HPT axis-independent TSHβ splice variant regulates the synthesis of thyroid hormone in mice

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Abstract. Thyroid stimulating hormone (TSH) consists of an α-subunit and a unique β-subunit. The first in-frame TSHβ splice variant produced by the cells of immune system was identified in 2009. The TSHβ splice variant and native TSHβ exhibit different expression profiles, and research has been conducted to elucidate the role of the TSHβ splice variant in different diseases. However, understanding of the fundamental physiological characteristics of the TSHβ splice variant is currently limited. To verify whether the TSHβ splice variant has the potential to induce thyroid follicular cells to synthesize thyroid hormone, *in vivo* and *in vitro* stimulation experiments were conducted in the present study. A total of 60 C57BL/6 mice were divided into control-, 5 and 10 μg TSHβ splice variant-treated groups at random. Mice were sacrificed at 0.5, 1 and 4 h after intraperitoneal injection, and serum levels of tri-iodothyronine (T3) and thyroxine (T4) were determined using a radioimmunoassay. Thyroid follicular cells were isolated from the thyroids of mice, and stimulated with 2 μg/ml TSHβ splice variant. Supernatants were collected, and the levels of T3 and T4 were detected. The protein expression levels of the sodium-iodide symporter, thyroperoxidase and thyroglobulin in thyroid follicular cells were quantified using western blot analysis. To verify whether the TSHβ splice variant expression was regulated by the hypothalamus-pituitary-thyroid (HPT) axis, similar to native TSHβ, a total of 60 C57BL/6 mice were equally divided into control, 2 mg/kg T3 intraperitoneal injection and 0.05 mg/kg thyroid-releasing hormone intraperitoneal injection groups at random. Mice were sacrificed at 1 and 4 h after injection. Alterations in the expression of the TSHβ splice variant in the pituitary, thyroid, peripheral blood leukocytes and spleen tissues were detected using western blot analysis. The present study demonstrated that the TSHβ splice variant is not regulated by the HPT axis and may affect thyroid hormone synthesis. Modifications in the expression of the TSHβ splice variant may occur in a uniquely regulated manner to provide peripheral immunological compartments with a source of activated cells, particularly under immune stress.

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Introduction

Thyroid stimulating hormone (TSH) is produced by the anterior pituitary, and is used for the regulation of thyroid hormone production, which subsequently controls metabolic activity (1). TSH consists of an α-subunit and a non-covalently bound β-subunit (TSHβ) (2). The former is also a subunit of luteinizing hormone, follicle stimulating hormone and chorionic gonadotropin. By contrast, TSHβ is unique and responsible for TSH activity and specific immunogenicity (3).

The mouse TSHβ gene consists of five exons, with the coding region located within exons 4 and 5 (4). The first functional alternatively spliced variant of mouse TSHβ was reported by Vincent *et al* (5) in 2009. The in-frame TSHβ splice variant involves the retention of a portion of intron 4 and the entire coding region of exon 5, but does not include exon 4. At present, the only evidence of alternative exon splicing of the mouse TSHβ gene involves exons 1, 2 and 3, all of which are located outside of the TSHβ-coding region (6,7). To the best of our knowledge, there is no evidence at present of alternative splicing of TSHβ within the coding region itself, or of splicing that affects the expression of the TSHβ protein. Previous studies have demonstrated that the TSHβ splice variant transcript is expressed in the mouse pituitary, thyroid, bone marrow, spleen, small intestine, large intestine, kidney, liver, heart and adipose tissues, whereas the full-length native TSHβ transcript is largely restricted to the pituitary gland (8).

These observations suggest that the extra-pituitary TSHβ splice variant may be markedly different from the full-length, native TSHβ. Various studies have focused on establishing a role for the TSHβ splice variant in different diseases. Previous studies have revealed that the extra-pituitary TSHβ splice variant serves a vital role in pathological processes, including autoimmunity, inflammation and bone remodeling (9,10). Despite this, the fundamental physiological characteristics of the TSHβ splice variant remain unknown, and an in-depth understanding of its physiological effects is required.

