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Negative Regulation of the Thyrotropin β Gene by Thyroid Hormone

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1. Introduction

Thyroid hormone (T3 and T4) is secreted from the thyroid gland, and is known to reduce the level of serum thyrotropin (thyroid-stimulating hormone, TSH) in the pituitary gland (Sarapura et al., 2002; Shupnik et al., 1989) (Fig. 1A). This is a typical example of negative feedback between the pituitary and endocrine organs, and is a key component of thyroid hormone homeostasis. TSH is one of the peptide hormones generated in the anterior pituitary, and is a heterodimer composed of an α chain (α-glycoprotein subunit, αGSU) and a β chain (TSHβ) (Shupnik et al., 1989). While αGSU is common to follicle stimulating hormone (FSH), luteinizing hormone (LH) and chorionic gonadotropin (CG), TSHβ is specific to TSH alone. Although the concentration of serum T4 is much higher than that of T3, T4 is converted to T3 by deiodinase (Dio) in the TSH-producing cells (thyrotrophs) of the pituitary (Christoffolete et al., 2006), and T3 exhibits biological activity as a thyroid hormone (Gereben et al., 2008). T3 inhibits expression of both *TSHβ* and *αGSU* at the transcriptional level (Shupnik et al., 1989). The magnitude of T3-induced repression of the *TSHβ* gene is greater than that of *αGSU*. Here, we provide an overview of the molecular mechanisms involved in T3-induced negative regulation of the *TSHβ* gene and its related genes.

2. Structure of T3 receptors (TRs)

T3 receptor (TR) belongs to the nuclear hormone receptor (NHR) superfamily, and is a ligand-dependent transcription factor (Cheng et al., 2010). TR is encoded by two separate alleles; *TRα* and *TRβ*. Through alternative splicing, the *TRα* gene generates TRα1 and TRα2, while the *TRβ* gene generates TRβ1 and TRβ2 (Fig. 2). While TRα1, TRβ1 and TRβ2 have T3-binding capacity, TRα2 does not bind T3. Hence, TRα1, TRβ1 and TRβ2 are thought to be the functional TRs. TRβ2 is expressed in limited organs including pituitary, hypothalamus and retina, while TRα1 and TRβ1 are ubiquitously expressed (Cheng et al., 2010). As in the case of other NHRs, TR consists of an N-terminal region (NTD), a central DNA binding domain (DBD), a hinge region and a C-terminal ligand binding domain (LBD) (Fig. 2).

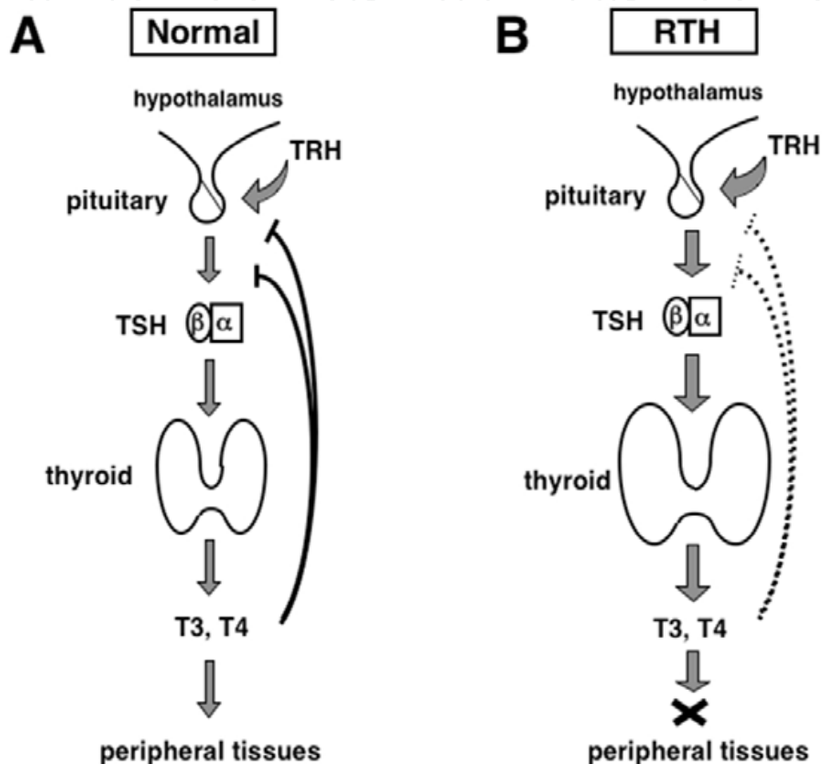

Fig. 1. Negative feedback loop in the hypothalamus-pituitary-thyroid axis and negative regulation of *TSHβ* and *αGSU*. A. The secretion of TSH (a heterodimer of TSHβ and αGSU subunits) in the anterior pituitary and TRH in the hypothalamus is inhibited by thyroid hormones (T3 and T4). β, TSHβ chain. α, αGSU chain. TRH, thyrotropin releasing hormone. Synthesis of TRH in hypothalamus is also negatively regulated by T3. B. In patients resistant to thyroid hormone (RTH), a negative feedback loop is impaired due to a defect in T3 receptor (TR) β. This finding provides the evidence for the involvement of TRβ in the negative regulation of the *TSHβ and αGSU* genes. Because of increased secretion of TSH, difuse goiters are often found in the patient with RTH.

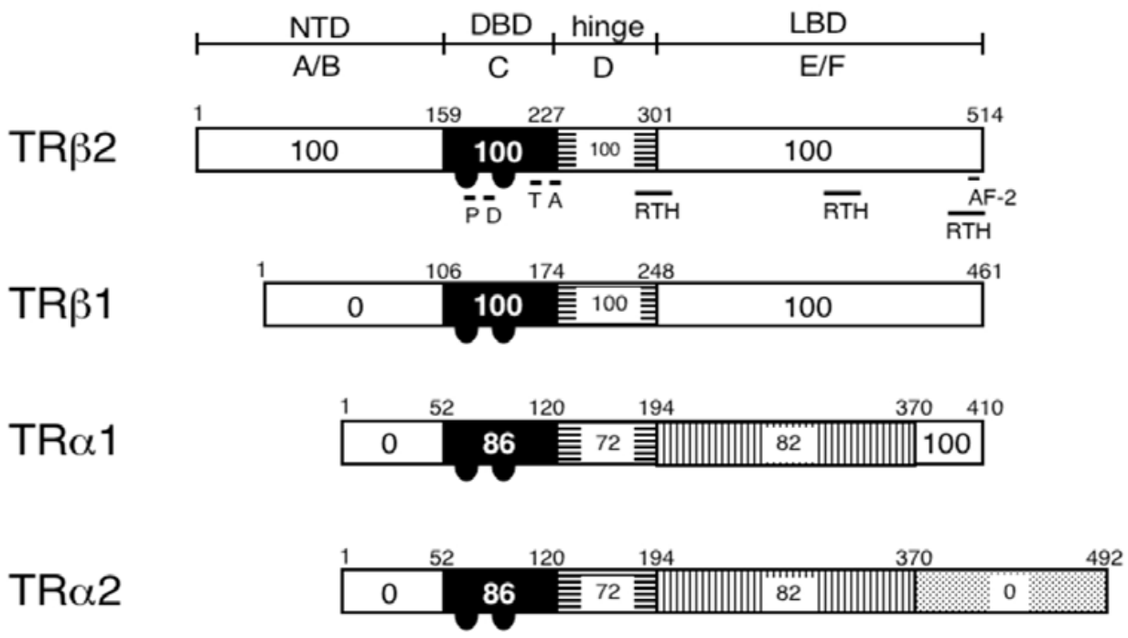

Fig. 2. Schematic representations of TR isoforms. TR consists of an N-terminal region (NTD, A/B domain), a central DNA-binding domain (DBD, C domain), a hinge region (D domain) and a C-terminal ligand binding domain (LBD, E/F domain). The numbers within the box represent the amino acid homology (%). While TRα1, TRβ1 and TRβ2 have T3-binding capacity, TRα2 does not bind T3. The P and D boxes are required for the recognition of half-site sequences (typically, AGGTCA) and the number of spacing nucleotides, respectively. The T and A boxes are involved in dimer formation and polarity of TR-RXR heterodimers on positive TRE (pTRE). P, Pbox. D, D box. T, Tbox. A, A box. RTH, three hot spots where mutations are frequently found in patients with RTH. AF-2, activation function 2.

3. Mechanism of positive regulation by T3

TR activates or inhibits the transcription of its target genes in a T3-dependent manner, and the molecular mechanism of T3-dependent activation (positive regulation) has been elucidated (Cheng et al., 2010) (Fig3A). Because findings in molecular mechanisms of positive regulation by T3 have greatly influenced the studies of negative regulation, it is necessary to outline the mechanism of T3-dependent positive regulation (Fig. 3A) before describing the negative regulation of the TSHβ gene (Fig. 3B). In the positive regulation, TR heterodimerizes with retinoid X receptor (RXR) at the T3-responsive element (TRE) of the gene, the transcription of which is positively regulated by T3-bound TR (T3/TR) (Cheng et al., 2010). In the absence of T3, TR-RXR heterodimers interact with co-repressors, including nuclear receptor co-repressor (NCoR) or silencing mediator for retinoid and thyroid hormone receptors (SMRT). These co-repressors recruit histone deacetylase (HDAC), which represses the transcription of the target genes. This repressive effect by unliganded TR is referred to as "silencing" and is thought to play an important role in the clinical symptoms in hypothyroidism (Astapova et al., 2008; Astapova et al., 2011). Upon T3 binding, the TR-RXR heterodimers release NCoR or SMRT and then recruit p160 family cofactors including

steroid receptor coactivator-1 (SRC-1). The TR-RXR-p160 complex also recruits an additional coactivator, CBP/p300 (Chen et al., 1999; Glass and Rosenfeld, 2000; Huang et al., 2003). Both the p160 family and CBP/p300 have intrinsic histone acetyltransferase (HAT) activity and modify chromatin structure, resulting in the transactivation of the target genes (Fig. 3A).

