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MULTIPLE ISOFORMS OF THYROID HORMONE RECEPTOR: AN ANALYSIS OF THEIR RELATIVE CONTRIBUTION IN MEDIATING THYROID HORMONE ACTION

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ABSTRACT

Thyroid hormone is essential for normal development and maintaining metabolic homeostasis. In mediating the thyroid hormone action, the thyroid hormone receptor (TR) plays a key role. Almost one decade ago, the cloning of TR was achieved, revealing the existence of at least two genes, TRα and TRβ, which encode TR. From these genes several TR isoforms can be generated by alternative splicing. They are designated as TRα1, TRα2 (inactive form), TRβ1 and TRβ2. Since the discovery of these TR isoforms, many studies have attempted to demonstrate their relative contribution to mediate thyroid hormone in various tissues. The distinct tissue distribution and the ontogenic expression of the TR isoforms, and the fact that TR gene abnormalities associated with the syndrome of resistance to thyroid hormone (RTH) have been found only in the TRβ gene, indicate that products of TRα and TRβ have distinct roles. However, no direct evidence of the distinct roles of the TR isoforms has been shown. Gene knockouts of either TR isoform would provide important information to understanding their specific roles. In this review, the history of the TR isoform discovery and studies attempting to demonstrate the specific roles of TR isoforms are summarized, and recent reports dealing with knockouts of TR isoforms are comprehensively presented.

Key Words: thyroid hormone, receptor, syndrome of resistance to thyroid hormone, gene knockout

INTRODUCTION

Thyroid hormones, the only known iodine-containing compounds with biological activity, are important for normal development of animals including human beings.[1] For example, a deficiency of the thyroid hormone in the early neonatal days causes severe mental and growth retardation as observed in cretinism.[2] Also, in amphibian metamorphosis, thyroid hormones are known to play a key role.[3] On the other hand, thyroid hormones act to maintain metabolic homeostasis in adults, affecting the function of virtually all organ systems. These functions can be understood from the signs and symptoms of adult patients with abnormal thyroid functions, such as hyper- or hypothyroidism.

Recent studies have uncovered the molecular and cellular events related to thyroid hormone action. As illustrated in Fig. 1, two active thyroid hormones, thyroxine (T_4) and 3,3′,5-triiodothyronine (T_3), enter the cell by a mechanism yet to be defined. In the cytoplasm, T_4 is