The aim of the present study was to perform a series of examinations to verify the following: i) Whether the TSHβ splice variant has the potential to induce thyroid follicular cells to synthesize thyroid hormone; and ii) whether the TSHβ splice variant is regulated by the hypothalamus-pituitary-thyroid (HPT) axis, similarly to the native form of TSHβ.

Materials and methods

Animals and sampling. C57BL/6 mice (6-8 weeks old, 50% male and 50% female, weighing 20-26 g) were purchased from the Experimental Animal Center of the Academy of Military Medical Sciences of China (Beijing, China). The animals were housed in a temperature-controlled room (21-23˚C) at 40±10% relative humidity under a 12:12-h light/dark cycle, with access to food and water *ad libitum*. All procedures used were in accordance with the Logistics University of Chinese People's Armed Police Force animal welfare guidelines. The animal protocols were approved by the Ethics Review Committee of the Logistics University of Chinese People's Armed Police Force (Tianjin, China).

A total of 156 C57BL/6 mice were used in the present study. Of these, 60 mice were randomly divided into the following groups: Control (n=20), where mice were administered with an intraperitoneal injection of saline; a 5 μg purified TSHβ splice variant protein (see *Generation of a TSHβ splice variant fusion protein*) intraperitoneal injection group (n=20); and a 10 μg purified TSHβ splice variant protein intraperitoneal injection group (n=20). The mice were sacrificed at 0.5, 1 and 4 h after intraperitoneal injection. Blood samples were collected, and the serum levels of free tri-iodothyronine (FT3) and free thyroxine (FT4) were determined using a radioimmunoassay (RIA) performed by the Radiology Institute of the Logistics University of Chinese People's Armed Police Force (Tianjin, China). The radioimmunodetection was repeated three times.

In another experiment, 60 mice were randomly divided into the following three groups: Control group (n=20), where mice were administered with an intraperitoneal injection of saline; a T3 group (n=20), where mice were administered with an intraperitoneal injection of 2 mg/kg T3 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany); and a thyroid-releasing hormone (TRH) group (n=20), where mice were intraperitoneally injected with 0.05 mg/kg TRH (Nanjing Peptide Biotech Ltd., Nanjing, China). The animals were sacrificed at 1 and 4 h after injection. Whole blood samples, anti-coagulated with 1% heparin sodium solution (0.1 ml; Lianxing Biotech Co., Ltd., Tianjin, China), were collected for the isolation of peripheral blood leukocytes (PBLs). In addition, pituitary gland, thyroid and spleen tissue samples were collected for the detection of TSHβ expression. All assays were repeated three times.

Mouse PBLs. Mouse whole blood samples, anti-coagulated with 1% heparin sodium solution, were collected. To process each blood sample, 1:3 (v/v) red blood cell:lysis buffer (Yuanpinghao Biotech Co., Ltd., Beijing, China) was added to the blood samples, and the sample tubes were mixed and placed on ice for 5 min. This was followed by centrifugation at 12,000 x g for 1 min at 4˚C. The clear, red supernatant was carefully removed, and the pellet that remained contained the PBLs. Total protein was extracted and quantified, and the levels of the TSHβ splice variant were detected by western blot analysis.

