Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology)
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.zju.edu.cn/jzus; www.springerlink.com
E-mail: jzus@zju.edu.cn

Perspective:

Recent advances in the study of testicular nuclear receptor 4*

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Received Dec. 24, 2012; Revision accepted Feb. 17, 2013
Crosschecked Feb. 18, 2013

doi:10.1631/jzus.B1200357
Document code: A **CLC number:** Q74; R34

Testicular nuclear receptor 4 (TR4), also known as NR2C2 (nuclear receptor subfamily 2, group C, member 2), is a transcriptional factor and a member of the nuclear receptor family. TR4 was initially cloned from human and rat hypothalamus, prostate, and testes libraries. For almost two decades, its specific tissue distribution, genomic organization, and chromosomal assignment have been well investigated in humans and animals. However, it has been very difficult to study TR4's physiological functions due to a lack of specific ligands. Gene knock-out animal techniques provide an alternative approach for defining the biological functions of TR4. In vivo studies of TR4 gene knockout mice (*TR4*$^{-/-}$) found that they display severe spinal curvature, subfertility, premature aging, and prostate prostatic intraepithelial neoplasia (PIN) development. Upstream modulators, downstream target gene regulation, feedback mechanisms, and differential modulation mediated by the recruitment of other nuclear receptors and coregulators have been identified in studies using the *TR4*$^{-/-}$ phenotype. With the establishment of a tissue-specific *TR4*$^{-/-}$ mouse model, research on TR4 will be more convenient in the future.

1 Introduction

Nuclear receptors include a large superfamily of at least 48 DNA-binding transcription factors whose target genes are important for many biological processes, including growth, differentiation, development, and metabolism (Robinson-Rechavi *et al*., 2003; Popov *et al*., 2007). Nuclear receptors share a common structure consisting of four domains: a variable N-terminal A/B transactivation domain, a highly conserved DNA-binding domain (DBD), a hinge domain, and a ligand-binding domain (LBD). The nuclear receptor superfamily can be divided into three subfamilies: (1) steroid hormone receptors, such as androgen receptors (ARs), estrogen receptors (ERs), glucocorticoid receptors (GRs), and progesterone receptors (PRs); (2) non-steroid hormone receptors, such as thyroid receptors (TRs), retinoic acid receptors (RARs), and vitamin D receptors (VDRs); (3) orphan receptors, such as testicular nuclear receptor 2 (TR2) and TR4 (also known as nuclear receptor subfamily 2, group C, member 2 (NR2C2)). Orphan receptors were initially cloned out by their structural homology to nuclear receptors without identified ligands. Some nuclear receptors were classified as orphan receptors before their physiological ligands were identified, including the retinoid X receptor (RXR), peroxisome proliferator-activated receptor (PPAR), and liver X receptor (LXR) (Aranda and Pascual, 2001).

TR4 was initially cloned from human and rat testes (Chang *et al*., 1994; Hirose *et al*., 1994). TR4 is ubiquitously expressed. Tissue distribution analyses showed that TR4 transcripts are expressed in many mouse tissues including the central nervous system and peripheral organs, such as the adrenal gland, spleen, thyroid gland, and prostate gland. Transcripts are most abundant in the testes and skeletal muscle (Lee *et al*., 2002; Yang *et al*., 2006). Functional studies of TR4 expression patterns, target gene

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* Project supported by the National Natural Science Foundation of China (No. 30973001), the National Basic Research Program (973) of China (No. 2012CB518304), the Zhejiang Provincial Natural Science Foundation of China (No. Y2110446), the Qianjiang Talents Project of Zhejiang Province (No. 2011R10039), and the PAO Yu-kong International Foundation for Scholars and Scientists, China

modulation, and knockout TR4 ($TR4^{-/-}$) mice suggested that TR4 might play important roles in growth, development, and metabolism.

