Pituitary NR4A1 is negatively regulated by thyroid hormone without direct binding of thyroid hormone receptors on the gene.

下垂体NR4A1の発現は甲状腺ホルモンにより甲状腺ホルモン受容体の遺伝子への直接結合なしに抑制される。

指導教員　山田　正信　教授
平成28年4月14日

群馬大学大学院医学系研究科
平成24年入学
代謝機能制御系・病態制御内科学

岡村　孝志

1. Introduction

Thyroid hormone is an important factor not only for the neuronal development of the fetus and growth, but also for the homeostasis of lipid and amino acid metabolism, cardiac function, and the coagulation pathway (Yen, 2001, Horacek et al., 2015; Shupnik et al., 1985; Costa-e-Sousa and Hollenberg, 2012: Brent, 1994). Thyroid hormone serum levels are mainly regulated by the hypothalamo (thyrotropin-releasing hormone, TRH) - pituitary (thyroid-stimulating hormone, TSH) - thyroid axis. Hypothalamic TRH stimulates the secretion of TSH from and its synthesis in the pituitary gland, and secreted TSH reaches the thyroid gland, in which it stimulates the synthesis and secretion of thyroid hormone. As reported by us and others, secreted thyroid hormone negatively regulates pituitary TSHβ and hypothalamic TRH synthesis, thereby establishing the TRH-TSH-pituitary axis (Shupnik et al., 1985; Costa-e-Sousa and Hollenberg, 2012; Gurr and Kourides, 1985; Segerson et al., 1987; Taylor et al., 1990; Shupnik, 2000; Chiamolera and Wondisford, 2009).

Since patients with resistance to thyroid hormone (RTH), who have a mutation in thyroid hormone receptor β (TRβ), exhibit enhanced TSH secretion, TRβ appears to play an important role in the negative regulation of the TSH gene (Ishii et al., 2004; Nakajima et al., 2010; Refetoff, 2008; Refetoff et al., 1993). Our previous study on GS125 mutant TR knock-in mice showing impaired DNA binding demonstrated that the DNA binding domain of TR was required for the negative regulation of the *TSHβ* gene (Shibusawa et al., 2003). We also reported the recruitment of TR to the region between -3 and +37 bp from the transcriptional start site of the human *TSHβ* gene using the avidin-biotin complex with DNA and chromatin immuno-precipitation (ChIP) assays (Shibusawa et al., 2003). On the other hand, Tagami et al. showed that negative regulation may be driven through protein-protein interactions in genes such as α-subunit (TSHα), TSHβ, and TRH using mutant TR (Tagami et al., 1997). We also

demonstrated that thyroid hormone negatively regulated the expression of the human *stearoyl-CoA desaturase-1 (SCD-1)* gene without the direct binding of TR to the promoter (Hashimoto et al., 2013). Therefore, it currently remains unclear whether the direct binding of TR with cognitive DNA, called negative thyroid hormone response elements (nTRE), is required for the T3-induced suppression of genes (Shibusawa et al., 2003).

The mechanisms responsible for the positive regulation of genes by thyroid hormone have recently been elucidated (Cheng et al., 2010; Oetting and Yen, 2007; Astapova et al., 2008; Astapova et al., 2011). In the absence of the ligand T3, TR binds to DNA on the promoter of positively regulated genes, and favors the recruitment of nuclear receptor co-repressors such as nuclear receptor CoRepressor (NCoR), which mainly associates with histone deacetylase 3 (HDAC3), leading to the repression of transcription. The presence of T3 induces the release of NCoR and recruitment of co-activators such as p160 family co-factors including steroid receptor co-activator 1 (SRC-1) and CREB binding protein and p300 (CBP/p300), which exhibit histone acetylase activity (HAT). TRAP/DRIP mediators are then recruited, followed by RNA polymerase II, resulting in the initiation of gene transcription (Cheng et al., 2010; Oetting and Yen, 2007; Astapova et al., 2008; Astapova et al., 2011).

Astapova et al. recently reported that mice expressing a mutant NCoR (NCoRΔID) that does not interact with the TR had normal TSH levels despite low circulating thyroid hormone levels (Astapova et al., 2011). Furthermore, a study on a mouse model that allowed for the induction of NCoRΔID expression postnatally demonstrated that NCoR played a critical role in the thyroid hormone-mediated regulation of the TSH gene in the pituitary gland by regulating the repressive function of TR (Astapova et al., 2011). These findings also suggest that the TR-NCoR interaction controls systemic thyroid hormone sensitivity as well as the set point of the hypothalamic-pituitary-thyroid axis (Astapova et al., 2011).

Nuclear receptor subfamily 4 group A, member 1 (NR4A1), also known as NUR77, NGFI-B, and TR3, is a member of the NR4A family of nuclear receptors, which belongs to a superfamily of nuclear receptors that do not have known specific ligands (Milbrandt, 1988). NR4A1 acts as an early response gene and is activated by many factors, including fatty acids, prostaglandins, growth factors, calcium, cytokines, peptide hormones, phorbol esters, and neurotransmitters (Milbrandt, 1988; Martínez-González and Badimon, 2005; Fu et al., 2007; Woronicz et al., 1994; Liu et al., 1994; Maxwell and Muscat, 2006). NR4A1 was previously reported to be distributed throughout organs and plays roles in hormone regulation, cell differentiation, proliferation, apoptosis, glucose, lipid metabolism, angiogenesis, vascular remodeling, and oncogenesis (Martínez-González and Badimon, 2005; Maxwell and Muscat, 2006; Zhao and Bruemmer, 2010; Kim et al., 2014; Pols, Bonta and de Vries, 2007; Wenzl et al., 2015). We recently reported using TRH knockout mice that TRH acts as a stimulant to specifically induce *NR4A1* mRNA in pituitary thyrotrophs (Nakajima et al., 2012). An immunohistochemical analysis of the pituitary gland in TRHKO mice demonstrated that the staining of NR4A1 was markedly weaker, particularly in thyrotrophs, and *in vitro* experiments revealed that TRH increased *NR4A1* mRNA levels by approximately 50-fold within 30 mins in the pituitary cell line, GH4C1 (Nakajima et al., 2012). We also reported that the overexpression of NR4A1 significantly stimulated the promoter activity of the human *TSHβ* gene in this cell line. Therefore, NR4A1 may regulate the expression of the *TSHβ* gene in conjunction with the pituitary-specific transcription factor, Pit1, and the hematopoietic transcription factor, GATA2, which have previously been identified as important factors for *TSHβ* gene expression (Steinfelder et al., 1991; Gordon et al., 1997).

Based on these findings, we hypothesized that pituitary NR4A1 levels may be regulated by thyroid hormone and affect the expression of the *TSHβ* gene. In the present study, we

investigated the regulation of *NR4A1* mRNA levels by thyroid hormone *in vivo* and *in vitro*, and demonstrated for the first time that thyroid hormone reduced the basal level, but not early responsiveness of *NR4A1* mRNA expression within 24 hr in the pituitary gland without the direct binding of TR to the gene. Our results also highlighted the importance of the release of TR-NCoR for this machinery.

2. Methods

2.1. Animals

Procedures for animal care and use in this study were approved by the Review Committee on Animal Use at Gunma University, Maebashi, Japan. Animals were maintained on a 12-hr light/ dark schedule (lights on at 6.00 h) and fed laboratory chow and tap water *ad libitum*. All experiments were performed between 11.00 and 13.00 h. In order to induce experimental thyrotoxicosis, mice were subcutaneously injected with a pharmacological dose of thyroxin (T4, 1.5 μg of L-thyroxine (L-T4)/100 g body weight) every 24 hrs for 2 weeks. Experimental hypothyroidism was induced by adding 0.1% MMI to drinking water and low-iodine chow with 1% propylthiouracil (PTU) for 14 days.