Mouse thyroid follicular epithelial cells. Previous studies have described methods to isolate thyroid cells from mice (11-13). To obtain follicular epithelial cells in the present study, 36 experimentally naïve mice were sacrificed. The animal protocols were approved by the Ethics Review Committee of the Logistics University of Chinese People's Armed Police Force. The fresh thyroid tissues were first minced with crossed razor blades into pieces <1.5 mm in diameter. The pieces were subsequently rinsed twice with Ham's F12 nutrient mixture without bovine serum (Thermo Fisher Scientific, Inc., Waltham, MA, USA), followed by disaggregation using dispersing reagents [103 kU/l collagen I and 1.35 kU/l dispase in Earle's balanced salt solution; pH 7.4 (Sigma-Aldrich; Merck KGaA)] for 40 min at 37˚C with agitation (100 oscillations/min). The samples were centrifuged at 200 x g for 10 min at room temperature. The supernatants were discarded, and the cells were resuspended in Ham's F12 nutrient mixture (Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific, Inc.), 2 mM L-glutamine, 10 mg/l insulin, 10 nM hydrocortisone, 5 mg/l transferrin and 1 U/l bovine TSH (all these elements were purchased from Sigma-Aldrich; Merck KGaA), 10 U/l penicillin and 100 mg/l streptomycin (Lianxing Biotech Co., Ltd.). Following gentle mechanical agitation, the disaggregated cell suspensions were transferred into 24-well Corning tissue culture plates (Corning Inc., Corning, NY, USA) and incubated in a humidified 5% CO_2 incubator. The medium was refreshed every 3 days with decreasing concentrations of FBS (from 10 to 5%). The mouse thyroid follicular epithelial cells were cultivated until 80% confluency, which was reached on day 7. On day 8, media was removed and replaced with Ham's F12 nutrient mixture lacking bovine TSH. Stimulation experiments were performed with the addition of purified TSHβ splice variant protein at a concentration of 2 μg/ml on day 9. Supernatants were collected for detecting the levels of T3 and T4. Cells were harvested at 0, 0.5, 1 and 4 h following TSHβ splice variant stimulation. Total protein was isolated from the cells and quantified. The experiments were repeated three times.

Generation of a TSHβ splice variant fusion protein. Total RNA was isolated from the mice PBLs using TRIzol® reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. RNA was reverse transcribed to synthesize cDNAs using a Reverse Transcription System (Promega Corporation, Madison, WI, USA). Polymerase chain reaction (PCR) amplification of the coding region of the TSHβ splice variant was conducted in a total reaction volume of 20 μl

containing 4 μl cDNA. PCR was conducted as follows: Initial denaturation at 95˚C for 5 min, then 30 cycles of denaturation at 95˚C for 30 sec, annealing at 60˚C for 30 sec and extension at 72˚C for 30 sec, and a final extension step at 72˚C for 5 min. The primer sequences were as follows: Forward, 5'-ATCATG TTAAGATCTCTTTTCTTT-3', reverse, 5'-AACCAGATT GCACTGCTATTGAA-3'. The coding region of the TSHβ splice variant was subcloned into the pcDNA3.1/V5-His-TOPO vector (Thermo Fisher Scientific, Inc.). Plasmid DNA was obtained using standard methods. Chinese hamster ovary (CHO) cells (GF113, Shanghai Gefan Biotechnology Co., Ltd.) were transfected with the generated plasmid DNA using an Amaxa electroporator (Amaxa Biosystems; Lonza Group Ltd., Basel, Switzerland) according to the manufacturer's instructions. Briefly, CHO cells were cultivated in Ham's F12 media and incubated at 37˚C with a humidified atmosphere of 5% CO_2 until they reached 90% confluency, at which point they were harvested by trypsinization (cells were incubated for 5 min at 37˚C with 0.5 mg/ml trypsin). Cells ($1x10^6$) were resuspended in 100 μl room-temperature Nucleofector® Solution (82 μl of Nucleofector Solution plus 18 μl of supplement; Amaxa Biosystems; Lonza Group Ltd.). The resulting cell suspension was combined with 2 μg recombinant DNA, and the cell/DNA suspension was transferred into an Amaxa certified cuvette, which was inserted into the Nucleofector Cuvette Holder. The U-023 Nucleofector program was performed, following which the cuvette was immediately taken out of the holder, and 600 μl of pre-equilibrated Ham's F12 medium was then added. Cells were gently transferred into 6-well plates (final volume 1.5 ml media/well). Transfected cells were incubated in humidified 37˚C/5% CO_2 incubator. Stable transfected cells were selected by continuous culture in medium containing 1.2 mg/ml neomycin. A total of $1x10^7$ cells were used to purify His-tagged recombinant proteins with the Ni-NTA Fast Start kit (Qiagen China Co., Ltd., Shanghai, China). The concentration of recombinant protein was determined using a Coomassie Plus-200 Protein assay (Pierce; Thermo Fisher Scientific, Inc.), and aliquots were stored at -80˚C.

