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Thyroglobulin (Tg) induces thyroid cell growth in a concentration-specific manner by a mechanism other than thyrotropin/cAMP stimulation

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ABSTRACT

Thyroglobulin (Tg), a major product of the thyroid gland, serves as a macromolecular precursor of thyroid hormone biosynthesis. In addition, Tg stored in the thyroid follicles is a potent regulator of thyroid-specific gene expression. In conjunction with thyroid stimulating hormone (TSH) and iodide, Tg regulates thyroid follicle function, which is the minimal functional unit of the thyroid gland. In the present study, we show that Tg stimulates growth of FRTL-5 thyroid cells in the absence of TSH, insulin and serum. Unlike TSH, Tg did not increase cellular cyclic AMP (cAMP) levels; rather, the TSH signal counteracted Tg-induced cell growth. A specific inhibitor of A-kinase, H-89, did not modulate the effect of Tg. Tg increased kinase activity of Akt to the same level as TSH, insulin and 5% serum, while LY294002 abolished Tg-induced growth. Interestingly, low Tg concentrations maximized growth-promotion activity and induction of the apical iodide transporter (PDS; SLC26A4), whereas high Tg concentrations suppressed both cell growth and the expression of thyroid-specific genes. These results suggest that a low levels of Tg in the follicular lumen might stimulates cell growth and iodide transport to accelerate the iodide organification process; however, elevated Tg levels in the follicle might then shut down all of these functions.

Introduction

Thyroglobulin (Tg) is a large glycoprotein produced and stored in the follicular lumen of the thyroid gland. Its primary function is to serve as a macromolecular substrate for the coupling of iodide to its tyrosine residues during thyroid hormone biosynthesis [1]. In addition to this conventional role, follicular Tg acts as a potent autocrine regulator of follicular function and mediates the transcriptional suppression of genes essential for iodide transport and hormone synthesis [2,3]. The addition of follicular Tg to the culture medium of FRTL-5 thyroid cells dramatically decreased the RNA levels of thyroid-specific genes such as Tg, thyroid peroxidase (TPO), and the sodium/iodide symporter (NIS; SLC5A5) [2,3]. Tg suppressed iodide uptake in a dose-dependent manner in FRTL-5 cells *in vitro,* and the *in vivo* accumulation of Tg in the follicular lumen correlates with low iodide uptake in the rat thyroid [3]. Tg concentration used in these studies is in the lower range of follicular Tg in different follicles of the normal thyroid, which varies from 0.1 mg/ml up to 250 mg/ml as measured by aspiration biopsy or micropuncture of a single follicle [4–6]. The relatively large range in estimates of follicular Tg concentration is the result of significant functional heterogeneity among follicles.

Follicular Tg not only exhibits suppressive activity, but also increases mRNA levels and promoter activity of major histocompatibility complex (MHC) class I genes, and significantly induces the Pendred syndrome gene (PDS; SLC26A4), an apical iodide transporter in the thyroid [2,7,8]. NIS and PDS, basal and apical iodide transporters, respectively, are differentially regulated by Tg concentration and exposure time. One of the interesting differences between the suppressive and activating effects of Tg is that the former seems to occur in a dose-dependent manner, while the latter maximizes at a lower concentration of Tg [2,7,8]. It was therefore proposed that accumulated follicular Tg within the colloid is a major regulator of follicular function [7,9–12]. The physiological turnover of follicular function seems to be dynamically and coordinately regulated by Tg and TSH.

In addition to the effects of Tg on modulation of thyroid gene expression and function, it is evident that Tg also regulates cell growth and gene transcription in various cell types. In mink lung epithelial cells, Tg is activated by disuccinimidyl suberate (DDS) to act as an agonist of transforming growth factor (TGF)-β and

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suppress DNA synthesis, while untreated Tg functions as a TGF-β antagonist [13]. A short variant form of Tg is expressed in the kidney [14], and Tg is a potent growth promoter for cultured kidney mesangial cells and has TGF-β-like transcriptional activity [15,16].

This study presents a further investigation of the role of Tg on thyroid cell growth, which resulted in the creation of a model for Tg-regulated follicular turnover.