2.2. RNA Extraction and Real-time PCR

Total RNA was prepared from the mouse pituitary gland using an RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. cDNA was then reverse-transcribed from 200 ng of total RNA (TaqMan Reverse Transcription Reagents, Applied Biosystems, Tokyo, Japan), and 0.5 μl was subjected to real-time PCR. All reactions were performed in triplicate using TaqMan probes and an Applied Biosystems 7500 sequence detection system (Nakajima et al., 2012). TaqMan probes for NR4A1 (Mm01300401), TSH*β*

(Mm03990915), NCoR (Hs0019620) and GAPDH (Mm99999915, Hs02758991) were purchased from Applied Biosystems. The expression level of each mRNA relative to that of GAPDH was calculated using a standard curve, and the relative quantification method was performed as described in ABI User Bulletin #2. All experiments were repeated at least twice.

2.3. Mammalian Cell Culture

CV-1 cells (a monkey kidney cell line) (JENSEN, GIRARDI, GILDEN et al., 1964) were kindly provided by Dr. Sakaki, Hamamatsu University School of Medicine, and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), as described previously (Lin, Sternsdorf, Tini et al., 2001).

2.4. Plasmid Constructions

Human $TR\beta_1$ cDNAs and human $RXR\alpha$ were subcloned into the vector $pKCR_2$. pCE4-NCoR was kindly provided by Prof. Rosenfeld, Howard Hughes Medical Institute (Hörlein et al., 1995). F455S and GS125 mutant human $TR\beta_1$ cDNAs were prepared by PCR mutagenesis and subcloned into the vector $pKCR_2$. The human NR4A1 promoter containing 1,295 bp of the 5' flanking sequence and 152 bp of exon 1 (pA3NR4A1(-1295~+152)-Luc) was fused to a firefly luciferase reporter plasmid (pA3Luc) (Nakajima et al., 2012). Series of a deletion mutants were made using specific PCR amplification and pA3NR4A1(-1295~+152)-Luc as a template, including pA3NR4A1(-337~+152)-Luc, pA3NR4A1(-147~+152)-Luc, pA3NR4A1 (-87~+152)-Luc, and pA3NR4A1(-27~+152)-Luc.

2.5. Cell Transfection and Luciferase Assay

Twenty-four hours before transfection, cells were split into 6-well plates at subconfluency. Transient transfection was performed using a calcium phosphate precipitation method, as described previously (Lin et al., 2001). The total amount of the transfected plasmid was adjusted in all experiments by adding an empty expression vector. Sixteen hours after transfection, medium was changed to DMEM supplemented with 10% FBS treated with AG1-X8 resin (Bio-Rad) and activated charcoal (Sigma) in order to remove thyroid hormone, and cells were incubated further in the presence or absence of T3 at the indicated concentration (Nakajima et al., 2012).

In order to determine luciferase activity levels, cell monolayers were rinsed twice with PBS, then lysed with 300 μl of 25 mM glycylglycine (pH 7.8) containing 15 mM $MgSO_4$, 4 mM EGTA, 1 mM dithiothreitol, and 1% v/v Triton X-100. Cells were scraped from the dishes and centrifuged at 12.000× g at 4°C for 5 min. Assays for Luc activity were performed using 150-μl aliquots of the cell lysate and 210 μl of 25 mM glycylglycine (pH 7.8) containing 15 mM $MgSO_4$, 4 mM EGTA, 3.3 mM KPO_4, 1 mM dithiothreitol, and 0.45 mM ATP. The reaction was initiated by the addition of 200 μl of 0.2 mM d-luciferin and light emission was measured for 10 seconds using a luminometer. Luciferase activity was expressed as arbitrary light units per microgram of cellular protein (Nakajima et al., 2012). All transfection experiments were repeated at least twice with triplicate determinants.

2.6. Electrophoretic Mobility Shift Assay (EMSA)

An EMSA was performed using radiolabeled probes and a thyroid hormone response element (TRE). The consensus sequence used as the TRE palindrome was 5'-aagattaaggtcatgacctgaggaga-3'. Double-stranded oligonucleotides were labeled with [α^{32}P]dCTP by a fill-in reaction using a Klenow fragment of DNA polymerase I. Human TRβ$_1$

and human RXRα were synthesized by *in vitro* transcription/translation from pKCR2-TR and pKCR2-RXRα using T7 RNA polymerase and the TNT-coupled reticulocyte lysate system (Promega Corporation).   The binding reaction, gel electrophoreses, and autoradiography were performed under previously described conditions (Nakajima et al., 2012).   Gel super-shift studies were performed with specific antibodies against TR (Thermo Fisher SCIENTIFICS).

2.7. ChIP Assay

ChIP assays were performed according to the manufacturer's protocol using a kit from Active Motif (Carlsbad, CA USA).   CV-1 cells were transfected with pA3NR4A1 ($-1295\sim+152$)-Luc and incubated in medium.   After an overnight incubation, medium was poured off the cells, fixation solution (0.54 ml of 37% formaldehyde was added to 20 ml minimal cell culture medium) was added, and the cells were then incubated at room temperature for 10 min in order to crosslink protein to DNA.   Cells were harvested using a ChIP-IT Express kit (Active Motif) using the manufacturer's protocol.   Chromatin was sonicated 3 times with 10-sec pulses using a sonicator set at 70% of the maximum power in order to reduce the DNA length to between 200 and 1500 bp. Chromatin-protein complexes were immunoprecipitated using the following antibodies: anti-Thyroid hormone receptor (Thermo Fisher SCIENTIFICS) and anti-NCoR, kindly provided by Dr. Lazar (Ishizuka and Lazar, 2005), which were added to magnetic beads.   Normal mouse IgG (RPN2124, Amersham Biosciences, UK) was used as a negative control. Samples were washed and reverse cross-linked. Real-time PCR was performed to amplify the regions expected to induce TR. The primers used for the region between -147~+148   bp   were   as   follows: forward   primer, 5'-GGGCCGCACCTCCCCCTGGCCGCGTCCC-3'   and   reverse   primer,   5'-

GCTCCTAGACTGGCGCCCCGAGTCTCA-3′.   Results were expressed as the ratio of IgG.

All ChIP assays were repeated at least three times.

2.8. Small-interfering RNA (siRNA) against NCoR

Pooled siRNA oligonucleotides targeting NCoR were designed and synthesized at Dharmacon

Research (siGENOME SMART pool NCOR1, M-003518-01). Pooled unrelated siRNA

(siCONTROL Non-Targeting siRNA pool, D-001206-13-20) was used as a control. These

siRNAs were introduced into CV-1 cells by the lipofection method as previously reported

(Lipofectamine RNAiMAX™; Invitrogen). Twenty-four hours after the first transfection, a

transient transfection of pA3NR4A1(-1295~+152)-Luc was performed using calcium phosphate

precipitation.   Cells were further incubated in the absence or presence of T3.

2.9. Statistical Analysis

Statistical analyses were performed with ANOVA and the Student's *t*-test or

Wilcoxon/Kruskal-Wallis test using JMP (SAS Institute Inc., Cary NC).

3. Results

3.1. Pituitary NR4A1 mRNA levels were negatively regulated by thyroid hormone *in vivo*.

In order to determine the effects of the thyroid status on pituitary NR4A1 levels *in vivo*,

experimental hypothyroidism and thyrotoxicosis were induced by antithyroid drugs (MMI and

PTU) in chow and daily injections of a pharmacological dose of T4 (L-T4 1.5 µg/100 g body

weight) for 2 weeks.   We then measured *NR4A1* mRNA levels in the pituitary gland by

real-time PCR with the TaqMan probe for NR4A1.   As shown in Figure 1A, pituitary *NR4A1*

mRNA levels were significantly lower, by 62.1%, in mice injected with T4 (n= 3, $p<0.05$) than

in control mice. On the other hand, no significant changes were observed in *NR4A1* mRNA levels between control and hypothyroid mice (Fig. 1A). Based on these results, we hypothesized that chronic changes in the thyroid status did not affect *NR4A1* mRNA levels, as observed in hypothyroid mice, whereas subacute changes such as those caused by daily injections of T4 may have altered *NR4A1* mRNA levels *in vivo*.

