

## TR4 orphan nuclear receptor functions as an apoptosis modulator via regulation of Bcl-2 gene expression

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Received 22 June 2007

Available online 10 July 2007

### Abstract

While Bcl-2 plays an important role in cell apoptosis, its relationship to the orphan nuclear receptors remains unclear. Here we report that mouse embryonic fibroblast (MEF) cells prepared from TR4-deficient ( $TR4^{-/-}$ ) mice are more susceptible to UV-irradiation mediated apoptosis compared to TR4-Wildtype ( $TR4^{+/+}$ ) littermates. Substantial increasing  $TR4^{-/-}$  MEF apoptosis to UV-irradiation was correlated to the down-regulation of Bcl-2 RNA and protein expression and collaterally increased caspase-3 activity. Furthermore, this TR4-induced Bcl-2 gene expression can be suppressed by co-transfection with TR4 coregulators, such as androgen receptor (AR) and receptor-interacting protein 140 (RIP140) in a dose-dependent manner. Together, our results demonstrate that TR4 might function as an apoptosis modulator through induction of Bcl-2 gene expression.

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**Keywords:** TR4; Bcl-2; AR; Caspase-3; Apoptosis

Members of the nuclear receptor superfamily are transcription factors that regulate gene expression important for growth, differentiation, and development. These nuclear receptors include receptors for steroids, thyroid, vitamin D3, retinoid, and a large number of orphan receptors with unknown ligands [1]. Among the nuclear receptor superfamily, TR2 and TR4 form a unique subfamily of orphan receptors, which is able to regulate the expression of target genes through binding to direct repeats (DR) of AGGTCA core motif with variable numbers of nucleotides [2–6]. TR4 is known to modulate the signaling pathway of various nuclear receptors, such as retinoic acid receptor (RAR), androgen receptor (AR), thyroid receptor (TR), and vitamin D3 receptor (VDR) through competition to

DNA binding or inactivation by protein–protein interaction. Previous studies have demonstrated that androgen and AR signal could down-regulate Bcl-2 gene expression. However, this suppressive effect of androgen and AR in Bcl-2 gene expression has not been determined yet.

Cell proliferation and apoptosis play an important role in development and cell homeostasis. The proto-oncogene Bcl-2 was first identified by its over-expression in non-Hodgkins B cell lymphoma with chromosomal translocation [7]. Bcl-2 is a membrane-bound protein with a molecular weight of 26 kDa and is located in the nuclear, endoplasmic reticular membranes as well as outer mitochondrial membranes [8]. The anti-apoptotic function of Bcl-2 and the over-expression of Bcl-2 gene was found in many tumors indicates its oncogenic property [9]. Bcl-2 plays its anti-apoptotic role by maintaining mitochondrial integrity, thereby blocking cytochrome c releasing and serial intrinsic cascade. The consequence of this serial

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cascade is activation of the downstream effector, caspase-3, which ultimately leads to cell death [10]. Previous reports showed that UV-induced apoptosis is attributed to activation of caspase-3 activity and is partially due to declination of Bcl-2 amount [11].

Two major promoter regions, P1 and P2, have been identified in the 5' promoter region of the *Bcl-2* gene. P1 is known as the promoter having a predominant role in B cells and many transcription factors, such as cAMP response element-binding protein (CREB), Sp1, and NF $\kappa$ B, have been known to regulate transcription of *Bcl-2* via the P1 promoter. The second region, P2, is located about 1.3-kb downstream of the P1 promoter [12], however, the regulation of P2 is relatively unidentified.

Recently, we have established a TR4 knockout (*TR4*<sup>-/-</sup>) mouse model to study the physiological roles of TR4. The *TR4*<sup>-/-</sup> mice showed several important phenotypes including defects in motor coordination and aberrant cerebellar developments with increased neuronal apoptosis [13]. Furthermore, we found that loss of *TR4* resulted in delayed adhesion and growth retardation of mouse embryonic fibroblast (MEF) cells in this study. This prompted us to investigate the cellular function of TR4 in cell apoptosis.