Western blotting. Total proteins from mouse thyroid follicular epithelial cells, and mouse PBLs, and pituitary gland, thyroid and spleen tissues were extracted using a ReadyPrep™ Protein Extraction kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Protein concentrations were determined using Coomassie Protein Assay reagent (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. The protein samples were subsequently denatured in SDS sample buffer (125 mM Tris-HCl, pH 6.8, 50% glycerol, 2% SDS, 5% b-mercaptoethanol and 0.01% bromophenol blue) at 100˚C for 10 min. Equal amounts of protein (30 μg/lane) separated by 18% SDS-PAGE were transferred to a polyvinylidene fluoride membrane at 15 V for 25 min using Trans-Blot SD (Bio-Rad Laboratories, Inc.). The membranes were blocked with 5% non-fat milk in TBS and Tween 20 (TBST; 25 mM Tris-base, 138 mM NaCl, 2.7 mM KCl, 0.2% Tween-20 and deionized water; pH 7.4) for 2 h at room temperature. The membranes were immunoblotted with primary antibodies overnight at 4˚C. The primary antibodies included anti-TSHβ antibody (1:500; cat. no. sc-7813), anti-sodium-iodide symporter (NIS) antibody (1:200; cat. no. sc-514487), anti-thyroperoxidase (TPO) antibody (1:200; cat. no. sc-376876; all purchased from Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-thyroglobulin (TG) antibody (1:200; cat. no. ab-187378; Abcam, Cambridge, UK) and anti-GAPDH antibody (1:800; cat. no. BM1623; Boster Biological Technology, Pleasanton, CA, USA). The membranes were subsequently washed with TBST, and incubated donkey anti-goat horseradish peroxidase (HRP)-conjugated secondary antibody (cat. no. sc-2020; Santa Cruz Biotechnology, Inc.) and goat anti-mouse HRP-conjugated secondary antibody (cat. no. ZB-2305; dilution, 1:5,000; OriGene Technologies, Inc., Rockville, MD, USA) for 2 h at room temperature. Following a final wash in TBST, the immunoreactive TSHβ splice variant bands were detected using an enhanced chemiluminescence system (Immobilon™ Western Chemiluminescent HRP Substrate; Merck KGaA), and the images were acquired using the Kodak Medical X-ray processor (Kodak, Rochester, NY, USA). Band densities were quantified using Image-Pro Plus v6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

The mouse TSHβ gene consists of five exons and the coding region of native TSHβ is located between exons 4 and 5. The molecular weight of native TSHβ protein is 17 kDa. The TSHβ splice variant consists of part of intron 4 and all of the coding region of exon 5, and the molecular weight of the TSHβ splice variant protein is 8 kDa (5). The anti-TSHβ antibody specifically binds to the protein sequence encoded by exon 5, and therefore detects both forms of the TSHβ protein.

Statistical analysis. All data are presented as the mean ± standard error. Comparisons between groups were performed using one-way analysis of variance, followed by a least significant difference post-hoc test. The statistical analyses were performed using SPSS software (v.13.0; SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Increased serum FT3 and FT4 levels are induced by TSHβ splice variant. Mice were injected with the purified TSHβ splice variant protein at different concentrations, and the serum levels of FT3 and FT4 were evaluated by RIA at 0.5, 1 and 4 h post-injection. As demonstrated in Fig. 1, serum levels of FT3 peaked at 1 h following injection, and declined to a normal level at 4 h post-injection. Serum levels of FT4 exhibited a similar trend (Fig. 2). The serum levels of FT3 in the 10 μg purified TSHβ splice variant protein-treated group were 1.56-fold higher than the the saline-injected control group at 1 h after injection. In addition, the serum levels of FT3 in the 5 μg purified TSHβ splice variant protein-treated group were 1.28-fold higher than the saline-injected control group at 1 h post-injection. Furthermore, the serum levels of FT4 in the 10 μg purified TSHβ splice variant protein-injected group were 2.19-fold higher than the saline-injected control group at 1 h post-injection, while the FT4 levels of the 5 μg-injected group were 2.05 fold higher relative to the control group. In summary, the results demonstrated that both serum FT3 and FT4 levels were significantly increased by TSHβ, and in a dose-dependent manner, compared with the saline-injected groups of mice.