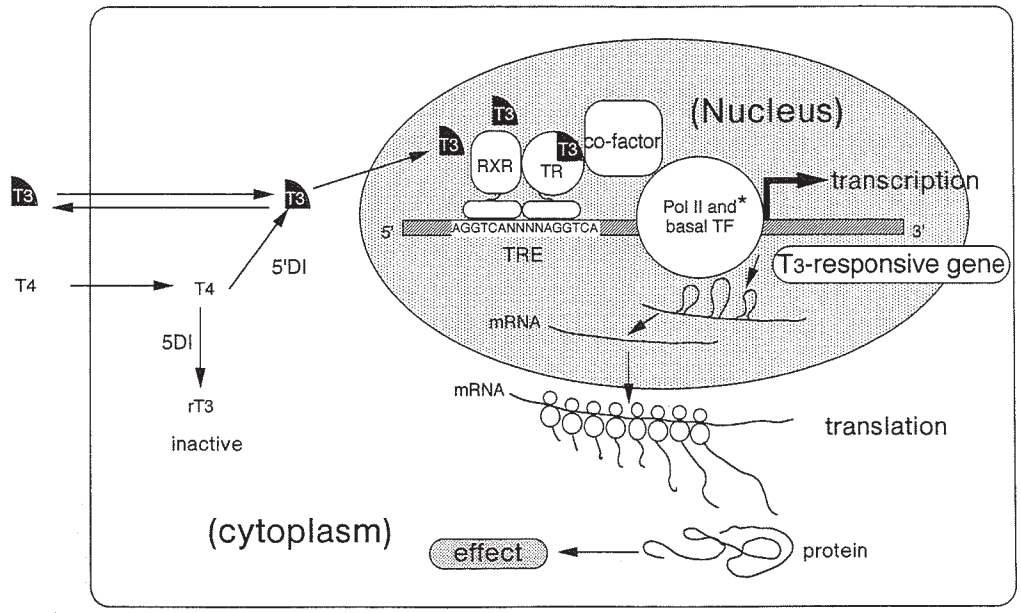

Fig. 1 Thyroid hormone action mechanism

converted to T_3 by the action of iodothyronine 5′ deiodinase.[4] Alternatively T_4 is converted to biologically inactive 3,3′,5′-triiodothyronine (reverse T_3, rT_3) by 5-deiodinase named type III iodothyronine deiodinase.[5] Only T_3 can be transported to the nucleus; it binds the T_3-receptor (TR), which usually heterodimerizes with retinoid-X receptor (RXR)[6-9] on the T_3-response element (TRE) of the regulatory region of a target gene for T_3. When T_3 binds to TR, co-factors are recruited and transmit signals to basal transcriptional machinery.[10-13] Accordingly, the transcriptions of the target genes are regulated by T_3 and, in this way, T_3 exerts its biological effects through the products of the transcripts. Although the thyroid hormone may have some important function through non-nuclear action,[14] those thyroid hormone actions mediated by nuclear TRs have been accepted as a major pathway. Therefore, TRs play a principal role in thyroid hormone action.

MOLECULAR CLONING OF THYROID HORMONE RECEPTORS

1986 remains a landmark year for researchers who are involved in the study of thyroid hormone action, because the first report of the molecular cloning of TRs was announced. In the same issue of *Nature*, two different research groups simultaneously reported that the previously isolated proto-oncogene, *c-erb-A*,[15,16] encodes TRs. Interestingly, they isolated two different genes encoding two distinct TRs. Sap et al.[17] cloned one TR from a chick embryo cDNA library, while the other was cloned from human placenta cDNA libraries by Weinberger et al.[18] One year later, when a rat TR with homology to the chick clone was isolated and its gene was mapped to human chromosome 17[19] and another type of TR was mapped to chromosome 3,[18] it became evident that the two different TR sequences did not reflect a species difference but indicated the existence of at least two TR isoforms. In addition, subsequent studies clearly

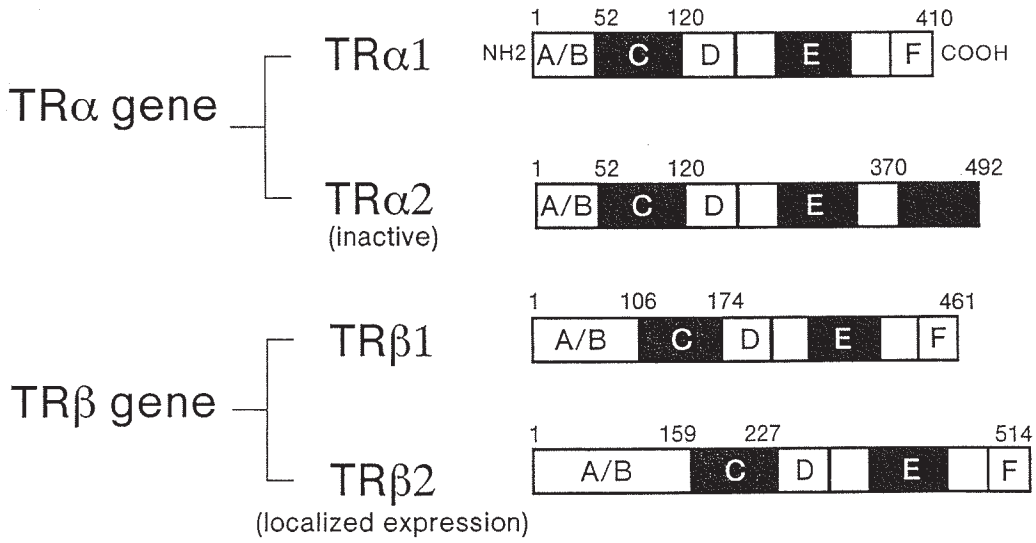

Fig. 2 TR isoform structures

demonstrated that the existence of TRα and TRβ extends across a variety of species. For reference, the TR isolated from chicken was designated alpha (TRα) because of its homology to previously isolated *erb-A* genes[15] while another TR originally from human placenta was designated beta (TRβ).[19]

The cloning of TR has also revealed that TR belongs to the steroid hormone receptor superfamily. Primary structures of TR and the steroid receptors are similar, consisting of 5 domains named as A/B, C, D, E, and F domain (Fig. 2). Among these domains, the C domain is a DNA-binding domain and is the most highly conserved region. The D-E-F domain is a hormone-binding domain.

