DEFICIENCY IN TR4 NUCLEAR RECEPTOR ABROGATES Gadd45a EXPRESSION AND INCREASES CYTOTOXICITY INDUCED BY IONIZING RADIATION

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Abstract: The testicular receptor 4 (TR4) is a member of the nuclear receptor superfamily that controls various biological activities. A protective role of TR4 against oxidative stress has recently been discovered. We here examined the protective role of TR4 against ionizing radiation (IR) and found that small hairpin RNA mediated TR4 knockdown cells were highly sensitive to IR-induced cell death. IR exposure increased the expression of TR4 in scramble control small hairpin RNA expressing cells but not in TR4 knockdown cells. Examination of IR-responsive molecules found that the expression of Gadd45a, the growth arrest and DNA damage response gene, was dramatically decreased in Tr4 deficient (TR4KO) mice tissues and could not respond to IR stimulation in TR4KO mouse embryonic fibroblast cells. This TR4 regulation of GADD45A was at the transcriptional level. Promoter analysis identified four potential TR4 response elements located in intron 3 and exon 4 of the GADD45A gene.

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Abbreviations used: ChIP – chromatin immunoprecipitation; DR – direct repeat; GADD45A – growth arrest and DNA-damage-inducible, alpha; GADDLuc – GADD45A gene controlled luciferase reporter; IgG – immunoglobulin G; IR – ionizing radiation; MEF – mouse embryonic fibroblast; MTT – 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; SC – cells expressing scramble control shRNA; shRNA – small hairpin RNA; shTR4 – cell expressing shRNA targeting TR4; TR4 – testicular nuclear receptor 4; TR4KO – TR4 knockout; TR4RE – TR4 response element; UV – ultraviolet light irradiation; WT – wild type
Reporter and chromatin immunoprecipitation (ChIP) assays provided evidence indicating that TR4 regulated the GADD45A expression through TR4 response elements located in intron 3 of the GADD45A gene. Together, we find that TR4 is essential in protecting cells from IR stress. Upon IR challenges, TR4 expression is increased, thereafter inducing GADD45A through transcriptional regulation. As GADD45A is directly involved in the DNA repair pathway, this suggests that TR4 senses genotoxic stress and up-regulates GADD45A expression to protect cells from IR-induced genotoxicity.

**Key words:** TR4, GADD45A, Ionizing radiation, Mouse embryonic fibroblast, Genotoxic stress, TR4 response element

**INTRODUCTION**

The testicular receptor 4 (TR4) was first cloned from prostate and testis cDNA libraries and is highly homologous to the TR2 nuclear receptor [1]. The molecular structure analyses indicated that TR4 belongs to the nuclear receptor superfamily that may control the expression of target genes involved in developmental, physiological, and behavioral responses from the cellular level to that of the whole organism. The structural features common to nuclear receptors include those required for ligand binding, dimerization, DNA binding, and transactivation. TR4 has been an orphan nuclear receptor without an identified ligand since it was cloned in 1994 [1]. Recently, Xie *et al.* and Tsai *et al.* found that the polyunsaturated fatty acid metabolites 15-HETE, 13-HODE, and γ-linoleic acid are able to activate TR4 [2, 3]. This suggests that TR4 may be able to function as a fatty acid sensor upon activation via these ligands/activators. Binding of TR4 to a specific DNA sequence, TR4 response element (TR4RE), within the promoter of its target genes is mediated by a region containing two zinc fingers in the DNA binding domain of TR4, which displays a high level of amino acid homology with other nuclear receptors [4].

*In vitro* data suggest that TR4 functions as a master regulator to modulate many signaling pathways, including induction of the ciliary neurotrophic factor alpha [5, 6], interfering with retinoic acid receptor/retinoid X receptor [7], thyroid receptor [8], androgen receptor [9], and estrogen receptor-mediated pathways [10], and facilitating viral infection and propagation of HPV-16 and SV40 [11]. Mice lacking Tr4 (TR4KO) have high rates of early postnatal mortality, show significant growth retardation [12], display reproductive defects in both genders [12, 13], abnormalities in spermatogenesis [14], reduced lipoprotein metabolism [15, 16], and reduced gluconeogenesis [17], as well as defects in cerebella development [18]. Furthermore, the premature aging phenotype of TR4KO mouse led to the finding of TR4’s role in defending oxidative stress and DNA damage response [19].

