMEM with 15% ES-fetal bovine serum (Life Technologies, Inc., Gaithersburg, MD) supplemented with 4.5 g/liter glucose, 0.1 mM β -mercaptoethanol, 4 mM L-glutamine, 1 mM NaHCO_3 , 100 IU/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin in 5% CO_2 /95% air at 37 C to allow blastocysts to hatch from the zona pellucida and attach on the slides. After culturing for 36 h, the attachment or outgrowth was observed, and the blastocysts grew out primary giant trophoblasts and formed ICMs. Mouse embryos from embryonic d 7.5 (E7.5) were collected and fixed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4). Fixed embryos were then embedded in paraffin for sectioning.

Fluorescent immunostaining

ES cells were grown on four-well chamber slides for 24 h, then subsequently fixed in ice-cold methanol (-20 C), and incubated for 15 min. After blocking by blocking buffer (2% BSA plus 2% fetal bovine serum in PBS), cells or *in vitro* cultured blastocysts were then incubated with anti-TR2 (M-85, Santa Cruz sc-9087; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or anti-TR4 (M-76, Santa Cruz sc-9086; Santa Cruz Biotechnology) antibody overnight at 4 C in a humidified chamber, followed by detection with biotinylated secondary antibodies and fluorescein isothiocyanate-conjugated avidin. Slides were dyed with 4',6-diamidino-2-phenylindole (DAPI) for 1 min in the dark at room temperature for nuclear staining. Samples were further analyzed by fluorescence microscopy (Olympus, Hamburg, Germany) equipped with a camera (SPOT; Diagnostic Instruments, Sterling Heights, MI) for photo-images.

Plasmids

The short hairpin RNA (shRNA) sequence used to silence the translation of TR2 was 5'-GCGTCATTACGGAGCAATAAC-3' and to silence the translation of TR4 was 5'-GGAGTCTGTACAGAGTGAACG-3'. The construction of pENTR/U6/TR2^{shRNA} and pENTR/U6/TR4^{shRNA} was performed according to the manufacturer's protocol (Invitrogen Corp., Carlsbad, CA). The pENTR/U6/LacZ^{shRNA} was used as a negative control.

Terminal deoxynucleotidyl transferase-mediated uridine 5'-triphosphate end labeling (TUNEL) assay

E7.5 embryos for TUNEL analysis were fixed, embedded, and sectioned. After deparaffinization and rehydration, sections were then treated with proteinase K in PBS containing 1% Triton X-100 and were subjected to the TUNEL assay according to the manufacturer's instructions (Oncogene Science, Siemens Healthcare Diagnostics, Deerfield, IL).

Luciferase reporter gene assays

The phosphoenolpyruvate carboxykinase (PEPCK)-LUC reporter plasmid was obtained from Dr. Y. F. Lee (Pathology Department, University of Rochester, Rochester, NY) (43). pCMV-TR4 and pCMV-TR2 that expressed human TR2 and TR4, respectively, were described previously (27, 30). For reporter gene assays, 1×10^6 ES cells were seeded in 100-mm tissue culture dishes. After 24 h, cells were transfected with the indicated constructs with Lipofectamine (Invitrogen) according to the manufacturer's protocol. Luciferase assays were measured with a luminometer and the Dual-Luciferase-Reporter Assay System (Promega Corp., Madison, WI). Firefly luciferase values were normalized to *Renilla* luciferase activity (from the transfection of 0.5 μg pRL-SV40 used to correct the differences in transfection efficiency). The data were ex-

pressed as relative luciferase activity as mean "fold induction" with respect to the normalized firefly luciferase activity of the empty vector pCMV transfected control. Luciferase activity measured from cell lysates obtained from cells cotransfected with reporter plasmids and empty expression vector was arbitrarily set at one. Data are displayed as mean \pm SD.

Quantitative real-time PCR analysis

Total RNA was extracted by using the TRIzol reagent (Invitrogen), and first-strand cDNA was synthesized from 2 μg total RNA in 20 μl reactions containing reverse transcriptase buffer with deoxynucleotide triphosphates, oligo-deoxythymidine, ribonuclease inhibitor, and Moloney murine leukemia virus reverse transcriptase according to the manufacturer's protocol (Invitrogen). The quantitative PCR was done as described in Ref. 44. The following primer pairs were used: TR2 forward 5'-CCGCATCTAATCGCTGGAGAG-3', reverse 5'-CGATGTAGTTGGCGAAGCG-3'; TR4 forward 5'-CATATTCACCACCTCGGACAA-3', reverse 5'-TGACGCCACAGACCACAC-3'; Oct-3/4 forward 5'-TGAGAACCTTCAGGAGATATGCAA-3', reverse 5'-CTCAATGCTAGTTCGCTTTCTCTTC-3'; and Nanog forward 5'-CAGAA-AAACCAGTGGTTGAAGACTAG-3', reverse 5'-GCAATGGATGCTGGGATACTC-3'.