2 TR4 and bone

Bone is a mineralized tissue that provides mechanical support to the body. Previous studies showed that TR4 is expressed ubiquitously in mice, including in bone tissue (Chang *et al*., 1994). Harada *et al*. (1998) found that TR4 inhibited ER-mediated transactivation in two osteoblastic cell lines (UMR-106 and ROS17/2.8). Similarly, Lin *et al*. (2012) reported that reduced osteoblast activity in mice lacking TR4 leads to osteoporosis. Lee *et al*. (2011) claimed that $TR4^{-/-}$ mice were born indistinguishable from their wild-type littermates, and that two- to three-month-old $TR4^{-/-}$ mice showed no significant skeletal abnormalities and had similar bone mineral density (BMD). However, radiograms revealed a severe kyphosis (curvature of the spine) at the age of six months. Dual energy X-ray absorptiometry (DEXA) scanning also showed an obvious reduction in BMD in the spine of six- to seven-month-old $TR4^{-/-}$ male and female mice. They also examined BMD in the whole body of all the $TR4^{-/-}$ mice, including the legs, tail, and skull, and found a significant difference in all these parts except the skull. In agreement with the BMD analyses by DEXA scanning, hematoxylin and eosin (H&E) staining showed that trabecular and cortical volumes in the thoracic and lumbar spine were obviously reduced in six-month-old $TR4^{-/-}$ mice. Furthermore, a large amount of fat was found in the bone marrow in the thoracic bone of the six-month-old $TR4^{-/-}$ mice, while age-matched $TR4^{+/+}$ mice showed normal bone with little adipocyte accumulation. These bone abnormalities in $TR4^{-/-}$ mice suggest that TR4 plays an important role in bone health.

Harada *et al*. (1998) found that TR4 had no effect on ER-mediated transactivation in kidney cell line COS-1 and breast cancer cell line MCF-7. So it looks like the suppression effect of TR4 on ER-mediated transactivation is bone-specific. Estrogen is very important to maintain female bone mass and some selective ER modulators (SEVMs) had been recommended for the prevention and treatment of ostmenopausal osteoporosis.

Another study reported that TR4 showed negative modulation of vitamin D signaling (Lee *et al*., 1999). Vitamin D signaling is crucial to regulate calcium homeostasis and thus bone density. This finding appears to contradict those of other studies described above. A possible explanation may be that TR4 regulates bone cell function through a complex downstream network, and the activation of different specific downstream pathways of TR4 could be dependent on the context and physiological conditions. So more research is necessary to explore the precise role of TR4 in bone health and the molecular mechanism through which TR4 affects bone metabolism. Also, it will be very interesting to study how the interactions between TR4 and ER or vitamin D signaling contribute to the regulation of bone remodeling.

3 TR4 and fertility

3.1 TR4 and male fertility

The highest relative expression of TR4 has been found in the testes. Mu *et al*. (2004) studied the detailed expression of TR4 during spermatogenesis. They found that in normal mice, TR4 shows particularly high expression in the primary spermatocytes at meiotic prophase, and that expression increases and reaches a peak at this stage, during the first wave of spermatogenesis. However, $TR4^{-/-}$ mice showed significantly delayed and interrupted development in meiotic prophase and subsequent meiotic divisions, resulting in a seriously delayed and disrupted first wave of spermatogenesis. Stages XI to XII, where late meiotic prophase and subsequent meiotic divisions take place, were also prolonged and disrupted in $TR4^{-/-}$ adult mice, resulting in a prolonged metaphase and the appearance of abnormal cells.

Collins *et al*. (2011) designed an experiment to observe the role of TR4 in mouse sexual behavior and fertility. Nine $TR4^{+/+}$ male mice were involved in the research on sexual behavior. All of them showed mounting behavior, five displayed intromission, and two achieved ejaculation. However, 11 $TR4^{-/-}$ adult male mice showed no sexual behavior during 90 min of initial pairing with a primed female mouse. To detect the effect of TR4 on fertility, five $TR4^{-/-}$ male mice were mated with mature $TR4^{+/+}$ female mice.

Four months later, only one produced offspring, with a total of two litters. In contrast, five adult $TR4^{+/+}$ male mice produced 22 litters in four months, giving an average of 4.4 litters per $TR4^{+/+}$ mouse. They concluded that not only were significantly fewer $TR4^{-/-}$ males able to produce offspring, but the number of litters generated by the fertile $TR4^{-/-}$ male was roughly half the average of the $TR4^{+/+}$ group. Thus, TR4 appears to play an essential role in normal spermatogenesis and male fertility.

3.2 TR4 and female fertility

Collins *et al*. (2004) found effects of TR4 on maternal behavior of female mice before their discovery of the effects of TR4 on male sexual behavior. They mated five $TR4^{-/-}$ females with normal adult $TR4^{+/+}$ males. During the 2.5-week study, only one produced a litter of six pups, whereas all $TR4^{+/+}$ females produced litters with an average of 6.4 pups per litter. Chen L.M. *et al*. (2008) confirmed the subfertility of $TR4^{-/-}$ female mice in a separate study.