Therefore, we examined changes in *NR4A1* mRNA levels in the pituitary gland 24 hr after a single peritoneal injection of T4. As shown in Figures 1B and C, *NR4A1* mRNA levels were significantly decreased to 51±9% of control levels 24 hours after the administration of T4 (T4 group, n=7; control, n=6; $p<0.01$), while *TSHβ* mRNA levels in the pituitary gland were also decreased to 49.1±10.3% of the control (T4 group, n=7 vs. control, n=6; $p<0.05$), indicating thyrotoxicosis in mice.

3.2. The promoter activity of the NR4A1 gene was negatively regulated by thyroid hormone *in vitro*: Possible involvement of NCoR with a TR-induced ligand-independent stimulation.

In order to examine the direct effects of thyroid hormone on the *NR4A1* gene, we investigated whether thyroid hormone stimulated the promoter activity of the *NR4A1* gene *in vitro* using CV-1 cells that do not express functional TR. As shown in Figure 2A, while an incubation with T3 without the expression of TRβ did not affect the promoter activity of the NR4A1 gene, the overexpression of TRβ in CV-1 cells increased NR4A1 promoter activity to 198.7± 19.3% of controls not expressing TRβ (n=3, $p<0.01$). Under the expression of TRβ, the incubation with 100 nM T3 induced a marked reduction in NR4A1 promoter activity, which was significantly less than basal promoter activity (without T3 or the expression of TRβ). Furthermore, the T3-induced suppression of the promoter activity of the *NR4A1* gene was

dose-dependent (Fig. 2B). An incubation with 0.1 nM T3 suppressed promoter activity by 7.1 ± 5.7% from that without T3; 1 nM T3, a further reduction of 37.4 ± 4.9% (n=3, $p<0.01$); 10 nM T3, 50.8 ± 2.3% (n=3, $p<0.01$); 100 nM T3, 43.1 ± 9.2% (n=3, $p<0.01$); 1000 nM T3, 15.0 ± 1.3% (n=3, $p<0.05$).

The co-repressor, NCoR is known to bind with TR in the absence of T3 in genes positively regulated by thyroid hormone, and a study on a mouse model with inducible NCoRΔID indicated that NCoR played a critical role in the thyroid hormone-mediated regulation of the TSH gene in the pituitary gland by regulating the repressive function of TR (Astapova et al., 2011). Since these findings suggested that the TR-NCoR interaction controls the set point of the hypothalamic-pituitary-thyroid axis (Astapova et al., 2011), we examined the effects of the overexpression of NCoR on the T3-induced repression of NR4A1 promoter activity. The overexpression of NCoR significantly enhanced the ligand-independent stimulation of TRβ (by 180.2 ± 16.9%, n=3, $p<0.01$), and led to a significant reduction in the T3-induced repression of NR4A1 promoter activity (Fig. 3A).

We further confirmed the effects of NCoR on the ligand-independent stimulation with TR by knocking down NCoR in CV1 cells. As shown in Figure 3B-1, the transfection of siRNA for NCoR in CV1 cells led to a reduction of 54.4% in mRNA and 65.7% in protein after 48 hr. Under this condition, the knockdown of NCoR completely abolished the ligand-independent activation of the *NR4A1* gene by TR, but did not affect its repression by T3 (Fig. 3B-2). These results further suggest that NCoR acts as a co-activator of TR on the NR4A1 gene.

3.3. Effects of TR mutants that impaired the release of NCoR by T3 and impaired DNA binding with the T3-induced suppression of NR4A1 promoter activity.

As shown in Figure 4, the ligand-independent transcriptional activation of the NR4A1

promoter by TR was significantly more potent with the F455S mutant TR, for which impaired

T3-dependent NCoR dissociation from TR was reported, than with the wild-type TR. The

repression of NR4A1 promoter activity by T3 was also impaired ($p<0.01$)

The NR4A1 promoter activity, examined using GS125 mutant TR, which has a mutated

DNA binding region, showed impairment of ligand-independent activation and inhibition of

T3-induced transcriptional repression, suggesting that the DNA-binding domain of TR was

required for the T3-induced repression of the NR4A1 gene.

3.4. The region responsible for suppressing the promoter activity of the *NR4A1* gene by T3 was located between -27 and +152 bp of the transcriptional site.

In an attempt to identify the region responsible for the T3-mediated suppression of the

promoter activity of the *NR4A1* gene, we generated a series of deletion mutants of the NR4A1

promoter and examined the effects of T3. The construct containing between -1295 and +152

bp from the transcriptional start site of the *NR4A1* gene (-1295-+152-Luc) exhibited strong

promoter activity in CV-1 cells, which was approximately 227% that of thymidine kinase (TK)

promoter activity (data not shown).

As shown in Figure 5A, a search for putative transcription binding sites by the web program,

TRANSFAC, demonstrated that the promoter region of the *NR4A1* gene contained putative

myocyte enhancer factor (MEF) 2 response elements (MRE) on the region between - 275 and

-309 bp, and cAMP response element (CREB) at -42 bp, -71 bp, -215 bp, and -235 bp series of

deletion mutants of the (-1295-+152-Luc) construct: (-337-+152)-Luc, (-147-+152)-Luc,

(-87-+152)-Luc, and (-27-+152)-Luc, exhibited weaker promoter activities than that of the

(-1295-+152)-Luc construct, suggesting that the region between -1295 and -337 bp exhibited

enhancer activity. In addition, the activity of (-337-+152)-Luc was weaker than that of

(-147-+152)-Luc, indicating silencer activity in the region between -337 and -147 bp. The promoter activity of (-87-+152-Luc) was stronger than that of (-27-+152)-Luc, which suggests that the CREB binding region between -87 and -27 bp is important for promoter activity (Figs. 5A and B).

Using these deletion mutants, we examined ligand-independent activation by TR and suppression by T3. As shown in Figure 5C, we detected significant ligand-independent activation and T3 induced suppression, even in the shortest construct, indicating that the region responsible for the T3-induced suppression of NR4A1 promoter activity was located in the region close to the transcription start site between -27 and +152 bp.

3.5. Lack of binding of TR to the region between -27 and +152 bp in the *NR4A1* gene.

We next determined whether TRβ directly binds to the region responsible for the T3-induced suppression of the promoter activity of the *NR4A1* gene, the region between -27 and +152 bp. We initially searched for the TRβ binding site in the region between -27 and +152 bp using the web program, TRANSFAC. We found 2 putative TRβ binding sites, AGGAGGGTCGG and AGGCTAC, in the regions between -29 and -8 bp and between +10 bp and +28 bp, respectively. We subsequently performed an EMSA study with the fragments containing these regions and synthetic TRβ and RXR generated by TNT systems. As shown in Figure 6, PAL-TRE, a typical TRE, palindromic TRE containing 5'-agcttcaggtcacaggaggtcagagag-3' and 5'-aagattaaggtcatgacctgaggaga-3', showed significant binding with the TRβ-RXR heterodimers (indicated by an arrow), and an incubation with T3 decreased binding with the TRβ-RXR heterodimers. Furthermore, the addition of an antibody against TRβ induced a clear super-shift, which confirmed that these bands were a complex containing TRβ.

Under the same conditions, the fragments containing the regions between -29 and -8 bp and

between +10 and +28 bp did not show any binding to either TRβ or RXR.

3.6. Recruitment of TRβ and NCoR, and their release by T3 into regions between -147 and +148 bp in the *NR4A1* gene.

Since no direct binding to TRβ was detected in the region responsible for T3-induced suppression, we performed a ChIP analysis in order to determine whether TRβ is recruited to the nuclear factor complex on this region.

As shown in Figure 7A, we detected the significant recruitment of TRβ into the fragment containing the region between -147 and +148 bp. Furthermore, the addition of T3 led to a significant reduction in the recruitment of TRβ into this region. No significant recruitment of TRβ was observed under any condition tested when IgG was used instead of the antibody against TRβ.

