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Follicular thyroglobulin enhances gene expression necessary for thyroid hormone secretion

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Abstract. We have previously shown that follicular thyroglobulin (Tg) has an unexpected function as an autocrine negative-feedback regulator of thyroid hormone (TH) biosynthesis. Tg significantly suppressed the expression of genes necessary for iodide transport and TH synthesis by counteracting stimulation by TSH. However, whether follicular Tg also regulates intracellular TH transport and its secretion from thyrocytes is not known. In the present study, we examined the potential effect of follicular Tg on TH transport and secretion by quantifying the expression of two TH transporters: monocarboxylate transporter 8 (MCT8) and μ-crystallin (CRYM). Our results showed that follicular Tg at physiologic concentrations enhanced both the mRNA and protein expression levels of MCT8 and CRYM in a time- and dose-dependent manner in rat thyroid FRTL-5 cells. Although both the sodium/iodide symporter (NIS), an essential transporter of iodide from blood into the thyroid, and MCT8, a transporter of synthesized TH from the gland, were co-localized on the basolateral membrane of rat thyrocytes *in vivo*, Tg decreased NIS expression and increased the expression of MCT8 by counteracting TSH action. Thus, the effect of Tg on TH secretion opposed its previously described negative-feedback suppression of TH synthesis. Our results indicate that Tg mediates a complex intrinsic regulation of gene expression that is necessary to balance two opposing vectorial transport systems: the inflow of newly synthesized TH and the outflow of TH by external secretion.

Key words: Thyroglobulin, Thyroid hormone, MCT8, CRYM

THYROGLOBULIN (Tg), the most abundant product synthesized by thyrocytes and stored in the thyroid follicles, serves as the macromolecular protein backbone for thyroid hormone (TH) biosynthesis through covalent incorporation of iodide into its tyrosyl residues [1]. In addition to this well recognized function, Tg stored in the follicle has been shown to have an unexpected role as a negative-feedback regulator of TH synthesis [2-7]. Thus, in rat thyroid FRTL-5 cells and rat thyroid glands *in vivo*, follicular Tg suppressed the expression of *Tg*, the sodium/iodide symporter (NIS; *Slc5a5*), thyroid peroxidase (*Tpo*), dual oxidase 2 (*Duox2*), dual oxidase maturation factor 2 (*Duoxa2*), the thyrotropin receptor (*Tshr*), thyroid transcription factor 1 (TTF1; *Nkx2–1*), thyroid transcription factor 2 (TTF2; *Foxe1*), and paired box gene 8 (*Pax8*), all of which are essential participants in the 'assembly line' of TH production [2-6]. Indeed, Tg inhibited TSH-stimulated iodine uptake and H_2O_2 generation, which are essential for TH synthesis [2, 5, 6]. Such negative-feedback mechanisms have also been observed in primary cultures of normal human thyrocytes [7]. Based on these studies, it has become apparent that the homeostasis of TH synthesis is physiologically balanced by TSH-induced stimulation from the basal side of the thyroid follicular cells and the negative-feedback action of follicular Tg from the apical side.

The question of whether Tg affects the secretion of TH from thyrocytes has not been investigated to date. In contrast to the iodide uptake and TH synthesis processes, the mechanisms involved in the transport and secretion of synthesized TH in thyrocytes are not well

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understood, although TSH is known to stimulate these processes [8, 9]. Recently, energy-dependent transporters that mediate TH secretion have been identified [10, 11]. Monocarboxylate transporter 8 (MCT8; *Slc16a2*) is highly specific for TH and has been shown to be involved in the secretion of TH from thyrocytes [12, 13]. Cytosolic nicotinamide adenine dinucleotide phosphate (NADPH)-regulated TH binding protein, also known as μ-crystallin (CRYM), has been identified as one of the TH-binding proteins involved in intracellular TH transport [14], although its role in the thyrocyte is not clear. Therefore, we evaluated the effects of Tg on the expression levels of MCT8 and CRYM as specific TH transporters to elucidate the role of accumulated follicular Tg in TH secretion.