## Materials and methods

**Preparation of mouse embryonic fibroblasts, UV-irradiation and cell culture.** Primary *TR4*<sup>+/-</sup> and *TR4*<sup>-/-</sup> MEFs were isolated from embryonic (E) 14–15 days littermates and cultured in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal bovine serum. *TR4*<sup>+/-</sup> and *TR4*<sup>-/-</sup> mice were housed and studied under the University Committee on Animal Resources-approved protocols in the animal facility of the University of Rochester Medical center. COS-1 and H1299 cells were cultured in DMEM plus 10% heat-inactivated fetal calf serum. Early passage (2–4) MEFs, COS-1, or H1299 cells were then plated at a density of  $5 \times 10^5$  cells per 6 cm dish, then 24 h later, cells were treated with UV-irradiation (200 J/m<sup>2</sup>, for MEF cells, 100 J/m<sup>2</sup> for H1299; Stratallinker, Stratagene) or (100 J/m<sup>2</sup>) combined either with 10  $\mu$ M ceramide (Sigma) or antisense-Bcl-2 (100  $\mu$ M; Sigma) for another 24 h. Cell death was determined by flow cytometric analysis, MTT assay or changes of proteins by immunoblotting.

**Plasmids.** pCMX-TR4 has been previously described [4]. pEF-receptor-interacting protein 140 (RIP140) and pRC/RSV-CREB, were kindly provided by Dr. M. Parker (Institute of Reproductive and Developmental Biology, Imperial College, UK) and Dr. R.H. Goodman (the Vollum Institute, Oregon Health and Science University, USA), respectively. The chloramphenicol acetyltransferase (CAT) reporter plasmid, which contains the *Bcl-2* P1 promoter (-2852 to -1288 bp), was a gift from Dr. J.C. Reed (The Burnham Institute, USA).

**Transient transfection and CAT assay.** COS-1 and H1299 cells were transfected using a modified calcium phosphate precipitation method [14]. For CAT assays, 4  $\mu$ g of CAT constructs were co-transfected with pCMX-TR4 or pCMX.  $\beta$ -Galactosidase expression vector was used to normalize transfection efficiency in all the experiments.

**Immunoblot analysis.** Proteins of total cell extracts (50  $\mu$ g) were separated on 10% SDS-PAGE and transferred to Immobilon P transfer membrane (Millipore). *Bcl-2* was resolved by enhanced chemiluminescence per manufacturer's instructions (ECL detection system, Amersham) using anti-Bcl-2 monoclonal antibodies (DAKO) and horseradish peroxidase-conjugated secondary antibodies (Santa Cruz).

**Determination of gene expression.** Total RNA was isolated from different tissues using Trizol reagent (Invitrogen), and cDNA was synthesized using SuperScript<sup>TM</sup> II and random hexamer primers (Invitrogen). Quantitative reverse transcription-polymerase chain reaction (RT-PCR)

was performed with iQ<sup>TM</sup> SYBR green supermix reagent using iCycler real-time PCR amplifier (Bio-Rad) according to the manufacturer's protocols. Sequences of the primers used for RT-PCR are available upon request.

**Detection of apoptotic cells.** Cells were stained with 7-amino actinomycin D (7-AAD, BD PharMingen) for 30 min on ice. After washing with ice-cold PBS, cells were resuspended in binding buffer at a concentration of  $10^5$ – $10^6$  cells/ml and incubated with 7-AAD (1:1000 diluted) for 15 min on ice. Without washing, the cells were immediately analyzed. Single staining of 7-AAD was used to determine positive signal. 7-AAD-negative cells were defined as viable, 7-AAD-positive cells as apoptotic. Cells were analyzed by flow cytometric analyses using a dual-laser FACSCalibur flow cytometer (Becton–Dickinson).

**Caspase-3 activity assay.** Cells at  $5 \times 10^5$  were resuspended in 1 ml Permeafix, (BD PharMingen) and incubated for 40 min at 20 °C. The suspension was then centrifuged, and the pellet was washed twice with washing buffer (0.2 mM EDTA, 5% FCS in PBS). Labeling was performed by adding 100  $\mu$ l washing buffer containing 5  $\mu$ l polyclonal phycoerythrin-conjugated anti-active caspase-3 antibodies (BD PharMingen). The samples were gently stirred for 1 h, washed in washing buffer, and analyzed by FACS analysis.