We next examined whether NCoR was recruited to this region using the ChIP assay with an anti-NCoR antibody. A previous study reported that the CV-1 cells used in this study express endogenous NCoR (Cheng et al., 2002). The results of the ChIP assay clearly demonstrated the significant recruitment of NCoR to the region between -147 and +148 bp without T3, and an incubation with T3 appeared to induce the release of NCoR from this region (Fig. 7B).

4. Discussion

We herein demonstrated that thyroid hormone clearly suppressed the basal expression level of *NR4A1* mRNA in the pituitary gland; a daily injection of T4 reduced *NR4A1* mRNA levels in the pituitary gland *in vivo*, whereas chronic hypothyroidism induced by MMI and PTU for weeks did not. Although many studies have reported the early-responsive effects of NR4A1 to many factors and hormones, including CRH, GnRH, and TRH, few have found reductions in

NR4A1 by a factor. The present results appear to show clear reductions in pituitary *NR4A1*

mRNA levels after an intraperitoneal injection of L-T4, which induced experimental

thyrotoxicosis.

We previously reported the strong (approximately 50-fold) and rapid (within 60 mins)

TRH-induced expression of *NR4A1* mRNA and its protein within 2 hr (Nakajima et al., 2012).

TRH stimulates the transcription of the NR4A1 gene through TRH receptors and the PKC and

MAPK pathways within 1 hr. In TRH knockout mice, the lack of NR4A1 was specifically

observed in thyrotrophs in the pituitary gland, suggesting TRH-specific effects on

TSH-producing cells in the pituitary gland. The overexpression of NR4A1 also stimulated

TSHβ promoter activity *in vitro*. Furthermore, the presence of NR4A1 increased the

responsiveness of TSHβ promoter activity to TRH. Taken together with the present results, we

speculate that thyroid hormone indirectly reduces the expression of TSHβ , at least in part, by

reducing that of NR4A1 (Fig. 8).

Genes such as the *TSHβ*, *αGSU*, and *TRH* have been reported to possess a negative TRE

(nTRE). However, the mechanisms by which T3 negatively regulates the transcription of these

genes currently remain unclear, as does the necessity of the direct binding of TR to nTRE. We

and others have shown that the co-activator and co-repressor, SRC-1 and NCoR, respectively,

induced histone modifications around nTRE (Umezawa et al., 2009; Kim et al., 2005). We

also demonstrated that the co-repressor, NCoR, acted as a co-activator on the *TRH* gene because

a ligand-independent stimulation by TR was completely abolished in the TRH gene when NCoR

was knocked down (Nakajima et al., 2010). Sung-Woo Kim et al also reported that the

promoter activity of CD44, an adhesion molecule in the extracellular matrix, gene was

negatively regulated by thyroid hormone, and NCoR acted as a co-activator to enhance

TR-mediated basal transactivation in the absence of T3 (Kim et al., 2005). The results of the

present study also support this finding; the overexpression of NCoR significantly enhanced the ligand-independent stimulation of TR, and the release of NCoR was induced by an incubation with T3.

Ramaddos et al. investigated TR binding sites in the hypothyroid and hyperthyroid mouse liver and hypothalamus using a genome-wide profiling study with ChIP-seq (Ramadoss et al., 2014). They found that TRβ bound to different sites or the same sites with different affinities between the hypo- and hyperthyroid statuses. They also reported that T3 mainly regulated transcription positively through the direct repeats (DR)4 motif and negatively through the DR0 motif. Furthermore, more than 60% of genes negatively regulated by thyroid hormone were not related to the binding of TRβ□(Ramadoss et al., 2014). We did not detect the DR0 motif in the NR4A promoter region or the direct binding of TR on the gene. On the other hand, a ChIP-ChIP analysis performed by Gagne et al. identified 85 sites that recruited TRE in the developing mouse cerebellum and included the *NR4A1* gene (Gagne et al., 2013). They also showed that NR4A1 mRNA levels in the mouse liver increased with experimental hypothyroidism induced by a PTU treatment, and that a NR4A1 binding site located far upstream of the transcriptional start site was responsible for the T3-induced suppression of promoter activity in the liver cell line, Hepg2 (Dong et al., 2007). In contrast, we found that the region responsible for the thyroid hormone-induced suppression of the NR4A1 gene was located close to the transcriptional start site, without the direct binding of TR in this region. This discrepancy may be due to tissue-specific differences in the machinery underlying the T3-induced suppression of NR4A1 mRNA levels between the liver and pituitary gland.

The promoter region of the NR4A1 gene has four CREB responsive elements (CREs) in the region between -42 and -235 bp and two MEF2 binding elements in the region between -275 and -309 bp. A previous study reported that CREB is necessary for activating NR4A1

transcription following depolarization in PC12 cells, and MEF2 mainly acts as a repressor in un-stimulated or non-depolarized PC12 cells in order to restrain the CREB-mediated expression of NR4A1 (Lam et al., 2010). Our deletion analysis of the promoter region showed no involvement of T3-induced reductions in promoter activity with these CREB and MEF2 sites because the shortest construct, -27-+152Luc, was negatively regulated by thyroid hormone.

The present ChIP analysis demonstrated that TR and NCoR form a complex on the promoter region in the NR4A1 gene in the absence of T3, following the addition of T3 to induce the release of TR and NCoR. We reported the similar dissociation of TR from the TRH gene after a 24-hr exposure to T3 (Umezawa et al., 2009). Furthermore, the knockdown of NCoR led to a decrease in the TR-induced ligand-independent stimulation, suggesting that NCoR may interact with TR on the NR4A1 gene. In addition, the F455S mutant that impaired the T3-induced release of NCoR increased the ligand-independent stimulation of the NR4A1 gene by TR, further supporting the interaction between NCoR and TR on the NR4A1 gene. These results suggest that a similar molecular machinery exists for the negative transcriptional regulation of the TRH and NR4A1 genes by thyroid hormone.

5. Conclusion

In conclusion, we demonstrated for the first time the novel effects of thyroid hormone on NR4A1, which is rather than up-regulator such as early responsive gene, negative regulation of its basal level by thyroid hormone. NR4A1 may be a key regulator of the TSHβ gene in the pituitary gland for regulation by hypothalamic TRH Therefore, and thyroid hormone (Fig. 8).

References

Astapova, I., Lee, L.J., Morales, C., Tauber, S., Bilban, M. and Hollenberg, A.N., 2008. The nuclear corepressor, NCoR, regulates thyroid hormone action in vivo, Proc Natl Acad Sci U S A. 105, 19544-9.

Astapova, I., Vella, K.R., Ramadoss, P., Holtz, K.A., Rodwin, B.A., Liao, X.H., Weiss, R.E., Rosenberg, M.A., Rosenzweig, A. and Hollenberg, A.N., 2011. The nuclear receptor corepressor (NCoR) controls thyroid hormone sensitivity and the set point of the hypothalamic-pituitary-thyroid axis, Mol Endocrinol. 25, 212-24.

Brent, G.A., 1994. The molecular basis of thyroid hormone action, N Engl J Med. 331, 847-53.

Cheng, S., Brzostek, S., Lee, S.R., Hollenberg, A.N. and Balk, S.P., 2002. Inhibition of the dihydrotestosterone-activated androgen receptor by nuclear receptor corepressor, Mol Endocrinol. 16, 1492-501.

Cheng, S.Y., Leonard, J.L. and Davis, P.J., 2010. Molecular aspects of thyroid hormone actions, Endocr Rev. 31, 139-70.

Chiamolera, M.I. and Wondisford, F.E., 2009. Minireview: Thyrotropin-releasing hormone and the thyroid hormone feedback mechanism, Endocrinology. 150, 1091-6.