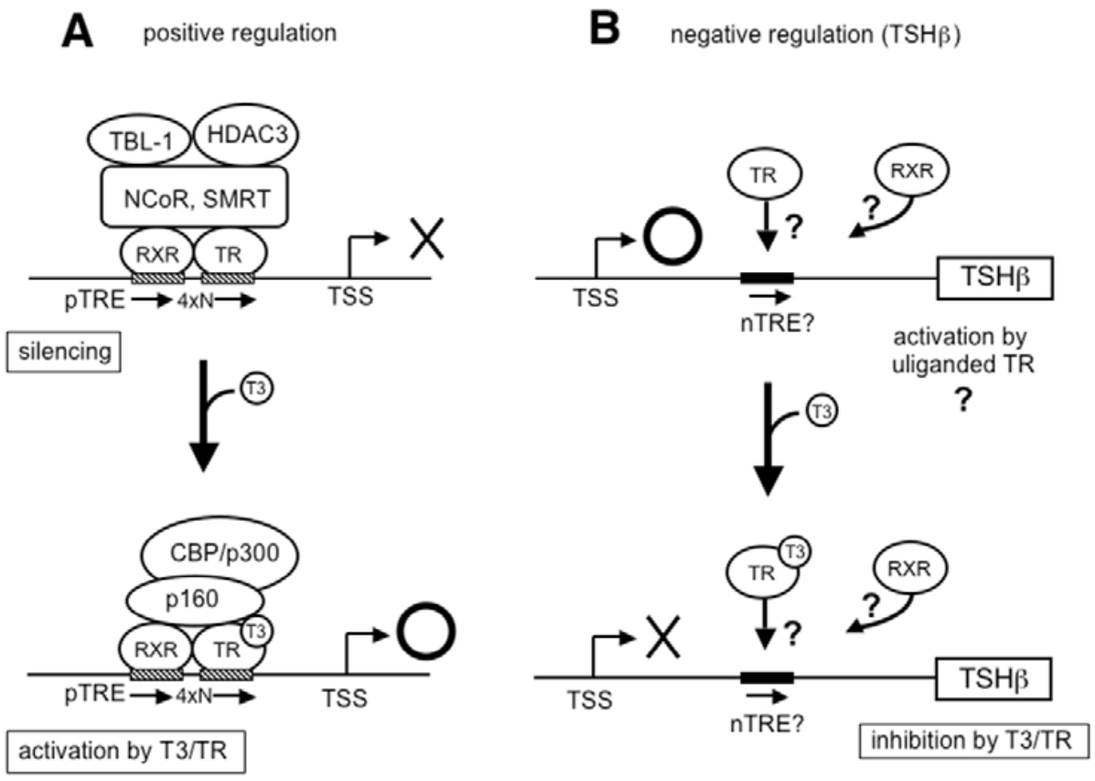

Fig. 3. Schematic representation of T3-dependent transactivation (positive regulation) (A) and transrepression (negative regulation) of the *TSHβ* gene (B). A. TR heterodimerizes with retinoid X receptor (RXR) on the T3-responsive element (TRE) of the gene, the transcription of which is positively regulated by T3-bound TR (T3/TR). Arrow, half-site sequence (typically, AGGTCA). 4xN, random four nucleotides for spacing. B. An nTRE (GGGTCA) has been postulated in the region immediately downstream to the transcription start site (TSS) of the *TSHβ* gene. In contrast to the mechanism for positive regulation, the molecular mechanism of negative regulation has been controversial (see text).

As shown in Fig. 3A, a typical TRE has a unique configuration designated as direct repeat 4 (DR4), in which the random four base pairs (spacer) are incorporated into tandem repeats of a hexameric half-site (Cheng et al., 2010; Umesono et al., 1991). Both the half-site sequence (typically AGGTCA) and the number of spacer nucleotides determine the specificity for DNA recognition by the TR-RXR heterodimer. Analogous to TR-RXR heterodimer binding at DR4, RXR heterodimerizes with vitamin D3 receptor (VDR) on direct repeats of half-sites spaced with 3 nucleotides (DR3) and it functions as a heterodimer partner for retinoic acid receptor (RAR) on direct repeats spaced with 5 nucleotides (DR5) (Cheng et al., 2010; Glass and Rosenfeld, 2000). In the studies of

positive regulation by T3, monkey kidney-derived CV1 cells (Jensen et al., 1964) have been often used (Naar et al., 1991; Tillman et al., 1993; Umesono et al., 1991) because they possess endogenous RXR but not TR.

4. Involvement of TR in negative regulation of the *TSHβ* gene: Syndrome of thyroid hormone resistance (RTH)

Although the molecular mechanism in negative regulation of the *TSHβ* gene has been disputed (Lazar, 2003; Shupnik, 2000; Weitzel, 2008), it is apparent that TR plays a crucial role in it. Syndrome of resistance to thyroid hormone (RTH) is characterized by a reduced tissue response to T3 (Fig. 1B). The majority of patients with RTH have mutations in the LBD of the *TRβ* gene, of which amino acid sequence is shared by TRβ1 and TRβ2 proteins (Refetoff et al., 1993) (Fig. 2). These mutant TRβ1s and TRβ2s have defects in their T3-binding capacity but have intact DBDs capable of recognizing the TRE (Fig. 3A). Thus, they bind to the TRE and constitutively interact with NCoR or SMRT, even in the presence of T3, resulting in silencing. In the majority of patients with RTH, inheritance is usually autosomal dominant, and mutant *TRβ* is thought to interfere with T3-induced activation by wild-type TR bound to the TRE (dominant negative effect). Of note, patients with RTH also exhibit elevated secretion of TSH (syndrome of inappropriate secretion of TSH, SITSH) (Refetoff et al., 1993) (Fig. 1B). This finding provides evidence for the involvement of *TRβ* in the negative regulation of the *TSHβ and αGSU* genes. However, the mechanism downstream to TR has been unknown (Lazar, 2003; Shupnik, 2000; Weitzel, 2008). With regard to the mechanism of negative regulation of *TSHβ* and *αGSU*, the central question has been whether TR directly interacts and recognizes the DNA sequence of the *TSHβ* promoter, as identified for the TRE in positive regulation. Some theories indicate the direct binding of TR with DNA, while others favor models that are independent of a direct binding with DNA.

5. Direct binding of TR with DNA: Negative TRE (nTRE) hypothesis

Following the identification of the role of the TRE in the positive regulation of genes (hereafter, positive TRE, pTRE), some researchers have postulated a so-called negative TRE (nTRE) in the *TSHβ* (Fig. 3B) and *αGSU* genes (Chin et al., 1993; Shupnik, 2000; Wondisford et al., 1989). The observation that serum TSH levels increase in hypothyroidism led to the idea that unliganded TR may be the transcriptional activator for *TSHβ* (Fig. 3B, upper panel) and *αGSU*. If unliganded TR is the transcriptional activator on the nTRE of these genes, one may able to identify the nTRE as the sequence required for the transcriptional activation by unliganded TR. Based on this hypothesis, deletion analysis of the *TSHβ* promoter was performed using human kidney-derived 293 cells (Wondisford et al., 1989) and it was reported that the transcriptional activity of the this promoter was abolished after deletion of a short DNA sequence immediately downstream to the transcription start site (TSS) (Wondisford et al., 1989) (Fig. 4). This sequence (GGGTCA) has been postulated as the nTRE because it has homology with the consensus sequence of a half-site (AGGTCA). The nTRE hypothesis has been regarded as one of the principal models to explain the molecular mechanism of negative regulation of the *TSHβ* gene (Chin et al., 1993; Cohen and Wondisford, 2005), and has been regarded as a potential mechanism of T3-dependent negative regulation of other genes (Edwards et al., 1994; Kim et al., 2005; Lin et al., 2000; Santos et al., 2006; Wright et al., 1999). However, this raised several questions, as discussed below.

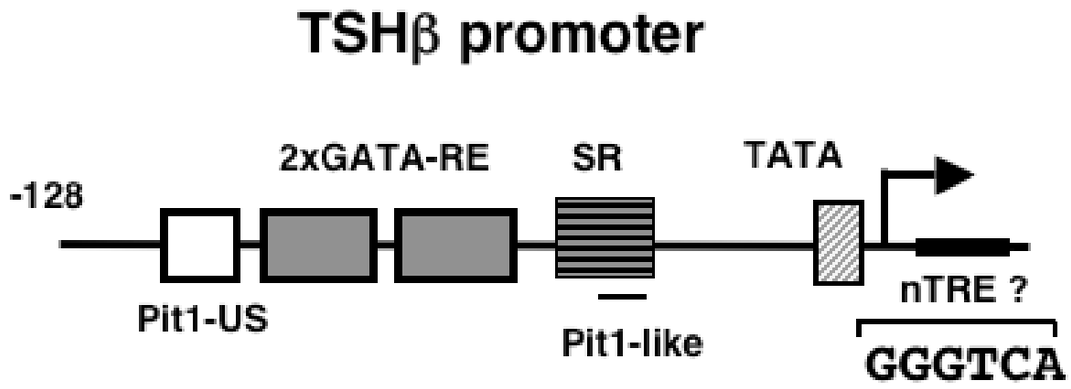

Fig. 4. Schematic representation of the *TSHβ* promoter. Pit1-US, functional Pit1-binding site. GATA-TRE, GATA-responsive element. SR, suppressor region. Pit1-like, the sequence similar to Pit1-binding site. TATA. TATA box. An nTRE (GGGTCA) has been postulated immediately downstream to the TSS.

5.1 Does TR heterodimerize with RXR on the nTRE?

While RXR is the obligate heterodimer partner for TR recognition of the pTRE (Fig. 3A), the involvement of RXR with the nTRE has not been determined (Fig. 3B). Although the nTRE sequence appears to be a single half-site, there remains the possibility that its flanking sequences may function as another half-site (Fig. 3B). However, if TR heterodimerizes with RXR on the nTRE, this configuration cannot be discriminated from that present on the pTRE (Fig. 3A), which may be functioning as a T3-dependent transcriptional activator but not an inhibitor. Previous results of reporter assays examining the effect of RXR overexpression on the *TSHβ* promoter have been controversial. Cohen et al. (Cohen et al., 1995) and Hallenbeck et al. (Hallenbeck et al., 1993) reported that RXR may antagonize inhibition of the *TSHβ* gene by T3/TR, while Safer et al. (Safer et al., 1997) reported that the requirement for RXR is different between TRβ1 and TRβ2. Nagaya et al. (Nagaya and Jameson, 1993) demonstrated that mutant TRβ1 (L428R) which is unable to dimerize with RXR failed to mediate T3-induced inhibition of the *αGSU* promoter, while Takeda et al. (Takeda et al., 1997) reported that overexpression of RXR had no effect on this promoter. According to Laflamme et al. (Laflamme et al., 2002) RXR enhances the negative regulation of the *TSHβ* gene by T3, and this effect is mediated by the RXR-LBD and but not the DBD, suggesting that RXR may act as a cofactor.