## Results

### Down-regulation of *Bcl-2* gene expression in *TR4* knockout (*TR4*<sup>-/-</sup>) MEF cells increases sensitivity to UV-irradiation

We have generated *TR4*<sup>-/-</sup> mice by insertion of an IRES  $\beta$ -gal MCI-Neo selection cassette between exons 4 and 5 of the *TR4* gene [13]. The RT-PCR analysis of total RNAs from *TR4*<sup>+/-</sup> and *TR4*<sup>-/-</sup> MEF cells confirmed deletion of *TR4* gene in exons 4 and 5 in homozygous *TR4*<sup>-/-</sup> MEF cells (Fig. 1A). To investigate whether loss of *TR4* affects MEF cell growth, MTT assays were carried out. *TR4*<sup>-/-</sup> MEF cells showed significant growth retardation as compared to *TR4*<sup>+/-</sup> MEF cells (Fig. 1B) with only little influence on basal apoptotic rate (*TR4*<sup>+/-</sup>: *TR4*<sup>-/-</sup> = 3.38% vs. 2.61%). To determine whether loss of *TR4* could affect cell survival upon apoptotic stimuli, we treated the cells with UV-irradiation, a well-known cell apoptotic challenge. *TR4*<sup>-/-</sup> MEF cells showed significantly increase in apoptosis upon UV-irradiation compared to *TR4*<sup>+/+</sup> MEF cells ( $13.2 \pm 2.5\%$  vs.  $21.78 \pm 0.53\%$ ) (Fig. 1C).

To determine whether *TR4* mediated cell apoptosis is linked to *Bcl-2* gene expression, we examined *Bcl-2* mRNA in *TR4*<sup>+/-</sup> and *TR4*<sup>-/-</sup> MEF cells derived from the same littermates. Q-PCR analysis showed that *Bcl-2* mRNA levels in *TR4*<sup>-/-</sup> MEF cells were reduced to 50% of those in *TR4*<sup>+/-</sup> MEF cells (Fig. 2A). Basal levels of *Bcl-2* protein in *TR4*<sup>-/-</sup> MEF cells are lower than those of *TR4*<sup>+/-</sup> MEF cells (Fig. 2B, lanes 1 and 4), while Bax protein levels remain similar (Fig. 2B, middle panel, lane 1 vs. 4). To test if TR4 effects cell apoptosis through the regulation of *Bcl-2* expression, we challenged MEF cells with either UV-irradiation (200 J/cm<sup>2</sup>) or sub-lethal UV-irradiation (100 J/cm<sup>2</sup>) with ceramide (10  $\mu$ g/ml) co-treatment. After 24 h treatments, *Bcl-2* protein levels in *TR4*<sup>+/-</sup> (Fig. 2B, lanes 2 and 3) and *TR4*<sup>-/-</sup> (Fig. 2B, lanes 5 and 6) MEF cells were decreased. In addition, we also examined TR4 effect on cell