Materials and methods

Culture and treatment of cells. Rat FRTL-5 thyroid cells (ATCC CRL8305) were provided by the Interthyr Research Foundation, Woodinville, WA 98072, and maintained as reported previously [2,7]. The effect of Tg on cell growth was analyzed using FRTL-5 cells cultured for at least three days. The cells were shifted to control medium containing 0.2% serum, but no TSH and insulin, for 5 days before each experiment (Quiescent FRTL-5 cells). TSH, forskolin, dibutyl-cAMP (db-cAMP), insulin, 5% serum, or bovine thyroglobulin (all from Sigma, St. Lois, MO) was added for the indicated period of time.

[^{3}H]-thymidine (^{3}H-TdR) incorporation. DNA synthesis was assessed by ^{3}H-TdR incorporation as previously described [17]. Briefly, ^{3}H-TdR was added to cells cultured in 48-well plates at a concentration of 74 kBq/well and further incubated for the indicated period of time. Cells were then washed twice with Dulbecco's phosphate buffered saline (DPBS) and incubated with ice-cold 5% trichloroacetic acid for 20 min at room temperature. Radioactivity was measured in a liquid scintillation counter after solubilization with 0.5 N KOH. Cell-associated radioactivity was counted using a liquid scintillation counter.

Measurement of cAMP levels. cAMP levels were measured in the culture supernatant by a non-acetylation EIA procedure [18] using the cAMP Biotrak enzyme immunoassay system according to the manufacturer's protocol (GE Healthcare Bio-Science Corp., Piscataway, NJ). Cells were treated by 0.5 M 3-isobutyl-1-methylxantine (IBMX), a phosphodiesterase inhibitor.

Akt kinase assay. Akt kinase activity was measured using the Akt Kinase Assay Kit (Cell Signaling Technologies, Danvers, MA) according to the manufacturer's protocol. Briefly, cell lysates were prepared in ice-cold 1× Cell Lysis Buffer with 1 mM phenylmethlsulfonyl fluoride (PMSF). Immunoprecipitation was performed with immobilized Akt monoclonal antibody beads slurry and incubated overnight at 4 °C. The mixture was centrifuged, washed with 1× Cell Lysis Buffer and resuspended in 1× Kinase Buffer supplemented with 10 mM ATP and GSK-3 fusion substrate protein. Phosphorylation of GSK-3 was detected by Western blotting using the phospho-GSK-3α/β (Ser21/9) antibody.

Luciferase assay. The TPO promoter 5′-6300 in pGL3-Basic was transfected into FRTL-5 cells and luciferase reporter activity was measured as previously described [19,20].

RNA isolation and Northern blot analysis. Total RNA was purified, cDNA was synthesized and Northern blot analysis was utilized as previously described [2,21].

Statistical analysis. All experiments were repeated at least three times using different batches of cells. Statistical significance was evaluated using Student's *t*-test, with statistical significance defined as a p value of less than 0.05 ($p < 0.05$).

Other reagents. Other reagents were purchased from Sigma unless otherwise noted.

Results

Thyroglobulin (Tg) increases cell number and [^{3}H]-thymidine (^{3}H-TdR) incorporation of FRTL-5 thyroid cells

In the course of studying the effect of Tg on the gene expression of rat thyroid FRTL-5 cells, we noted an increase in cell number following the addition of Tg to culture medium in the absence of TSH [22]. The effect of Tg on the growth of thyroid cells was further elucidated using quiescent FRTL-5 cells maintained with no TSH, no insulin, and 0.2% rather than 5% serum. Arrest of the cell cycle at the G_0/G_1 phase in this condition was previously confirmed by ^{3}H-TdR incorporation and flowcytometric analysis [23]. The quiescent cells were stimulated with 5 mg/ml of bovine Tg or with TSH and insulin plus 5% serum and cell number was counted using a hemocytometer for up to 5 days after treatment. The increase in the number of cells treated with bovine Tg was comparable to treatment with TSH and insulin plus 5% serum (Fig. 1A). Additionally, evaluation of ^{3}H-TdR incorporation confirmed that the DNA synthesis induced by bovine Tg was comparable to that observed in complete medium containing TSH–insulin and 5% serum (Fig. 1B). However, the addition of Tg to the culture medium did not affect growth of the cancer cell lines (Fig. S1).

The specificity of Tg-induced growth promotion was examined by immunoprecipitating Tg with specific anti-Tg bound to protein G Sepharose beads. ^{3}H-TdR incorporation was significantly reduced when Tg was removed (Fig. S2A). Furthermore, when Tg was fractionated by gel filtration as described [24], the strongest effect on ^{3}H-TdR incorporation was obtained by 27S Tg tetramer, followed by the 19S dimer (Fig. S2B). These results support the evidence that the growth of FRTL-5 thyroid cells induced by bovine Tg is specific to the Tg protein itself, and is not the product of a contaminated unknown fraction in the Tg preparation.