As a matter of course, the cloning of TRs has resulted in great advances in the research field of thyroid hormone action. However, at the same time, a new question has been raised, namely whether TRα and TRβ have distinct roles in the mediation of thyroid hormone action.

MULTIPLE TR ISOFORMS

It is now generally accepted that several TR isoforms are generated from each TRα and TRβ gene by alternative splicing. Although various TR isoforms have been reported, these isoforms can be categorized into either TRα1, TRα2, TRβ1 or TRβ2. Schematic structures of these isoforms are illustrated in Fig. 2. TRα1 and TRβ1 are the major products from TRα and TRβ genes, respectively, and are widely distributed in the body. Alternative splicing of the 3′-most exon of TRα1 results in the generation of TRα2.[20,21] TRα2 lacks the 40 amino acids of TRα1 at its C-terminus but contains an additional 120 (human) or 122 (rat, mouse) amino acids with no homology to other known sequences.[22] This C-terminal region is critical for T_3-binding, hence, TRα2 is inactive for T_3-binding. TRα2 is widely expressed, and in some tissues, the expression is more abundant than that of TRα1. Although there was a report that TRα2 has an inhibitory action against active TRs,[23] this dominant negative effect has not

always been demonstrated. It thus remains to be clarified how TRα2 is functioning in the body.

Alternative splicing of the N-terminus region of TRβ1 results in the generation of TRβ2.[24] TRβ2 shows distinct tissue distribution. In the first report of TRβ2, the abundant expression of TRβ2 mRNA was demonstrated in rat anterior pituitary while the mRNA was not detected in liver, heart, cerebrum, or brown adipose tissue.[24] However, in the following reports[25] the use of reverse transcription coupled with polymerase chain reaction (RT-PCR) or immunohistochemistry, showed TRβ2 expression in other tissues as well, especially in the central nervous system. The existence of TRβ2 was originally reported in rat, but was also confirmed in mouse,[26] chicken[27] and human.[28] TRβ2 can bind to T_3 and is thought to be functionally identical to TRβ1. However, the expression of TRβ2 is highly regulated by T_3 itself at least in the anterior pituitary. The expression was almost 85% suppressed by T_3 in the rat pituitary cell line (GH3).[24] The exact physiological role of TRβ2 and its regulation by T_3 has not been clarified yet.

TISSUE DISTRIBUTION AND ONTOGENIC PROFILE OF THE EXPRESSION OF TR ISOFORMS

Up until now, no fundamental differences in function between TRα1 and TRβ1 or TRβ2 has been demonstrated *in vitro*. Though differences in the affinity of *in vitro* synthesized TRα1 and TRβ1 for the acetic acid analogue of T_3 was shown, their binding affinity for T_3 was similar.[29] Also *in vitro* DNA-binding analyses and cell transfection studies have generally indicated that the TRα1, TRβ1 and TRβ2 exhibit similar ligand dependent transcriptional activity.[22]

Despite these similarities *in vitro* function, there are several findings which imply differential *in vivo* roles between TRα1, TRβ1, major isoforms generated from TRα and TRβ genes, respectively. One is the unique tissue distribution of TRα1 and TRβ1. Shown in Table 1 is a summary of the isoform distribution in rat tissue. In the brain, dominant expression TRα1 is seen both at mRNA and protein levels whereas the expression of TRβ1 is dominant in the liver. In the heart and kidney, both isoforms are almost equally expressed. An ontogenic profile of TR expression in the rat brain suggests that TRα1 and TRβ1 play specific roles in brain development. TRα1 mRNA is widely distributed in the rat brain throughout the developmental period.[30] The expression is detectable as early as embryonic day 11.5 (E11.5),[31] several days before the onset of fetal thyroid function. Wide distribution of TRα1 expression is already seen on E14 and the expression increases throughout early fetal development. The mRNA reaches a peak during the neonatal period and decreases markedly to adult levels.[30] On the other hand, the expression of TRβ isoforms localize in specific brain regions. Both TRβ1 and TRβ2 are detectable as early as E12.5 in the portion of the ventral pole of the optic vesicle that gives rise to cochlea,[32] but localization of the expression remains restricted to the area including cochlear cell lineage during the gestational days.[31,32] Another feature of the TRβ1 expression profile is a dramatic surge around the perinatal period. Strait et al.[33] reported that a 40-fold increase in TRβ1 mRNA occurred in the transition between 19-day gestational fetus and the 10-day-old neonate. This increase corresponds well to the increase in T_3 contents in the brain.[33] Since thyroid hormone effects on rat brain development appear to occur largely in the first 10–15 days of life, they hypothesized that TRβ1 may play a primary role in mediating T_3 effects in developing and adult animals. The validation of their hypothesis must await successful preparation of TRβ1 knockout mouse.