Further dissection showed that TR4 plays an important role in normal folliculogenesis and ovarian function in female mice. Wang *et al*. (2006) reported that TR4 might stimulate oxytocin (OXT) gene expression through the OXT element in a dose-dependent manner. Chen L.M. *et al*. (2008) found that $TR4^{-/-}$ female mice had smaller ovaries, fewer oocytes, and significantly fewer and smaller corpora lutea. They also found higher granulosa cell apoptosis in $TR4^{-/-}$ ovaries. All these data indicate that $TR4^{-/-}$ female mice have defective ovarian function. However, all stages of primary, preantral, and antral follicles were present and histologically normal. By immunohistochemistry (IHC) staining, they found reduced luteinizing hormone receptor (LHR) expression in luteal cells of $TR4^{-/-}$ female mice. Both 5′ promoter assays and chromatin immunoprecipitation (CHIP) assays demonstrated that TR4 could stimulate LHR gene expression via direct binding to its TR4 response element located on the LHR gene sequence on the 5′ promoter side (Zhang and Dufau, 2000). They concluded that TR4 might modulate LHR gene expression to control estradiol and progesterone production and play an important role in normal ovarian function. This effect of TR4 may be related to the subfertility of $TR^{-/-}$ female mice.

4 TR4 and metabolism

Metabolic syndrome is a combination of medical disorders, which increases the risk of developing cardiovascular disease and diabetes. The exact mechanisms of the complex pathways involved in metabolic syndrome remain unknown. Yan *et al*. (1998) showed regulation of peroxisome proliferator-activated receptor alpha (PPARα)-induced transactivation by TR4. Lee *et al*. (1998) found negative feedback control of the retinoid-retinoic acid/retinoid X receptor pathway by the human TR4. Lee *et al*. (2001) claimed that TR4 represses human steroid 21-hydroxylase gene expression through the monomeric AGGTCA motif. Thus, many findings suggest a link between TR4 and metabolism.

In studies of lipid metabolism, Kim *et al*. (2003; 2005) demonstrated that TR4 induces apolipoprotein E (apoE) expression in HepG2 hepatoma cells via the TR4RE-DR1-apoE element in HCR-1. Using $TR4^{-/-}$ mice, they confirmed the importance of TR4 in the regulation of apoE expression, and in the expression of other apolipoproteins, apoC-I and apoC-II in the same gene cluster. Choi *et al*. (2011) showed that TR4 facilitates lipid accumulation in 3T3-L1 adipocytes via induction of the FATP1 gene. Xie *et al*. (2009) reported that TR4 functions as a fatty acid sensor to modulate CD36 expression and foam cell formation. TR4 can be transactivated via its activators, such as polyunsaturated fatty acid (PUFA) metabolites and thiazolidinediones (TZDs). Tsai *et al*. (2009) found that TR4 can be activated by fatty acids. The correlation between TR4 and these small metabolic molecules suggests that TR4 might be an important gene in metabolism.

TR4 also plays a crucial role in carbohydrate metabolism and insulin sensitivity. Liu *et al*. (2007) published a paper on diabetes in which they reported that TR4 increases glucose production in primary hepatocytes and hepatoma cells, but the glucose homeostatic response is altered in $TR4^{-/-}$ mice. Kim *et al*. (2011) demonstrated a pathway of metformin→adenosine monophosphate-activated protein kinase (AMPK)→TR4→stearoyl-CoA desaturase-1 (SCD1)→insulin sensitivity, and concluded that TR4 may function as an important modulator to control lipid metabolism. This finding sheds light on the use

of small molecules to modulate TR4 activity as a new approach for treating metabolic syndrome. Kang *et al*. (2011) found that TR4-deficient mice are protected against obesity-linked inflammation, hepatic steatosis, and insulin resistance. This is the most important in vivo discovery which links TR4 directly to not only carbohydrate metabolism and insulin sensitivity, but also metabolism.

To sum up, these discoveries about TR4 may provide a platform to screen new drugs to battle the metabolic syndrome and diabetes.