The Tg-induced growth does not utilize the cAMP/PKA mediated pathway

To test whether Tg might increase the cAMP levels that mediate TSH action in thyroid cells, cAMP levels were determined in the cells treated with Tg in the presence or absence of TSH or insulin. Although TSH increased cAMP levels, Tg did not (Fig. 1C). The possible role of protein kinase A (PKA), which is regulated by cAMP, in Tg-mediated cell growth was studied by evaluating the effect of PKA inhibitor H-89, a synthetic isoquinoline sulfonamide [25]. H-89 significantly suppressed TSH-induced ^{3}H-TdR incorporation in FRTL-5 cells, but did not modulate Tg-induced ^{3}H-TdR incorporation (Fig. 1D).

Insulin/IGF-1 signaling is essential for thyroid cell growth [26,27]. Therefore, the effect of Tg on Akt, a downstream member of the insulin/IGF-1 signaling cascade was examined. Akt was first immunoprecipitated using a monoclonal anti-Akt antibody and an *in vitro* kinase assay was performed using GSK-3 fusion protein as a substrate. Tg was a strong activator of Akt in 30 min, an effect comparable to that of TSH, insulin, and serum, (Fig. 1E). Moreover, the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 not only abolished FRTL-5 cell growth induced by TSH, insulin, and serum, but also suppressed Tg-promoted growth (Fig. 1F). These results indicate that the effect of Tg is not mediated by the cAMP/PKA pathway, which is downstream of TSH action, but instead utilizes PI3K/Akt signaling downstream of insulin/IGF-1 action.

TSH/cAMP suppresses Tg-induced cell growth

TSH and insulin/IGF-1 are considered the major regulators of thyroid cell growth [26,27]. Although insulin and Tg worked together in an additive fashion to increase ^{3}H-TdR incorporation, TSH suppressed ^{3}H-TdR incorporation to lower levels than those induced by Tg alone or Tg plus insulin (Fig. 2A). Forskolin and db-cAMP were used to determine if this effect of TSH is mediated by cAMP. Both forskolin (Fig. 2B) and db-cAMP (Fig. 2C) suppressed ^{3}H-TdR incorporation of FRTL-5 cells. Especially, high concentrations of db-cAMP significantly inhibited insulin-induced ^{3}H-TdR