As described above, the tissue distribution and ontogenic profile of TRα1 and TRβ1 imply distinct roles for these TR isoforms; however, TR abnormality in the syndrome of resistance to

Table 1. Tissue distribution of TR isoforms in adult rats

	TRα1		TRβ1		TRβ2	
	mRNA	Protein	mRNA	Protein	mRNA	Protein
Brain	++++	+++ (61%)	++	++ (29%)	-	+ (10%)
Pituitary	++	nd	+	nd	++	nd
Kidney	++	+ (45%)	+++	+ (41%)	nd	+ (15%)
Heart	+++	++ (41%)	+++	++ (41%)	-	+ (18%)
Liver	+	+ (13%)	+++	++++ (71%)	-	++ (17%)
Spleen	+	nd	-	nd	nd	nd

analysis:
mRNA by Northern blot
protein by immunoprecipitation
nd: not determined

references:
(mRNA): Murray, M.B. et al. J. Biol. Chem. 1988
(TRβ2): Hodin, R.A. et al. Science 1989
(protein): Schwartz, H.L. et al. J. Biol. Chem. 1994

thyroid hormone (RTH) would provide even stronger evidence for the distinct roles of TRα1 and TRβ1.

THE SYNDROME OF RESISTANCE TO THYROID HORMONE (RTH)

RTH is characterized by reduced clinical and biochemical manifestations of thyroid hormone action relative to the circulating hormone levels.[34]. RTH was first reported by Refetoff et al. in 1967.[35] Since the first report, an increasing number of cases has been reported, covering 347 patients as of 1993.[34] Most patients have persistent elevation of serum free T_4 and free T_3 with inappropriately non-suppressed thyrotropin (TSH). Patients present goiter almost exclusively, and sometimes short stature, hyperactivity and learning disability in children or adolescents. Administration of a supraphysiological dose of thyroid hormone fails to produce the expected suppressive effect on the secretion of TSH and/or to induce metabolic responses in peripheral tissues.

Until the cloning of TR, the etiology and the pathogenesis of RTH had been left open to speculation. Defective transport, metabolism of thyroid hormones, or antagonism by another substance had been ruled out and the authenticity of thyroid hormones in patients with RTH was confirmed.[34] An intracellular defect in thyroid hormone action was postulated soon after the discovery of the syndrome.[35] Since the demonstration of a putative nuclear TR,[36] several attempts were made to identify the TR abnormality in patients with RTH using circulating mononuclear cells and cultured skin fibroblasts. However, the results were inconsistent, although defective responsiveness to T_3 was clearly demonstrated in the fibroblasts from patients with RTH.[37,38] Therefore, until the cloning of TR, it had been presumed that RTH was caused by a

number of defects in T_3 action at various stages.

TR ABNORMALITIES IN RTH

Almost three years after the identification of TR genes, the first report showing a TR abnormality in the patient with RTH was published by Sakurai et al.[39] They found a point mutation in the ligand binding domain of TRβ in the patient. More importantly, they demonstrated that the mutation results in the loss of T_3-binding activity in the mutant TRβ. Since the first report about TRβ mutation, a number of reports demonstrating similar mutations in the TRβ gene have been released. Except for one case,[40] the defect always involved one of the two TRβ alleles. This fact is compatible with the dominant mode of inheritance in most RTH cases. An exceptional TR abnormality was found in one family with on RTH inherited recessive trait.[41] This family was the same family reported as the first case of RTH,[35] and it was found that the coding sequence of both alleles of TRβ gene was completely deleted in the affected members of this family. Heterozygotes of this family, expressing a single TRβ gene, were clinically and biochemically normal. Thus, the inheritance patterns of RTH can be categorized in two ways; autosomal dominant and autosomal recessive.