Recently, we reported that FOXO3a protects cells from oxidative stress via inducing the expression of TR4 as one major mechanism [20]. TR4 also protects cells from ultraviolet radiation via regulating transcription-coupled DNA repair
protein, Cockayne syndrome B [21]. Following these discoveries, the protective mechanism of TR4 in cells facing stress was further explored. By using DNA Damage Signaling Pathway focused PCR Array (SABiosciences™), we found that expression of several genes controlling cell cycle arrest, including Gadd45a (growth arrest and DNA-damage-inducible, alpha), was reduced in TR4 knockdown cells (data not shown). Gadd45a is reported to be induced by multiple types of genotoxic stress including ultraviolet radiation and ionizing radiation (IR) [22, 23], and is downstream of the FOXO3a stimulated DNA repair pathway when cells are exposed to genotoxic stress [24]. We therefore suggest that TR4 is the mediator through which FOXO3a regulates GADD45A to promote DNA repair and protect cells from IR-induced cell death. The following study investigated whether TR4 regulates GADD45A at the transcriptional level.

MATERIALS AND METHODS

Plasmids
The plasmids pCMX and pCMX-TR4 have been described previously [16]. pRL-TK (TK Renilla luciferase) was purchased from Promega. GADDLuc and GADDLuc-3 reporters are generous gifts from Dr. Wenlong Bai as described in his publication [25]. The pSuperior.retro.puro. plasmids containing scramble small hairpin RNA (shRNA) and shRNA targeting TR4 were described in a previous publication [2].

Experimental animals, genotyping, MEF and mPrE cell culture
TR4KO mice were obtained from Lexicon Genetics Incorporated and were generated and genotyped as described previously [12]. Animals were maintained and experimental procedures on animals were conducted in accordance with guidelines outlined by the Guide for the Care and Use of Laboratory Animals following the legal requirement of the United States and approved by the University Committee of Animal Resources. Primary cultures of mouse embryonic fibroblast (MEF) cells were prepared from embryos at embryonic day 14.5 (E14.5) bearing wild type (WT) or TR4KO genotypes. The head and internal organs were removed, and the torso was minced and dispersed in 0.1% trypsin (45–60 min at 37°C). Cells were grown for two population doublings (considered as one passage) and then stored in liquid nitrogen. These MEFs were used for all subsequent experiments. MEFs were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS; GIBCO) and subcultured 1:4 upon reaching confluence. The spontaneously immortalized mouse prostatic epithelial cell line, mPrE, was a generous gift from Dr. Min Jiang [26]. The cell line was maintained in RPMI 1640 medium (GIBCO) supplemented with 5% fetal bovine serum and 1% Antibiotic-Antimycotic (Invitrogen). Stable mPrE cell lines expressing scramble shRNA (SC) and shRNA against TR4 (shTR4) were established by transfecting
pCDNA6/TR and pSuperior.retro.puro plasmids into mPrE and selected for stable cell lines by treatment with puromycin (1.2 µg/ml) and blasticidin (12 µg/ml) for two weeks. Tetracycline (1 µg/ml) was applied to cells one day before IR to induce shRNA expression and treated every day until harvesting.

Cell viability assay
Cell survival rate was determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. IR was delivered by a γ-ray source irradiator (Model 8114, 3200 Curie Shepherd Cs137 Irradiator). Four days after IR exposure, MTT (5 mg/ml, Sigma) was added into each well at 10% volume of medium and incubated for 2-3 h at 37°C. Cells were dissolved in 0.04 M HCl in isopropanol and absorbency was read at wavelength 570 nm subtracting absorbency read at wavelength 660 nm (O.D.570-660). IR-treated surviving cells were calculated as the ratio of O.D.570-660 in treated group to non-treated group.

Western blot analysis
Whole-cell extracts were isolated in RIPA buffer supplemented with protease inhibitors. Protein samples were separated by electrophoresis in 10% SDS-PAGE and subjected to immunoblotting. The anti-TR4 antibody, N15, was produced as described previously [9]. The anti-GADD45A, anti-α-tubulin, alkaline phosphatase-conjugated and horseradish phosphatase-conjugated anti-mouse, goat, and rabbit IgG antibodies were from Santa Cruz Biotechnology. The immunoblotted membrane was subjected to quantification analysis by VersaDoc (BioRad).