RNA interference

ES cells were transfected with 24 μg pENTR/U6 entry (Invitrogen) plasmids containing double-stranded oligos encoding shRNA targeting TR2, TR4, or LacZ (*i.e.* pENTR/U6/TR2^{shRNA}, pENTR/U6/TR4^{shRNA}, or pENTR/U6/LacZ^{shRNA}) by Lipofectamine, following the manufacturer's protocols. RNAs were prepared 48 h after transfection and assayed with quantitative RT-PCR (qRT-PCR). The cells transfected with pENTR/U6/LacZ^{shRNA} were used as a negative control.

Chromatin immunoprecipitation (ChIP) assay

The ChIP was done with ES cells treated with 1 μM tRA for 24 h. Cells were cross-linked with 1% formaldehyde for 10 min at 37 C, harvested in sodium dodecyl sulfate lysis buffer to obtain nuclear extracts, and DNA was sheared to fragments by sonication. Antibodies against TR2, TR4, or RAR β were added to each aliquot of precleared chromatin and incubated overnight. Protein G agarose beads were added and incubated for 2 h at 4 C. After washing and reversing the cross-links, DNA was isolated and used for PCRs. The primer sets 5'-CCCCACCCCGCGT-TCCAATCT-3' and 5'-GCGCCTCGAGCCTTCTCTG-3' were used to amplify a 189-bp region of the Oct-3/4 promoter harboring the TR2/4-RE.

Statistical analysis

Results are expressed as the mean \pm SD. Statistical significance was evaluated by one-way ANOVA or a *t* test (SPSS, Inc., Chicago, IL).

Results

The generation of TR2^{-/-}:TR4^{-/-} mice

To study the physiological function of TR2 and TR4, we have generated TR2 (33) and TR4 knockout mice (34). Here, we crossed heterozygous TR2 and TR4 mice to generate TR2/4 compound heterozygous (TR2^{+/-}:TR4^{+/-}) mice, from which we produced TR2^{-/-}:TR4^{+/-} mice. We then used TR2^{-/-}:TR4^{+/-} mice to generate mice that lack both the TR2 and TR4 (TR2^{-/-}:TR4^{-/-}) to study further the role of TR2 and TR4 in physiology. The detailed process demonstrated in Fig. 1 shows the PCR genotyping analysis to identify TR2^{-/-}:TR4^{-/-} mice.

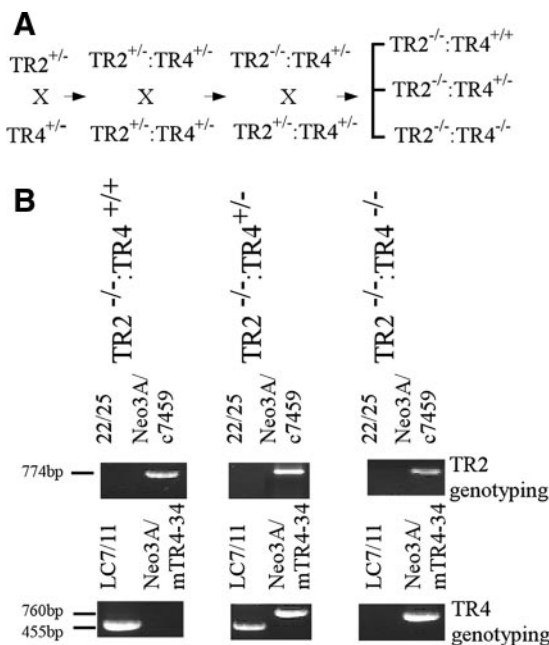


FIG. 1. The generation of $TR2^{-/-};TR4^{-/-}$ mice. **A**, The scheme of the generation process for $TR2^{-/-};TR4^{-/-}$ mice. $TR2$ ($TR2^{+/-}$) and $TR4$ ($TR4^{+/-}$) heterozygous mice were crossed to generate $TR2$ and $TR4$ compound heterozygous mice ($TR2^{+/-};TR4^{+/-}$). By crossbreeding $TR2$ and $TR4$ compound heterozygous mice ($TR2^{+/-};TR4^{+/-}$), we produced $TR2$ knockout and $TR4$ heterozygous mice ($TR2^{-/-};TR4^{+/-}$). We then crossbred $TR2$ knockout and $TR4$ heterozygous mice ($TR2^{-/-};TR4^{+/-}$) to generate $TR2$ and $TR4$ double-knockout mice ($TR2^{-/-};TR4^{-/-}$). **B**, The representative gel of PCR genotyping on genomic DNA isolated from embryos. Primers (22/25) amplified a 498-bp fragment from the $TR2$ wild-type allele. Primers (Neo 3A/c7459) amplified a 774-bp fragment from the $TR2$ mutant allele. Primers (LC7/11) amplified a 455-bp fragment from the $TR4$ wild-type allele. Primers (Neo 3A/mTR4-34) amplified a 760-bp fragment from the $TR4$ mutant allele. The sizes of PCR products are indicated.