As shown in Fig. 3, in a RTH case caused by a point mutation of a single TRβ gene allele, there are three intact genes which encode functional TRs. As a matter of fact, it has been shown that a normal and the mutant TRβ1 genes are equally expressed in fibroblasts from patients with RTH.[42] The expression of the TRα1 gene is also intact. These results indicate that both intact TRα1 and TRβ1 are expressed as well as mutant TRβ1 in patients with RTH, and raises a question as to why the existence of the mutant TRβ1 or TRβ2 causes clinical and biochemical manifestations of RTH. To answer this question, many studies has been carried out. It has recently been proposed that the mutant TRβ1 inhibits the normal TR function in a dominant negative manner.[43] One subject with homozygous TRβ mutation exhibited the most severe clinical manifestations. His resting heart rate was 190 beats/min and his T_3, T_4, and TSH serum levels were the highest ever seen. He exhibited the most sever delay in growth and central nervous system development among individuals with RTH. He died from cardiogenic shock complicating

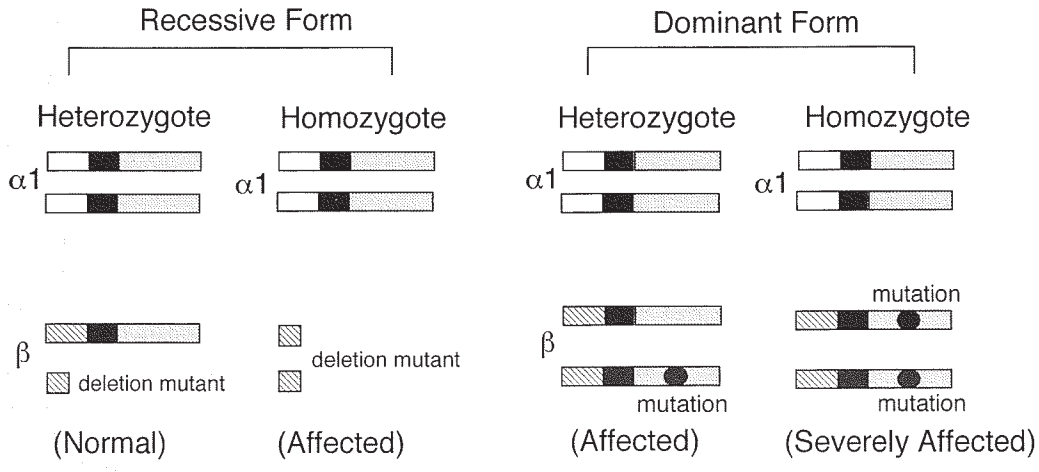

Fig. 3 TR gene anomalies in RTH and its mode of inheritance

staphylococcal septicemia, and it has been believed that this is the only case in which RTH contributed to the patient's death.[34] This case strengthens the case for a dominant negative effect by mutant TRβ, because the clinical manifestation was much more severe than in the case of homozygous TRβ deletion. Interestingly, there has been no reported case of RTH due to a TRα abnormality even though a case without any obvious TRα nor TRβ abnormality was reported.[44]

The fact that the homozygous patients with TRβ deletion also exhibited RTH indicates that TRβ is necessary to maintain normal thyroid function and the peripheral effects of thyroid hormone. Then what about TRα? Why have we not found a RTH case with defective TRα? Two completely opposite hypotheses can be asserted. One is that a TRα mutation or deletion becomes fetal so that the subject with defective TRα cannot exist. Another is that a TRα abnormality does not exhibit any clinical manifestations. The preparation of TRα knockout mouse would be the only way to answer these questions. In addition, preparation of either a TRα or TRβ knockout mouse would provide an ideal model to understand how TRα and TRβ play their distinct roles in mediating thyroid hormone action.