Quantitative PCR analysis
Total RNAs were isolated by using TRizol (Invitrogen) following the manufacturer's protocol. 5 µg RNA were reverse-transcribed by Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol. Oligo-dT was used as the primer for first strand synthesis. cDNA was subjected to PCR, or real-time PCR using the SYBR Green PCR Reagents kit (Bio-Rad). Experiments were performed in triplicate for each data point. β-actin was used as a control for normalization. Quantitative analysis was performed using iCycler analysis software (Bio-Rad Laboratories). The quantification of each sample relative to the control was calculated using the 2-ΔΔCT method. PCR primers were as follows: Tr4 forward, 5'-CATATTTCACACCTCGGACAAC-3', Tr4 reverse, 5'-TGACGACCAGACCACAGAC-3'; Gadd45a forward, 5'-AGATCCAT TTCACCTCTATCC-3', Gadd45a reverse, 5'-GGCGTGTTCTCAGTGACGCT-3'; β-Actin forward, 5'-TGTGCCCATCTACAGGAGGTAG-3', β-Actin reverse, 5'-GGTACA TGGTGTTGCCGCGCCAGACA-3'.

Luciferase reporter assays
The CV-1 cells were cultured in 12-well plates (Falcon), at a concentration of 10⁵ cells/well, transfected with 1.2 µg DNA/well combining pCMX or pCMX-TR4 and indicated reporter constructs by SuperFect (Qiagen) according to the
manufacturer’s manual. The internal control plasmid pRL-TK (Promega) was cotransfected in all transfection experiments. After 48 h, transfected cells were lysed in passive lysis buffer and luciferase activity was analyzed using a luminometer (Turner design) and Dual-Luciferase assay kit (Promega) according to the manufacturer’s instructions. Results were obtained from at least three sets of transfection and were presented as the mean ± S.D.

**Chromatin immunoprecipitation (ChIP) assay**

ChIP assays were performed according to the literature [27]. Briefly, 10^7 cells were fixed with 1% formaldehyde, pelleted, lysed, and nuclear fractions were sonicated. Immunoprecipitation was performed overnight at 4°C using 2 µg monoclonal antibody N-15 against NH₂ termini of TR4 or control mouse immunoglobulin G (IgG). The immunocomplexes were collected using 30 µl salmon sperm DNA-protein A agarose beads, washed, then DNA-protein complexes were collected in elution buffer (1% SDS, 0.1 M NaHCO₃) and incubated at 65°C for 5 h in the presence of 312 mM NaCl and 0.06 µg/µl RNase A. Proteins were removed by QIAquick Spin kit (Qiagen) and DNA fragments were analyzed by PCR. The following primer pairs were used to amplify DNA fragments containing region I: sense 5’-GGTTGCCTGATTGTGGATCTGTG-3’, antisense 5’-GCTGACTCCTTAATGAGGGGTGAG-3’; region II-III: sense 5’-ACAGCCCAGATTATTGGCTACTCC-3’, antisense 5’-TTTCTTCAAGGTAGTTGGGTTCCC-3’; region IV: sense 5’-TGAACGGGTGATCTGATTG-3’, antisense 5’-TTTCCCGTCCAGATGGTTC-3’; and 5’- promoter: sense 5’-TGTGTGGGTGTCAGATGGTTGTC-3’, antisense 5’-TTATTTCGGTGCCCTGATGG-3’.

**RESULTS**

**TR4 senses and controls cell response to IR**

In our previous studies, we demonstrated that TR4 responded to oxidative stress, thereby protecting cells from oxidative stress-induced cell death [20]. Here, we tested whether TR4 could also respond to IR, and if loss of TR4 changed the cell sensitivity to IR-induced genotoxic stress. We first examined responses of SC and shTR4 mPrE cells to IR from 3 to 9 Gy. After IR challenge, cells were allowed to recover for 4 days before measuring cell viability. IR can induce apoptosis and cell cycle arrest, and decrease clonogenicity of cells. These events could be independent processes [28]. Therefore, MTT assay was performed to evaluate whether the impact of IR on overall cell survival and growth could be altered by TR4 status. As shown in Fig. 1A, shTR4 cells were more sensitive to IR-induced cell death and growth inhibition than SC cells. The expression of TR4 proteins was transiently induced by IR by 6 h and returned to the basal level at 24 h. On the other hand, the IR-induced TR4 protein expression was lost in shTR4 cells (Fig. 1B). Therefore, the expression level of TR4 responds to genotoxic challenges and TR4 defends cells from IR-induced cell death and/or growth inhibition.
Fig. 1. TR4 protects cells from IR-induced death and its expression is induced by IR. A – TR4 knockdown cells are more sensitive to IR. SC and shTR4 cells were seeded at 1000 cells/well in 24-well plates. One day later, SC and shTR4 cells were treated with tetracycline (1 µg/ml) 24 h before IR and until harvested. Cells were then exposed to IR at 3, 6, and 9 Gy, and the cells were measured by MTT assay 4 days post-IR. The surviving cells were calculated as the percentage of non-IR exposed cells and mean ± S.D. from three independent experiments plotted. B – IR induces TR4 protein expression. Cells were seeded and treated with tetracycline as described in (A) one day before IR. Cells were exposed to 6 Gy IR, and were harvested at 0, 2, 6, and 24 h post-IR. Western blotting with a specific mouse monoclonal antibody against TR4 was used to determine TR4 protein expression. The levels of TR4 were quantified, normalized by α-tubulin, and relative expression levels were calculated by setting the 0 h level at 1.