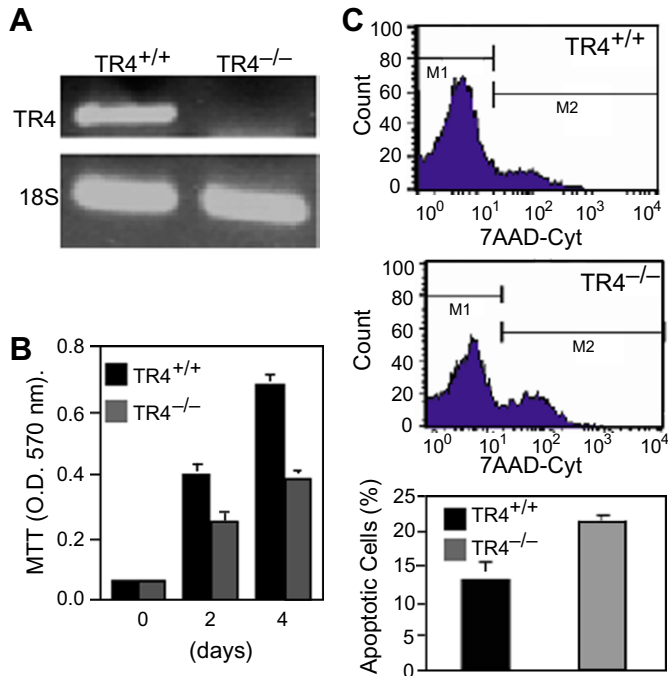


Fig. 1.  $TR4^{-/-}$  MEF cells show increased sensitivity to UV treatment. (A) RT-PCR analysis of  $TR4^{+/-}$  and homozygous  $TR4^{-/-}$  MEF cells using specific primer amplifying exon 3–5 to distinguish  $TR4^{+/-}$  and  $TR4^{-/-}$ . (B) Relative growth rate of  $TR4^{+/-}$  and  $TR4^{-/-}$  MEF cells. Values are means  $\pm$  SD of MTT assay readings from three-independent wells. (C) Flow cytometric measurement of MEF cell death induced by UV (200 J/cm<sup>2</sup>) as indicated. Cells were stained with 7-AAD and analyzed by FACSCalibur flow cytometer (upper and middle panel). Live cells were gated as M1 and apoptotic cells were gated as M2 population in readings. Relative percentage of apoptotic cells are shown in lowest panel as the means  $\pm$  SD of three individual experiments.

survival upon UV-irradiation. As shown in [Supplementary Fig. 1](#), TR4 partially rescued UV-mediated cell apoptosis and anti-Bcl2 dsDNA diminished the cell survival effect of TR4. Furthermore, Bcl-2 protein level of  $TR4^{-/-}$  MEF cells was much lower than that of  $TR4^{+/-}$  MEF cells ([Fig. 2B](#), lanes 5 and 2; lanes 6 and 3), suggesting that decreased Bcl-2 protein levels may contribute to enhanced apoptosis in  $TR4^{-/-}$  MEF cells. Bcl-2 has been shown to inhibit caspase-3 mediated intrinsic pathway, which ultimately leads to cell death. To determine whether down-regulation of *Bcl-2* gene expression in  $TR4^{-/-}$  MEF cells causes increased to caspase activity, we compared caspase-3 activity between  $TR4^{+/-}$  and  $TR4^{-/-}$  MEF cells after UV-irradiation. As shown in [Fig. 2C](#),  $TR4^{-/-}$  MEF cells showed more than 2-fold increase of caspase-3 activity compared to that of  $TR4^{+/-}$  MEF cells, indicating that reduced Bcl-2 protein might result in an increase of caspase-3 activity in  $TR4^{-/-}$  MEF cells. Since Bcl-2 mRNA level was reduced in  $TR4^{-/-}$  MEF cells, we determined whether TR4 could induce *Bcl-2* gene expression through the stimulation of *Bcl-2* promoter region. pBcl-2-CAT, the plasmid containing *Bcl-2* P1 promoter (–2852 to –1288 bp) linked to CAT reporter gene was co-transfected to COS-1 and H1299 cells with TR4 expression vector. As