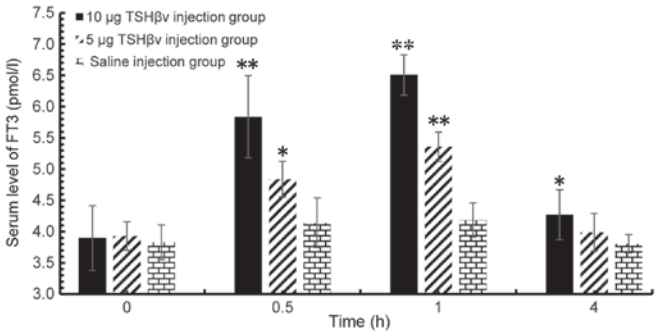

Figure 1. Altered serum FT3 levels among the control group, 5 and 10 μg TSHβv injection groups at 0.5, 1 and 4 h following injection evaluated by radioimmunoassay. TSHβv-injected (5 μg) vs. control animals: 0.5 h, P=0.037; 1 h, P<0.001; and 4 h, P=0.362. TSHβv-injected (10 μg) vs. control animals: 0.5 h, P<0.001; 1 h, P<0.001; and 4 h, P=0.032. *P<0.05 and **P<0.001 vs. respective control. FT3, free tri-iodothyronine; TSHβv, thyroid-stimulating hormone β splice variant.

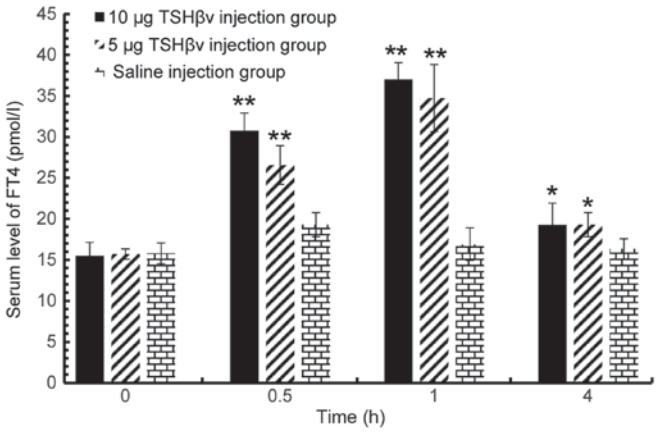

Figure 2. Altered serum FT4 levels among the control group, 5 and 10 μg TSHβv injection groups at 0.5, 1 and 4 h following injection evaluated by radioimmunoassay. TSHβv-injected (5 μg) and control animals: 0.5 h, P<0.001; 1 h, P<0.001; and 4 h, P=0.030. TSHβv-injected (10 μg) and control animals: 0.5 h, P<0.001; 1 h, P<0.001; 4 h, P=0.031). *P<0.05 and **P<0.001 vs. respective control. FT4, free thyroxine; TSHβv, thyroid-stimulating hormone β splice variant.