Materials and Methods

Cell culture and treatment

Rat thyroid FRTL-5 cells were grown in Coon's modified Ham's F-12 medium supplemented with 5% bovine serum (Invitrogen, Waltham, MA, USA) and a six-hormone mixture (1 mU/mL TSH, 10 μg/mL insulin, 10 ng/mL somatostatin, 0.36 ng/mL hydrocortisone, 5 μg/mL transferrin, and 2 ng/mL glycyl-L-histidyl-L-lysine acetate) as described [2, 6, 15]. Culture medium that did not contain TSH was also used in some experiments. Bovine Tg (Sigma-Aldrich, St. Louis, MO, USA) was used at a final concentration of 0.1-10 mg/mL [2, 3]. Bovine serum albumin (BSA) (Sigma-Aldrich) at the same concentrations as Tg was used as a control.

Total RNA isolation and real-time PCR

Total RNA was purified using RNeasy Plus Mini Kit (Qiagen, Hilden, Germany), and cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA, USA) as described previously [6]. Real-time PCR was performed using Fast SYBR Green Master Mix (Applied Biosystems) and StepOnePlus Real-Time PCR System (Applied Biosystems) according to the manufacturer's instructions. The sequences of PCR primers were as follows:
Slc5a5 forward, 5′-CTACCGTGGGTGGTATGAAGG-3′;
Slc5a5 reverse, 5′-TGCCACCCACTATGAAAGTCC-3′;
Slc16a2 forward, 5′-AACATGCGTGTATTTCGCCAGC-3′;
*Slc16a2*reverse,5′-GCAGGAATGAGAGGACCTGCAAG-3′;
Crym forward, 5′-AAGGAGGTGAGAATGTGGAACC-3′;
Crym reverse, 5′-CTACTGGCTCCAACAGCATTGA-3′;
Gapdh forward, 5′-ACAGCAACAGGGTGGTGGAC-3′;
and *Gapdh* reverse, 5′-TTTGAGGGTGCAGCGAACTT-3′.
The relative mRNA expression levels were normalized against *Gapdh* expression. Real-time PCR analysis was carried out at least in triplicate and repeated at least three times.

Protein preparation and Western blotting

Preparation of cell protein and Western blot analysis were performed as described [2, 6, 7]. Briefly, cells were lysed in a buffer containing 50 mM HEPES, 150 mM NaCl, 5 mM EDTA, 0.1% NP40 and 20% glycerol, followed by addition of protease inhibitor cocktail tablets (Complete Mini; Roche Diagnostics, Basel, Switzerland) at 4°C for 1 h. The mixture was centrifuged at 4°C for 20 min to recover cell protein. The protein concentration was determined using DC protein assay reagents (BIO-RAD, Hercules, CA, USA) and a VMax Kinetic Microplate Reader (Molecular Devices, Sunnyvale, CA, USA) according to the manufacturer's instructions. Western blotting was performed using 10 μg of protein extracts separated on NuPage 4-12% Bis-Tris gels (Invitrogen) and transferred to PVDF membranes as described previously [2, 6, 7].

The membranes were washed with PBS containing 0.1% Tween 20 (PBST), blocked with PBST containing 5% nonfat milk for 1 h, and then incubated with rabbit anti-NIS antibody (provided by Dr. N. Carrasco; dilution 1:1000), rabbit anti-MCT8 antibody (LSBio, Seattle, WA, USA; 1:1000), rabbit anti-CRYM antibody (Proteintech, Chicago, IL, USA; 1:200), or goat anti-β-actin antibody (Santa Cruz Biotechnology, Dallas, TX, USA; 1:2000) at 4°C for 12 h. After washing with PBST, membranes were incubated with either donkey anti-rabbit IgG-biotin conjugates (GE Healthcare, Little Chalfont, UK 1:2000) or donkey anti-goat IgG-biotin conjugates (Millipore, Bellerica, MA, USA; 1:2000) for 1 h. After washing, the membranes were incubated with streptavidin horseradish peroxidase (GE Healthcare; 1:20000) for 1 h. Specific bands were visualized using Immunostar LD reagent (Wako, Osaka, Japan) and analyzed using a C- DiGit blot scanner (LI-COR, Lincoln, NE) according to the manufacturer's instructions.