TRβ KNOCKOUT MOUSE

In 1996, Forrest et al.[45] reported the gene knockout of TRβ in a mouse. They targeted a part of the TRβ gene encoding the first zinc finger of the DNA-binding domain. This gene targeting was predicted to disrupt the DNA-binding and T_3-binding domains. As a result of the homologous recombination with the targeting gene, a mRNA approximately 100 bp shorter than that of wild type was generated. The mutated mRNA had an aberrant open reading frame that terminated after 8 bp in exon 4. Thus, the protein product lacked the DNA-binding and T_3-binding domains of TRβ, and as a matter of fact, the mutation precluded expression of functional TRβ1 or TRβ2 in all tissues examined. No gross compensatory increase in TRα expression was observed in the TRβ deficient ($TR\beta^{-/-}$) mouse.

As summarized in Table 2, the phenotype of $TR\beta^{-/-}$ was quite similar to that observed in the patient with RTH due to the deletion of TRβ gene. High T_4 and T_3 without TSH

Table 2. Phenotype of TRβ knockout mice (quoted from ref. 45)

1. Thyroid function

	Wild type (TRβ+/+)	TRβ-/-
T4 (μg/dl)	3.3-4.8	8.9-27.9
T3 (ng/dl)	93-125	155-387
TSH (%)	-40/0	+113/+391

2. Goiter

3. Hearing disturbance

4. No major anomaly, normal development, fertile

suppression and goiter were noted. These findings indicate that TRβ is essential for the regulation of TSH secretion and TRα cannot compensate for such regulation. Interestingly, deaf mutism observed in patients with TRβ gene deletion was reproduced in the $TR\beta^{-/-}$ mouse. The fact that a patient with TRβ gene deletion had associated auditory dysfunction and that specific expression of TRβ gene in cochlear cell lineage was observed during rat development, strongly suggested that TRβ1 and TRβ2 play an important role for the development of the auditory system. So the findings in a TRβ deficient mouse has proven that TRβ isoforms are essential for auditory development and indicate that distinct TR genes serve certain unique functions.[46]

A distinct profile of TRβ gene expression during rat brain development implies that TRβ isoforms play a key role in rat brain development. However, $TR\beta^{-/-}$ mice displayed no overt abnormality in neuroanatomy, behavior or in hippocampal long-term potentiation. Also in a human case, the deletion of the TRβ gene did not cause mental and neurological disorders.[35] Brain is the tissue where TRα1 is predominantly expressed. Since levels T_3 and T_4 levels are high in $TR\beta^{-/-}$ mice, TRα1 might be saturated with T_3 more than in the wild type and might compensate for the loss of TRβ.

In contrast to the brain, TRβ is predominantly expressed in the liver. In fact, by TRβ knockout, the TR number was reduced to 24% in $TR\beta^{+/+}$ mice.[47] As a result, several parameters showed that the liver became resistant to thyroid hormone. In $TR\beta^{-/-}$ mice the serum cholesterol level was significantly higher than that in wild type mice and it remained high even if a supraphysiological dose of T_3 was administered.[47] The increase in serum alkaline phosphatase was also blunted in $TR\beta^{-/-}$ mice. Resistance to thyroid hormone in the liver of $TR\beta^{-/-}$ mice was also demonstrated in the expression of T_3-responsive genes. Spot 14[48] and malic enzyme[49] have been studied as hepatic T_3-responsive genes. The expression of these genes was greatly enhanced by the administration of T_3, however in $TR\beta^{-/-}$ mice, significant increases were missing.[47] A T_3-dependent increase in another T_3-responsive genes, 5′DI,[50,51] was also blunted in $TR\beta^{-/-}$ mice, even though a slight but significant increase in T_3 was observed (unpublished data). On the other hand, heart rates and energy expenditure were not different between $TR\beta^{-/-}$ and wild type mice.[47] These results suggest that TRβ abnormalities are reflected mainly in the organ where TRβ is predominantly expressed. The heterogeneity of refractoriness to T_3 among tissues in RTH may therefore be dependent to a variable degree on the presence of TRβ.