The phenotypes of $TR2^{-/-};TR4^{-/-}$ mice

To clarify their function *in vivo*, we intercrossed $TR2^{-/-};TR4^{+/-}$ compound mice because $TR2$ knockout mice have normal reproduction ability. After examining the genotypes of newborn pups and embryos, we found that no $TR2^{-/-};TR4^{-/-}$ pups were born, and embryos at E12.5 were determined to be $TR2^{-/-};TR4^{+/+}$ and $TR2^{-/-};TR4^{+/-}$ genotypes only. Embryos from E9.5–11.5 were also dissected for genotype analysis, which showed that four resorbed embryos, with abnormal shrunken morphology indicating severe developmental defects (Fig. 2A), were $TR2^{-/-};TR4^{-/-}$ double knockout. E7.5 embryo genotypes were distributed approximately according to mendelian ratios (Table 1). These results show that the loss of both $TR2$ and $TR4$ results in early embryonic lethality. Because early embryonic lethality was observed in $TR2^{-/-};TR4^{-/-}$ compound mutant mice, we further determined whether the loss of both $TR2$ and $TR4$ causes developmental defects resulting from increased apoptosis by performing the TUNEL assay on wild-type and $TR2^{-/-};TR4^{-/-}$ compound mutant E7.5 embryos. The result (Fig. 2B) revealed that $TR2^{-/-};TR4^{-/-}$ compound mutant E7.5 embryos displayed more fluorescence-positive apoptotic cells than wild-type embryos. Therefore, early embryonic lethality and increased apoptosis were observed in the $TR2^{-/-};TR4^{-/-}$ compound mutant embryos.

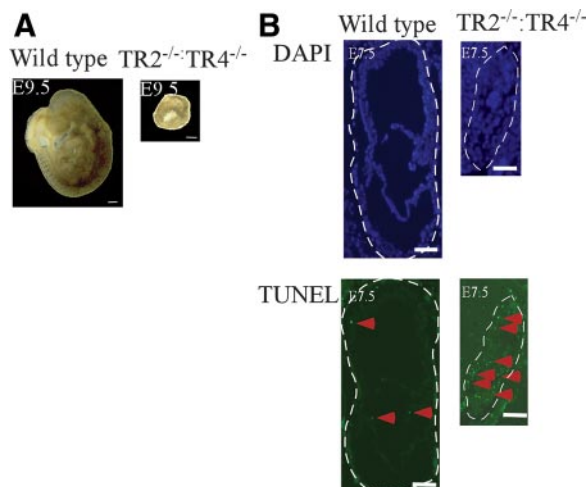


FIG. 2. The phenotypes of $TR2^{-/-};TR4^{-/-}$ mice. **A**, Defected growth and differentiation in $TR2^{-/-};TR4^{-/-}$ embryos. E9.5 wild-type embryos begin organogenesis, but $TR2^{-/-};TR4^{-/-}$ E9.5 embryos are in resorption with no distinguishable structures. **B**, TUNEL assay on E7.5 embryos. Apoptotic cells were measured by performing a TUNEL assay on either wild-type (left) or $TR2^{-/-};TR4^{-/-}$ E7.5 embryos. Arrowheads indicate fluorescein isothiocyanate-labeled apoptotic cells (bright green, lower panel). DAPI was used for nuclear staining (blue, upper panel). Dotted lines in **B** outline the embryos.

In vitro cultured mouse blastocysts, mouse ES cells, and EBs express both $TR2$ and $TR4$

To investigate whether $TR2$ and $TR4$ play a role in early embryogenesis, we first examined whether $TR2$ and $TR4$ are present in stem cells existing in early embryos and ES cells. *In vitro* cultured blastocysts and ES cells were stained with anti- $TR2$ or anti- $TR4$ antibodies. We found that in blastocysts, the expression of $TR2$ and $TR4$ was weakly detected in the trophoblast but was strongly expressed in the ICM (Fig. 3A). ES cells were also shown to express $TR2$ and $TR4$ in the nucleus (Fig. 3B). To prove further the presence of $TR2$ and $TR4$ in early embryos, we used RT-PCR to perform mRNA analysis on ES cells and EBs that are ES cell multicellular aggregates. They resemble early postimplantation embryos and are an essential stage in ES cell differentiation (42). The results demonstrated the presence of $TR2$ and $TR4$ RNA transcripts in ES cells and EBs (Fig. 3C). Testis was used as a positive control for the expression of $TR2$ and $TR4$. These results show that $TR2$ and $TR4$ are present in stem cells whether they are in early embryos, derived cell lines, or cell aggregates. As transcription factors, $TR2$ and $TR4$ contain the ability to modulate their target gene transcription. To examine whether this ability is functional in ES cells, we performed luciferase reporter gene assays to determine whether $TR2$ and $TR4$ function as transcriptional factors to activate