Double immunofluorescence staining

Paraffin-embedded rat thyroid sections were deparaffinized in xylene and rehydrated through ethanol,

followed by washing with deionized H_2O. Antigen retrieval was performed by heating the sections in 0.001 N NaOH at 121°C in an autoclave for 5 min as described [15-17]. The sections were blocked with PBST containing 5% donkey serum (Cosmo Bio Co, Tokyo, Japan) for 1 h and incubated simultaneously with rabbit anti-NIS antibody (produced by our laboratory; dilution 1:50) and goat anti-MCT8 antibody (Santa Cruz Biotechnology; 1:50) for 1 h. After washing with PBST, sections were incubated simultaneously with Alexa Fluor 488-conjugated chicken anti-rabbit IgG antibody (Life Technologies, Waltham, MA, USA; 1:1000) and Alexa Fluor 594-conjugated donkey anti-goat IgG antibody (Life Technologies; 1:1000) for 1 h, and then the nuclei were counterstained with Hoechst 33258 (Life Technologies; 1:1000) for 15 min. The sections were washed with deionized H_2O and mounted with fluorescence mounting medium (Dako, Tokyo, Japan). Immunofluorescence labeling was visualized and the images were captured with an all-in-one fluorescence microscope BZ-9000 Generation II (KEYENCE, Osaka, Japan).

Results

Follicular Tg induces mRNA and protein expression of MCT8 and CRYM in rat thyroid FRTL-5 cells

To investigate the potential effect of follicular Tg on TH transporters, rat thyroid FRTL-5 cells maintained in the presence of TSH were incubated for 72 h with 10 mg/mL Tg, a concentration employed to mimic the reported concentrations in normal rat thyroid follicles and not those in serum [18-20]. BSA at the same concentrations as Tg was used as a control for the possible effects of non-specific proteins. Real-time PCR was performed to evaluate mRNA expression levels. *Slc5a5* (NIS) mRNA was found to be significantly suppressed after 6 h and even more so after 72 h (Fig. 1A). Consistent with previous reports [2, 5], the effect was dependent on Tg concentration (Fig. 1B). In contrast, the *Slc16a2* (MCT8) mRNA level was significantly increased 48 h after addition of Tg in a concentration-dependent manner (Fig. 1C and 1D). The *Crym* mRNA level was significantly increased by Tg in a time- and dose-dependent manner, which was evident as early as 6 h after Tg addition (Fig. 1E and 1F). BSA had virtually no effect on the expression of these three genes, which is similar to our previous results on other genes [3]. Although high concentrations of BSA slightly modified *Slc16a2* expression (Fig. 1C and 1D), the effect of Tg was clearly stronger than that of BSA (Fig. 1C, 48 h: $p < 0.001$, 72 h: $p < 0.001$, Fig. 1D, 5 mg/mL: $p < 0.01$, Fig. 1D, 10 mg/mL: $p < 0.001$).

We then examined the changes in the levels of NIS, MCT8 and CRYM proteins by Western blot analysis after Tg addition to FRTL-5 cell culture medium. The intensities of specific bands were quantified and expressed in bar graphs relative to the corresponding β-actin levels. In line with the changes in mRNA expression, and in conformity with previous reports [2, 5], the protein level of NIS was significantly lowered by Tg (Fig. 2). In contrast, the expression of MCT8 protein and CRYM protein were significantly increased in a time-dependent manner (Fig. 2). The induction of MCT8 or CRYM protein levels was not replicated by BSA (Fig. 2, hatched bars).

