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Review

Thyroid-Specific Transcription Factors

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Introduction

The thyroid is the unique organ that synthesizes thyroid hormones. At least four thyroid-specific proteins are involved in the synthesis of the hormones: thyroglobulin (TG), a precursor of thyroid hormones; thyroperoxidase (TPO) that catalyzes the iodination and coupling of the specific tyrosine residues within the TG molecule; a receptor for thyroid-stimulating hormone (TSHR), and sodium-iodide symporter that transports iodide into the cells. TSH regulates the expression of these four proteins thereby controlling the production of thyroid hormones.

Tissue-specific gene expression is often regulated at the transcriptional level by DNA-binding, *trans*-acting factors that are themselves tissue-specific. Recently two thyroid-specific transcription factors, TTF-1 and Pax-8, have been identified and demonstrated to play an important role in the thyroid-specific expression of TG, TPO and TSHR genes. It has also been shown that these transcription factors participate in hormonal regulation of the expression of the thyroid-specific genes. In this review we summarize the physiological roles of thyroid-specific transcription factors, especially TTF-1 and Pax-8.

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Cloning of Thyroid-Specific Transcription Factors

A 170-base pair promoter region of the TG gene containing the transcription start site was demonstrated to be necessary and sufficient for the thyroid-specific expression of the TG gene [1]. In this minimal TG promoter, three binding sites for TTF-1 were identified [2] as shown in Fig. 1. By sequence-specific DNA affinity chromatography, rat TTF-1 protein was purified, and then its cDNA was cloned by screening a library with a degenerate oligonucleotide corresponding to the amino acid sequence of the purified protein [3].

Rat TTF-1 consists of 372 amino acids and contains a homeodomain that was originally identified as a sequence-specific DNA-binding domain of homeotic gene products in *Drosophila* [3]. The TTF-1 homeodomain is homologous to those of NK-2 in *Drosophila* [4] and of Dth-1 and Dth-2 in *planaria* [5]. TTF-1 is also called NKx-2.1, since TTF-1 is the first homolog to the NK-2 gene family in mammals. Thyroid-enhancer binding protein (T/EBP) that was identified as binding to the thyroid-specific enhancer in human TPO gene [6] is identical to TTF-1 [7].

The TTF-1 gene is located on chromosome 14q13 in human and 12pc in mouse [3]. The rat TTF-1 gene consists of two exons and a single intron, and the coding sequence is separated by an intron [8]. The structure of the human TTF-1 gene is similar to that of the rat one [9]. In the 5′-flanking region of the rat TTF-1 gene, a putative TTF-1 binding site is present, suggesting that the transcription of the TTF-1 gene is regulated by its own gene product [8]. The expression of TTF-1 gene in adult rat is restricted to the thyroid and the lungs [3].