It was reported that the ligand for RXR (rexinoids) has inhibitory effect on *TSHβ* expression (Sherman et al., 1999). However, subsequent analysis revealed that the signaling pathway is mediated via a nt. -200/-149 region of the mouse *TSHβ* gene, which is different from the reported nTRE (GGGTCA at nt. +1/+6) (Sharma et al., 2006). While T3 inhibits the synthesis of thyrotropin releasing hormone (TRH) in hypothalamus (Fig 1A) and a nTRE is postulated in this gene (Hollenberg et al., 1995), rexinoids has no effect on its production (Sherman et al., 1999). It was reported that the transcription of the *TSHβ* gene is repressed in the CV1 cells treated with retinoic acid (RA) (Breen et al., 1995); however, its precise mechanism is unknown. Although all three RXRs, α, β and γ, are expressed in TSHoma cells, TtT97 (Sharma et al., 2006), double immunostaining studies of the hypothyroid rat pituitary using an antibodies against pituitary hormones and RXR suggest that RXRγ is predominantly expressed in thyrotrophs (Sugawara et al., 1995). Barros et al. (Barros et al., 1998) reported

that no alteration in the serum level of TSH, T3 or T4 was observed in RXRα $^{(-/+)}$ mice or RXRγ $^{(-/-)}$ mice. The authors suggested that ablation of RXRs has little effect on the negative regulation of the *TSHβ* gene. Currently, there are few rationales to confirm the recognition of the nTRE by TR-RXR heterodimers.

5.2 Does TR bind to the nTRE as a monomer?

Gel shift assays indicate that TR monomers bind with the nTRE and that this interaction is abolished by RXR (Cohen et al., 1995). However, the hypothesis that TR binds with the nTRE as a monomer (Fig. 3B) also raises other questions which are difficult to answer. First, for the direct recognition of DNA sequence by DNA-binding transcription factors including NHRs, formation of a homo- or heterodimer is usually required. As expected, gel shift assays revealed that TR-monomer binding with single half-sites (i.e. nTRE) is much weaker than that of TR-RXR heterodimer binding to DR4 (Cohen et al., 1995). It should be noted that TSH synthesis in severe hypothyroidism is dramatically high (Fisher et al., 2000). Given that unliganded TR may maintain the basal activity of the *TSHβ* promoter in hypothyroidism, it is difficult to imagine that such a weak binding of TR monomer with the nTRE can achieve this high level of transcriptional activity. Second, there is ligand/NHR selectivity, i.e., the negative regulation of the *TSHβ* gene is clinically specific to T3 and partially estrogen (E2), but not other NHR ligands (Cohen and Wondisford, 2005). While, in positive regulation, the number of spacing nucleotides between half-sites is a critical factor in determining receptor specificity, it is unknown as to how TR selectively recognizes the nTRE DNA sequence in the *TSHβ* gene. Finally, it is not easy to explain why TR monomers on a single half-site can exhibit reverse functions, i.e. recruitment of co-activators in the absence of T3 and association with co-repressors in the presence of T3 (Fig. 3B). We proposed previously a model where TR is able to bind with reported nTRE only in the presence of T3 (Sasaki et al., 1999). Although the nTRE in the *TSHβ* gene was originally defined on the basis of the experiments with non-pituitary 293 cells (Wondisford et al., 1989), we and other investigators suggested the possibility that an unknown thyrotroph-specific factor may switch T3/TR on the nTRE from a transcriptional activator to an inhibitor (Sasaki et al., 1999; Shupnik, 2000; Wondisford et al., 1993). However, its existence has not been confirmed because of the limited number of cultured thyrotroph cell lines available (Ooi et al., 2004; Sarapura et al., 2002).

6. Models that do not postulate direct DNA binding of TR

The following hypotheses proposed models for T3-induced negative regulation without the involvement of direct DNA binding of TR.

6.1 NCoR or SMRT

There are many studies with regard to the involvement of NCoR or SMRT in the negative regulation by T3. It was reported that co-expression of NCoR and SMRT may enhance basal stimulation of the *TSHβ*, *aGSU* and *prepro-TRH* promoters in a TR-dependent manner (Tagami et al., 1997). With regard to the T3-induced negative regulation of the Rous sarcoma virus-derived 5' truncated terminal repeat (RSV-LTR) (Berghagen et al., 2002) and the rat *CD44* gene (Kim et al., 2005), it was reported that NCoR and SMRT may function as transcriptional co-activators in the transcriptional regulation of these genes. According to

Tagami et al. (Tagami et al., 1999), unliganded TR in solution may squelch NCoR or SMRT from the transcription factor on the target promoter. Upon T3 binding, TR may release these co-repressors, resulting in their association with the DNA-binding transcription factor, which maintains the basal activity of the promoter of the target gene. However, this notion was tempered by following questions. First, the mechanism involved in the association of NCoR or SMRT with the target promoters is unknown. Second, it was undetermined whether the majority of NCoR and SMRT are sequestered by the relatively limited amount of intracellular TR (5000 to 10000 molecule/cell) (Oppenheimer et al., 1974). Third, NCoR and SMRT also interact with unliganded RAR and peroxisome proliferator-activated receptors (PPARs) (Nofsinger et al., 2008; Suzuki et al., 2010). It is unlikely that these NHRs also inhibit *TSHβ* or *aGSU* expression in the presence of cognate ligands. Finally, it was reported that the negative regulation by T3 is mediated by the mutant TRβ, AHT, of which interaction with co-repressors is impaired (Nakano et al., 2004). Using an experimental model similar to the mammalian two-hybrid assay, Wulf et al. (Wulf et al., 2008) also demonstrated that negative regulation by T3/TR is possible via the interaction of TR with a co-repressor. However, their experimental setting was completely artificial.

Amino acid sequence of TRβ2-NTD is unique and has low homology with that of TRβ1 or TRα1 (Fig. 2). The N-terminal domain of TRβ2 is known to neutralize the silencing activity of co-repressors in the context of TR-RXR heterodimers on the pTRE (Hollenberg et al., 1996; Yang et al., 1999), and the NTD of TRβ2 may have some role in the negative regulation of the *prepro-TRH* gene (Guissouma et al., 2002). However, the physiological relevance in the negative regulation of *TSHβ* gene is unknown. Of note, in vitro experiments showed that not only TRβ2 but also TRβ1 and TRα1 can inhibit the transcription of the *TSHβ* gene in a T3 dependent manner (Nakano et al., 2004). The findings in *TRβ/TRa*-double knockout mice (Gothe et al., 1999) indicates that TRα1 is partially involved in the negative regulation of *TSHβ* gene although its expression level is less than that of TRβ2 (see below).

The amino acid sequence required for the interaction of NCoR and SMRT with unliganded TR has been identified. Based on this information, mice harboring mutant SMRT (SMRTmRID) (Nofsinger et al., 2008) or mutant NCoR (NCoRΔID) (Astapova et al., 2011), which results in defective interaction with unliganded TR, were established. Importantly, the negative regulation of the *TSHβ* gene by T3 was not impaired in knock-in mice with SMRTmRID or NCoRΔID, although the latter exhibited a reduced amount of TR protein in the pituitary gland and reduced sensitivity for TSH by the thyroid gland. Likewise, TRH expression in the hypothalamus was not affected in NCoRΔID-knock-in mice (Astapova et al., 2011). This is supported by an *in vivo* study, which showed that the overexpression of these co-repressors is incompatible with physiological regulation of TRH (Becker et al., 2001). As in the cases of the *TSHβ* and *aGSU* genes, mRNA expression of the myosin heavy chain β subunit (*MHCβ*) gene in the heart is also repressed by T3/TR (Gupta, 2007). In NCoRΔID mice, T3-induced inhibition of *MHCβ* is maintained (Astapova et al., 2011). Moreover, Astapova et al, (Astapova et al., 2008) established a liver-specific NCoRΔID knock-in mouse. According to them, of 326 genes that are negatively regulated by T3 in the liver, only 3 genes were repressed in hypothyroid conditions, suggesting little effect of NCoR on the majority of the negatively regulated T3-target genes. While SMRTmRID- or NCoRΔID-knock-in mice survive, the global deletion of both the *NCoR* and *SMRT* genes are embryonic lethal (Jepsen et al., 2007). These findings suggest that NCoR and SMRT have important roles other than interaction with liganded NHRs. For example, they interact with

p53, Myc, MyoD Ptx1 and Foxo1 (Nofsinger et al., 2008). Thus, it is unlikely that T3-binding with TR affects the transcriptional regulation of all of the genes regulated by NCoR or SMRT *in vivo*.

6.2 CBP/p300 and p160 family

cAMP-response-element-binding protein-binding protein (CBP)/p300 is required for transactivation by multiple DNA-binding transcription factors including NFkB and AP-1, and functions as a coactivator for liganded NHRs (Kamei et al., 1996). As a model for the ligand-dependent inhibition by NHRs, it was proposed that liganded NHRs may attenuate the transactivation by DNA-binding transcription factors via interference of CBP/p300 function (Kamei et al., 1996). However, subsequent studies reported that inhibition by liganded NHR is not rescued by overexpression of these co-activators (De Bosscher et al., 1997; De Bosscher et al., 2001; Wu et al., 2004). It was also suggested that the CBP/p300-interacting surface on the NHR-LBD may be different from that required for T3-dependent inhibition (Saatcioglu et al., 1997; Valentine et al., 2000).

Although inappropriate overexpression of TSH is reported in SRC1-deficient mice (Takeuchi et al., 2002), it is difficult to determine what adaptive processes have occurred during pituitary development in SRC-1$^{(-/-)}$ mice since ablation of the SRC-1 gene also affects the expression of other p160 family members and TRs (Sadow et al., 2003; Xu et al., 1998). While p160 proteins are known to interact with multiple NHRs other than TR in a ligand-dependent manner, inhibition of the *TSHβ* gene or the *aGSU* gene is specific to T3, and partially estrogen (Cohen and Wondisford, 2005). In some patients with RTH, there are no mutations of the *TRβ* or *TRa* gene (non-TR RTH) (Refetoff and Dumitrescu, 2007; Refetoff et al., 1993). Although defects in cofactors that may mediate the negative regulation of the *TSHβ* gene have been postulated in these cases, linkage analyses with polymorphic markers showed that the involvement of SRC-1, NCoR, SMRT or RXRγ is unlikely (Reutrakul et al., 2000).

6.3 Protein-protein interaction of TR with DNA-binding transcription factors: Tethering model

In vitro binding assays, including gel shift assays, are limited in that the amount of TR and/or RXR used may not always reflect the *in vivo* situation. To overcome this problem, Shibusawa et al. (Shibusawa et al., 2003a; Shibusawa et al., 2003b) established mice in which TRβ is unable to bind DNA due to a mutation in its DBD. They reported that the negative regulation of the *TSHβ* gene is relieved in these mice. One may assume that this result provides evidence for the direct binding of TRβ with the nTRE because, in positive regulation, the pTRE is recognized by the TR-DBD. However, the function of the DBD of NHR is not limited to DNA recognition. It is known that the DBD also interacts with other DNA-binding transcription factors including NFkB (De Bosscher et al., 2003; Kalaitzidis and Gilmore, 2005; Kalkhoven et al., 1996; Ray and Prefontaine, 1994; Scheinman et al., 1995; Stein and Yang, 1995; Tao et al., 2001; Wissink et al., 1997), AP-1 (typically Jun/Fos heterodimers) (De Bosscher et al., 2001; Heck et al., 1994; Lopez et al., 1993; Schule et al., 1990; Webb et al., 1995), Nur77 (Martens et al., 2005) and GATA family transcription factors (Clabby et al., 2003; Matsushita et al., 2007), resulting in their inhibition by NHRs in a ligand-dependent fashion.