TSHβ splice variant induces thyroid follicular cells to synthesize thyroid hormone. For *in vitro* analysis, mice thyroid follicular cells first isolated and cultured. Following induction with a recombinant TSHβ splice variant protein, the expression levels of NIS, TPO and TG in mice thyroid follicular cells were detected via western blot analysis. In addition, the levels of T3 and T4 in the supernatant were detected via RIA. As demonstrated in Fig. 3A, T3 levels in the supernatant peaked at 1 h post-induction, and sharply declined to a normal level at 4 h following induction. The levels of T4 in the supernatant similarly peaked at 1 h post-induction and then declined; however, T4 levels in the induction group remained significantly above control levels at 4 h following induction (Fig. 3B). Overall, the *in vitro* fluctuations in levels of the T3 and T4 appear to reflect those observed *in vivo*, thus attesting to success of the recombinant TSHβ splice variant. In addition, NIS protein expression levels increased and peaked at 0.5 h post induction, and gradually decreased to a normal level at 4 h following induction. TG and TPO protein expression levels peaked at 0.5 h post-induction, and slowly decreased thereafter; however, the levels remained significantly increased at 4 h post-induction compared with baseline expression prior to induction (Fig. 4). The expression levels of NIS in the TSHβ splice variant protein-treated group were 2-fold higher than in the control group at 0.5 h following treatment. TG expression levels at 0.5 h post induction in the TSHβ splice variant protein group were 1.85-fold higher than the control group at the same time point. In addition, TPO expression levels in the TSHβ splice variant protein-treated group were 1.92-fold higher than the control group at 1 h post-induction.

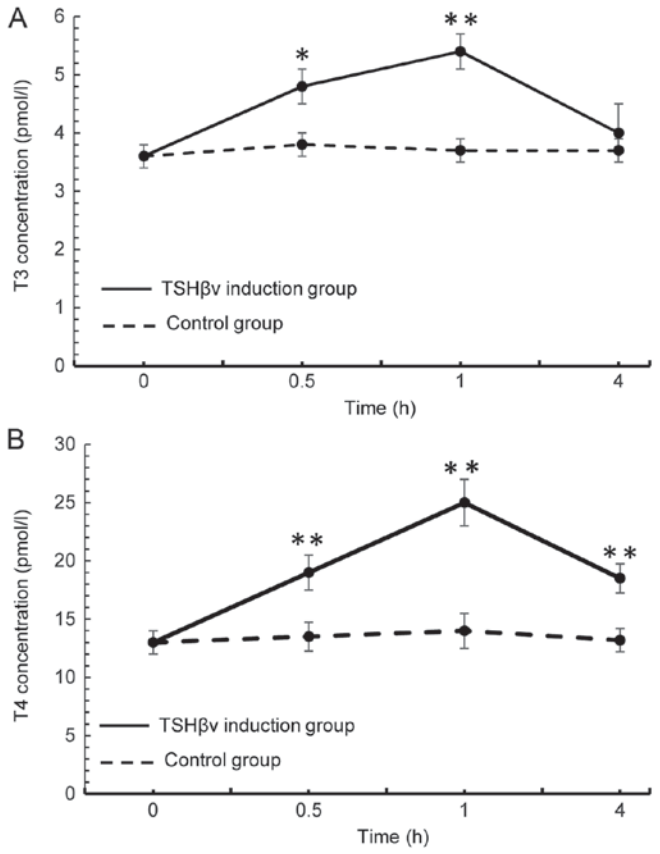

Figure 3. Following induction of mouse thyroid follicular cells with purified TSHβv protein, alterations in the concentrations of (A) T3 and (B) T4 in the supernatant were detected using a radioimmunoassay at 0, 0.5, 1 and 4 h following induction. T3 levels, TSHβv-treated vs. control cells: 0.5 h, P=0.006; 1 h, P<0.001; and 4 h, P=0.091. The T4 levels, TSHβv-treated vs. control cells: 0.5 h, P<0.001; 1 h, P<0.001; and 4 h, P<0.001. *P<0.01 and **P<0.001 vs. respective control. T3, tri-iodothyronine; T4, thyroxine; TSHβv, thyroid-stimulating hormone β splice variant.