Pax-8 belongs to the paired-domain transcription

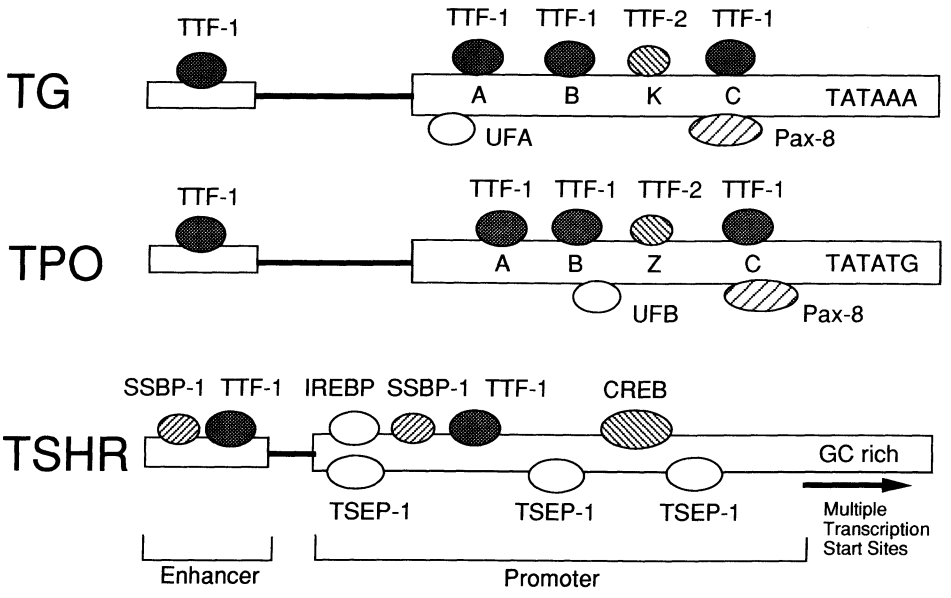

Fig. 1. Thyroid-specific expression of TG, TPO and TSHR genes. The *cis*-acting elements present in the enhancer and promoter sequences of TG, TPO and TSHR genes, and their binding proteins are schematically represented. TTF-1 binds to three different sites named A, B and C sites, in the TG and TPO promoters. TTF-2 binds to K and Z sites in the TG and TPO promoters, respectively. The Pax-8-binding site overlaps to the C site for TTF-1. TTF-1 binding sites are also present in the enhancers of both genes. In the TSHR gene, TTF-1 binding sites are present in the enhancer and promoter sequences. The binding sites for CREB, SSBP-1 (single-strand DNA-binding protein-1), TSEP-1 (TSHR suppressor element-binding protein-1), and insulin-responsive element-binding protein (IREBP) are indicated.

factors called Pax [10]. The paired domain, a DNA-binding domain [11], was originally identified in *Drosophila* segmentation gene products, and its amino-acid sequence is well conserved among Pax families reported [10, 12, 13]. Pax-8 cDNA was isolated from a mouse-embryo library by the method of low stringent screening with a cDNA encoding the paired-domain as a probe [14]. Mouse Pax-8 consists of 457 amino acids and contains a 128-amino-acid paired domain at the N-terminal of the molecule [14]. Pax proteins usually have a homeodomain as well as a paired-domain [13], but Pax-8 only possesses a partial homeodomain, the first α-helix of the domain. This is a common characteristic of Pax-2, Pax-5 and Pax-8 [13]. They thus belong to a subclass in Pax proteins. The Pax-8 genes in mouse [14] and in human [15] are mapped onto chromosomes 2p and 2q, respectively. The human Pax-8 gene contains at least 12 exons and 11 exons encode the translated region of Pax-8 [16].

Since the expression of Pax-8 in adult mouse was detected only in the thyroid and kidneys [14], the possible involvement of Pax-8 in the expression of thyroid-specific genes was studied. It was demonstrated that Pax-8 binds to the TG and TPO promoters and increases the transcription from the promoters [17, 18]. The transactivation domain exists in the C-terminal region of the Pax-8 molecule [19]. Recently several Pax-8 mRNA isoforms were identified in human thyroid, kidney and placenta [16, 20]. These isoforms are generated by alternative splicing, resulting in the inclusion or exclusion of exon 8 and/or exon 9 sequences. The products of these isoforms have a complete paired domain, but different transactivation domains, and thus display different transactivating properties. In addition, the alternative splicing of Pax-8 primary transcripts is temporally and spatially regulated during early mouse development [20].

Involvement of Thyroid-Specific Transcription Factors in the Expression of Thyroid-Specific Genes

A, B and C sites in the rat minimal TG promoter in Fig. 1 represent the binding sites for TTF-1. The unique binding site for Pax-8 overlaps to the C site for TTF-1, and the binding of Pax-8 and TTF-1 is mutually exclusive [17]. Another thyroid-specific transcription factor TTF-2 and ubiquitous factor UFA recognize K and A sites, respectively [2]. The cDNAs of both factors have not been cloned yet. If any one of the binding sites is disrupted, no full promoter activity is observed [2] so that all of these tissue-specific and ubiquitous transcription factors are required for the maximal transcription of the TG gene in the thyroid cells.

Interestingly it has been demonstrated that the binding sites for TTF-1, TTF-2 and Pax-8 align in a similar manner on both TG and TPO promoters (Fig. 1) [18]. Ubiquitous factor UFB binds to the B site of the TPO promoter. This similar alignment of *cis*-acting elements indicates a common mechanism for thyroid-specific expression of TG and TPO genes. An *in vitro* study showed that Pax-8 activated TPO promoter better than TG promoter, whereas TTF-1 activated TG rather than TPO promoter [17]. Recently it was shown that Hepatocyte Nuclear Factor-3β (HNF-3β), a member of the fork-head family of transcription factors, participates in the expression of TPO promoter [21].