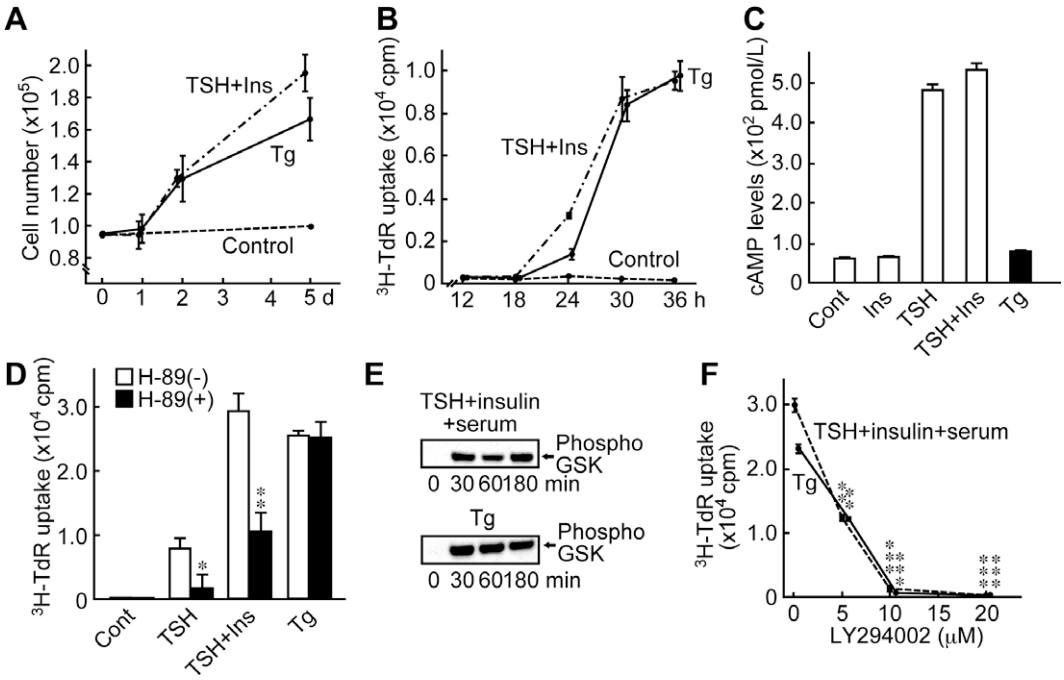

Fig. 1. Tg induces cell growth and DNA synthesis in FRTL-5 thyroid cells without using the cAMP/PKA mediated pathway. Quiescent FRTL-5 cells were treated with 5 mg/ml of bovine Tg or complete medium for up to 5 days and the number of cells were counted using a hemocytometer (A). Cells were similarly prepared and treated with Tg, complete medium or Tg plus complete medium for the indicated period of time before ^{3}H-TdR incorporation was measured (B). Quiescent FRTL-5 cells were treated with insulin (Ins) alone, TSH alone, TSH plus insulin or 5 mg/ml of bovine Tg for 12 h before cAMP levels were measured in culture supernatants (C). The effect of 1 μM of A-kinase inhibitor H-89 was assessed by ^{3}H-TdR incorporation 12 h later (D). Quiescent FRTL-5 cells were treated with complete medium or 5 mg/ml of bovine Tg for 30–180 min. Akt was immunoprecipitated using monoclonal anti-Akt antibody, then kinase activity was assessed using phosphorylation of GSK-3 fusion protein by Western blotting as described in the Materials and methods (E). Quiescent FRTL-5 cells were treated with either complete medium or 5 mg/ml of bovine Tg in the presence or absence of the PI3K inhibitor LY2094002 at the indicated concentration. ^{3}H-TdR incorporation was measured 12 h later (F). Data are expressed as the mean ± SD (n = 3). One asterisk (∗) indicates a value of $p < 0.05$, two asterisks (∗∗) indicate a value of $p < 0.01$ and three asterisks (∗∗∗) indicate a value of $p < 0.001$.

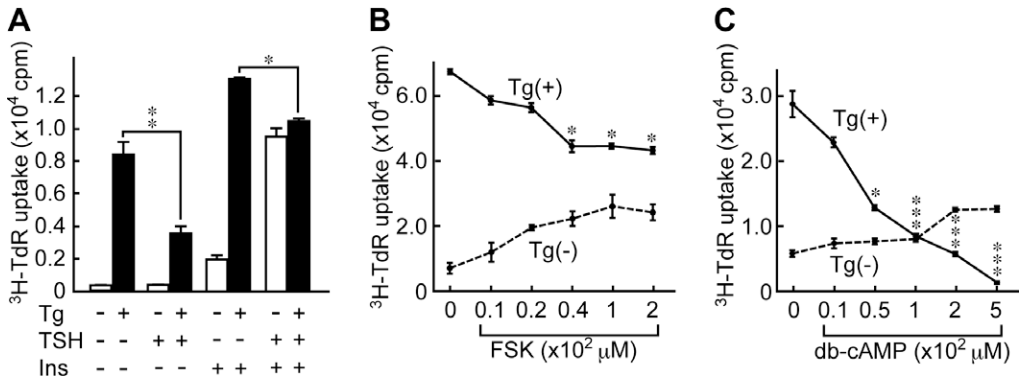

Fig. 2. TSH/cAMP signaling counteracts Tg-induced growth. Quiescent FRTL-5 cells were treated with either TSH or insulin in the presence or absence of 5 mg/ml of bovine Tg. ^{3}H-TdR incorporation was measured 12 h later (A). Quiescent FRTL-5 cells were cultured with insulin plus 5% serum in the presence or absence of 5 mg/ml of Tg with the indicated amount of forskolin (FSK). ^{3}H-TdR incorporation was measured 12 h later (B). Quiescent FRTL-5 cells were cultured with insulin plus 5% serum in the presence or absence of 5 mg/ml of Tg with the indicated amount of db-cAMP. ^{3}H-TdR incorporation was measured 12 h later (C). Data show the mean ± SD (n = 3). One asterisk (∗) indicates a value of $p < 0.05$, two asterisks (∗∗) indicate a value of $p < 0.01$ and three asterisks (∗∗∗) indicate a value of $p < 0.001$.

incorporation in the presence of Tg (Fig. 2C). These results suggest that the intracellular signaling cascade activated by TSH/cAMP inhibits the Tg-induced signaling pathway that stimulates thyroid cell growth.