5 TR4 and oxidative stress, gene reparation, and aging

Li *et al*. (2008) identified a daf-16 family protein-binding element (DBE; 5′-TGTTTAC-3′) in the promoter region of the TR4 gene that can be recognized by the forkhead transcriptional factor FOXO3a, a key stress-responsive factor. The interaction between DBE in the TR4 promoter region and FOXO3a was confirmed using electrophoretic mobility shift assay (EMSA) and CHIP assays. Functional assays further demonstrated that TR4 gene expression is activated via this DBE. Activation of FOXO3a by oxidative stress and phosphatidylinositol 3-kinase inhibitor also induced TR4 expression. In contrast, suppression of FOXO3a by short hairpin RNA (shRNA) decreases oxidative stress-induced TR4 expression. The biological consequence of the FOXO3a-induced TR4, activated by oxidative stress, is protection against stress-induced cell death. Cells with reduced FOXO3a are less resistant to oxidative stress, and addition of TR4 can increase stress resistance. Thus, Li *et al*. (2008) claimed that the stress-FOXO3a-TR4 pathway is a fundamentally important mechanism regulating stress resistance and cell survival.

Aging is the accumulation of changes in a person over time (Bowen and Atwood, 2004). Aging in humans refers to a multidimensional process of physical, psychological, and social change. Roughly 100000 people worldwide die each day of age-related causes. However, the biological basis of aging is unknown. DNA damage is thought to be the common pathway causing aging. Liu *et al*. (2011b) reported that TR4 modulates ultraviolet (UV) sensitivity by promoting the transcription-coupled NER (TC-NER) DNA repair pathway through transcriptional regulation of CSB. Yan *et al*. (2012) found that deficiency of TR4 abrogates growth arrest and DNA-damage-inducible protein GADD45 alpha (Gadd45α) expression, and increases cytotoxicity induced by ionizing radiation. A more direct link to aging comes from another study. Yan *et al*. (2012) reported that $TR4^{-/-}$ mice suffered from premature aging. Almost all $TR4^{-/-}$ mice displayed premature aging phenotypes, including cachexia, short lifespan, and kyphosis. Further study found that $TR4^{-/-}$ mice had a deficient oxidative stress defense system and a compromised genomic. They claimed that the stress-FOXO3a-TR4 pathway may be responsible for a deficient oxidative stress defense system. But further investigation is needed to clarify which specific genes are responsible for the impaired genomic integrity of $TR4^{-/-}$ mice.

6 TR4 in erythrocytes and hemoglobin

Tanabe *et al*. (2002) demonstrated that a TR2/TR4 heterodimer forms a core of a larger direct repeat erythroid-definitive (DRED) complex that represses embryonic and fetal globin transcription in definitive erythroid cells. Therefore, this suppression of activity might be an attractive intervention point for treating sickle cell anemia. Shyr *et al*. (2009) claimed that TR4 may have an important role in early embryonic development by regulating key genes involved in stem cell self-renewal, commitment, and differentiation. Campbell *et al*. (2011) found that forced TR2/TR4 expression in sickle cell disease mice conferred enhanced fetal hemoglobin synthesis and alleviated disease phenotypes. Cui *et al*. (2011) found that TR2 and TR4 recruit many epigenetic transcriptional corepressors which associate specifically with the embryonic β-type globin promoters in differentiated adult erythroid cells. However, research on the relationships between TR4 and erythrocytes and hemoglobin is limited and further study is needed. This may lead to a new and helpful way to cure erythrocyte-related disease.

7 Conclusions

This review clearly indicates that TR4 plays essential roles in bone, fertility, metabolism, oxidative stress, gene reparation, aging, erythrocytes, and hemoglobin. TR4 was also found to interact with some receptors or be modulated by some genes having roles in many physiological functions (Chen Y.T. *et al*., 2008; Huang *et al*., 2010; Liu *et al*., 2011a; Xie *et al*., 2011; Zhou *et al*., 2011). However, there are still many questions that need to be answered regarding the detailed mechanisms and the cross-talk between TR4 effects in different tissues. Fortunately, a tissue-specific TR4 knockout mouse model has been established recently. This mouse model will further clarify TR4's role in different tissues without concerns about primary and secondary effects. Future studies in the tissue-specific TR4 knockout mouse model will improve our understanding of TR4's physiological and pathological functions. This may lead to the application of TR4 in the development of new diagnostic and therapeutic approaches for treating human diseases.

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Introducing editorial board member:

Dr. Chawnshang CHANG is a George Hoyt Whipple Distinguished Professor of Pathology in University of Rochester, USA. He earned his PhD degree from the University of Chicago, USA in 1985. In 1988, Dr. CHANG succeeded in becoming the first scientist to clone the complete human and rat androgen receptors (AR). It is the landmark discovery in the Androgen-AR field. Over the next 25 years, he continued his research on AR and three nuclear orphan receptors TR2, TR3, and TR4 he isolated from human testis, and had more than 350 papers published, including *Science*, *Nature*, and so on.

Chawnshang CHANG