Costa-e-Sousa, R.H. and Hollenberg, A.N., 2012. Minireview: The neural regulation of the hypothalamic-pituitary-thyroid axis, Endocrinology. 153, 4128-35.

Dong, H., Yauk, C.L., Williams, A., Lee, A., Douglas, G.R. and Wade, M.G., 2007. Hepatic gene expression changes in hypothyroid juvenile mice: characterization of a novel negative thyroid-responsive element, Endocrinology. 148, 3932-40.

Fu, Y., Luo, L., Luo, N., Zhu, X. and Garvey, W.T., 2007. NR4A orphan nuclear receptors modulate insulin action and the glucose transport system: potential role in insulin resistance, J Biol Chem. 282, 31525-33.

Gagne, R., Green, J.R., Dong, H., Wade, M.G. and Yauk, C.L., 2013. Identification of thyroid hormone receptor binding sites in developing mouse cerebellum, BMC Genomics. 14, 341.

Gordon, D.F., Lewis, S.R., Haugen, B.R., James, R.A., McDermott, M.T., Wood, W.M. and Ridgway, E.C., 1997. Pit-1 and GATA-2 interact and functionally cooperate to activate the thyrotropin beta-subunit promoter, J Biol Chem. 272, 24339-47.

Gurr, J.A. and Kourides, I.A., 1985. Thyroid hormone regulation of thyrotropin alpha- and beta-subunit gene transcription, DNA. 4, 301-7.

Hashimoto, K., Ishida, E., Miura, A., Ozawa, A., Shibusawa, N., Satoh, T., Okada, S., Yamada, M. and Mori, M., 2013. Human stearoyl-CoA desaturase 1 (SCD-1) gene

expression is negatively regulated by thyroid hormone without direct binding of thyroid hormone receptor to the gene promoter, Endocrinology. 154, 537-49.

Horacek, J., Maly, J., Svilias, I., Smolej, L., Cepkova, J., Vizda, J., Sadilek, P., Fatorova, I. and Zak, P., 2015. Prothrombotic changes due to an increase in thyroid hormone levels, Eur J Endocrinol. 172, 537-42.

Hörlein, A.J., Näär, A.M., Heinzel, T., Torchia, J., Gloss, B., Kurokawa, R., Ryan, A., Kamei, Y., Söderström, M. and Glass, C.K., 1995. Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor, Nature. 377, 397-404.

Ishii, S., Yamada, M., Satoh, T., Monden, T., Hashimoto, K., Shibusawa, N., Onigata, K., Morikawa, A. and Mori, M., 2004. Aberrant dynamics of histone deacetylation at the thyrotropin-releasing hormone gene in resistance to thyroid hormone, Mol Endocrinol. 18, 1708-20.

Ishizuka, T. and Lazar, M.A., 2005. The nuclear receptor corepressor deacetylase activating domain is essential for repression by thyroid hormone receptor, Mol Endocrinol. 19, 1443-51.

JENSEN, F.C., GIRARDI, A.J., GILDEN, R.V. and KOPROWSKI, H., 1964. INFECTION OF HUMAN AND SIMIAN TISSUE CULTURES WITH ROUS SARCOMA VIRUS, Proc Natl Acad Sci U S A. 52, 53-9.

Kim, S.W., Ho, S.C., Hong, S.J., Kim, K.M., So, E.C., Christoffolete, M. and Harney, J.W., 2005. A novel mechanism of thyroid hormone-dependent negative regulation by thyroid hormone receptor, nuclear receptor corepressor (NCoR), and GAGA-binding factor on the rat cD44 promoter, J Biol Chem. 280, 14545-55.

Kim, Y.D., Kim, S.G., Hwang, S.L., Choi, H.S., Bae, J.H., Song, D.K. and Im, S.S., 2014. B-cell translocation gene 2 regulates hepatic glucose homeostasis via induction of orphan nuclear receptor Nur77 in diabetic mouse model, Diabetes. 63, 1870-80.

Lam, B.Y., Zhang, W., Ng, D.C., Maruthappu, M., Roderick, H.L. and Chawla, S., 2010. CREB-dependent Nur77 induction following depolarization in PC12 cells and neurons is modulated by MEF2 transcription factors, J Neurochem. 112, 1065-73.

Lin, R.J., Sternsdorf, T., Tini, M. and Evans, R.M., 2001. Transcriptional regulation in acute promyelocytic leukemia, Oncogene. 20, 7204-15.

Liu, Z.G., Smith, S.W., McLaughlin, K.A., Schwartz, L.M. and Osborne, B.A., 1994. Apoptotic signals delivered through the T-cell receptor of a T-cell hybrid require the immediate-early gene nur77, Nature. 367, 281-4.

Martínez-González, J. and Badimon, L., 2005. The NR4A subfamily of nuclear receptors: new early genes regulated by growth factors in vascular cells, Cardiovasc

Res. 65, 609-18.

Maxwell, M.A. and Muscat, G.E., 2006. The NR4A subgroup: immediate early response genes with pleiotropic physiological roles, Nucl Recept Signal. 4, e002.

Milbrandt, J., 1988. Nerve growth factor induces a gene homologous to the glucocorticoid receptor gene, Neuron. 1, 183-8.

Nakajima, Y., Yamada, M., Horiguchi, K., Satoh, T., Hashimoto, K., Tokuhiro, E., Onigata, K. and Mori, M., 2010. Resistance to thyroid hormone due to a novel thyroid hormone receptor mutant in a patient with hypothyroidism secondary to lingual thyroid and functional characterization of the mutant receptor, Thyroid. 20, 917-26.

Nakajima, Y., Yamada, M., Taguchi, R., Shibusawa, N., Ozawa, A., Tomaru, T., Hashimoto, K., Saito, T., Tsuchiya, T., Okada, S., Satoh, T. and Mori, M., 2012. NR4A1 (Nur77) mediates thyrotropin-releasing hormone-induced stimulation of transcription of the thyrotropin β gene: analysis of TRH knockout mice, PLoS One. 7, e40437.

Oetting, A. and Yen, P.M., 2007. New insights into thyroid hormone action, Best Pract Res Clin Endocrinol Metab. 21, 193-208.

Pols, T.W., Bonta, P.I. and de Vries, C.J., 2007. NR4A nuclear orphan receptors: protective in vascular disease?, Curr Opin Lipidol. 18, 515-20.

Ramadoss, P., Abraham, B.J., Tsai, L., Zhou, Y., Costa-e-Sousa, R.H., Ye, F., Bilban, M., Zhao, K. and Hollenberg, A.N., 2014. Novel mechanism of positive versus negative regulation by thyroid hormone receptor β1 (TRβ1) identified by genome-wide profiling of binding sites in mouse liver, J Biol Chem. 289, 1313-28.

Refetoff, S., 2008. Resistance to thyroid hormone: one of several defects causing reduced sensitivity to thyroid hormone, Nat Clin Pract Endocrinol Metab. 4, 1.

Refetoff, S., Weiss, R.E. and Usala, S.J., 1993. The syndromes of resistance to thyroid hormone, Endocr Rev. 14, 348-99.

Segerson, T.P., Kauer, J., Wolfe, H.C., Mobtaker, H., Wu, P., Jackson, I.M. and Lechan, R.M., 1987. Thyroid hormone regulates TRH biosynthesis in the paraventricular nucleus of the rat hypothalamus, Science. 238, 78-80.

Shibusawa, N., Hashimoto, K., Nikrodhanond, A.A., Liberman, M.C., Applebury, M.L., Liao, X.H., Robbins, J.T., Refetoff, S., Cohen, R.N. and Wondisford, F.E., 2003. Thyroid hormone action in the absence of thyroid hormone receptor DNA-binding in vivo, J Clin Invest. 112, 588-97.

Shibusawa, N., Hollenberg, A.N. and Wondisford, F.E., 2003. Thyroid hormone receptor DNA binding is required for both positive and negative gene regulation, J Biol

Chem. 278, 732-8.