This kind of ligand-induced repression via protein–protein interactions is referred to as the "tethering mechanism" (De Bosscher et al., 2003; Herrlich, 2001; Nissen and Yamamoto, 2000; Pfahl, 1993). Thus, while mutation of the TRβ DBD abrogates the negative regulation of the *TSHβ* gene by T3, it does not always imply a direct interaction of the TR with DNA. Of note, there are ligand/receptor specificities in the repression by liganded NHRs via the tethering mechanism (Caldenhoven et al., 1995; Liden et al., 1997; Matsushita et al., 2007). Moreover, dimer formation is not always required for ligand-dependent inhibition via the tethering mechanism. A mutant glucocorticoid receptor (GR), A458T, is known to have a defect in dimer formation and therefore in glucocorticoid-responsive element-dependent transactivation. It was reported that functions that require cross-talk with other transcription factors, such as transrepression of the AP-1-driven genes, remain intact in this mutant GR (Herrlich, 2001; Reichardt et al., 1998). This raises again the question whether heterodimer formation of TR with RXR is necessary for the negative regulation of the *TSHβ* gene by T3.

7. Other possible mechanisms

Although T3 treatment is known to reduce the stability of TSHβ mRNA (Krane et al., 1991), the role of TR has not been clarified, and the involvement of similar mechanisms in the regulation of the αGSU mRNA have not been reported (Staton et al., 2000). A mechanism operating via anti-sense RNA was proposed to be involved in negative regulation of the MHCβ gene by T3 (Danzi and Klein, 2005; Haddad et al., 2003). In rat chromosome 15, the MHCβ gene is located upstream to the MHCα gene, which has a classic pTRE. It was reported that, in a T3-dependent manner, TR-RXR heterodimers at pTRE of the MHCα promoter may activate not only MHCα transcription but also synthesis of anti-sense RNA against the MHCβ gene, resulting in the antagonism of MHCβ expression. However, this kind of mechanism has not been reported in other negatively regulated genes including the TSHβ or the αGSU genes.

8. Artificial negative regulation by T3/TR

There have been at least two technical problems that have hindered the elucidation of the mechanism of negative regulation by T3/T.

8.1 pUC/pBR322-derived AP-1 site

As shown above, liganded NHRs, including T3/TR and liganded GR, inhibit the transcriptional activity of AP-1 via the tethering mechanism. Unexpectedly, a functional AP-1 site was identified in nt. 1/138 of pUC-derived plasmids and its activity is repressed by T3/TR (Lopez et al., 1993). More than 2000 plasmid constructs bearing the sequence identical to nt. 1/138 in the pUC18/19 vector were detected in the BLAST database. Interestingly, our computer search revealed that this site is also included in the pBR322 vector (Yanisch-Perron et al., 1985). In early molecular biology studies, both vectors were often utilized in “home-made plasmids”. Unfortunately, this AP-1 site was contaminated in some of the plasmids used for the analysis of *TSHβ* negative regulation by T3 (Hallenbeck et al., 1993; Wondisford et al., 1989).

8.2 Firefly luciferase gene

The firefly luciferase assay has been utilized in a variety of analyses of transcriptional regulation, including negative regulation of the *TSHβ* and the *αGSU* genes due to its advantage over the CAT assay (Misawa et al.). However, at least in CV1 cells (Tillman et al., 1993), JEG3 cells (Maia et al., 1996) and Hepa1-6 mouse hepatoma cells (Chan et al., 2008), firefly luciferase cDNA has been reported to function as a transcriptional regulatory sequence that mediates artificial negative regulation by T3/TR. The length of firefly luciferase cDNA (1653 bp) is much longer than that of the CAT gene (657 bp). A computer search predicts more than 250 potential sites for DNA-binding transcription factors in firefly luciferase cDNA (Liu and Brent, 2008). Misawa et al. (Misawa et al.) recently found that firefly luciferase cDNA behaves as a transcriptional enhancer that can be stimulated by the protein kinase C activator, phorbol 12-O-tetradecanoate-13-acetate (TPA), and that this activity is inhibited by T3/TR in CV1 and JEG3 cells. The cDNA sequences of modified firefly luciferase (luc+) (Annicotte et al., 2001; Paguio et al., 2005) and conventional Renilla luciferase (RL) (Ho and Strauss, 2004; Osborne and Tonissen, 2002; Zhuang et al., 2001) also harbor numerous short sequences that can be recognized by a variety of transcription factors. Modified luciferase genes, including hRluc (Zhuang et al., 2001) and Luc2 (Paguio et al., 2005), may be more reliable than firefly luciferase (Misawa et al.), presumably because the majority of predicted transcription factor binding sites were mutated.

8.3 Problems with artificial negative regulation in the identification of the nTREs by reporter assays

When a strong promoter is fused to firefly luciferase cDNA, the activity of this DNA sequence as a transcriptional regulatory element can be negligible. Nonetheless, one should remember that sequential deletion or mutation of the promoter sequence often reduces its transcriptional activity. Once the activity of the promoter becomes lower than that of the activity via the firefly luciferase gene, the overall activity of the reporter gene may represent that of the firefly luciferase cDNA, which can be artificially inhibited by T3/TR. For example, a deletion analysis to identify a nTRE in the *αGSU* gene was also carried out using the firefly luciferase reporter system in JEG3 cells (Madison et al., 1993). However, nTRE was not identified because T3-induced negative regulation was detected even a promoter that only has a TATA box and a TSS. There is the possibility that sequential deletion of DNA might reduce the transcription activity of the *αGSU* promoter, thereby permitting the firefly luciferase cDNA to function as a transcriptional regulatory element, resulting in artificial suppression by T3/TR. Using the luciferase reporter gene, it was previously proposed that the nTRE of the *TSHβ* gene may have a direct repeat configuration without spacing nucleotides (DR0) (Naar et al., 1991). The authors reported that a reduction in spacing nucleotide number may convert DR4 from a pTRE to a nTRE. Unexpectedly, this was not reproduced by the CAT-based reporter system (private communication from Dr. Kazuhiko Umesono). According to Tillman et al. (Tillman et al., 1993), deletion of spacing nucleotides might destroy the T3-dependent activation and allowed artificial repression by T3/TR via firefly luciferase cDNA.

Whereas firefly luciferase assay has broad linearity, careful interpretation and appropriate control are necessary in particular when the promoter activity before T3 addition is reduced in the course of deletion or mutation analysis (Misawa et al.). Although several nTREs in different genes have been suggested in the vicinity of TSSs, few of these reports excluded the possibility of T3-mediated artificial negative regulation by a reporter plasmid backbone.

9. TRβ2 is the main mediator for the negative regulation of the TSHβ gene by T3

There is one family with RTH in which the *TRβ* allele was globally deleted (Takeda et al., 1992). Elevated synthesis of TSH, i.e. SITSH, was found in a homozygote in this family, presumably due to the defect in TR signaling in the thyrotrophs. Likewise, mice deficient for the *TRβ* gene exhibit increased expression of *TSHβ* and *aGSU* in thyrotrophs (Forrest et al., 1996). These findings imply the involvement of TRβ in the negative regulation of these genes. Moreover, Abel et al. (Abel et al., 1999) reported that TRβ2-null mice develop a similar degree of central resistance to T3 similarly to TRβ-null mice, suggesting that, among TRβ1, TRβ2 and TRα1, TRβ2 is the main mediator for the inhibition of the *TSHβ* gene. This notion is compatible with our findings that the expression of TRβ2 is much higher than TRα1 or TRβ1 (Nakano et al., 2004) in the thyrotroph cell line, TαT1 (Yusta et al., 1998). The fact that no resistance to T3 is observed in mice deficient for the *TRa* gene (Fraichard et al., 1997; Wikstrom et al., 1998) indicates that TRα1 in thyrotrophs has a limited role in the negative feedback of the *TSHβ* gene by T3. It should be noted, however, that serum TSH levels in *TRβ*/*TRa*-double knockout mice (Gothe et al., 1999) is higher than *TRβ*-null mice (Forrest et al., 1996). Hence, the negative regulation of the *TSHβ* gene is partially mediated by TRα1 *in vivo*.

10. What is the mechanism that maintains the basal transcriptional activity of the *TSHβ* gene before T3 addition?

It is apparent that the negative regulation of the *TSHβ* gene by T3/TR can be observed only when its promoter is activated prior to T3 addition. However, previous studies have paid little attention to the mechanism of activation because some of the hypotheses mentioned above regard the basal transcriptional activity of the *TSHβ* gene to be maintained by unliganded TR. In addition, due to the limitation of the cell lines that recapitulate the thyrotroph phenotype (Ooi et al., 2004; Sarapura et al., 2002), the nTRE of the *TSHβ* gene has been studied using either kidney-derived 293 cells (Wondisford et al., 1989), COS cells (Carr et al., 1992) or somatotroph-derived GH3 cells (Sasaki et al., 1999). Of note, even in the presence of overexpressed TR, the magnitude of the basal transcriptional activity of the *TSHβ* gene prior to T3 addition is extremely low in these cell lines (Sasaki et al., 1999; Wondisford et al., 1989), and is almost negligible compared with that observed in the presence of the thyrotroph-specific transcription factors, Pit1 and GATA2 (Nakano et al., 2004) (see below).

11. Unliganded TR per se is not a transcriptional activator for the *TSHβ* gene

Because negative regulation has been regarded as the mirror image of the positive regulation, unliganded TR was thought to be a transcriptional activator (Wondisford et al., 1989). If this were the case, *TSHβ* and *aGSU* expression would be reduced in mice lacking the *TR* gene irrespective of serum T3 and T4 levels. However, as described above, their expression was not reduced but rather was increased in mice deficient for the *TRβ* gene (Abel et al., 1999; Forrest et al., 1996; Weiss et al., 1997) or both *TRa* and *TRβ* genes (Gothe et al., 1999). In the family with RTH, in which the *TRβ* allele is globally deleted (Takeda et al., 1992), elevation of serum TSH was also found in the homozygotes of this family. This again

suggests that unliganded TRβ is not necessary for the activity of the *TSHβ* promoter in human and that the *TSHβ* gene is activated by factors other than unliganded TR.