TRα KNOCKOUT

1) Knockout of both TRα1 and TRα2

In a sense, researchers want a TRα knockout mouse more than a TRβ knockout mouse, because the function of TRβ is partially predicted from the phenotype of RTH patients with TRβ gene deletion. Also, the question as to why a case of RTH with abnormal TRα has not been found could be answered by the inactivation of the TRα gene. By homologous recombination, the TRα gene was inactivated in mouse embryonic stem cells and it has been shown at the cellular level that neural differentiation induced by retinoic acid was inhibited.[52] It has therefore been suggested that TRα influences neural differentiation by affecting retinoic acid action. However, we needed to wait another 3 years to know the consequence of a TRα gene knockout at the whole animal level. In 1997, the first report of TRα knockout mouse was made by Fraichard et al.[53] They designed the targeting vector to eliminate the expression of both TRα1 and TRα2. By intercrossing heterozygous mice, approximately 25% of the resulting offspring were homozygous ($TR\alpha^{-/-}$). This report indicated that homozygous disruption of the TRα gene was not deleterious to embryonic development. However, the genetic disruption caused fatal changes in

the phenotype of homozygous mice in the post-natal period. The growth of TRα$^{-/-}$ offspring stopped completely 2 weeks after delivery, they lost 30–50% of their weight, and they died between postnatal days 20–35. Another peculiar phenotype of TRα$^{-/-}$ mice was hypothyroidism, probably due to the reduced secretion of TSH. Their thyroid gland showed hypoplasia and serum concentrations of T_4 and T_3 were markedly reduced (Fig. 4B). TRα$^{-/-}$ mice also exhibited delayed maturation of the small intestine and delayed bone development, findings that are compatible with hypothyroidism.[54] These results strongly indicate that products of TRα gene positively regulate the production of thyroid hormones. Otherwise, there were no gross anatomical or behavioral anomalies. While wide distribution of TRα1 in the early embryonic day and findings of TRα knockout at a cellular level suggest the importance of TRα1 in neural development,[52] TRα$^{-/-}$ mice did not show any obvious cellular and morphological abnormalities in the brain except reduced size.

One interesting observation of TRα$^{-/-}$ mice is that their lethal growth retardation was rescued by one week of T_3 administration (Fig. 4). Surprisingly, T_3 administration also rescued their thyroid function. The authors proposed two hypotheses to explain the T_3 rescue. One is that the injected T_3 can activate genes that are responsible for the production of thyroid hormone via a TRβ dependent pathway. Alternatively, the injected T_3 may transiently cure