Tg opposes TSH action to induce Slc16a2 and Crym gene expression

A notable feature of Tg action revealed in the previous studies was that Tg could suppress or even reverse gene expression induced by TSH, which has been thought to be a master regulator of the thyroid [2, 3, 5, 15]. To investigate the possible role of Tg on TSH action in the regulation of *Slc16a2* and *Crym* expression, FRTL-5 cells were first maintained in the absence of TSH for 1 week, then treated with TSH for 48 h, followed by addition of 10 mg/mL Tg or BSA, in the presence of TSH for a further 72 h. *Slc5a5* (NIS) mRNA expression was significantly induced by TSH, but this effect was completely counteracted by Tg (Fig. 3A), as reported previously [2, 5]. In contrast, *Slc16a2* (MCT8) expression was significantly suppressed by TSH in a time-dependent manner. This TSH-induced suppression was completely abolished by Tg, the level of *Slc16a2* becoming even greater than that before TSH stimulation (Fig. 3B). *Crym* mRNA levels were not significantly affected by TSH by 48 h but were greatly increased by Tg stimulation in a time-dependent manner (Fig. 3C) in accordance with the result shown in Fig. 1E. BSA did not mimic the effect of Tg; although the expression of *Slc16a2* was slightly affected (Fig. 3B), the effect was significantly weaker than that of Tg.