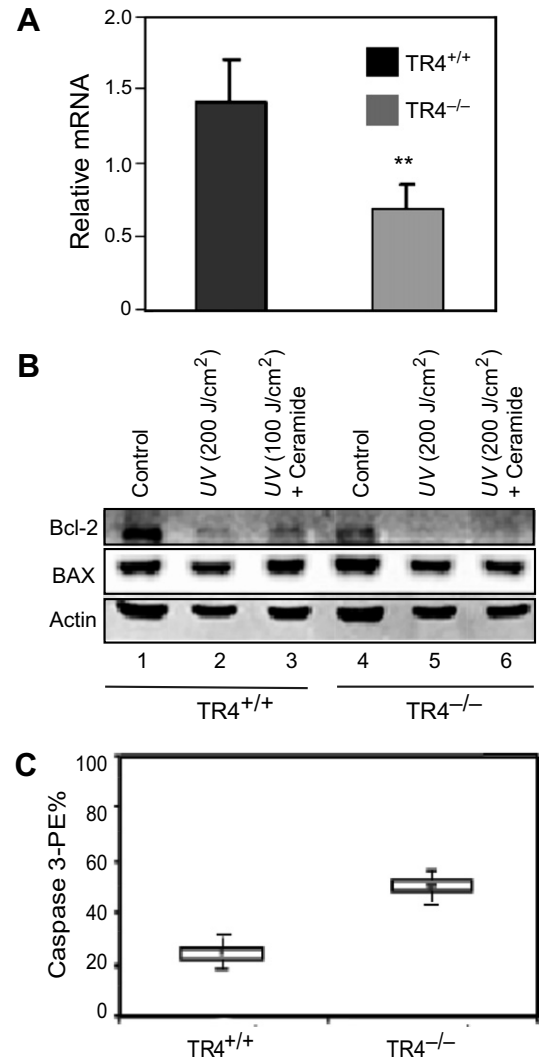


Fig. 2. Expression of the *Bcl-2* gene is reduced in  $TR4^{-/-}$  MEF cells. (A) *Bcl-2* mRNA expression in  $TR4^{-/-}$  MEF cells. Data are shown as means  $\pm$  SD from four individual assays. \*\* $p < 0.01$  as compared with  $TR4^{+/-}$  MEF cells. (B) Immunoblot analysis of Bcl-2 and Bax expression in  $TR4^{+/-}$  and  $TR4^{-/-}$  MEF cells under UV (200 J/m<sup>2</sup>) or combined treatment of UV (100 J/m<sup>2</sup>) and ceramide (10  $\mu$ g/ml). Representative blotting shown here is from two individual experiments with similar result. (C) Activation of Caspase-3 in UV-treated  $TR4^{+/-}$  and  $TR4^{-/-}$  MEF cells. Percentage of caspase-3 positive cells are represented as values of means  $\pm$  SD from three-independent sets of experiments and have reached to significant difference ( $p < 0.05$ ).

shown in [Supplementary Fig. 2a](#), *Bcl-2* promoter activity was up-regulated to around 14-fold in COS-1 and 7-fold in H1299 cells with TR4 transfection. [Supplementary Fig. 2b](#) also demonstrated a dose-dependent effect of TR4 on *Bcl-2* promoter activity in COS-1 cells. These results strongly suggest that *Bcl-2* expression could be directly regulated by TR4.

To determine whether this TR4 effect on *Bcl-2* expression is through direct binding of TR4 to *Bcl-2* promoter, we performed gel mobility shift assay. The specific region (–1562 to –1487 bp) of *Bcl-2* promoter was able to bind TR4 ([Fig. 3A](#)). Considering the band appeared only when

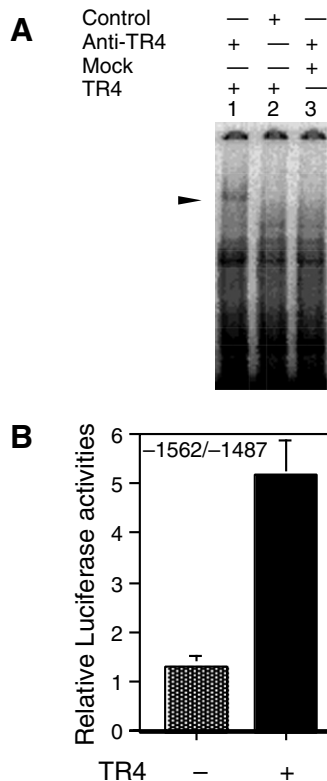


Fig. 3. Positive regulation of *Bcl-2* gene expression by TR4. (A) Induction of *Bcl-2* gene is mediated by TR4 binding to *Bcl-2* promoter.  $^{32}\text{P}$ -labeled *Bcl-2* DNA fragment (–1562 to –1487 bp) was used in electrophoretic mobility shift assay without or with *in vitro* translated TR4 (lanes 1 and 2). Supershift of specific complex was induced by the presence of TR4 and anti-TR4 antibodies (lane 3) but was not by mock translated lysate and anti-TR4 antibodies (lane 1) or by TR4 with unrelated monoclonal antibodies (anti-RXR antibodies, lane 2). (B) The sequence of –1562 to –1487 bp of *Bcl-2* promoter is enough for the promoter activation by TR4. The pBcl-2-Luc containing –1562 to –1487 region of the *Bcl-2* promoter with pCMX-TR4 or pCMX (1  $\mu\text{g}$ ) was transfected to COS-1 cells. All results of reporter gene assays were expressed as means  $\pm$  SD of three separate experiments.

TR4 and anti-TR4 antibodies were added, and not in the mock lysate with anti-TR4 antibodies or TR4 with unrelated monoclonal antibodies, we strongly believe this band is specific for TR4. We could not find any consensus sequence in this region; therefore, we assumed that there are TR4 binding sites with low affinities and these binding sites may cooperatively contribute to TR4 response. The specific region of –1562 to –1487 bp was constructed into pGL3-Luciferase vector and examined for promoter activity in COS-1 cells with TR4 co-expression. TR4 can activate this reporter gene (Fig. 3B), suggesting that TR4 regulates *Bcl-2* gene expression via direct binding to this promoter region.