12. GATA2 and Pit1 maintain basal *TSHβ* expression in thyrotrophs

It is known that a pituitary-specific transcription factor, Pit1 (Fig 5A), plays a critical role in *TSHβ* expression since its mutation causes combined pituitary hormone deficiency (CPHD), where the syntheses of TSHβ, prolactin and growth hormone are crippled or abolished (Cohen and Radovick, 2002).

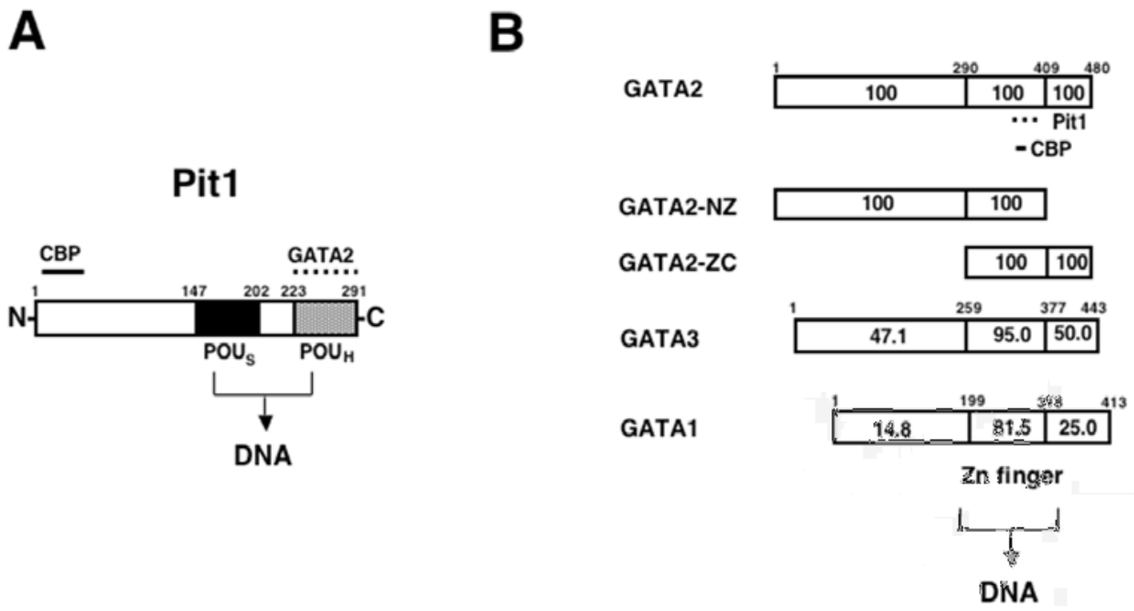

Fig. 5. Schematic representations of Pit1 (A) and GATA1, 2 and 3 (B). A. CBP, amino acid sequence interacting with CBP. POU_S, POU specific domain. POU_H. POU homeodomain. GATA, amino acid sequence interacting with GATA2. B. Structure of GATA1, 2, 3, GATA2-NZ and GATA2-ZC (see text). The numbers within the box represent the amino acid homology (%).

Promoter analysis of the *TSHβ* gene in TSHoma cells, TtT97, revealed that nt. -269 from the TSS is sufficient for thyrotroph-specific expression of this gene (Wood et al., 1990). As shown in Fig. 4, a functional Pit1-binding site is included in this region (Haugen et al., 1993) and was designated as Pit1-US (Kashiwabara et al., 2009). Interestingly, comparison of the pattern of DNA foot printing using nuclear extracts from TtT97 cells and that from GH3 cells revealed that this promoter region also has binding sites for the transcription factor, GATA2 (Fig 4 and 5B) (Gordon et al., 1997), which was originally identified to be involved in the gene regulation of a hematopoietic cell lineage (Shimizu and Yamamoto, 2005). Indeed, there are two GATA-responsive elements (GATA-REs) immediately downstream of the Pit1-US (Gordon et al., 1997). Subsequent analysis with various transgenic mice revealed that co-expression of Pit1 and GATA2 is crucial for the differentiation of thyrotrophs (Dasen et al., 1999).

13. GATA2, not Pit1, is the true activator that drives the promoter activity of *TSHβ*

Kashiwabara et al. (Kashiwabara et al., 2009) reported that the co-operation of Pit1 with GATA2 is strictly determined by the number of nucleotides between the Pit1-US and GATA-REs (Fig 4), and suggested the possibility that a configuration of the Pit1-US and GATA-REs may be critical for the recruitment of CBP/p300 (Fig 6).

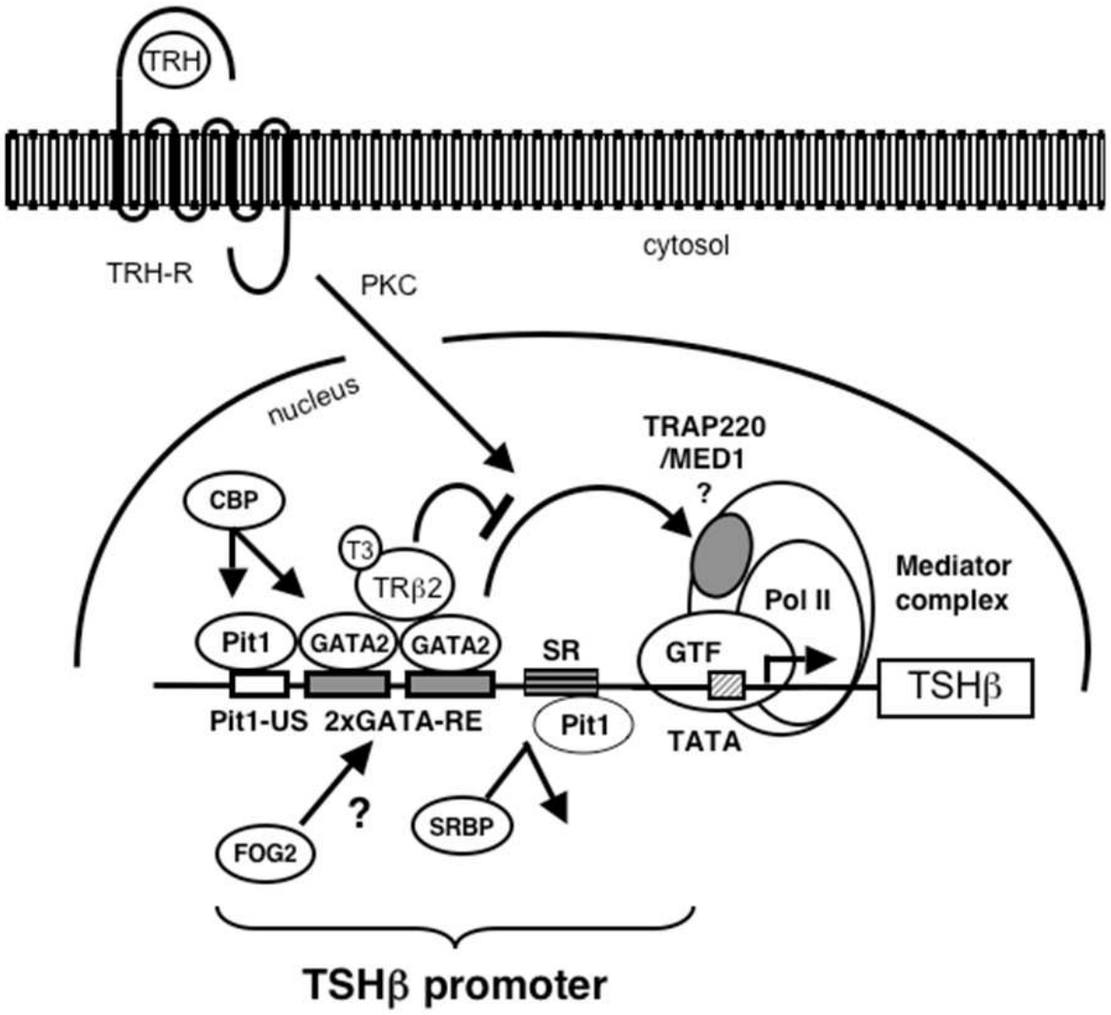

Fig. 6. Molecular mechanism of the transcriptional regulation of the TSHβ gene. A configuration of the Pit1-US and GATA-REs may be critical for the recruitment of CBP/p300. T3/TR represses the GATA2-dependent activation of the *TSHβ* promoter via a tethering mechanism. Pit1 binds with the Pit1-like element in SR and competes with SR binding protein (SRBP), resulting in protection of GATA2 functionality from inhibition by SR (de-repression). TRH-R, TRH receptor, FOG2, friend of GATA 2.

The authors also noticed that a 30bp region downstream from the GATA-REs is highly conserved among rat, mice and humans, and includes a sequence similar to the Pit1-binding site (Pit1-like element, Fig. 4). This sequence was designated the suppressor region (SR) because its deletion increased the transactivation by GATA2 and Pit1. Interestingly, deletion of the SR enabled GATA2 to transactivate the *TSHβ* gene without Pit1. Detailed analysis revealed that Pit1 binds with the Pit1-like element in the SR (Fig. 4) and competes with binding of SR binding protein (SRBP), resulting in protection of the GATA2 function from inhibition by SRBP (Fig 6). Thus, cooperation of Pit1 with GATA2 is not synergistic, but Pit1 protects GATA2 from inhibition by SR (de-repression). These findings not only provide an insight as to why *TSHβ* expression is restricted in thyrotrophs where Pit1 and GATA2 co-exist, but also imply that the true activator that drives the *TSHβ* promoter activity is GATA2 but not Pit1 (Kashiwabara et al., 2009).

14. Negative regulation of the *TSHβ* gene is not the mirror image of positive regulation

Gordon et al. (Gordon et al., 1997) reported that the *TSHβ* gene can be activated by Pit1 and GATA2 in CV1 cells. Because CV1 cells are kidney derived and has been utilized in the studies of positive regulation by T3/TR (Naar et al., 1991; Tillman et al., 1993; Umesono et al., 1991), Nakano et al. (Nakano et al., 2004) tested whether negative regulation of *TSHβ* by T3 may be simulated when TR is co-expressed with Pit1 and GATA2 in this cell line. They employed the CAT-based reporter gene, *TSHβ*-CAT (Sasaki et al., 1999), in which the pUC/pBR322-derived AP-1 element (Lopez et al., 1993) was deleted. Using this experimental system, Nakano et al. (Nakano et al., 2004) found the following results. First, T3-induced inhibition of the *TSHβ* gene was readily observed in CV1 cells transfected with Pit1, GATA2 and TRβ2. This implies that T3-induced negative regulation of the *TSHβ* gene does not require so-called thyrotroph specific factors except for Pit1, GATA2 and TR. Second, T3-induced inhibition was also detected with all three functional TRs, TRβ1, TRβ2 and TRα1, with TRβ2 exhibiting the most potent T3-dependent inhibition among them. This observation again supports the notion that TRβ2 is the principal TR that mediates negative regulation of the *TSHβ* gene (Abel et al., 1999). Third, without Pit1 or GATA2, unliganded TR did not transactivate the *TSHβ* promoter at all. This implies that unliganded TR alone is not the transcriptional activator. This notion is in line with the results of data from *TRβ*-knockout mice (Forrest et al., 1996) and *TRβ/TRa*-double knockout mice (Gothe et al., 1999). Therefore, in negative regulation of the *TSHβ* gene, T3/TR is a transcriptional repressor, but unliganded TR per se is not an activator. This is in contrast with positive regulation (Fig. 3A), where the T3-target genes are activated by T3/TR while it is repressed by unliganded TR (silencing). These findings run counter to the hypothesis that the negative regulation of this gene may be a mirror image of its positive regulation (Wondisford et al., 1989).