The TTF-1 binding sites were also identified in the enhancer regions in human TPO gene [6], and bovine [22] and human [23] TG genes. The binding of TTF-1 to these enhancers results in an increase in the promoter activities.

The molecular mechanisms for TSHR gene expression have also been studied in detail. As shown in Fig. 1, several *cis*-acting elements and their binding proteins are defined in the rat TSHR gene [24]. The cAMP-responsive element (CRE) in the promoter is the essential element for the constitutive transcription of the gene. The transcription factors interacting with all other elements appear to exert their effects on CRE thereby regulating the TSHR gene expression. The TTF-1 binding sites are present in both promoter and enhancer regions [25, 26]. When the TTF-1 binding site in the promoter was mutated, the promoter showed decreased activity in thyroid cells [27]. TTF-1 is thus required for the maximal expression of the TSHR gene in the thyroid cells. Taken together, these findings indicate that thyroid-specific transcription factors play a pivotal role in the expression of thyroid-specific genes.

TSH-Dependent Regulation of the Expression of Thyroid-Specific Genes via Thyroid-Specific Transcription Factors

The expression of TG and TPO genes in rat thyroid FRTL-5 cells is increased by TSH at the transcriptional level [28]. Most, if not all, TSH actions are mediated by the cAMP-protein kinase A (PKA) pathway which is activated after the interaction of TSH with TSHR. However, no canonical CRE appears to be present in rat TG and TPO promoters. It was reported that the DNA binding to A and C regions of the TG promoter was increased by TSH and cAMP, and that the proteins that bind to the regions were neither CREB (CRE-binding protein) nor AP-1 [29, 30]. We examined which transcription factors was involved in the hormonal regulation of TG promoter activity in FRTL-5 cells [31]. Our results showed an increase in the binding activities of Pax-8, TTF-1 and TTF-2 by TSH. It was also reported that the TTF-2 binding was increased by TSH, but TTF-1 binding was not changed [32, 33].

On the other hand, despite the increased DNA binding, we observed a decrease in the TTF-1 and Pax-8 mRNA levels, suggesting that their binding activities might be regulated in the posttranslational steps. A similar finding was obtained in a study on the effect of interferon (IFN)-γ on TTF-1 [34]. The IFN-γ-dependent decrease in TSHR gene expression was associated with a decrease in TTF-1 binding activity, but the mRNA and protein levels of TTF-1 were not altered by IFN-γ.

The expression of the TSHR gene is transiently increased and then decreased by the addition of TSH to FRTL-5 cells [35, 36]. The transient increase seems to be mediated by TTF-1 [25]. Its DNA binding is increased by TSH via phosphorylation of the molecule by PKA (see below). The subsequent decrease in the expression of the TSHR gene is explained, at least in part, by a decrease in the binding activity of TTF-1 [25] whose mRNA

level is decreased by TSH [7, 25].

These studies indicate that thyroid-specific transcription factors are important for the hormonal regulation of the expression of TG, TPO and TSHR genes as well as for their tissue-specific expression. However, the hormonal effects on TTF-1 binding are controversial and therefore remain to be defined.

As mentioned above, TSH decreases the TTF-1 mRNA level in FRTL-5 cells, but in a primary culture of dog thyrocytes, forskolin did not alter the TTF-1 gene expression [37]. It was also shown that forskolin increased the Pax-8 mRNA and protein levels in dog thyrocytes [38]. These results indicate the species difference in the regulation of TTF-1 and Pax-8 expression by TSH/cAMP.

TSH promotes the growth of thyroid cells. It was shown that the introduction of antisense DNA for TTF-1 or Pax-8 into FRTL-5 cells inhibited the increase in thymidine-incorporation and in the number of cells caused by TSH, indicating that thyroid-specific transcription factors play a role in the TSH-dependent proliferation of the thyroid cells [39].

Posttranslational Modification of Thyroid-Specific Transcription Factors

Numerous studies have shown that posttranslational modification such as phosphorylation is important in modifying protein function. It was shown that treatment of the extracts of FRTL-5 cells cultured in the absence of TSH with catalytic subunit of PKA resulted in an increase in the DNA-binding activity of TTF-1 [25]. In addition, overexpression of the catalytic subunit of PKA in the cells increased the TTF-1-dependent transactivation. These results indicate that the phosphorylation of TTF-1 by PKA enhances its binding activity and *trans*-activating function. Nevertheless, a recent report showed that the binding activity and *trans*-activating ability of TTF-1 were not altered by the phosphorylation, although five to seven serine residues on the TTF-1 molecule were phosphorylated in FRTL-5 cells cultured in the presence of TSH [40]. In dog thyrocytes, an increase in cAMP did not alter the phosphorylation state of TTF-1 [37] or Pax-8 [38]; so it is still unclear whether phosphorylation actually plays a role in the hormonal regulation of TTF-1 and Pax-8 functions.