MCT8 co-localizes with NIS on the basolateral membrane of rat thyrocytes in vivo

Although the subcellular distribution of MCT8 has

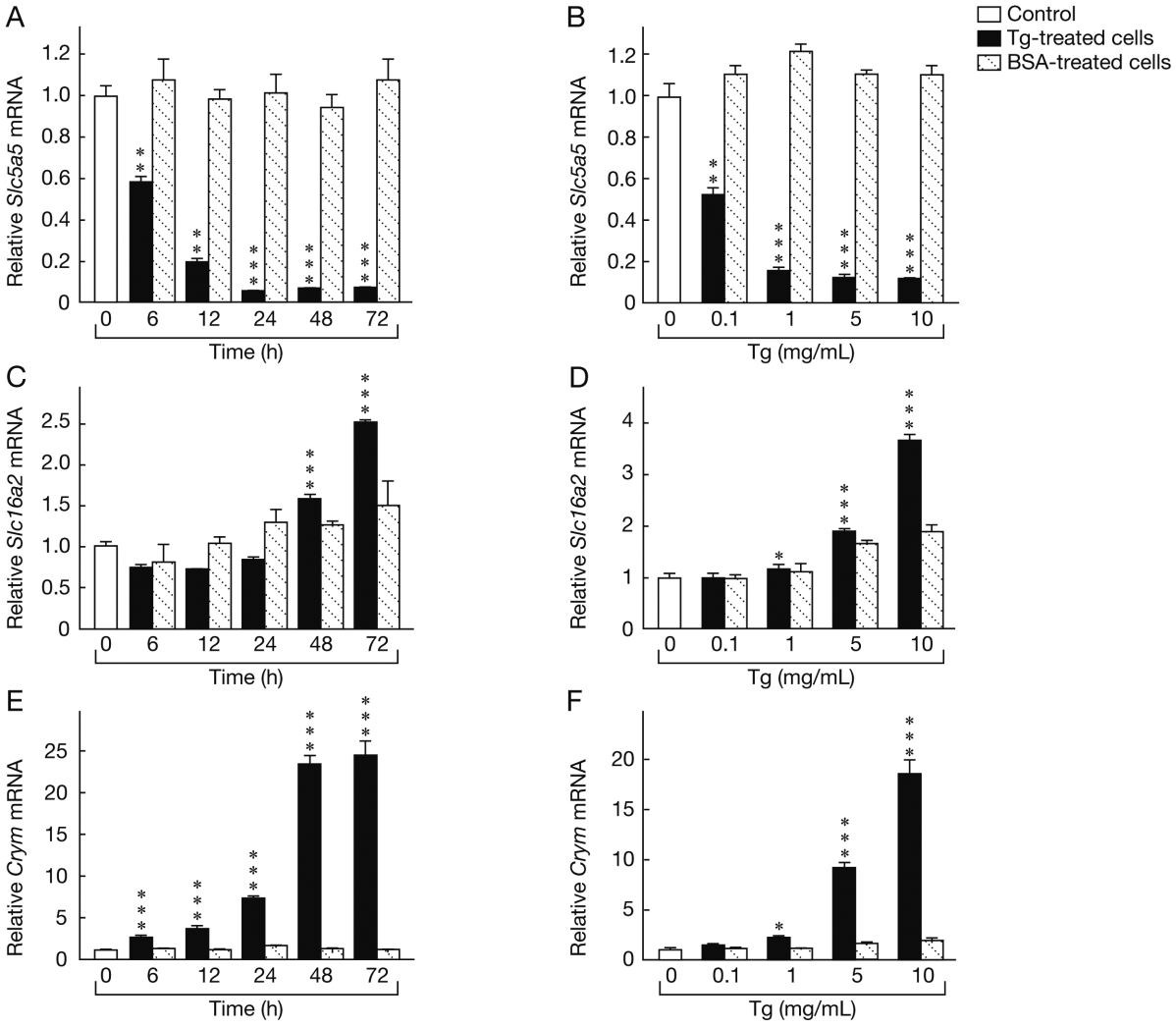

Fig. 1 Follicular concentrations of Tg regulate *Slc16a2* (MCT8) and *Crym* mRNA expression. FRTL-5 cells maintained in the presence of TSH were treated with 10 mg/mL Tg or BSA for the indicated periods up to 72 h (A, C and E), or with 0.1 to 10 mg/mL Tg or BSA for 72 h (B, D and F). Total RNA was purified from cells and subjected to real-time PCR analysis to determine the relative mRNA expression levels of *Slc5a5* (A and B), *Slc16a2* (C and D) and *Crym* (E and F). mRNA levels of each gene were normalized against *Gapdh* and expressed relative to the control. The results of the Tg-treated cells are shown by closed bars and that of the BSA-treated cells by hatched bars. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, compared with control (open bars).

been examined in mouse and human thyroid glands [12, 21], to our knowledge its localization in rat thyroid has never been reported. Immunofluorescence staining was therefore performed to visualize the localization of MCT8 and NIS in rat thyroid follicles using paraffin-embedded tissue sections. MCT8 was found to be localized on the basolateral membrane of the follicular epithelium, similar to its localization in mice and humans (Fig. 4). Moreover, MCT8 also clearly co-localized with NIS (Fig. 4, 'Merge'), indicating that the action of Tg is independent of the localization of the target protein, and that it suppresses or induces gene expression in accordance with the function of each gene product.

Discussion

In this study we explored the effect of follicular Tg on the expression levels of MCT8 and CRYM, essential transporters of TH. Our results showed that Tg at physiologic follicular concentrations counteracted TSH action and enhanced both the mRNA and protein expression of MCT8 and CRYM.