Optimal Tg concentration shows biphasic pattern for growth promotion and suppression of gene expression

To evaluate the optimal concentration of Tg required for the thyroid cell growth, different concentrations of Tg were tested for their effect on ^{3}H-TdR incorporation in FRTL-5 cells. ^{3}H-TdR incorporation maximized at 5 mg/ml of Tg, but it was reduced at a higher concentration (Fig. 3A). In contrast, the thyroid-specific gene transcription accessed by TPO promoter activity was suppressed by increasing amounts of Tg (Fig. 3B). Similarly to the ^{3}H-TdR incorporation, mRNA expression levels of the apical iodide transporter PDS were maximal at 5 mg/ml of Tg and were suppressed at higher Tg concentrations (Fig. 3C). These data and previously reported results [2,7] suggest that the action of Tg on thyroid cell function as measured by gene expression and the growth, appears to be regulated by Tg in a concentration-dependent fashion.

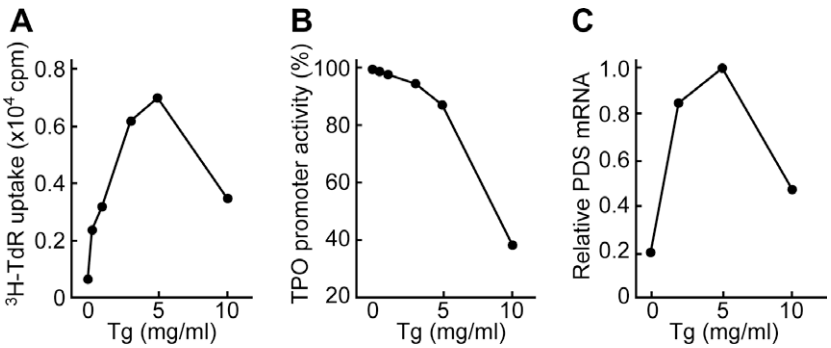

Fig. 3. The effect of Tg differs based on its concentration. Quiescent FRTL-5 cells were treated with the indicated concentration of bovine Tg. ^{3}H-TdR incorporation was measured 12 h later (A). FRTL-5 cells maintained in complete medium were transiently transfected with a luciferase reporter gene containing the TPO promoter (5′-6300 TPO pGL3-Basic) [19,20] and treated with the indicated concentration of Tg to obtain a measure of TPO promoter activity (B). FRTL-5 cells maintained in complete medium were treated with the indicated concentration of Tg. RNA was purified 24 h later and Northern blotting was performed and quantitated to assess PDS mRNA levels. Representative data from three independent experiments are shown.

Discussion

Thyroid cell growth is tightly regulated by the coordinate action of TSH and insulin/IGF-1 signaling [26,27]. Therefore, it was surprising to observe that Tg stimulated cell growth and ^{3}H-TdR uptake as strongly as TSH, insulin and serum. In such a situation, several possibilities should be carefully examined: (1) are similar pathways utilized for Tg-induced growth and TSH- or insulin-induced growth; (2) is the Tg action specific as opposed to an effect produced by contamination of the bovine Tg preparation; and (3) what is their relationship when present together, as they must be *in vivo*.

Although Tg stimulated cell growth, it did not increase cAMP levels in FRTL-5 cells. An inhibitor of cAMP-dependent protein kinase, H-89, reduced TSH-induced ^{3}H-TdR incorporation, but had no effect on Tg action. These data clearly show that Tg does not activate the cAMP signaling cascade in thyroid cells. Moreover, TSH/cAMP counteracted the action of Tg to induce cell growth or the converse. Therefore, the intracellular signaling utilized by Tg, which is still largely unknown, appears to be quite different from that used by TSH. Tg signaling in relation to insulin/IGF-1 is, in contrast, a more complex issue. Both Tg and insulin/IGF-1 activated Akt, a key downstream molecule that mediates insulin/IGF action. Similarly, a PI3K inhibitor LY294002 significantly suppressed Tg-induced ^{3}H-TdR incorporation. However, the cell growth induced by Tg was comparable to the maximal level induced by TSH, insulin, and serum, whereas insulin/IGF-1 in the absence of TSH could not induce such strong cell growth [26,27]. Furthermore, there is an additive action between Tg and insulin to stimulate cell growth. This suggests that Tg may utilize PI3 K/Akt to induce cell growth, but possibly by a mechanism that involves an as yet unknown signaling cascade in this process.