TABLE 1. Genotypes of neonates and embryos from $TR2^{-/-};TR4^{+/-} \times TR2^{-/-};TR4^{+/-}$ mice intercrosses

Age/ genotype	$TR2^{-/-};TR4^{+/+}$	$TR2^{-/-};TR4^{+/-}$	$TR2^{-/-};TR4^{-/-}$	Total
E7.5	4 (31)	6 (46)	3 (23)	13
E9.5–11.5	9 (33)	14 (52)	4 (15%, resorbed)	27
E12.5	5 (30)	12 (70)	(0)	17
Newborn	127 (38)	208 (62)	(0)	335

Values are expressed as number (%).

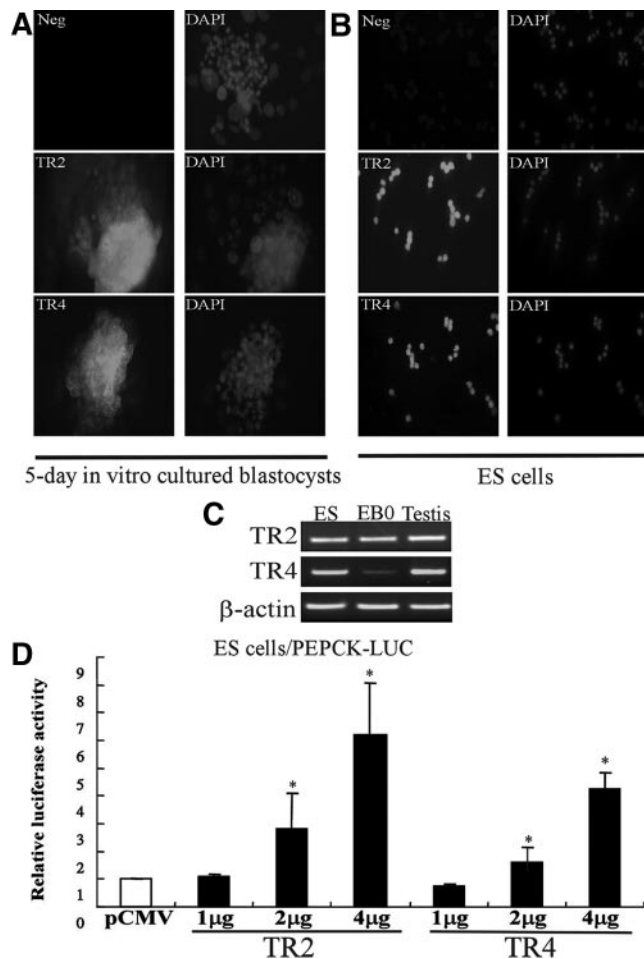


FIG. 3. The expression and action of TR2 and TR4 in *in vitro* cultured blastocysts and ES cells. **A**, E3.5 blastocysts were isolated and cultured on chamber slides for 5 d to let them hatch and attach on slides. The slides were then fixed and incubated with PBS as negative control (Neg) (upper panel), anti-TR2 (middle panel), or TR4 antibodies (lower panel). **B**, ES cells were placed on slides coated with poly-L-lysine for 2 h for attachment. The slides were then fixed and incubated as in **A** to perform immunofluorescent staining. DAPI was used for nuclear staining. Scale bars represent 250 μ m in **A** and 60 μ m in **B**. **C**, RT-PCR analysis of TR2 and TR4 in ES cells, EBs, and testes. **D**, The transcriptional activity of TR2 and TR4 in ES cells. ES cells were transfected with pPEPCK-LUC along with pCMV empty vector or an increasing amount of pCMV vector expressing TR2 or TR4 for 48 h. Cell lysates were harvested to perform dual luciferase assay. To determine the relative luciferase activity, the level of ES cells transfected with pPEPCK-LUC was set at one. Data represent means \pm SD from at least three experiments. *, $P < 0.05$ by ANOVA, *post hoc* Tukey's test against the vector control group.

transcription. The luciferase reporter plasmid contains the 5' proximal promoter of the PEPCK, the key gene in gluconeogenesis to respond to quick nutritional depletion and/or hormonal alteration. We transfected ES cells with the plasmids encoding TR2 and TR4, as well as the pPEPCK-LUC plasmids containing the DR1 sequence that can be bound by TR2 and TR4 and mediates the transactivational ability of TR2 and TR4 (43). As increasing amounts of TR2 or TR4 were exogenously expressed in ES cells, the luciferase activity was dose dependently increased by approximately 7-fold for TR2 and 4-fold for TR4 (Fig. 3D). This result shows that TR2 and TR4 function in ES cells as transactivators to turn on gene expression.