15. T3/TR represses GATA2-dependent activation of the *TSHβ* promoter via the tethering mechanism

The putative nTRE was defined by analysis of the *TSHβ* promoter in the absence of T3 (Wondisford et al., 1989). Because this was based on the hypothesis that unliganded TR

may be a transcriptional activator, Matsushita et al. (Matsushita et al., 2007) re-evaluated the function of nTRE by deletion analysis of this promoter (Fig. 4) in the presence of Pit1, GATA2 and TR. Unexpectedly, we found that repression of the *TSHβ* promoter by T3/TRβ2 was maintained after the nTRE is completely deleted or mutated. Thus, the reported nTRE (Fig. 4) is dispensable for T3/TR-dependent inhibition. Moreover, repression by T3/TR was also observed even in a deletion construct that has only Pit1-US and two GATA-REs. These findings suggest that direct DNA binding of TR is unnecessary and that the mechanism for T3-dependent inhibition may be mediated by the crosstalk of T3/TR with Pit1 or GATA2 (Matsushita et al., 2007).

As mentioned above, the true activator that drives the TSHβ promoter is GATA2 but not Pit1 (Kashiwabara et al., 2009) and the deletion of SR enables GATA2 to transactivate the *TSHβ* promoter without Pit1. Using the reporter gene lacking for SR, Matsushita et al. (Matsushita et al., 2007) found that T3/TRβ2 inhibits the transactivation by GATA2 alone. Thus, GATA2 is thought to be the target of inhibition by T3/TR (Fig. 6). This notion is supported by the observation that T3/TRβ2 inhibits GATA2-induced activation of the *αGSU* promoter and the endothelin-1 (*ET-1*) promoter, both of which are known to bear a functional GATA-RE (Jorgensen et al., 2004; Steger et al., 1994; Dorfman et al., 1992). In addition, T3/TR inhibited the *CD34* gene-derived GATA-RE fused to a heterologous thymidine kinase promoter (Matsushita et al., 2007). Co-immunoprecipitation experiments and GST-pull down assays demonstrated that the DBD of TRβ2 interacts with the Zn finger domain of GATA2 *in vivo* in a T3-independent manner. Thus, the TR-DBD is involved in protein-protein interactions with GATA2 but not in direct binding of DNA (Matsushita et al., 2007). These results indicate that negative regulation of the *TSHβ* gene is mediated by tethering of T3/TR by GATA2 (Fig. 6).

16. Ligand/receptor specificity in negative regulation of the *TSHβ* gene

As discussed above, ligand/receptor specificity has been reported in ligand-dependent inhibition via the tethering mechanism (Caldenhoven et al., 1995; Liden et al., 1997). Matsushita et al. (Matsushita et al., 2007) found that GATA2-induced activity of the *TSHβ* promoter was specifically inhibited by T3/TR but not by RA/RAR or vitamins D3 (VD3)/VDR. This may reflect ligand selectivity *in vivo* in negative regulation of the *TSHβ* gene (Cohen and Wondisford, 2005). Of note, it is known that estrogen (E2) inhibits expression of the *TSHβ* gene (Cohen and Wondisford, 2005) at the transcriptional level, although its magnitude is smaller than that by T3. E2 is also known to reduce expression of the *αGSU* gene (Chaidarun et al., 1994; Shupnik et al., 1988), the promoter of which has a functional GATA-RE (Jorgensen et al., 2004; Steger et al., 1994). In agreement, the serum level of TSH in women has a tendency to elevate after the menopause (Nagayama et al., 2008). To explore the molecular mechanism underlying inhibition by E2, Nagayama et al. (Nagayama et al., 2008) tested the effect of E2-bound ERα (E2/ERα) using CV1 cells cotransfected with Pit1 and GATA2, and found that E2/ERα significantly inhibits activity of the *TSHβ* promoter. As predicted, the magnitude of inhibition by E2/ERα was approximately half of that by T3/TRβ2. They also found that E2/ERα directly interacts with GATA2, as shown for GATA1. Testosterone was reported to have the effect similar to estrogen, presumably due to the conversion of testosterone to estrogen (Ahlquist et al.,

1987). This may explain why elevation of serum TSH level is also found in aged men (Surks and Hollowell, 2007).

17. The role of GATA2 and TR in TRH signaling in thyrotrophs

TRH is processed from prepro-TRH and secreted from the hypothalamus (Fig. 1A). TRH signaling not only stimulates TSH secretion but also enhances expression of *TSHβ* and *aGSU* (Franklyn et al., 1986; Shupnik et al., 1986). To clarify the role of TRH-induced transactivation and T3/TR mediated inhibition in the hypothalamic-pituitary-thyroid (H-T-P) axis, various in *vivo* studies, including genetic ablation of these genes, have been performed (Forrest et al., 1996; Friedrichsen et al., 2004; Gothe et al., 1999; Mittag et al., 2009; Nikrodhanond et al., 2006; Shibusawa et al., 2000). Although the *in vivo* evidence observed in these experiments is definitely important, the experimental system using cultured cells has the advantage that the effect of individual hormone can be analyzed in detail without influence by a negative feedback loop.

17.1 TRH signaling-promoted *TSHβ* expression is mediated by GATA2

Although serum TSH levels are reduced in patient with CPHD who have mutations in the Pit1 gene (Cohen and Radovick, 2002), the involvement of Pit1 downstream of TRH signaling has been controversial (Ohba et al., 2011; Steinfelder et al., 1992a; Steinfelder et al., 1992b). Unfortunately, previous analyses have been performed without consideration of GATA2. Interestingly, the increase in *TSHβ* expression in hypothyroidism was impaired in mice with pituitary-specific ablation of the GATA2 gene (Charles et al., 2006). Because TRH synthesis in the hypothalamus is expected to increased in hypothyroidism (Fig. 1A) (Abel et al., 2001; Kakucska et al., 1992), this finding suggested the involvement of GATA2 in the TRH signaling pathway.

Using CV1, GH3 and TαT1 cells, Ohba et al. (Ohba et al., 2011) recently reported that TRH signaling potentiates GATA2/Pit1-induced transcriptional activity of the *TSHβ* gene. Additionally, experiments with a *TSHβ* promoter that lacks SR revealed that GATA2 but not Pit1 is the target of TRH signaling (Fig. 6). Similar results were obtained with GATA2-induced activation of the *aGSU* and *ET-1* promoters. It is known that the signal from TRH receptor activates protein kinase C (PKC) (Gershengorn and Osman, 1996; Sun et al., 2003). The PKC pathway is also known to enhance DNA binding of GATA2 with GATA-RE in the *aGSU* (Fowkes et al., 2002) and the *V-CAM1* promoters (Minami et al., 2003). Gel shift assays also suggested that DNA binding of GATA2 with the *TSHβ* promoter is facilitated by the TRH/PKC pathway (Ohba et al., 2011). Thus, GATA-REs seem to be the point of convergence for both activation and inhibition signals controlling TSHβ transcription. Although it has been postulated that TRH signaling in transactivation of the *TSHβ* gene may be mediated by unliganded TR on nTRE (Wondisford et al., 1993), Ohba et al. (Ohba et al., 2011) showed that unliganded TR without Pit1 or GATA2 failed to mediate the stimulating effect by TRH on this promoter and that reported nTRE (Fig. 4) is dispensable for activation of the TRH-induced transcription.

17.2 TRH-dependent activation vs. T3/TR-induced repression

The in vitro data demonstrated by Ohba et al. (Ohba et al., 2011) correlate well with the *in vivo* findings (Fig. 7).

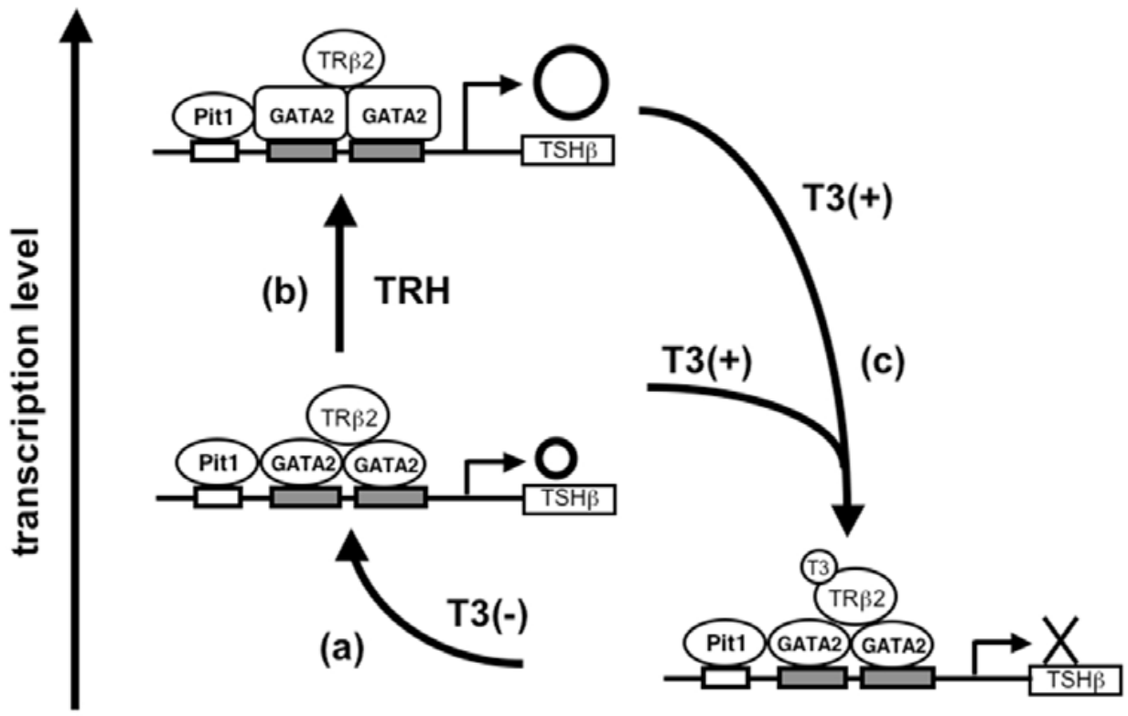

Fig. 7. Schematic representation of transcriptional regulation of the *TSHβ* gene by TRH signaling and T3/TR. With support by Pit1, GATA2 maintains the basal transcription of the *TSHβ* gene and mediates TRH/TRH-R1 signaling in hypothyroidism, while unliganded TR alone is not a transcriptional activator. Inhibition by T3/TR is dominant over activation by GATA2 even in the presence of TRH signaling. The release of T3/TR-induced repression (a) is more crucial for *TSHβ* expression than TRH signaling (b) since the inhibition by T3/TR is dominant over the stimulation by TRH (c). DNA binding of GATA2 with the *TSHβ* promoter is facilitated by the TRH pathway (b).