Regulation of follicular function by Tg stored in

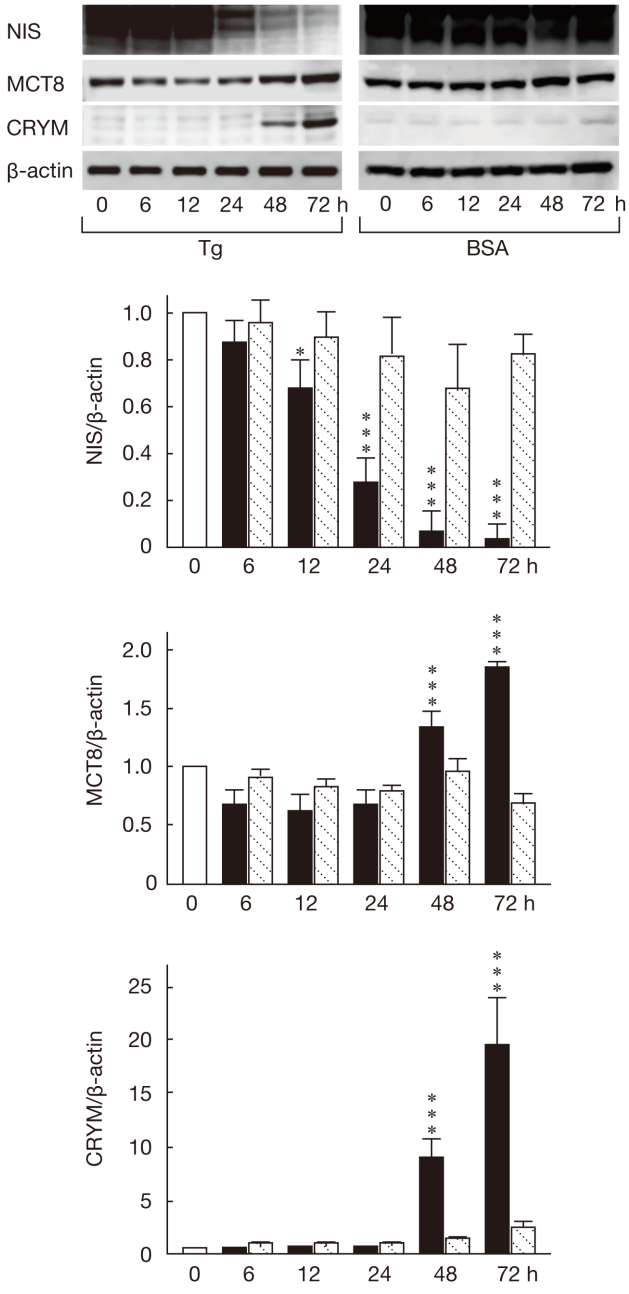

Fig. 2 Follicular concentrations of Tg regulate MCT8 and CRYM protein expression. FRTL-5 cells maintained in the presence of TSH were treated with 10 mg/mL Tg or BSA for the indicated period of time up to 72 h. Whole cell protein was extracted and subjected to Western blot analysis to determine the protein expression levels of NIS, MCT8, CRYM and β-actin. The densities of specific bands were determined, and the relative protein expression levels normalized against β-actin were presented as bar graphs. The results of the Tg-treated cells are shown by closed bars and that of the BSA-treated cells by hatched bars. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, compared with control (open bars).

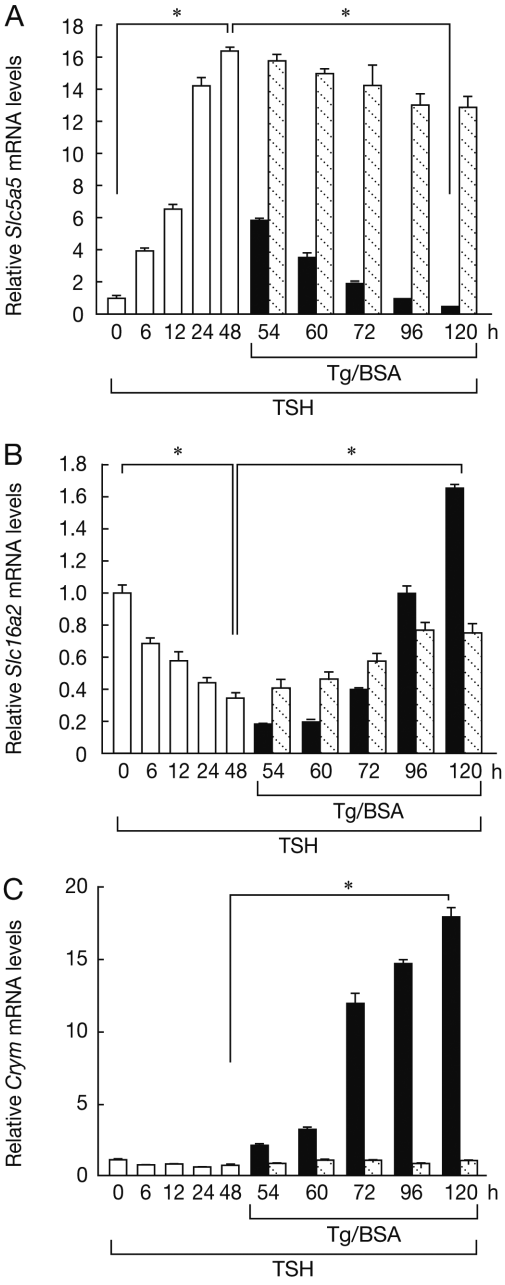

Fig. 3 Follicular concentration of Tg counteracts TSH action to induce *Slc16a2* (MCT8) and *Crym* mRNA expression. FRTL-5 cells maintained in the absence of TSH for 1 week were first incubated with 1 mU/mL of TSH for 48 h (open bars) and then treated with 10 mg/mL Tg or BSA in the presence of TSH for another 72 h (120 h after initial TSH stimulation). Total RNA was extracted at the indicated time points and subjected to real-time PCR analysis to determine the relative mRNA levels of *Slc5a5* (A), *Slc16a2* (B), and *Crym* (C). Relative mRNA levels were normalized against *Gapdh*. Changes in mRNA expression induced by TSH alone were shown by open bars; the results of the Tg-treated cells are shown by closed bars and that of the BSA-treated cells by hatched bars. * $p < 0.001$.