The dysregulation of Gadd45a expression in TR4KO mice
To investigate the molecular mechanism through which the temporary induction of TR4 protects cells from IR-induced cell death, we conducted a small scale gene profiling array, and found that the expression of Gadd45a was reduced in TR4 knockdown cells (data not shown). We therefore hypothesized that GADD45A is regulated by TR4 to protect cells from genotoxic stress. To test whether TR4 is responsible for altered Gadd45a expression, we first examined the expression of Gadd45a in TR4KO mice, and found a reduction of Gadd45a mRNA in TR4KO mice muscle and liver (data not shown), in 6-month-old mice as compared to TR4 WT littermates (Fig. 2A). The reduction of GADD45A protein was also found in TR4KO mice cerebellum at 6 months and 21 months old compared to WT (Fig. 2B). The observation of more severe reduction in older mice indicated that accumulation of GADD45A during the aging process could be lost in TR4KO mice. We also found that expression of Gadd45a mRNA in WT MEFs was induced by IR at 10 h, which is later than the induction of Tr4 at 4 h post-IR (Fig. 2C). We therefore suspected that expression of Gadd45a might be regulated by TR4 at the transcriptional level. Importantly, we found that Gadd45a expression could be induced by IR in WT, but not in TR4KO MEF cells, after exposure to 6 Gy IR (Fig. 2D). Together, these results suggest that the presence of TR4 plays important roles for IR-modulated Gadd45a expression.
TR4 regulates GADD45A expression through transcriptional regulation

TR4 is a transcriptional factor that recognizes the DNA element composed of the direct repeat (DR) motif located regulatory regions of its target genes, including DR-3 [8]. As shown in Fig. 3A, four DR-3 type TR4 response elements (TR4REs) have been identified in the third intron and fourth exon of the GADD45A gene, which further supports our hypothesis that TR4 may be able to bind to these putative TR4REs to regulate GADD45A gene expression at the transcriptional level. A GADD45A gene controlled luciferase reporter (GADDLuc)
containing the putative TR4REs was then co-transfected with pCMX-TR4 or vector control to examine the Luc activity in the CV-1 cells that express little TR4 to reduce the basal level of reporter activity. We first found that ectopic expression of TR4 led to significant induction of TR4 proteins (Fig. 3B inserted panel), and GADDLuc activity was transactivated by TR4 in a TR4-dose dependent manner, but not the GADDLuc-3 containing only TR4RE IV (Fig. 3B). The above results were further supported by direct in vivo binding ChIP assays showing that TR4 could directly bind to the GADD45A genomic regions containing TR4RE I, II, and III located in intron 3, but not the region containing TR4RE IV located in exon 4 (Fig. 3C). We used primers amplifying 5’ promoter regions, which do not contain TR4REs, as a control of optimal fragmentation of genomic DNA (Fig. 3C, first row), and IgG as a control of pull-down specificity.

![Fig. 3. TR4 regulates expression through transcriptional regulation. A – Several DR3-motifs indicated as I, II, III, IV are found in GADD45A intron 3 and exon 4. B – TR4 activates the GADD45A reporter gene (GADDLuc). CV-1 cells were co-transfected with GADD45A reporter (GADDLuc or GADDLuc-3), different amounts of TR4 expressing plasmid (pCMX-TR4), and transfection control plasmid pRL-tk. GADD45A gene controlled Luc activities were measured then normalized with internal control Renilla Luc activity. The relative luciferase activity compared to vector was calculated and mean ± S.D. was plotted. * – p < 0.05 compared to vector transfected CV-1 cells. The protein expression of TR4 in vector and pCMX-TR4 transfected CV-1 cells was examined by Western blotting and is shown in the upper panel. C – TR4 binds to the GADD45A gene. ChIP assay was performed to pull down TR4-DNA complex by TR4 antibody (N-15). Control IgG was used as a negative control. PCR was performed to amplify DNA fragments containing regions I, II-III, or IV. A pair of primers amplifying 5’ promoter, where no TR4RE was found, served as a negative control. Lane 1 – input control, Lane 2 – IgG pull-down complex, Lane 3 – TR4 antibody pull-down complex.](image-url)
Due to the limitation of ChIP, we could not separate TR4RE II and III as those are located within 100 bp. It is possible that either both or only one of them can be bound by TR4. Nevertheless, these findings concluded that TR4 could directly induce Gadd45a expression at the transcriptional level.