First, they showed that as long as T3 is at low concentrations or absent, expression of the *TSHβ* gene is maintained by Pit1 and GATA2 without stimulation by TRH signaling (Fig. 7(a)) (Gordon et al., 1997; Kashiwabara et al., 2009; Ohba et al., 2011). In agreement with this, the signaling and the number of TSHβ-positive cells in the pituitary of TRH-deficient mice were comparable with those of wild-type mice (Shibusawa et al., 2000). Second, given that unliganded TR is not a transcriptional activator, elevation of *TSHβ* expression in hypothyroidism should depend on TRH signaling but not on unliganded TR (Fig. 7(b)). Nikrodhanond et al. (Nikrodhanond et al., 2006) compared *TSHβ* expression in wild-type, TRH-, TRβ- and TRH/TRβ-double knockout mice and found that, in hypothyroidism, TSH expression predominantly depends on TRH signaling but not by unliganded TRβ. Since the authors regarded unliganded TRβ as the stimulator for the *TSHβ* gene, they mentioned that their findings were unexpected. However, their results are in agreement with the notion that unliganded TR is not the activator or mediator for the *TSHβ* gene in the absence or presence of TRH signaling. Finally, our data suggested that, in *TSHβ* transcription, the inhibitory effect by T3/TR is dominant over the TRH-induced stimulation (Fig. 7(c)) (Ohba et al., 2011). In accordance with this, an earlier study with human subjects indicated that continuous injection of TRH cannot release the inhibition of serum TSH in thyrotoxicosis (Chan et al.,

1979). Taking advantage of the fact that the Pax8-null mouse is an excellent animal model for congenital hypothyroidism (Friedrichsen et al., 2004), Mittag et al. (Mittag et al., 2009) demonstrated that thyrotroph differentiation in Pax8/TRH-R double-knockout mice is comparable with that in the hypothyroidism of mice homologous for a Pax8-null allele. Their results support the notion that release of T3/TR-induced inhibition (Fig. 7(a) is more critical for *TSHβ* expression than TRH signaling (Fig. 7(b)) because inhibition by T3/TR is dominant over stimulation by TRH (Fig. 7(c)).

18. Mechanism of T3/TR interference with GATA2 transactivating function

Negative regulation of the *TSHβ* gene is expected to provide an excellent experimental model to study transcriptional regulation since this promoter is activated by TRH signaling and repressed by T3/TR (Fig. 6). An important next step would be to investigate how T3/TR interferes with the transactivation function of GATA2. As pointed out above, the involvement of RXRs, TR-related coactivator (p160) or co-repressors (NCoR, SMRT) has been controversial. We favor another possibility; that TR may regulate the function of GATA2-related cofactors in a T3-dependent manner.

In pituitary-specific GATA2-null mice (Charles et al., 2006), the defect in *TSHβ* expression was partial and GATA3 expression was increased. Thus, GATA3 may be able to compensate for the reduction in GATA2 expression and there may be functional redundancy between GATA2 and GATA3. Amino acid homology between the Zn-finger domains of GATA1, GATA2 and GATA3 is well conserved (Fig. 5B) and plays a pivotal role in DNA recognition as well as cofactor interaction (Bates et al., 2008; Shimizu and Yamamoto, 2005). Consistent with this, our *in vitro* results show that GATA1, GATA2 and GATA3 have the capacity to mediate cooperation with Pit1 (Kashiwabara et al., 2009), TRH signaling-induced transactivation (Ohba et al., 2011) and inhibition by T3/TR (Matsushita et al., 2007). All these properties were also observed in the deletion mutant of GATA2 that lacks an N-terminal domain (GATA2-ZC) or a C-terminal domain (GATA2-NZ) (Fig. 5B). These findings suggest a critical role of the Zn-finger domain in GATA2 (GATA2-Zf) in *TSHβ* gene regulation. Besides CBP/p300, TR-associated protein (TRAP) 220/MED1 and Friend of GATA (FOG) 1 or 2 are known to interact with this domain. Of course, there is the possibility that other unknown factors may play a critical role in negative regulation by T3/TR and that there is interplay among various histone modifications to achieve local control of *TSHβ* gene transcription. Although the chromatin immunoprecipitation (ChIP) assay is expected to provide important information, the amount of endogenous GATA2 in TαT1 cells may not be sufficient for this approach (Ohba et al., 2011).

18.1 TRAP220/MED1 and Mediator complex

TRAP220/MED1 is a constituent of the Mediator complex that directly regulates the function of RNA polymerase II (Pol II) (Fig. 6) (Chadick and Asturias, 2005). The following findings indicate the involvement of TRAP220/MED1 in transactivation by the GATAs (Fig. 6). First, *in vitro* experiments show that TRAP220/MED1 interacts with GATA2-Zf (Gordon et al., 2006). Second, homozygous TRAP220/MED1-null mice are embryonic lethal due to an abnormality in cardiac function and its phenotype is reminiscent of that observed in mice deficient for the GATA family transcription factors (Crawford et al., 2002). Third, expression

of the *TSHβ* gene is reduced in heterozygous TRAP220/MED-knockout mice (Ito et al., 2000), suggesting that TRAP220/MED1 is required for expression of the *TSHβ* gene, which is GATA2-dependent. Interestingly, TRAP220/MED1 possesses two LXXLL motives, which functions as an interface for interaction with the TR-LBD in a T3-dependent manner. Mutant TRAP220/MED1, which only has two LXXLL motives but lacks other transactivation domains, is reported to function as a dominant negative inhibitor against wild-type TRAP220/MED1 in T3-dependent positive regulation via TR-RXR heterodimers on the pTRE (Yuan and Gambee, 2000). Matsushita et al. (Matsushita et al., 2007) found that mutant MED1/TRAP220 also attenuates T3-induced inhibition of the *TSHβ* gene. It was reported that mutant TRAP220/MED1 specifically interferes with the activity of wild-type TRAP220/MED1 but not other LXXLL-type co-activators including the p160 family and CBP/p300 (Acevedo and Kraus, 2003). Thus, there is the possibility that TR may regulate the activity of a complex containing GATA2 and TRAP220/MED1 in a T3-dependent fashion. Given that inhibition by T3/TR targets the final step of GATA2-induced transactivation, i.e. TRAP220/MED1-Pol II complex, repression might occur downstream of or at the same point as TRH-induced activation. This may account for the findings that inhibition by T3/TR is dominant over TRH-induced activation of the *TSHβ* gene (Mittag et al., 2009; Ohba et al., 2011). *In vivo* experiments with mouse embryonic fibroblasts showed that the requirement of TRAP220/MED1 may be specific to TR, but not to RAR or VDR (Ito et al., 2000). Intriguingly, the binding of different activators triggers a specific conformational change in the Mediator complex, which may have a critical role in the regulation of Pol II (Chadick and Asturias, 2005).

18.2 FOG2 and chromatin-remodeling factors

The co-repressor, friend of GATA (FOG) 1 or 2 (Cantor and Orkin, 2005) may be involved in T3/TR-induced negative regulation via GATA2. FOG1 and FOG2 interact with the N-terminal Zn finger of the GATAs and recruit chromatin-remodeling factors (Fig. 6) (Hong et al., 2005; Roche et al., 2008; Rodriguez et al., 2005). In addition, FOG2 is expressed in non-hematopoietic tissues and interacts with TRβ (Rouf et al., 2008) and other NHRs (Clabby et al., 2003; Huggins et al., 2001). Matsushita et al. (Matsushita et al., 2007) generated a mutant GATA2 (C295A) which is predicted to have impaired interaction with the FOGs. Although the basal transcriptional activity of this mutant was also reduced (by approximately half) compared with wild-type GATA2, inhibition by T3/TR (fold repression) was significantly relieved in mutant GATA2.

19. Molecular mechanism of SITSH in RTH

As described above, patients with RTH exhibit SITSH (Refetoff et al., 1993). The mutant TRβ found in RTH patients is supposed to interact with GATA2 because it has an intact DBD, which is the interface for the Zn-finger domain of GATA2. Nakano et al. (Nakano et al., 2004) tested whether mutant TRβ2s identified in RTH patients exhibit a dominant negative effect on the negative regulation of the *TSHβ* gene using CV1 cells cotransfected with Pit1, GATA2 and wild-type TRβ2. As predicted, mutant TRβ2s blunted the T3-induced inhibition of the *TSHβ* gene by wild-type TRβ2. Although these findings are likely to be the result of dominant-negative interference of wild-type TRβ function by mutant TRβ, further studies are required with regard to its molecular mechanism. Following questions also remain. First,

in patients with non-TR RTH (see above), no genetic abnormalities in the *TRβ* or *TRa* genes have been identified. Although linkage analysis of these patients showed no relation with NCoR, SMRT, SRC-1 or RXRγ (Reutrakul et al., 2000), an understanding of the transcriptional control mechanisms underlying non-TR RTH may provide an insight into the molecular basis of negative regulation of the *TSHβ* gene. Second, RTH is clinically classified as a generalized resistance to thyroid hormone (GRTH) and resistance of the pituitary to thyroid hormone (PRTH) (Refetoff et al., 1993). Patients with PRTH possess mutations similar or identical to those found in GRTH; however, PRTH patients display greater resistance to thyroid hormone in thyrotrophs compared to peripheral tissues, resulting in thyrotoxicosis. Currently, the mechanism of pituitary-dominant resistance in PRTH is unknown (Nakano et al., 2004).

20. Mechanism of logarithmic alterations in serum TSH by linear changes of T3/T4

TSH synthesis in the pituitary is dramatically altered by subtle changes in serum T3 and T4. Indeed, linear changes in the concentration of serum T4 and T3 correspond to logarithmic changes in serum TSH (Fekete and Lechan, 2007; Fisher et al., 2000; Kakucska et al., 1992). Such a sensitive alteration of *TSHβ* expression may be necessary for thyroid hormone homeostasis (Fig. 1A) because TSH signaling is thought to be one of the most critical determinants of T3 and T4 synthesis in the thyroid gland. In other words, serum TSH level has been regarded as most sensitive clinical marker for thyroid gland function. Indeed, SITSH is an important indicator for RTH.