Shupnik, M.A., 2000. Thyroid hormone suppression of pituitary hormone gene expression, Rev Endocr Metab Disord. 1, 35-42.

Shupnik, M.A., Chin, W.W., Habener, J.F. and Ridgway, E.C., 1985. Transcriptional regulation of the thyrotropin subunit genes by thyroid hormone, J Biol Chem. 260, 2900-3.

Steinfelder, H.J., Hauser, P., Nakayama, Y., Radovick, S., McClaskey, J.H., Taylor, T., Weintraub, B.D. and Wondisford, F.E., 1991. Thyrotropin-releasing hormone regulation of human TSHB expression: role of a pituitary-specific transcription factor (Pit-1/GHF-1) and potential interaction with a thyroid hormone-inhibitory element, Proc Natl Acad Sci U S A. 88, 3130-4.

Tagami, T., Madison, L.D., Nagaya, T. and Jameson, J.L., 1997. Nuclear receptor corepressors activate rather than suppress basal transcription of genes that are negatively regulated by thyroid hormone, Mol Cell Biol. 17, 2642-8.

Taylor, T., Wondisford, F.E., Blaine, T. and Weintraub, B.D., 1990. The paraventricular nucleus of the hypothalamus has a major role in thyroid hormone feedback regulation of thyrotropin synthesis and secretion, Endocrinology. 126, 317-24.

Umezawa, R., Yamada, M., Horiguchi, K., Ishii, S., Hashimoto, K., Okada, S., Satoh, T. and Mori, M., 2009. Aberrant histone modifications at the thyrotropin-releasing hormone gene in resistance to thyroid hormone: analysis of F455S mutant thyroid hormone receptor, Endocrinology. 150, 3425-32.

Wenzl, K., Troppan, K., Neumeister, P. and Deutsch, A.J., 2015. The nuclear orphan receptor NR4A1 and NR4A3 as tumor suppressors in hematologic neoplasms, Curr Drug Targets. 16, 38-46.

Woronicz, J.D., Calnan, B., Ngo, V. and Winoto, A., 1994. Requirement for the orphan steroid receptor Nur77 in apoptosis of T-cell hybridomas, Nature. 367, 277-81.

Yen, P.M., 2001. Physiological and molecular basis of thyroid hormone action, Physiol Rev. 81, 1097-142.

Zhao, Y. and Bruemmer, D., 2010. NR4A orphan nuclear receptors: transcriptional regulators of gene expression in metabolism and vascular biology, Arterioscler Thromb Vasc Biol. 30, 1535-41.
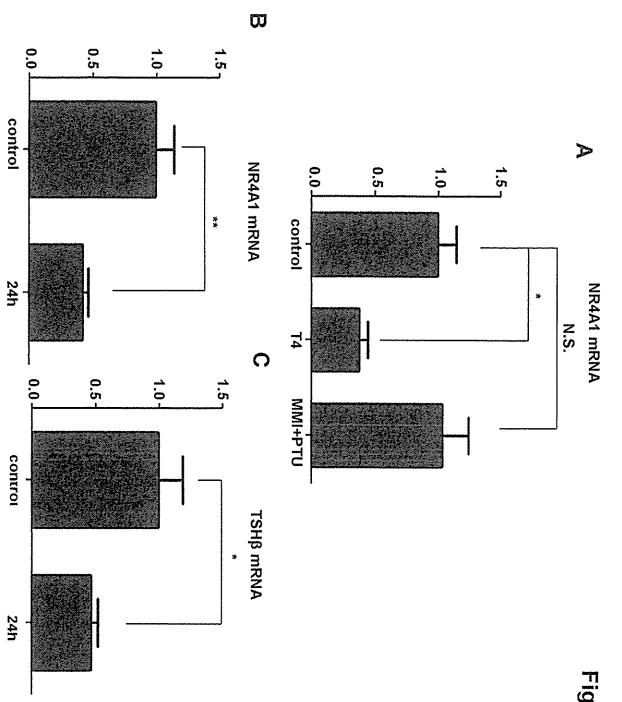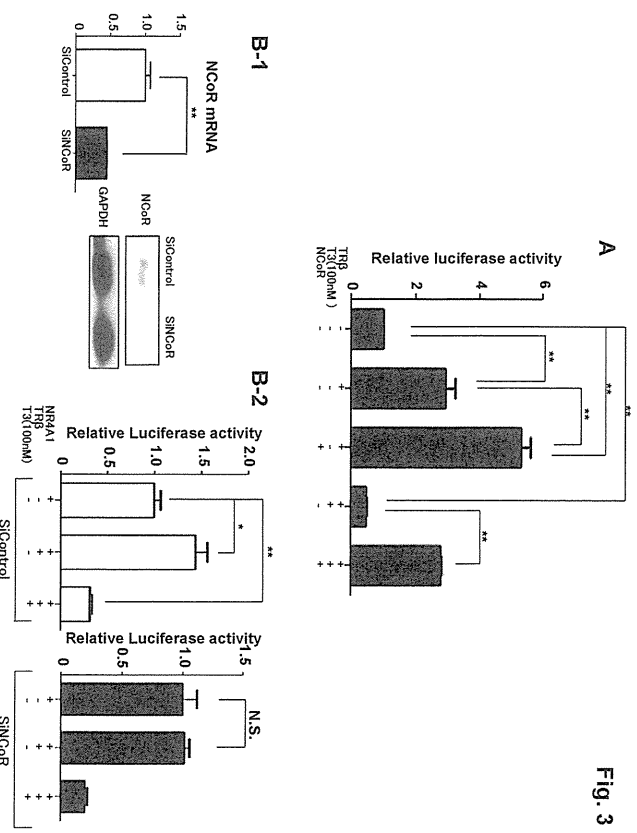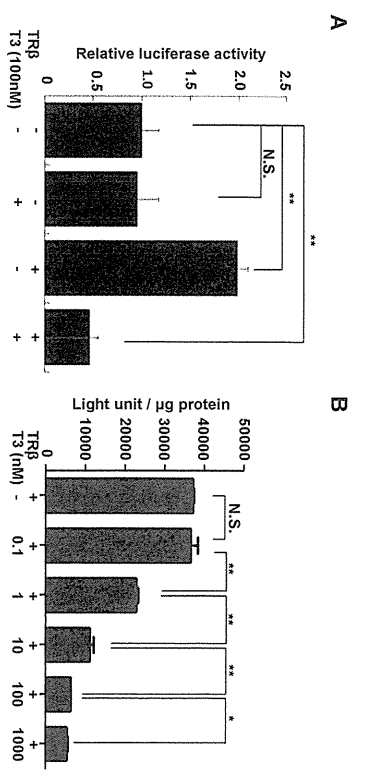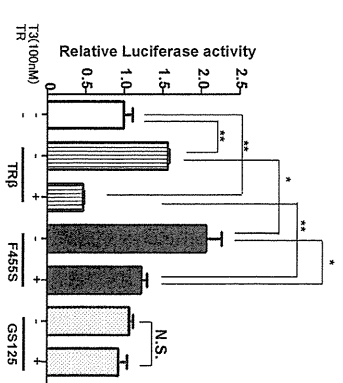
Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