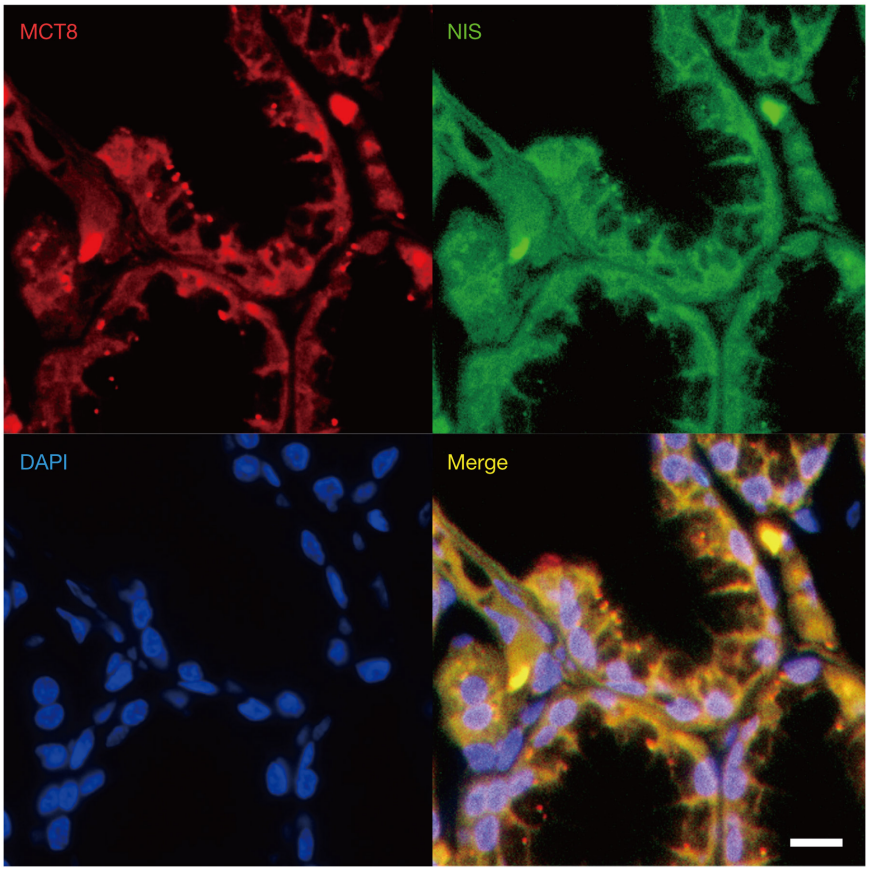

Fig. 4 MCT8 and NIS proteins co-localize on the basolateral membrane of rat thyrocytes *in vivo.* Rat thyroid tissue sections were stained with anti-MCT8 antibody (red) and anti-NIS antibody (green). Nuclei were counterstained with Hoechst 33258 (blue). Immunofluorescence images were captured using an all-in-one fluorescence microscope BZ-9000 Generation II. Original magnification: x1000. Bars: 10 μm.

the thyroid follicle, or the 'follicular cycle model', well explains the nature of follicular heterogeneity [2, 4, 5, 19, 22, 23]. Thus, the activities of the follicles, which are the minimum functional units of the gland, are not synchronized but heterogeneous despite having similar TSH supplies [24-29]. The model hypothesizes that each follicle repeats 'synthesis phase', 'storage phase' and 'secretion phase' without apparent synchronization, which primarily depends on the variable follicular Tg accumulation [19]. In the present study, Tg was found first to 'induce' thyroid functional genes, which is in striking contrast to the previously known negative-feedback 'suppression' [2, 3, 5-7, 15]. However, taking into consideration that the action of follicular Tg is to maintain the physiologic cycle of synthesis, storage and secretion of TH, initiation of TH secretion by follicular Tg seems to be reasonable. The role that Tg plays in the 'follicular cycle' seemingly is to prevent cells from overproducing TH by an amount that exceeds the maximum capacity of storage by antagonizing the action of TSH. From this perspective, stimulation of TH secretion by the induction of MCT8 and CRYM expression levels when follicular Tg has reached a high enough concentration, *i.e.* 'secretion phase', is quite rational. Tg exerts potentially opposite effects on MCT8 and NIS in accordance with their functions despite their common basolateral localization. Thus, Tg differentially regulates, rather than merely suppresses, the thyroid functional genes to maintain efficient running of the 'follicular cycle'.