The expression of TR2 and TR4 during ES cells neural, osteogenic, or adipogenic differentiation

ES cells are capable of differentiating into several type of cells derived from three germ layers, including neuron, bone, and fat. This pluripotency makes ES cells an ideal tool to study the cell differentiation and tissue development, which are multiple processes involving activation and repression of many essential genes, including nuclear receptors. To investigate whether TR2 and TR4 are involved in ES cell differentiation, we induced ES cell into either neural, adipogenic, or osteogenic differentiation with factors needed for induction, and examined the relationship between specific cell lineage cell differentiation and the expression of TR2 and TR4. By using qRT-PCR, as shown in Fig. 4A, compared with the TR2 expression in undifferentiated ES cells, TR2 expression level increased at the EB stage by 1.7- and 2.5-fold when cells were induced into neural differentiation. When cells were induced into adipocytes and osteoblasts, TR2 mRNA was expressed 50 and 45% higher, respectively, compared with the RNA level in undifferentiated ES cells. For TR4 expression pattern, compared with its expression in undifferentiated ES cells, TR4 mRNA level decreased at EB stage by 80 and 70% when cells were induced into neural differentiation. TR4 mRNA level remained unchanged when cells were induced into adipocytes and osteoblasts (Fig. 4B). Marker genes for neurons, adipocytes, or osteoblasts were also assayed to demonstrate the differentiation of cells (Fig. 4, C–E). The expression of these genes was increased after induction.

RA affects the expression of TR2 and TR4

Previous studies demonstrate that a mutual interaction exists between RAR/RXR and TR2 or TR4 (26, 39). On one hand, TR2 and TR4 modulate RA-RAR/RXR signaling through the competition for the same DNA site, and on the other hand, the expression of TR2 and TR4 is regulated by RA, suggesting an intricate regulatory network between RA/RXA and TR2 or TR4. Therefore, we tested whether TR2 and TR4 expression was also affected by RA in ES cells. By conducting q-RT-PCR analysis on ES cells treated with RA at different time points (1, 2, and 4 d), we found that TR2 and TR4 mRNAs were expressed increasingly after treatment with RA in a time-dependent manner compared with their expression in ES cells in the absence of RA. TR2 expression increased 1.7-fold, and TR4 RNA transcripts were also up-regulated to 1.9-fold after 72 h RA treatment (Fig. 5, A and B). As shown in Fig. 5, C and D, the Oct-3/4 and Nanog mRNA both decreased when ES cells were treated with RA, indicating the ability of RA to differentiate ES cells. These results show that RA represses Oct-3/4 and Nanog transcript production but induces the expression of TR2 and TR4 in ES cells.

TR2 and TR4 regulate Oct-3/4 and Nanog expression in ES cells

Because Oct-3/4 controls the fate of ES cells, therefore, it becomes a central switch targeted by differentiation inducers like RA that binds RAR/RXA to repress Oct-3/4 expression (40, 45, 46). In addition to Oct-3/4, Nanog is another transcriptional factor that is involved in deciding the direction for ES cells toward self-renewal or differentiation (15, 16). To determine