A

```
-337
GCCCCCGCGGCGCGCGTCTCTACACGCGCCTTCTCGGGGCTCTGGCTCCCGCTTGGTTTATTCTGGGACCTGGGGGCCCCCAG
CTGGGACCCAGTCCGGTGCGGGGGAGCCTAGTGGGGCCTGGGAGTGTGCTATTTTTTAGCGCGGGGCCCGGGCCCGAGGAG
                                              MRE
CCTATTTATAGATCAAACAATCCGCACACTCCCTGCGTCAATGAAACCCCGCGTCGTCAGCGCGCAGACATTCCAGGCCCCC
    MRE                            CREB
                      -147
CCTCCTCGGCCCGCCCCCCTCGGGGGTCCCCGGGGTCCCCGCCCTGCCCGTCGACCTCCCCCCTTGGGCGCGCCCGCCCCCA
                                        CREB
       -87
CGCGCCCCTTTGATGGCCAAAAGCTCGACGGAGCGGCCCTGCGTCAGTGCGCGCCCCCTCCCGTGCGTCACGGAGCG
              -27    +1                CREB
CTTAAGAGAGAGGGGTCGGGCCCGGGGGAGTCCCAGTGGCGGGGAAGGCTACGAAACTTGGGGGAGTGCACAGAAAACTT
CGGGAGCGGCACGCGGGGACCAGGGGAGCTGAGAGACTCGGGGCGCCCAGTCCGGGGGCGGGCAGCGGGGGAGCCCGGCCG
GGTAGGTTCCCCTCGGGAAACGTGCA
                        +152
```
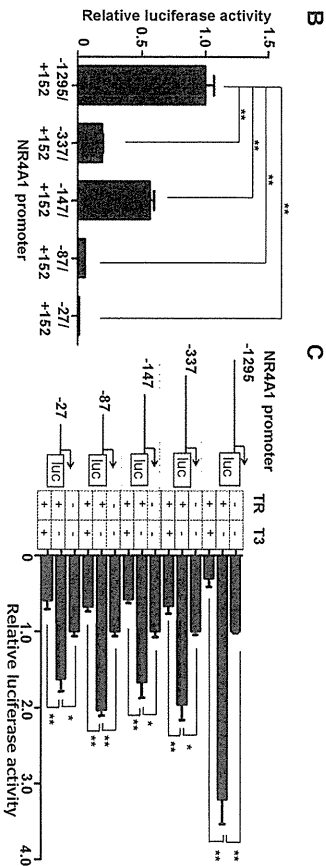
B

Relative luciferase activity

NR4A1 promoter: -1295/+152, -337/+152, -147/+152, -87/+152, -27/+152

C

NR4A1 promoter
-1295, -337, -147, -87, -27 (luc)

TR / T3

Relative luciferase activity

Fig. 6

TNT
TRβ
RXRα
T3(nM)
αTRβ

NR4A1promoter -29/-8   1 2 3 4 5
NR4A1 promoter +10/+28   6 7 8 9 10
PAL-TRE   11 12 13 14 15

N.S.
N.S.

← TRβ-RXRα
← Super-shift

Fig. 7

A αTR-ChIP

Relative enrichment

IgG
αTR
T3 (100nM)

-147/+148 NR4A1 promoter

B αNCoR-ChIP

Relative enrichment
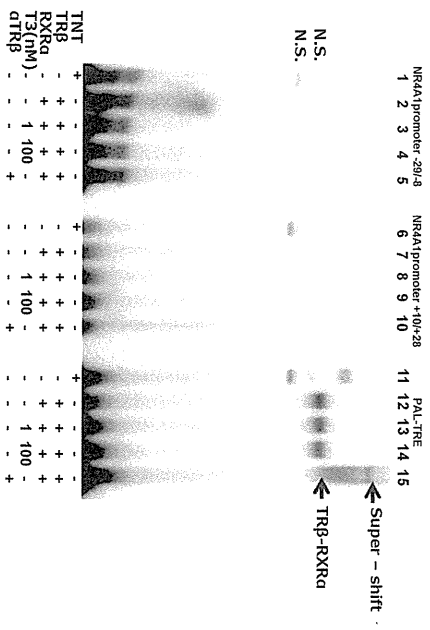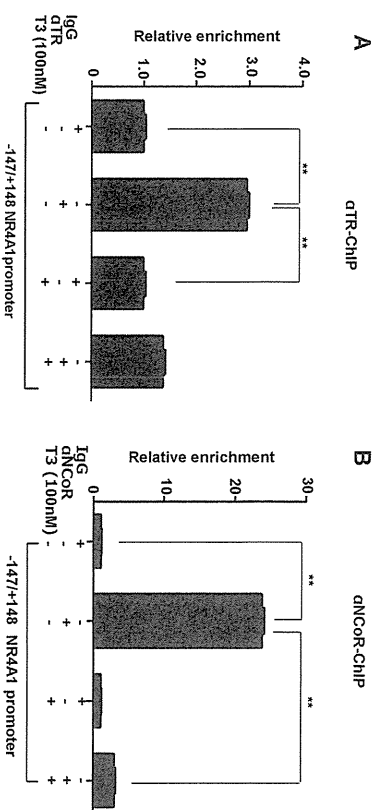
IgG
αNCoR
T3 (100nM)

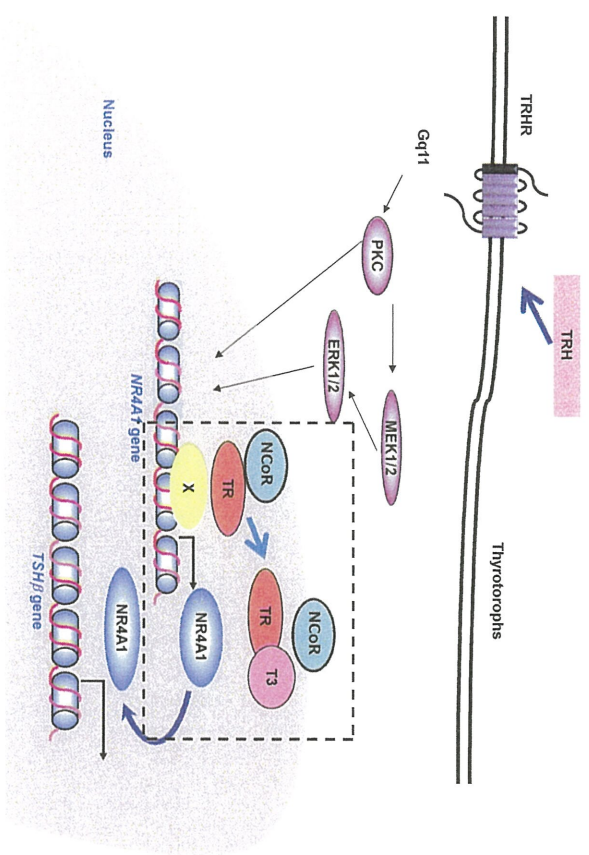-147/+148 NR4A1 promoter

Fig. 8

Figure legends

Fig. 1. Subacute thyrotoxicosis reduced pituitary NR4A1 mRNA levels in mice.

A) *NR4A1* mRNA levels were measured in the pituitary glands of wild-type mice and experimental hypothyroid and thyrotoxicosis mice. Experimental hypothyroid and thyrotoxicosis were induced by anti-thyroid drugs (MMI and PTU) in chow and daily peritoneal injections of a pharmacological dose of T4 for 2 weeks. Although daily injections of T4 reduced NR4A1 mRNA levels, chronic hypothyroidism had no effects.

NR4A1 mRNA levels (B) and *TSHβ* mRNA levels (C) were measured 24 hr (indicated as 24 h) after a single injection of the same dose of T4 as that described above. Both levels were significantly lower than those in the control (control), indicating that the single injection of T4 caused thyrotoxicosis in 24 hr that was sufficient to depress pituitary TSHβ mRNA levels, which led to reductions in NR4A1 mRNA levels. Data are expressed as mRNA levels relative to the control set as 1.0. Control indicates the injection of vehicle, saline: T4, injection of T4; MMI+PTU, experimental hypothyroidism induced by the MMI and PTU treatment.

**, $p<0.01$; *, $p<0.05$; N.S., not significant.

Fig. 2. Thyroid hormone suppressed the promoter activity of the NR4A1 gene in vitro.

A) An incubation with 100 nM T3 did not any change the promoter activity of the *NR4A1* gene containing the promoter region between -1295 and +152 bp of the transcriptional start site in CV-1 cells that did not express functional TR. An incubation with T3 without the expression of TRβ did not affect the promoter activity of the NR4A1 gene. The overexpression of TRβ led to a marked increase in NR4A1 promoter activity to 198.7± 19.3% that of controls. Under the expression of TRβ, the incubation with 100 nM T3 induced a significant reduction in NR4A1 promoter activity, which was less than basal promoter activity

(without T3 or the expression of TRβ). Data were expressed as activity relative to that of

controls (without T3 or the expression of TRβ).