Slc16a2 (MCT8)-knockout mice exhibited lower serum thyroxine (T4) levels with high TH contents in thyrocytes compared with their wild type littermates. Thus, after ^{125}I administration, the rates of labeled iodine secretion by the thyroid and its appearance in the serum were remarkably reduced in *Slc16a2*-knockout mice [12]. In patients carrying a *SLC16A2* inactivating mutation, low serum T4 levels with characteristic fol-

licular hyperperfusion and hyperplasia have been noted, potentially explicable by the defect in TH secretion [21]. CRYM is highly specific to triiodothyronine (T3), and its essential function is believed to be to transport T3 in the cytosol in the presence of NADPH [14]. *Crym*-knockout mice showed significantly reduced serum TH levels despite a euthyroid state. After intravenous injection, radiolabeled T3 did not remain long in the tissues of *Crym*-knockout mice, indicating that CRYM is necessary for maintaining the normal turnover and access rate of TH in target cells [30]. However, the role of CRYM in TH secretion in the thyroid was not clear. Since all the genes that have been shown to be tightly regulated by follicular Tg are essential for thyroid function [2, 3, 5-7, 15, 16], our present results suggest that CRYM also plays an important role in TH binding and transportation in thyrocytes.

One of the limitations of the present study is the lack of evidence of an actual increase in TH secretion by Tg, which is not possible to demonstrate in monolayers of FRTL-5 cells, as it needs a three-dimensional follicular structure. Furthermore, at present we know little about the underlying molecular mechanisms of Tg action. Unanswered questions include how Tg is recognized by follicular cells, and which signal transduction pathways are used that are strong enough to counteract TSH action.

Several proteins, including the asialoglycoprotein receptor (ASGPR) [31], an N-acetylglucosamine (GlcNAc) receptor [32], megalin/gp330 [33, 34], and mannose 6-phosphate/IGF-II receptor [35], have been postulated to bind Tg on the apical membrane of thyrocytes. However, none of these receptors have been firmly established to mediate Tg signaling [19]. Nevertheless, the autoregulatory effect on gene expression appears to be highly restricted to Tg protein at follicular concentrations [3, 19], and to be limited to normal thyroid cells but not to goiters, follicular adenomas or papillary carcinomas [7].

Similarly, signal transduction pathways used in Tg action, which are strong enough to counteract TSH effects in many genes, are not known. The effects of Tg under chronic TSH stimulation (Fig. 1) and 48 h stimulation (Fig. 3) on *Slc5a5* and *Slc16a2* appeared to be slightly different. Since follicles are exposed to rather constant TSH stimulation and fluctuating Tg stimulation from basolateral and apical surfaces, respectively [2, 19], the interaction of TSH and Tg signaling is certainly an important subject needing clarification to understand follicular physiology and pathology. Based on such strong effects of follicular concentrations of Tg on normal thyroid cells, it seems reasonable to speculate that a malfunction in Tg regulation (such as hyperactivity or dysfunction) has consequences that likely cause abnormal thyroid function. Therefore, it might be appropriate to reconsider our present concepts of thyroid pathology from the point of view of Tg.

In summary, in this study we showed that Tg at physiologic follicular concentrations induces both mRNA and protein expression of two specific Tg transporters: MCT8 and CRYM. The present and previous results indicate that follicular Tg is as an intrinsic regulator of both TH synthesis and TH secretion and transport that seems to coordinate with TSH action to ensure that follicles function in concert.

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Disclosure

None of the authors has any potential conflicts of interest in relation to this research.

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