With regard to the molecular mechanism of logarithmic changes in serum TSH, there are the following possibilities. First, T3/TR negatively regulates not only *TSHβ* and *aGSU* but also the prepro-TRH gene (Fig. 1A). Thus, the dual inhibitory mechanism at the hypothalamus and the pituitary may be important for the non-linear change of serum TSH level. Second, Pit1 expression may be negatively regulated by T3 (Sanchez-Pacheco et al., 1995). Because this was found in a somatotroph cell line, GH3 (Ooi et al., 2004), which lacks endogenous GATA2 (Gordon et al., 1997), an unknown pituitary factor may be involved in this inhibition. Third, expression of GATA2 in thyrotrophs may also be negatively regulated by T3. It was reported that there are two promoters in the *GATA2* gene, and that the distal one contains a GATA-RE (Kobayashi-Osaki et al., 2005). Therefore, there may be a positive feedback mechanism in the expression of *GATA2* and this mechanism is thought to be crucial in the differentiation of hematopoietic cell lineages. There is the possibility that, in thyrotrophs, T3/TR may interfere with the transactivation function of GATA2 not only on the *TSHβ* promoter but also on the GATA2 promoter. In addition, GATA2 protein may be quickly degraded by the ubiquitin system (its half life is approximately 30 min) (Minegishi et al., 2005). This may also contribute the drastic regulation of serum TSH level. Finally, there is the possibility that T3-induced inhibition of prepro-TRH expression in the hypothalamus may also be logarithmic (Fekete and Lechan, 2007; Kakucska et al., 1992). Although an nTRE was postulated in the prepro-TRH promoter (Hollenberg et al., 1995), the molecular mechanism of logarithmic inhibition of prepro-TRH expression by T3 is unknown.

The diagnoses of both subclinical hypo- and subclinical hyper-thyroidism also depend on the sensitive change in serum TSH level. Although serum free T3 and T4 levels are within normal range, subclinical hypo- or hyperthyroidism influence lipid metabolism (Walsh et

al., 2005b) and cardiovascular function (Walsh et al., 2005a). In their pathogeneses, there may be the abnormality in the transcription of the gene which is negatively regulated by T3 as robustly as the TSHβ gene.

21. Other genes negatively regulated by T3/TR

Microarray analyses revealed that approximately 30 to 50% of T3-target genes are negatively regulated (Feng et al., 2000; Weitzel, 2008). Therefore, elucidation of negative regulation by T3 is thought to be the next frontier.

21.1 Reported nTREs in other T3-negatively regulated genes

In addition to the *TSHβ* gene, negative regulation by T3/TR has been reported in the genes for αGSU (Chatterjee et al., 1989; Pennathur et al., 1993), MHCβ (Edwards et al., 1994; Wright et al., 1999), prepro-TRH (Hollenberg et al., 1995; Satoh et al., 1996), RSV-LTR (Saatcioglu et al., 1993), rat Na, K-ATPase α3 subunit (Chin et al., 1998), Nm23-H1 (Lin et al., 2000), phospholamban (PBL) (Belakavadi et al., 2010), rat CD44 (Kim et al., 2005), superoxide dismutase-1 (Santos et al., 2006), deiodinase type 2 (Dio2) (Christoffolete et al., 2006) and β-amyloid precursor protein (Villa et al., 2004). In some of these genes, the existence of single half-sites homologous to the *TSHβ* nTRE have also been postulated (Chatterjee et al., 1989; Chin et al., 1998; Edwards et al., 1994; Hollenberg et al., 1995; Kim et al., 2005; Lin et al., 2000; Pennathur et al., 1993; Saatcioglu et al., 1993; Santos et al., 2006; Villa et al., 2004; Wright et al., 1999). However, there are few experimental studies that show the molecular mechanism by which these putative nTREs reverse the function of T3/TR from transcriptional activator to repressor. In *TRβ*- and/or *TRα1*-deficient mice, the expression of *αGSU* (Forrest et al., 1996; Gothe et al., 1999) in the pituitary, *prepro-TRH* in the hypothalamus (Abel et al., 2001; Dupre et al., 2004) and *MHCβ* in the heart (Mansen et al., 2001) are maintained, suggesting that, as in the case of the *TSHβ* gene, the basal activities of these genes are also maintained by a transcriptional activator other than unliganded TR. Thus, existence of nTREs in these genes should also be reconsidered.

21.2 Possible involvement of the tethering mechanism

If T3-dependent inhibition of these genes occurs via a tethering mechanism between a DNA-binding transcription factor and T3/TR, identification of such a transcription factor may provide an insight into the molecular mechanism of T3-induced inhibition. As discussed above, a functional GATA-RE in the *αGSU* promoter may be the target of suppression by T3/TR. GATA-REs are also predicted in the promoters of *Dio2* (Dentice et al., 2003), *MHCβ* (Hasegawa et al., 1997; Morimoto et al., 1999) and *PBL* (Belakavadi et al., 2010). *Dio* activity in the thyrotrophs regulates the intracellular concentration of T3, which is the determinant of negative regulation of the *TSHβ* gene (Escobar-Morreale et al., 1996). Although *Dio1* and *Dio2* are expressed in thyrotrophs, the inhibitory effect on TSH by T3 was relieved in mice deficient for *Dio2* but not *Dio1* (St Germain et al., 2005), indicating the crucial role of *Dio2* in the regulation of T3 concentration in the thyrotroph. Further studies may clarify the role of the predicted GATA-RE in the *Dio2* promoter (Dentice et al., 2003) and the complexity of the T3 sensing mechanism in regulation of the *TSHβ* gene (Christoffolete et al., 2006). Although it was previously reported that the GATA-RE in the *MHCβ* gene plays a role in its transcriptional activity (Hasegawa et al., 1997; Morimoto et al., 1999), other investigators suggested that it may not be functional (Vyas et al., 1999). Another study of the *MHCβ*

promoter using rat neonatal cardiomyocytes suggests that the M-CAT site in this promoter is critical for its expression (Flink et al., 1992). M-CAT is the recognition site for the TEF family of transcription factors (Yoshida, 2008). TEF family transcription factors are the major target of α1 adrenaline signaling (Chen et al., 2004), which is known to mimic the cardiac phenotype seen in heart failure (Yoshida, 2008). Consistently, overexpression of TEF-1 in vivo exhibits a phenotype similar to that of chronic heart failure (Tsika et al., 2010). Our preliminary data suggests that T3/TR inhibits TEF-dependent transactivation of the *MHCβ* gene.

22. Negative regulation by liganded NHRs other than TR

A tethering mechanism has been reported in genes that are negatively regulated by liganded NHRs other than T3/TR. For example, the proopiomelanocortin (POMC) gene is activated by a transcription factor, Nur77, which is also the mediator of corticotropin-releasing hormone (CRH) signaling (Maira et al., 2003). Liganded GR interferes with this activity via a tethering mechanism (Martens et al., 2005). Moreover, a recent report suggested the involvement of chromatin remodeling factors in inhibition of the POMC gene by liganded GR (Bilodeau et al., 2006). Expression of parathyroid hormone (PTH) is inhibited by liganded VDR. PTH expression is maintained in the mice deficient for the VDR gene (Kim et al., 2007), suggesting that unliganded VDR is not the transcriptional activator for the PTH gene. A DNA-binding transcription factor, VDR interacting repressor (VDIR), binds with the promoter region of the *PTH* gene and activates its transcription (Kim et al., 2007). It was reported that VDR associates with VDIR (Kim et al., 2007; Murayama et al., 2004), resulting in VD3-dependent inhibition. A tethering mechanism between liganded VDR and VDIR also plays a role in negative regulation of human 1α(OH)ase (CYP27B1) expression by VD3 (Murayama et al., 2004). In this scenario, a chromatin remodeling factor complex (Kitagawa et al., 2003), and a DNA methylation-related proteins (Kim et al., 2009) may play crucial roles.

23. Conclusion

Negative feedback is the key component in homeostasis of hormones. A typical example is the inhibition of TSH synthesis by T3/TR. Although serum TSH levels are increased in hypothyroidism, observations in TR-knockout mice (Forrest et al., 1996; Gothe et al., 1999), human subjects with a deletion of the *TRβ* gene (Takeda et al., 1992) and *in vitro* experiments (Nakano et al., 2004) provide evidence that unliganded TR is not a transcriptional activator (Fig. 7). Moreover, deletion analysis of the *TSHβ* gene with co-expression of GATA2 and Pit1 revealed that a putative nTRE (Fig. 4) is dispensable for inhibition by T3/TR (Matsushita et al., 2007). Study of the *TSHβ* gene suggests the importance of a transcription factor that maintains the basal transcriptional activity of the promoter before ligand addition (Fig. 7). Identification of the factor required for the basal promoter activity may also be important and the first step in the analysis of other promoters that are repressed by T3/TR or other liganded NHRs. The factor may interact with NHRs. Once such a transcription factor is identified, it will be possible to study negative regulation using cells that express the factor and to carry out reporter analysis with co-transfection of its expression plasmid. For example, it will be possible to compare the mechanisms of positive and negative regulation using same cell line, for instance CV1 (Nakano et al., 2004). Information of the

factor required for the basal promoter activity would be helpful to avoid artificial negative regulation mediated by plasmid backbones (for example a pUC/pBR322-derived AP-1 site or firefly luciferase cDNA). We are only just beginning to unravel some of complexities involved in negative regulation by liganded NHR including T3/TR.

24. References

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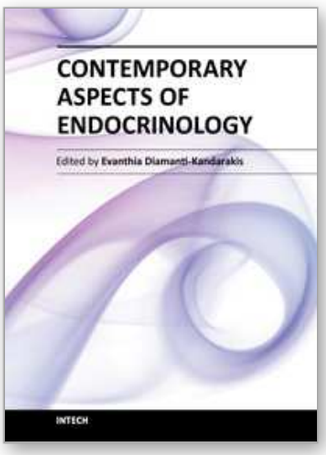

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This book aims to provide readers with a general as well as an advanced overview of the key trends in endocrine disorders. While covering a variety of topics ranging from thyroid carcinogenesis and pituitary adenomas to adrenal tumors and metabolic bone disease, this book also focuses on more specific issues not yet fully elucidated (e.g. the molecular pathways involved in thyrotropin beta gene regulation or monogenic phosphate balance disorders). Readers of different fields and background will have the opportunity to update their knowledge and more importantly to clarify areas of uncertainty and controversies in several topics of endocrine disorders.

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