It is interesting that the stimulation of thyroid cells (cell growth and gene expression of PDS) is maximized by 5 mg/ml of Tg, while follicular function and growth is suppressed by higher concentration of Tg. These results suggest that there are at least two distinct mechanisms of Tg action that have different kinetics. Therefore, it might be plausible to speculate that different recognition systems exist rather than the possibility that the two different domains of Tg protein are responsible for the biphasic action. Unfortunately, the Tg recognition system and the detailed signaling pathways are still largely unknown. An understanding of these mechanisms will be particularly important because such machineries can be attractive targets for the development of new drugs to modulate thyroid function and growth.

Based on the results of this work and previous studies showing Tg-mediated regulation of thyroid-specific gene expression and iodide transport, we propose a revised model in which follicular function and growth is regulated by follicular Tg (Fig. 4). When follicular Tg storage levels are very low, thyroid-specific gene expression, such as NIS and Tg, are maximal (Fig. 4, top follicle). As a low concentration of Tg accumulates in the follicular lumen, cell growth and pendrin expression are turned on (Fig. 4, right follicle). This allows efficient transportation of iodide into the follicular lumen and expands the size of the follicle in order to synthesize and store thyroid hormone precursors in the follicle. When enough Tg accumulates in the follicle, the processes of gene expression, iodide transportation and cell growth are shut down (Fig. 4, left follicle). The follicle is full and ready to secrete thyroid hormones. The follicle must shrink after the decrease in follicular Tg content. Possible apoptosis or another mechanism for this process is yet to be determined. Nevertheless, the model also explains the basis of follicular heterogeneity.

It might also be worth noting that high concentrations of db-cAMP almost shut down Tg-induced ^{3}H-TdR incorporation. This effect might be construed to cause limited thyroid cell growth in Graves' disease or other thyrotoxicoses where cellular cAMP levels

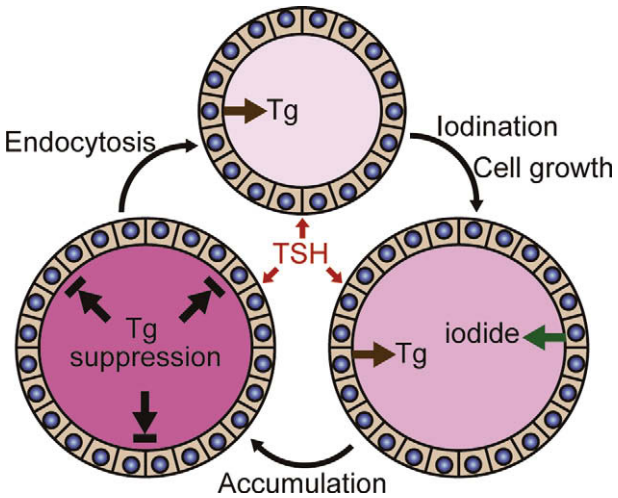

Fig. 4. Revised model for the regulation of follicular function and growth by accumulation of follicular Tg. When a low concentration of Tg accumulates in a follicle (lower right), cell growth is induced that will increase follicular size. Iodide transport increases due to maximal PDS gene expression. When sufficient Tg accumulates within a follicle (lower left), cell growth terminates and all gene expression necessary for iodide transport and hormone synthesis is suppressed. Such a follicle is then ready to supply thyroid hormones by a process of resorption, degradation and secretion, which results in a decrease of follicular Tg storage (top).

are significantly high, however Tg is very depleted in those follicles because of accelerated degradation, conversion to thyroid hormones, and secretion. This is therefore no apparent contradiction in function or the proposed model. Although the molecular mechanisms underlying such a strong growth stimulation by Tg are unknown, an imbalance or a dysregulation of such a mechanism could, nevertheless, relate to aberrant thyroid cell growth. For example, a gain-of-function mutation of the TSH receptor or Gsα subunit of G proteins leads to thyrotoxicosis due to toxic adenomas, toxic goiters or McCune-Albright syndrome [28,29]. Not all of these effects are as readily explained as above. Therefore, some of the thyroproliferative disorders with unknown etiology might be due to the abnormality of Tg-regulated cell growth. Studies of the molecular mechanism of Tg action might lead to a better understanding of thyroid physiology and pathology.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.11.158.

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