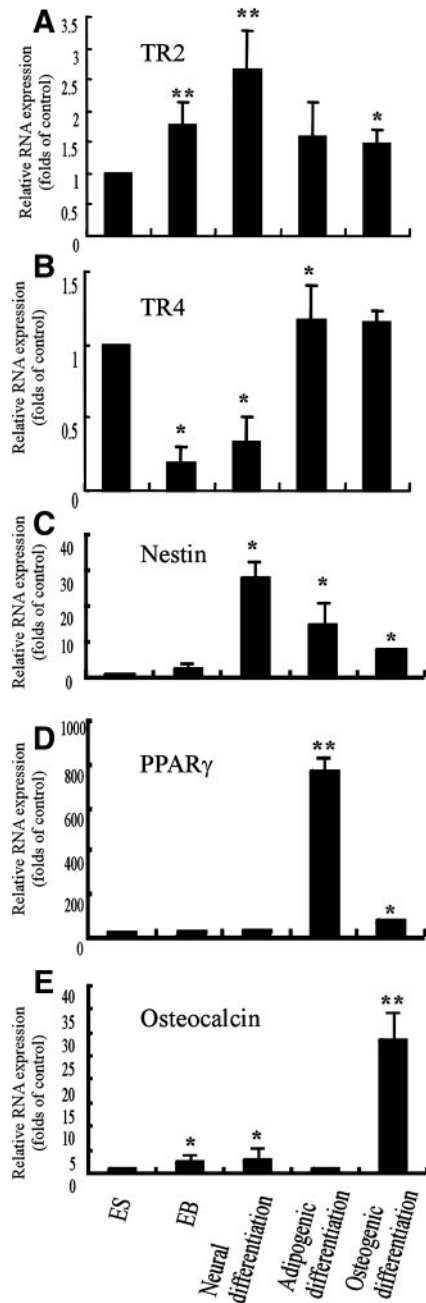


FIG. 4. The differential expression pattern of TR2 and TR4 during the neural, adipogenic, or osteogenic differentiation of ES cells. ES cells aggregated to form EBs that were induced for neural, osteogenic, or adipogenic differentiation. At the end of differentiation, the cells were harvested and assayed for TR2 (A) and TR4 (B) mRNA transcripts by qRT-PCR. Marker genes for neurons (C), adipocytes (D), or osteoblasts (E) were also examined. Results are shown as fold increase of expression compared with control ES cells and normalized to β -actin. Data represent means \pm SD from at least three experiments. *, $P < 0.05$; **, $P < 0.01$ by ANOVA, *post hoc* Tukey's test against the ES cells control group. PPAR, peroxisome proliferator activated receptor.

whether TR2 and TR4 play a role in the ES cells decision to self-renew or differentiate by modulating key genes in those events, we tested the requirements of TR2 or TR4 expression on Oct-3/4 and Nanog RNA level by knocking down TR2 or TR4 expression in ES cells with plasmids expressing shRNA for TR2 or TR4. Compared with the RNA transcripts in the cells transfected with LacZ shRNA plasmids, TR2 expression (Fig. 6A,

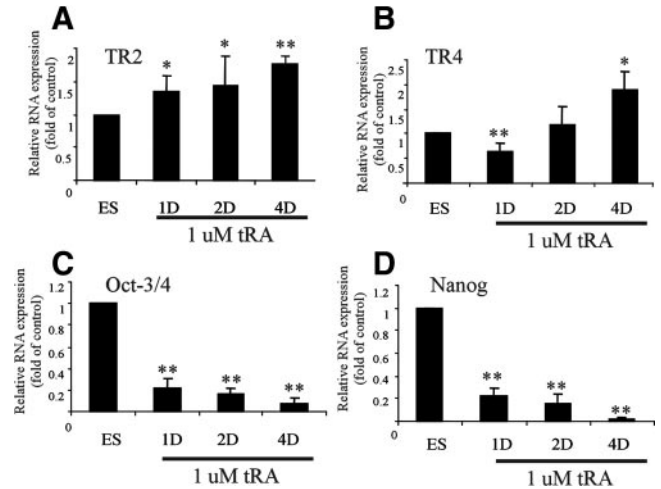


FIG. 5. The effects of RA on the expression of TR2 and TR4. qRT-PCR analysis of TR2 (A), TR4 (B) Oct-3/4 (C), and Nanog (D) mRNA levels were performed on RNAs isolated from ES cells stimulated with 1 μ M tRA at indicated time points. Results are shown as fold increase of expression compared with ES cells control and normalized to β -actin. Data represent means \pm SD from at least three experiments. *, $P < 0.05$; **, $P < 0.01$ by ANOVA, *post hoc* Tukey's test against the ES cells control group.

upper panel) was repressed by 70% by TR2 shRNA plasmid transfection, which also caused the expression of Oct-3/4 to decrease 55% (Fig. 6A, middle panel) and reduced Nanog mRNA by 54% (Fig. 6A, lower panel). Similarly, the expression of Oct-3/4 decreased 40% (Fig. 6B, middle panel), and Nanog mRNA reduced 30% (Fig. 6B, lower panel) after knocking down TR4 expression by 60% (Fig. 6B, upper panel) with TR4 shRNA plasmids. Thus, the reduced expression of TR2 and TR4 causes the repression of Oct-3/4 and Nanog expression. The expression of Oct-3/4 was repressed in TR2 and TR4 knockdown ES cells, indicating that TR2 and TR4 may regulate Oct-3/4 expression. Furthermore, Oct-3/4 expression is repressed by RA-RAR/RXR signaling pathway that is mutually affected by TR2 and TR4. To define the role of TR2 and TR4 on Oct-3/4 expression, we searched the Oct-3/4 promoter sequence to find the target DNA binding site for TR2 and TR4. We found a DR1 element, the DNA binding sequence for TR2 and TR4, in the Oct-3/4 promoter. We designated it as TR2/4 responsive element (TR2/4-RE). Incidentally, this DNA element was also identified as a RA-responsive element, which is composed of three tandemly arranged direct repeats with 1- and 0-nucleotide spacers (47). We then examined the binding of TR2, TR4, and RAR/RXR on this DR1 sequence (TR2/4-RE) in the Oct-3/4 promoter with ChIP assay in ES cells with or without RA treatment. As shown in Fig. 6C, with the ability to regulate Oct-3/4 expression, RAR β binding to DR1 element in the Oct-3/4 promoter was enhanced with the RA treatment. In the absence of RA in ES cells, the recruitment of TR2 and TR4 to the DR1 sequence in the Oct-3/4 proximal promoter was detected, but in RA treated ES cells, TR2 and TR4 decreased the binding to DR1. This result shows that TR2 and TR4 are associated with the DR1 in the Oct-3/4 promoter, which is repressed by RA. Thus, the ChIP assay demonstrates that TR2 and TR4 have a binding site in the promoter of Oct-3/4 gene, which is shared with RAR β . In addition, these bindings can be influenced by the presence of RA.