It has recently been shown that DNA-binding activities of a number of transcription factors are regulated by the reduction-oxidation (redox) state of the cells [41–47]. Reversible transition between oxidation and reduction of the critical cysteine residue(s) in the factors modifies their binding activities. We therefore examined a possible involvement of redox regulation as a posttranslational modification of thyroid-specific transcription factors [48]. Our study showed that treatment of Pax-8 and TTF-1 with oxidizing or reducing agents drastically altered their binding activities *in vitro*. It was also demonstrated that the increased DNA-binding activities of Pax-8 and TTF-1 due to their reduction were at least partially responsible for the TSH-dependent increase in the TG promoter activity in FRTL-5 cells. We next examined whether an intracellular reducing catalyst thioredoxin (TRX) is involved in the redox regulation of Pax-8. In FRTL-5 cells, TRX mRNA was increased by TSH. Co-transfection of the plasmids expressing TRX and Pax-8 increased the TG promoter activity, demonstrating that TRX could be a candidate in TSH-mediated redox regulation of Pax-8.

Arnone *et al.* showed the redox regulation of TTF-1 and defined Cys-87 and Cys-363 as redox-sensitive cysteines [49]. The redox regulation of TTF-2 was also demonstrated [50]. The binding of purified TTF-2 was increased by the reduction. It is of particular interest that all known thyroid-specific transcription factors are modified by the redox control mechanism. Considering that TSH induces hydrogen peroxide production [51, 52], the redox regulation could be important for thyroid cell development and function.

Organogenesis and the Thyroid-Specific Transcription Factors

As shown in Fig. 2, the thyroid rudiment develops from a median endodermal thickening of the ventral wall of the primitive pharynx (cranial gut) between days 10.5 and 11.5 p.c. (post coitum) in rat embryo [53]. This thickening, located beneath the posterior part of the tongue anlage, then migrates caudally. The migration is completed by

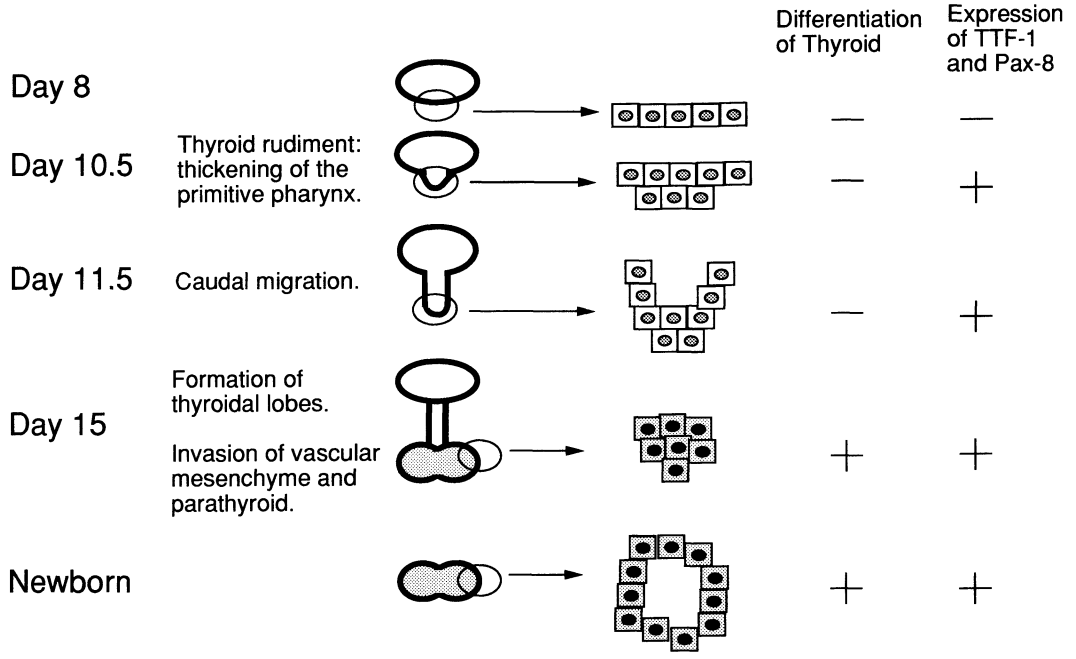

Fig. 2. Thyroid development and the expression of TTF-1 and Pax-8 in rat.