B) The suppression of the promoter activity of the *NR4A1* gene by T3 was dose-dependent.

The suppression of the NR4A1 gene was initially observed from 0.1 nM T3 and a reduction of

85.6 ± 1.4% was noted with 1,000 nM T3.

Values are represented as the mean ± SEM. **, $p<0.01$; *, $p<0.05$

Fig. 3. Effects of the overexpression of NCoR on T3-induced repression of NR4A1 promoter

activity in vitro.

A) We examined the effects of NCoR on the T3-induced repression of NR4A1 promoter

activity using pA3-Luc(-1295~+152). The overexpression of NCoR led to a significant

increase in the ligand-independent stimulation of NR4A1 promoter activity by TR and

impaired reductions by T3-induced repression (far right lane).

B-1 and 2) Effects of the knockdown of NCoR by siRNA on the T3-induced suppression of the

NR4A1 gene in CV1 cells.

B-1) A real-time PCR analysis showed that the transfection of siRNA for NCoR (SiNCoR)

led to 54.4% lower mRNA levels than those by the control (SiCONTROL) after 48 hrs (the left

panel). A Western blot analysis demonstrated that the level of the NCoR protein, ~270kDa in

size, was also decreased to 65.7% that of the control, whereas the expression of GAPDH was

not changed as a control (right panel).

B-2) The promoter activity and effects of the overexpression of TR and T3-induced

suppression of the NR4A1 gene were examined in CV-1 cells using pA3-Luc(-1295~+152). In

the control vehicle with siCONTROL RNA, the significant ligand-independent activation of the

NR4A1 gene by TR and repression by 100 nM T3 were observed (left panel). However, the

knockdown of NCoR abolished ligand-independent activation, but did not affect repression by T3 (right panel). Data were expressed as luciferase activity relative to the control (in the absence of the overexpression of TR and T3).

Data are presented as the mean ± SE of three experiments (*, $p<0.05$; **, $p<0.01$).

Fig. 4. Effects of TR mutants on T3-induced repression of the promoter activity of the NR4A1 gene in CV-1 cells.

The promoter activity of the NR4A1 gene (pA3-Luc (-1295~+152)) was examined under the expression of mutant TR instead of wild-type TR.   When transfected F455S mutant TRβ that impaired T3-induced release of NCoR in CV-1 cells, a ligand-independent activation by TR was increased as compared to that of the wild-type TR, and repression by T3 was also impaired ($p<0.01$). The NR4A1 promoter activity, examined using GS125 mutant TR, which has a mutated DNA binding region, showed impairment of ligand-independent activation and inhibition of T3-induced transcriptional repression. Data were expressed as luciferase activity relative to the control set as 1.0 (in the absence of the expression of TR or T3).

Data are presented as the mean ± SE of three experiments (*, $p<0.05$; **, $p<0.01$).

Fig. 5. The region responsible for T3-induced suppression was located close to the transcriptional start site of the NR4A1 gene.

A) The sequence of the promoter region of the human *NR4A1* gene.

The promoter region of the NR4A1 gene contains putative myocyte enhancer factor (MEF) 2 response elements (MRE) in the region between -275 and -309 bp, and a putative cAMP response element (CREB) at -42 bp, -71 bp, -215 bp, and -235 bp of the transcription start site.

B) A series of deletion mutants of the NR4A1 promoter construct showed weaker promoter

activity than that of the (-1295-+152)-Luc construct, suggesting that the region between -1295 and -337 exhibits enhancer activity. The activity of (-337-+152)-Luc was weaker than that of (-147-+152)-Luc, suggesting silencer activity in the region between -337 and -147 bp.

C) Using these deletion constructs, the ligand-independent activation of NR4A1 promoter activity by TR and suppression by T3 were examined. We detected significant ligand-independent activation and T3 suppression in all constructs examined, indicating that the region responsible for the T3-induced suppression of the NR4A1 promoter was located close to the transcriptional start site (-27bp ~ +152 bp).

Data were expressed as luciferase activity relative to the (-1295-+152)-Luc construct set as 1.0 (in the absence of expression of TR or T3) (Fig. 5B). In Figure 5C, each control (in the absence of the expression of TR and T3) was set as 1.0. Data are presented as the mean ± SEM of three experiments (*, $p<0.05$; **, $p<0.01$).

Fig. 6. The EMSA demonstrated that neither TRβ nor RXR bound to putative TR binding sites.

The EMSA was performed with the fragments containing the putative TR binding sites, AGGAGGGTCGG and AGGCTAC, in the regions between -29 and -8 bp and between +10 and +28 bp, respectively, indicated as a probe in the upper column. The typical thyroid hormone response element, palindromic TRE (PAL-TRE), containing 5'-agcttcaggtcacagga ggtcagagag-3' and 5'-aagattaaggtcatgacctgaggaga-3', showed significant binding with the TRβ-RXR heterodimers (lane 12). An incubation with T3 decreased the binding of TRβ-RXR heterodimers (indicated as TRβ-RXRα) (lane 13 with 1 nM T3, lane 14 with 100 nM T3). Furthermore, the addition of an antibody against TRβ induced a clear super-shift (indicated as

an arrow with Super-shift) (lane 15), confirming that the bands were a complex containing TRβ.

Under the same conditions, the fragments containing the regions between -29 and -8 bp indicated as the NR4A1 promoter -29/-8 and between +10 and +28 bp (NR4A1 promoter +10/+28) did not show any binding to either TRβ or RXR.   N.S. indicated non-specific binding by the TNT lysate alone.

Fig. 7. ChIP assays demonstrated that TRβ and NCoR were recruited to the region between -147 and +148 bp of the NR4A1 promoter.

A) ChIP assays were performed on CV1 cells transfected with pA3-Luc (-1295~+152) and TRβ using an antibody against TRβ.   The significant recruitment of TRβ was observed into the fragment containing the region between -147 and +148 bp.   Furthermore, the addition of T3 induced a significant reduction in the recruitment of TRβ into this region.   No significant recruitment of TRβ was found under any condition tested when IgG was used instead of the antibody against TRβ.

B) The ChIP assay with pA3-Luc (-1295~+152) and an anti-NCoR antibody was performed using CV-1 cells that expressed endogenous NCoR.   A ChIP assay demonstrated that NCoR was recruited to the region between -147 and +148 bp without T3, while an incubation with T3 induced the release of NCoR from that region.

Values are represented as the mean ± SEM.   *, $p < 0.01$.

Fig. 8. Proposed machinery for the involvement of NR4A1 with TRH- and T3-mediated regulation of the TSHβ *gene in the pituitary gland.*

NR4A1 stimulates the promoter activity of the *TSHβ* gene in a dose-dependent manner in pituitary thyrotrophs as previously reported (Nakajima et al., 2012). TRH stimulates *NR4A1* mRNA levels through TRH receptors and the PKC and ERK signal transduction pathways. In the present study, we demonstrated that thyroid hormone negatively regulated the level of NR4A1 mRNA, possibly via the release of TR and NCoR from the promoter of the NR4A1 gene, and then suppressed its promoter activity (in the dotted line of the frame). NR4A1 may be a key regulator of the *TSHβ* gene in the pituitary gland for regulation by hypothalamic TRH and thyroid hormone.

発表予定論文

Pituitary NR4A1 is negatively regulated by thyroid hormone without direct binding of thyroid hormone receptors on the gene.

Molecular and Cellular Endocrinology（投稿中）

著者名 :
岡村孝志、中島康代、渋沢信行、堀口和彦、松本俊一、山田英二郎、登丸琢也、石井角保、小澤厚志　石塚高広、橋本貢士、岡田秀一、佐藤哲郎、山田正信

発表予定論文