Discussion

To clarify the exact roles of TR2 and TR4 in development and physiology, we wanted to observe the phenotypes of mice lacking both receptors, and our results show that embryos losing both the TR2 and TR4 gene are developmentally defective and die in an early stage of embryogenesis. TR2^{-/-}:TR4^{-/-} compound mutant embryos died around E7.5 and exhibited high numbers of apoptotic cells in E7.5 embryos (Fig. 2). These observations indicate that TR2 and TR4 are involved in the early events of embryogenesis. The loss of both receptors may cause primitive cells, including ES cells, and subsequent progenitor cells to fail to differentiate and submit to apoptosis, causing increased cell death in early embryos. Therefore, TR2 or TR4 has an essential role in early embryo survival.

We have also demonstrated the expression of TR2 and TR4 on blastocysts, ES cells, and EBs (Fig. 3, A–C), as well as the transcriptional activity of TR2 and TR4 in ES cells (Fig. 3D), suggesting that TR2 and TR4 are capable of acting as transcriptional factors to regulate gene expression in ES cells and early embryos. Many nuclear receptors, including steroid receptors, receptors for RA, and thyroid hormone (41, 48, 49), as well as orphan receptors such as estrogen receptor-related proteins, chicken ovalbumin upstream promoter-transcription factors (Coup-TFs) I and II, germ cell nuclear factor (GCMF) and liver receptor homolog-1 (47, 49–51) were found to exist in early stages of embryonic development. However, their exact roles in regulating cell proliferation, differentiation, and apoptosis during early embryogenesis are not fully understood. Orphan nuclear receptors, such as Coup-TFs I and II, GCMF, and liver receptor homolog-1 are shown to modulate the Oct-3/4 expression (47, 49–51). Previous studies have shown the expression of TR2 and TR4 in embryos and embryonic carcinoma cells (27, 52) but with little information about their biological functions in embryonic development. Our knowledge of how genes direct the development of embryos will be advanced if we can determine the roles of nuclear receptors in embryos. Our report provides the evidence that the TR2 and TR4 nuclear receptors are involved in early embryo development and may help us to further understand how these two nuclear receptors act in early embryonic development to influence stem cell proliferation, differentiation, or apoptosis.

The expression pattern of TR2 and TR4 varies with different cell differentiations (Fig. 4), suggesting that they may have their own specific physiological functions in development and cell differentiation despite their similarities in biochemical properties such as DNA binding and expression patterns.

The physiological relevance of the presence of TR2 and TR4 in ES cells and early embryos for their differentiation and development was further explored by examining the interaction between RA signaling and TR2 or TR4 RA repression, which shows that the expression of TR2 and TR4 is regulated by RA (Fig. 5), and furthermore, TR2 and TR4 collaborate with RA signaling to regulate the Oct-3/4 gene (Fig. 6, A and B), possibly for ensuring its accurate and precise expression required for self-renewal or differentiation. Further investigation found that TR2 and TR4 were able to bind the Oct-3/4 promoter by the ChIP assay (Fig. 6C), suggesting their direct regulation of the Oct-3/4 gene. A recent study showed that TR2