#### TR4 coregulators, RIP140 and AR, modulate TR4 transcriptional activity of *Bcl-2* promoter

RIP140 has been shown to be a common coregulator for several members of the nuclear receptor superfamily

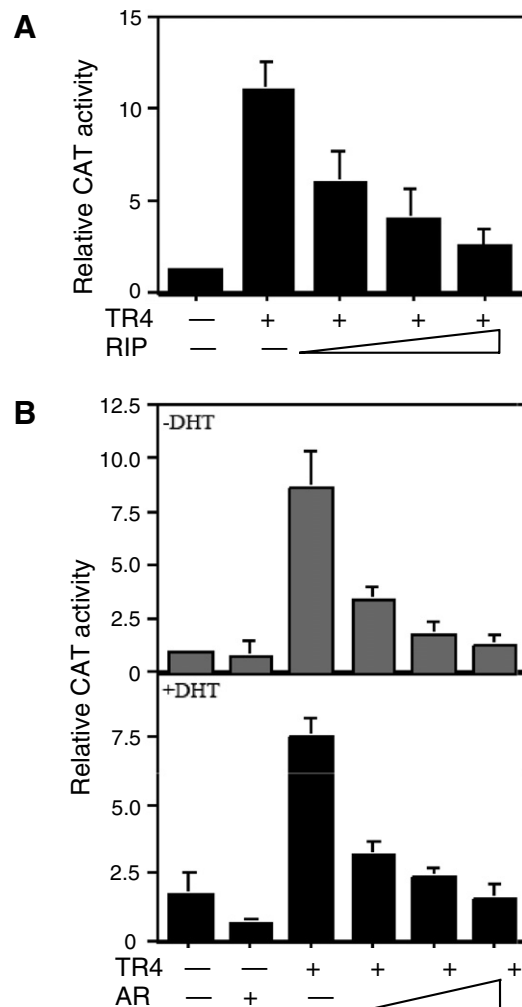


Fig. 4. Modulation of TR4-mediated transcriptional activity of *Bcl-2* promoter by coregulators. (A) COS-1 cells were co-transfected with 2  $\mu\text{g}$  pBcl-2-CAT and 1.5  $\mu\text{g}$  pCMX-TR4 with increasing amounts of RIP140 (0, 0.25, 0.5, and 1  $\mu\text{g}$ ) and appropriate amount of empty vector (1, 0.75, 0.5, and 0  $\mu\text{g}$ ) as a balance. The relative CAT activities were compared with the CAT activities with empty vector alone (set as 1-fold). (B) Suppressive effect of AR on TR4-mediated induction of *Bcl-2* promoter activity. COS-1 cells were co-transfected with 2  $\mu\text{g}$  pBcl-2-CAT and 1.5  $\mu\text{g}$  pCMX-TR4 with increasing amounts of pCMV-AR (0, 1.5, 3, and 4.5  $\mu\text{g}$ ) and appropriate amount of empty vector (4.5, 3, and 1.5  $\mu\text{g}$ ) as a balance. After 24 h transfection, the cells were treated with 10 nM DHT. After another 24 h, the cells were harvested for CAT assay. The relative CAT activities were compared with the CAT activities with empty vector alone in the absence of DHT (set as 1-fold). All the promoter CAT assays were from three individual sets of experiments and the difference were reached statistical significance using *T*-test ( $p < 0.05$ ).

[15,16]. We co-transfected RIP140, TR4 expression vector and pBcl-2-CAT into COS-1 cells to observe RIP140 effect. As shown in Fig. 4A, RIP140 suppressed TR4-mediated transcriptional activity in a dose-dependent manner. Recently, we reported that TR4 and AR have cross-suppressive effects on their transcriptional activities and this repression may go through TR4-AR heterodimer formation [14]. We were interested in determining whether AR also represses TR4-mediated transcriptional activity of *Bcl-2* promoter. As shown in Fig. 4B, in the absence of

DHT, basal *Bcl-2* promoter activity was not affected by AR. While DHT alone, in the absence of AR, increased basal promoter activity about 2-fold, cotreatment of DHT and AR reduced *Bcl-2* promoter activity to basal level. This may indicate that DHT alone may affect *Bcl-2* promoter activity in an AR-independent manner and in the presence of AR, DHT may suppress *Bcl-2* gene expression via AR. Further study will be needed to determine DHT effect in the absence of AR on *Bcl-2* gene expression. We also co-transfected TR4 expression vector into COS-1 cells with different amounts of AR expression vectors and assayed AR effects on TR4-mediated induction of *Bcl-2* promoter activity. As shown in Fig. 4B, AR suppressed TR4-induced-*Bcl-2* promoter activity in a dose-dependent manner under DHT treatment. This is consistent with the previous report and suggests that AR might regulate *Bcl-2* gene expression through suppression of TR4-mediated transcription activity.