day 13.5 p.c. The parathyroids and vascular mesenchyme join the thyroid at day 14.5 p.c. At this stage, the thyroid forms a lobular shaped structure. Between days 15 and 17 p.c., primitive follicular structures appear in the thyroid lobes. TG, TPO and TSHR mRNAs are detected by day 15.5 p.c. TSH secreting cells appear in the anterior pituitary gland at day 16–17 p.c. Synthesis of thyroid hormones begins at day 17 p.c. TTF-1 mRNA and protein [53], and Pax-8 mRNA [14] are detected at the onset of the thyroid morphogenesis at day 10.5 p.c., suggesting that both factors may play an important role in the commitment of the thyroid cell precursors.

The lung also originates in the cranial gut. TTF-1 expression is detected in the lung bud at day 10.5 p.c., suggesting an essential role of TTF-1 in the development of the lung [53]. Several studies have demonstrated the involvement of TTF-1 in the expression of pulmonary-specific proteins. The promoter of surfactant protein-B gene contains two binding sites for TTF-1 [54]. These TTF-1 sites and a neighboring HNF-3 binding site are necessary for the tissue-specific expression of the gene. In the promoter of surfactant protein-A gene, four functional TTF-1 binding sites are identified [55].

In the gene of Clara cell secretory protein (CCSP), another lung cell-specific protein, TTF-1 binding sites are defined both in the promoter [56] and enhancer [57]. TTF-1 and ubiquitous transcription factors Sp1 and Sp3 increase the promoter activity [56]. Interestingly, it is shown that a cardiac muscle-specific homeobox protein (CSX) [58], which is also called NKx2.5 because of its similarity to TTF-1 (NKx2.1) [59], binds to the regulatory elements for TTF-1 in the CCSP gene, and transactivates the promoter in a similar manner to that of TTF-1 [57].

It was recently reported that TTF-1 and HNF-3β are coexpressed in respiratory epithelial cells in human fetal lung and that HNF-3β activates the transcription of the TTF-1 promoter [60]. On the other hand, it was also shown that ubiquitously expressed Octamer-binding protein-1 (Oct-1), one of the POU-domain transcription factors, recognizes the TTF-1 promoter [61].

The expression of Pax-8 is also observed in the developing excretory system [14, 15]. Its expression is increased at the stage of condensed mesenchyme, comma-shaped and S-shaped body, following a decline in the Pax-2 expression.

TTF-1 and Pax-8 expressions are demonstrated in the central nervous system. The expression of TTF-1 is restricted to the hypothalamic area of the

diencephalon and to the infundibulum at the earliest stage of their differentiation at day 10.5 p.c. [53]. The infundibulum forms the posterior lobe of the pituitary, the neurohypophysis. No signal is detected in the Rathke's pouch. Pax-8 is expressed transiently between days 11.5 and 12.5 p.c. along the rostrocaudal axis extending from the myelencephalon throughout the length of the neural tube [14]. It was recently shown that Pax-8 can activate the promoter of the neural cell adhesion molecule (N-CAM) gene [62]. The N-CAM gene is the first target gene for Pax-8 identified in cells other than the thyroid cells.

Last year TTF-1 (T/EBP) null mouse was established [63]. Heterozygous mice developed normally, whereas mice homozygous for the disrupted TTF-1 gene were born dead. The null mice lacked the lung parenchyma, the thyroid gland, the ventral region of the forebrain, and the entire pituitary. Since the expression of TTF-1 is detected in the infundibulum but not in Rathke's pouch in normal development [53], the absence of the entire pituitary implied that the presence of the infundibulum and/or TTF-1 expression in the infundibulum might induce Rathke's pouch to fully develop into the anterior and intermediate pituitary. The parathyroid was intact in the null mouse.