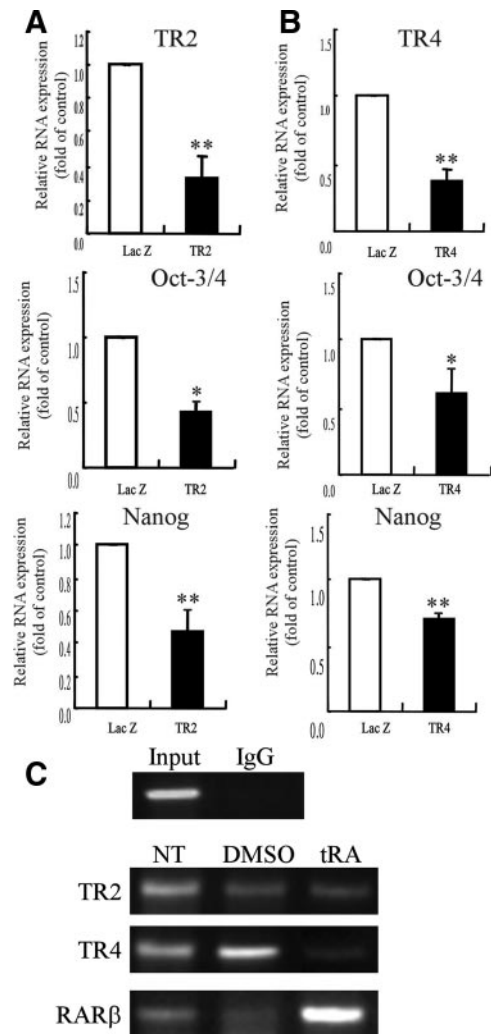


FIG. 6. The effect of the repression of TR2 or TR4 RNA level by RNA interference knockdown on Oct3/4 and Nanog expression in ES cells. ES cells were transfected with vectors expressing shRNA for knocking down TR2 (A) or TR4 (B). The cells were harvested for qRT-PCR analysis for TR2, TR4, Oct3/4, or Nanog. The plasmid encoding a shRNA target LacZ gene was used as a transfection control. C, ChIP assay of the Oct-3/4 promoter. Cell lysates from ES cells treated with 1 μ M tRA, vehicle control dimethylsulfoxide (DMSO), or without treatment (NT) were immunoprecipitated with anti-TR2, TR4, or RAR β antibodies. DNA was extracted from immunoprecipitation lysates and examined by PCR with primers that amplify the TR2/4-RE (DR1) in the Oct-3/4 promoter. Similar results were obtained in three independent experiments. *, $P < 0.05$; **, $P < 0.01$ by the t test against the control group.

was shown to enhance or repress the Oct-3/4 expression and cell proliferation of P19 cells, which depends on its small ubiquitin-related modifier (SUMO)ylation status. When TR2 is abundant, TR2 is SUMOylated, and SUMOylated TR2 exchanges its coregulators from coactivators to corepressors, which switches its up-regulatory function to down-regulatory action on Oct-3/4 gene (38). These results suggest a network of nuclear receptors that interact with RAR/RXR by sharing either the same DNA binding elements or the coregulators (53).

It is likely that TR2, TR4, and RAR/RXR delicately control the Oct-3/4 gene expression by either activating or repressing its promoter activity through coordinated action among them. TR2 and TR4 may play a fundamental role in RA-induced differentiation of ES cells and in regulating expression of transcription

factors, such as the Oct-3/4 gene, whose function is critical for normal embryogenesis. Because Oct-3/4 serves as a master regulator of pluripotency and lineage commitment, its actions depend on its quantitative expression controlled by sophistication of critical transcriptional regulators (45) and the activation or repression of a complex cascade of regulatory genes (46, 54). Based on the interaction among receptors, TR2 and TR4 possibly participate in building up and regulating a hierarchy of a network of transcription factors that regulate genes essential for pluripotency, as well as lineage commitment and differentiation to establish normal development, defining their roles in early embryogenesis.

Another gene essential for ES cells remaining in pluripotency is Nanog, which functions as a strong positive regulator of self-renewal and suppresses the multilineage differentiation due to the removal of leukemia inhibitory factor and bone morphogenetic protein (15, 16). We found that knocking the TR2 and TR4 expression down with the RNA interference method decreased the Nanog expression, suggesting the up-regulatory roles of TR2 and TR4 for Nanog (Fig. 6, A and B). In contrast, another orphan nuclear receptor, GCNF, was reported to repress Nanog expression because RA lost the ability to repress Nanog in GCNF^{-/-} ES cells (55, 56). This again demonstrates that orphan nuclear receptors work closely to monitor the expression of genes that are critical in determining stem cell fate/commitment.

The orphan receptors such as Coup-TFs, GCNF, steroidogenic factor-1, TR2, and TR4, whose ligands remain unknown, were shown to have a role in regulating early embryonic development and stem cell differentiation (27, 38, 50, 51). These findings may imply that these orphan nuclear receptors without known ligands are part of a network that comprises orchestration of transcription factors that control stem cell self-renewal and lineage progression in early embryos by eliciting cascades of gene expression. Subsequently, this suggests that early embryos are ligand sources for these orphan receptors. Indeed, in embryo extracts, endogenous benzoate metabolites were reported to act as ligands for the benzoate X receptor (57).

With the continuing search for ligands that activate orphan nuclear receptors, early embryos need further investigation to find ligand targets within them. Because ligands for nuclear receptors are lipophilic molecules that are ideal for diffusible cues in affecting embryonic development, finding novel ligands for orphan receptors in early embryos may allow us to manipulate ES cells, which constitute a renewable source to provide differentiated cells for replacing diseased or damaged tissues by cellular transplantation (58).

Determining the roles and endogenous ligands for TR2 and TR4 in early embryos not only will facilitate an understanding of nuclear receptor signaling but also will provide a basis for the development of programs in manipulating ES cells that can be used for cell replacement therapy to treat diseases such as diabetes, Parkinson's disease, and Huntington's disease.

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