## Discussion

Loss of *TR4* in MEF cells resulted in delayed adhesion, growth retardation, and significant sensitization to apoptosis-inducing agents. Interestingly, the *Bcl-2* protein expression is nearly absent in slowly adhering cells [17]. This indicates that TR4 may participate in cell survival via regulation of *Bcl-2* level. Previous reports have shown that UV-irradiation causes a decline of *Bcl-2* protein level and this decline enhances caspase-3 activity leading to cell death [11]. The expression of *Bcl-2* was down-regulated in *TR4*<sup>-/-</sup>, further, UV treatment decreased *Bcl-2* to an even lower level. Recently, enhanced caspase-3 activity was shown to contribute to *Bcl-2* degradation. *TR4*<sup>-/-</sup> MEF cells could increase caspase-3 activity after UV-irradiation, suggesting the other mechanisms might contribute to down-regulation of *Bcl-2*.

Global loss of *TR4* caused aberrant cerebellar developmental with increased apoptosis in the granule cells [18]. However, Zhang et al. reported that *TR4* deficiency resulted in impaired myelination of forebrain neurons with no obvious increase in oligodendrocyte apoptosis [19], indicating that increased apoptosis due to *TR4* deficiency may not be a universal phenomena. Thus, TR4 may modulate cell apoptosis in a complex network in different cells although further study is necessary to determine the mechanisms that control TR4 regulation of apoptosis in a cell-specific manner.

It is now well established that nuclear receptors may coordinate with coregulators for the proper and maximal functions [20–22]. Few coregulators were identified as corepressors of nuclear receptor function. Here we show that both RIP140 and AR can suppress TR4 function in *Bcl-2* gene expression. Recently, it was reported that RIP140 could be recruited to the AF1 domain of TR4 and this interaction is dependent on MAPK-mediated phosphorylation of TR4 [23], suggesting that RIP140 may suppress TR4-mediated *Bcl-2* gene expression by inhibiting recruitment of other coactivators via interaction with TR4 AF1

domain. We have demonstrated previously that AR interacts with TR4 to down-regulate TR4-mediated transactivation in the presence or absence of DHT [14]. These two findings support that AR suppresses TR4-mediated regulation of *Bcl-2* gene.

Previous studies suggest that the androgen-independent prostate cancer may be the result of de-repression of *Bcl-2* gene expression. This de-repression might be a cause of the blocking of apoptosis induced by androgen withdrawal. Despite the suppression role of androgen in *Bcl-2* gene expression, the involvement of AR in *Bcl-2* gene expression has not yet been defined. In contrast to PC-3 cells, LNCaP cells are androgen-responsive and have a higher amount of endogenous AR. PC-3 cells show higher survivability than LNCaP cells in the absence of trophic factors and this higher survivability may be correlated with *Bcl-2* expression level [24]. Recently, we compared endogenous *TR4* expression levels in prostate cancer cell lines as well as other cancer cell lines [25]. LNCaP and PC-3 cells have high level of endogenous *TR4* compared to DU145 cells. This may explain why PC-3 cells have higher endogenous *Bcl-2* than LNCaP cells even though there is a similar level of endogenous TR4, suggesting that AR play a suppressive role in TR4-mediated up-regulation of *Bcl-2* gene expression.

In summary, our report demonstrated that TR4 modulates cell apoptosis via regulation of *Bcl-2* gene expression and caspase-3 activity. Our discovery of the new regulation pathway in apoptosis by TR4 might provide a new foothold toward the clarification of the regulation mechanisms of orphan nuclear receptors in apoptosis.

## Acknowledgments

We thank Drs. M. Parker, R.H. Goodman, and J.C. Reed for kindly providing plasmids. This work was supported by the National Institutes of Health Grant and the George Whipple Professorship Endowment. The *TR4*<sup>-/-</sup> mice were generated in collaboration with Lexicon Genetics, Inc. We also thank K. Wolf for help in manuscript preparation.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.06.168.

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