Group 3 Hox genes (HoxA3, HoxB3 and HoxD3) are expressed in the anterior neuroectoderm, branchial arches and their derivatives, including the thyroid and lung rudiments in mouse embryo. Mice homozygous for a HoxA3 knockout mutation exhibited abnormalities similar to those of the human congenital disorder DiGeorge's syndrome: a wide range of throat and craniofacial abnormalities including reduced thyroid tissues [64]. It was therefore examined whether the products of group 3 Hox genes regulate the TTF-1 gene expression [65]. It was shown that HoxB3 gene product can increase the TTF-1 promoter activity.

Differentiated Thyroid-Phenotype and the Thyroid-Specific Transcription Factors

Exposure of FRTL cells to v-K-ras induced inactivation of the TG gene expression and loss of the TTF-1 binding [66, 67]. When the temperature-sensitive variant of ras was denatured by warming the cells to 39 °C, TTF-1 binding and TG promoter activity were restored. Treatment of the nuclear extracts with PKA reactivated TTF-1, but after a four-week exposure to ras, denaturation of ras no longer restored TTF-1 binding or reactivated TG promoter. Treatment of the cells with 5-azacytidine reactivated TTF-1 and TG promoter, suggesting that the effect of persistent exposure to ras might be imprinted by methylation of the TTF-1 gene. It was subsequently reported that the PKC activated by v-K-ras inhibited the translocation of the catalytic subunit of PKA and, as a result, the activity of PKA was reduced to half [68]. This could account for the ras-dependent inactivation of TTF-1.

Transformation of FRTL-5 cells with v-H-ras resulted in a decrease in the TG and TPO gene expression in spite of the presence of active DNA binding form of TTF-1, but there was no TTF-2 binding in the cells [69]. This showed that TTF-2 also contributes to the expression of differentiated phenotypes in the thyroid cells.

p53 is a nuclear DNA-binding protein which plays a role in the negative control of cellular proliferation. In thyroid tumors, p53 mutations correlate with loss of the differentiated phenotype. Overexpression of mutated p53 in a differentiated thyroid cell line (PCCl3) abrogated TSH-dependency of this cell line and reduced iodide uptake and the expression of TG, TPO and Pax-8 genes, but TTF-1 expression was not affected [70]. Transfection of Pax-8 expressing plasmid into the cells restored the transcription from the TPO promoter. Conversely, when wild type p53 was introduced into a poorly differentiated papillary carcinoma cell line (NPA), Pax-8 was reexpressed together with TPO [71]. These results indicate that various factors are involved in the maintenance of differentiated phenotypes of the thyroid cells.

Human Diseases and the Thyroid-Specific Transcription Factors

TTF-1, Pax-8, TG and TPO mRNA levels were measured in nonmalignant and malignant human thyroid tissues [72]. The expression of TTF-1 and Pax-8 was not sufficient for the expression of a thyroid-differentiated phenotype. In follicular

adenomas, Pax-8 mRNA levels were strictly related to the TPO mRNA levels. TTF-1 mRNA was always detectable in papillary carcinomas and, in contrast, always absent from anaplastic carcinomas.

The expression of TTF-1 in various histopathological types of lung cancers was also examined [73]. TTF-1 expression was found in 7 of 23 cases of non-small cell lung carcinomas, but the expression did not correlate with the histological degree of differentiation. In small cell lung carcinomas, TTF-1 was always expressed, indicating that they might originate in the endodermal cell lineage.

Congenital human TG defect associated with low expression of TTF-1 was recently reported [74]. The propositus had a large goiter with low T4 and T3 levels and high TSH levels. Analysis of her goiter revealed that it contained a large amount of iodinated albumin instead of TG, and that the TG mRNA and protein levels were extremely reduced. The TTF-1 mRNA level and its binding activity were also reduced. In contrast, the levels of Pax-8 and TPO mRNAs were normal and increased, respectively. Although the cause of the low TTF-1 expression was not evaluated, it was considered that the defect in TG synthesis was due to impaired TTF-1 activity.

Conclusions

We summarized the studies of thyroid-specific transcription factors reported since their discovery in 1990. These studies allowed us to outline the molecular mechanisms for the development of the thyroid and lungs, and for the maintenance and regulation of their differentiated phenotypes: in which various transcription factors belonging to homeo-, forkhead- and paired-domain families, and ubiquitous factors play an important role in the expression and control of tissue-specific phenotypes.

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Addendum

After this paper was accepted for publication, cloning of TTF-2 cDNA was reported (*EMBO J* 16: 3185–3197, 1997).