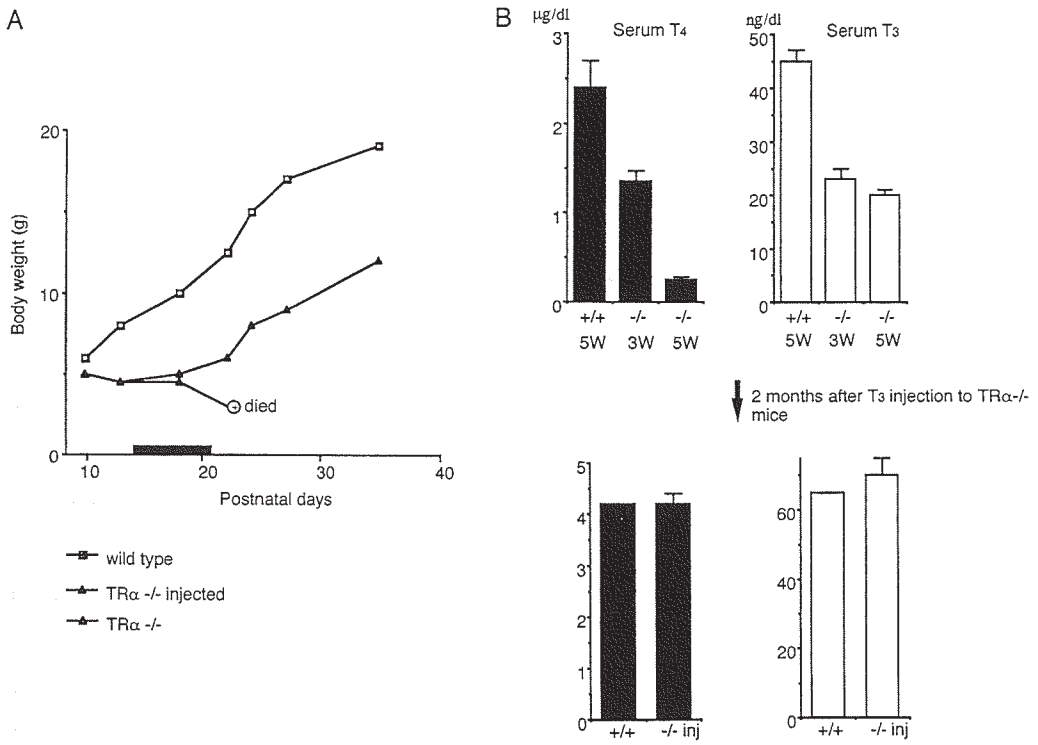

Fig. 4 TRα$^{-/-}$ mice were rescued by T_3-injection (by Fraichard et al. EMBO J., 1997)
(A) Growth rate of animals: Three-week-old TRα$^{-/-}$ mice were injected subcutaneously with 1 μg of T_3 daily for 7 days.
(B) Impaired production of thyroid hormones in TRα$^{-/-}$ mice was rescued by the T_3-injections for 1 week.

animals before the onset of delayed TRα-independent production of thyroid hormone. The inactivation of both TRα and TRβ genes may provide the answer to these hypotheses.

This report seems to answer the question of why RTH with abnormal TRα has not been found, as TRα knockout causes lethal damage in mice. However, results from the specific inactivation of either TRα1 or TRα2 create another controversy.

2) Specific knockout of TRα1, TRα2, or rev-erb Aα

Almost one year after the report by Fraichard et al., specific knockout of TRα1 was reported by Wikström et al.[55] A targeting vector was designed to inactivate TRα1 specifically so that a functional TRα1 was deleted but the splicing variant, TRα2 and the related orphan receptor, rev-erb Aα (transcribed on the opposite strand), were still expressed in homozygous mice ($TR\alpha1^{-/-}$). Surprisingly, the specific inactivation of TRα1 did not cause any lethal abnormality. $TR\alpha1^{-/-}$ mice were fertile and did not show any gross anatomical abnormalities with normal locomotor activity. The only abnormalities that they exhibited were bradycardia with prolonged QT duration, a very mild hypothyroidism and reduced body temperature. This report is truly surprising because TRα1 is the only functional gene product of TRα in terms of mediating T_3 action. In addition, the report gave an alternative answer to why a case of RTH with a TRα abnormality has not been found, namely because a TRα abnormality does not show distinct clinical manifestations.

How then can we explain the difference in results between the TRα common knockout and the TRα1 specific knockout? Does TRα2 or rev-erb Aα play a critical role in maintaining normal development in a mouse? As a matter of fact, a Swedish group which prepared the TRα1 knockout have succeeded in specific knockout of TRα2 (presented during 70th annual meeting of the American Thyroid Association in Colorad Springs, CO.). However, the results failed to explain the discrepancies. $TR\alpha2^{-/-}$ mice showed mild hypothyroidism with reduced weight gain but their life span was normal. They also prepared rev-erb $A\alpha^{-/-}$ mice but their life span was also normal. The question of why common knockout of TRα products resulted in lethal damage whereas the individual knockouts did not cause any serious abnormality remains to be answered.

3) Double knockout of TRα1 and TRβ

Finally, the Swedish group prepared TRα1 and TRβ double knockout mice by intercrossing TRα1 and TRβ deficient mice (also presented during 70th annual meeting of the American Thyroid Association). Since functional TRs are either TRα1, TRβ1 and TRβ2, this double knockout would yield TR deficient mice. The phenotype of the TR deficient mice is even more surprising than that of TRα1 knockout mice. The mice showed very high T_3 and T_4 levels and deaf mutism as observed in TRβ deficient mice and shorter bones and reduced body weight, however, they could survive without any functional TR.

Clinical evidence, as well as animal experiments, clearly show that thyroid hormones are indispensable for the normal development of tissue, especially the brain. TR is supposed to play a key role in mediating thyroid hormone action. So what does the phenotype of TR deficient mice indicate? Are there any unidentified TR genes, such as TRγ? Or, do other nuclear receptors substitute for the function of TR? Experiments with TR knockout mice have given us further important questions to answer.

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