**DISCUSSION**

This study was initiated by the finding that TR4 deficient cells exhibit hypersensitivity to IR-induced cell death. IR-induced temporary increase of TR4 mRNA and protein suggests that TR4 expression is tightly regulated in response to IR. The fact that presence of TR4 protects cells from IR-induced death led us to further explore the molecular mechanisms by which TR4 protects cells from IR. We found that IR-induced GADD45A requires TR4 that binds to TR4RE in the intron 3 of GADD45A to activate transcription. The temporary association of TR4 and Gadd45a expression in cells exposed to IR suggests that GADD45A is one key molecule mediating the hyper-sensitivity to IR in TR4 deficient cells. In our previous publication TR4 was up-regulated upon the oxidative stress challenges by FOXO3a. Here we find that IR can transiently induce TR4 expression that supports the role of TR4 in sensing genotoxic stress. Moreover, TR4 knockdown cells display increasing sensitivity to IR-induced cell death and/or growth inhibition. This indicates the protective effect of TR4 against IR. Since we did not completely deplete TR4 in mPrE cells used in the study shown in Fig. 1, cells can possibly recover from IR but the recovery is delayed. Further examination of IR sensitivity in TR4 knockout cells by colony forming assay will determine the importance of TR4 in protecting cell survival from IR challenges.

Next, we demonstrated that TR4 controls not only the basal but also stress-induced expression of Gadd45a. Noticeably, the expression of Gadd45a before IR is similar between WT and TR4KO MEFs. We suspect that the discrepancy of Gadd45a expression between WT and TR4KO tissues (Fig. 2A, B) and MEFs (Fig. 2D) is due to age. Since Gadd45a is a stress-responsive gene, the reduction of Gadd45a in TR4KO mice could be amplified during the aging process. Therefore, the difference of Gadd45a expression in MEFs isolated from E14.5 embryos is discernable, but becomes obvious in tissues from 6-month-old WT and TR4KO mice. It has been shown that FOXO3a can directly up-regulate the expression of Gadd45a during genotoxic stress [24]. Here, we find that IR poorly induces Gadd45a expression in TR4KO cells and TR4 can directly regulate transcription of Gadd45a. Our observation suggests that TR4 is another essential mediator for inducing GADD45A upon genotoxic stress besides FOXO3a. Previous reports describe faster and transient induction of GADD45A by IR (starting from 30 min and reaching a plateau within 4 h) while IR induction of Gadd45a mRNA level in MEF cells started at 10 h and lasted until 24 h [23, 29]. The difference in time-point of induction observed could be due to lower sensitivity of Q-PCR assay than RNase protection assay applied in previous reports. This might be because the amplification efficiency of Q-PCR
primers for Gadd45a is not optimal. Another possibility is that there could be variations between species and tissues as IR did not induce GADD45A in CHO as significantly as in human cells [23]. The induction of GADD45A in human fibroblasts is observed in early (1-12 h), intermediate (24-48 h), and late (72 h) phases [30]. Therefore, our observation (10-24 h) is within the response time range.

Fig. 4. Illustration of TR4 roles in stress-induced cellular defense signaling.

The known functions of GADD45A include inducing G2/M phase cell cycle arrest [31] upon DNA damage, DNA repair [32], and DNA demethylation [33, 34], which is still controversial [35]. The fact that Gadd45a null mice exhibit genomic instability and increased radiation-induced carcinogenesis [36] implies that several premature aging phenotypes observed in TR4KO mice could result from loss of Gadd45a expression. Although Gadd45a null mice do not have hyper-sensitivity to IR-induced death [36], Gadd45a null hematopoietic cells are sensitive to genotoxic stress-induced apoptosis [37]. Therefore, the importance of GADD45A and whether there are other molecules involved in mediating TR4 protecting against IR-induced cell death demands further investigation.

In conclusion, TR4 senses IR challenges by increasing expression to initiate subsequent gene activation to guard cells from IR-induced death. Gadd45a is one of the guardian genes that TR4 induces when cells face IR challenges. TR4 directly binds to its response element located in the Gadd45a gene and activates transcription. As illustrated in Fig. 4, TR4 responds to genotoxic stress to protect genome integrity and is potentially involved in epigenetic modification by regulation of Gadd45a. The importance of GADD45A in guarding genome integrity links TR4 to cancer and aging, which are tightly linked to genome instability. Currently, the role and mechanism of TR4 in protection of genome integrity in preventing cancer are under investigation.
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