TSHβ splice variant expression is not regulated by the HPT axis. Mice were administered with T3 or TRH. The expression levels of the TSHβ splice variant in pituitary, thyroid, spleen tissues and PBLs were assessed by western blot analysis at 1 and 4 h following treatment. The native form and TSHβ splice variant were detected in the pituitary (Figs. 5 and 6, respectively), as both forms are expressed in the pituitary gland. The TSHβ splice variant was detected in PBLs (Fig. 7), spleen (Fig. 8) and thyroid (Fig. 9), as the native form of TSHβ is known to not be expressed in these tissues (2-4). As expected, the results indicated that the native form of TSHβ expression was upregulated by TRH stimulation and downregulated by

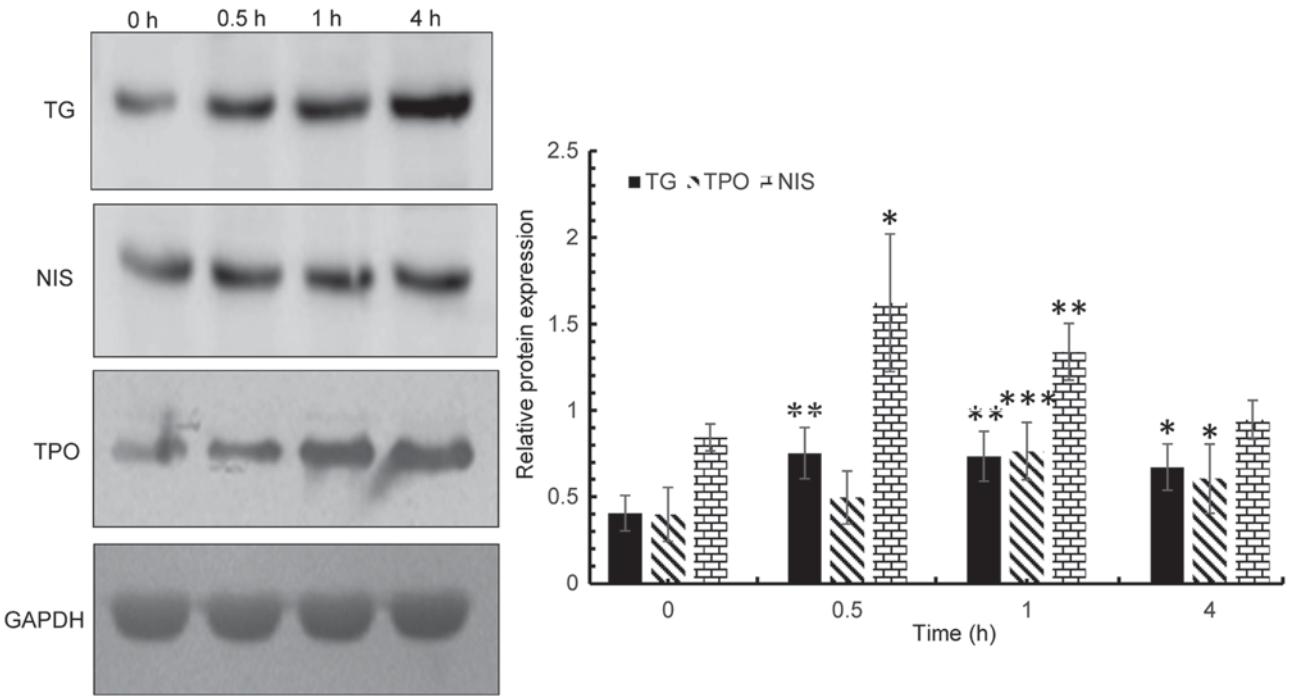

Figure 4. Following induction of thyroid follicular cells with purified thyroid-stimulating hormone β splice variant protein, the expression levels of NIS, TG and TPO were detected via western blot analysis at 0, 0.5, 1 and 4 h post induction. NIS expression vs. 0 h: 0.5 h, P=0.016; 1 h, P=0.001; and 4 h, P=0.367. TG expression vs. 0 h are: 0.5 h, P=0.004; 1 h, P=0.003; and 4 h, P=0.015. TPO expression vs. 0 h are: 0.5 h, P=0.092; 1 h, P<0.001; and 4 h, P=0.010. *P<0.05, **P<0.01 and ***P<0.001 vs. respective control. NIS, sodium iodide symporter; TG, thyroglobulin; TPO, thyroperoxidase.

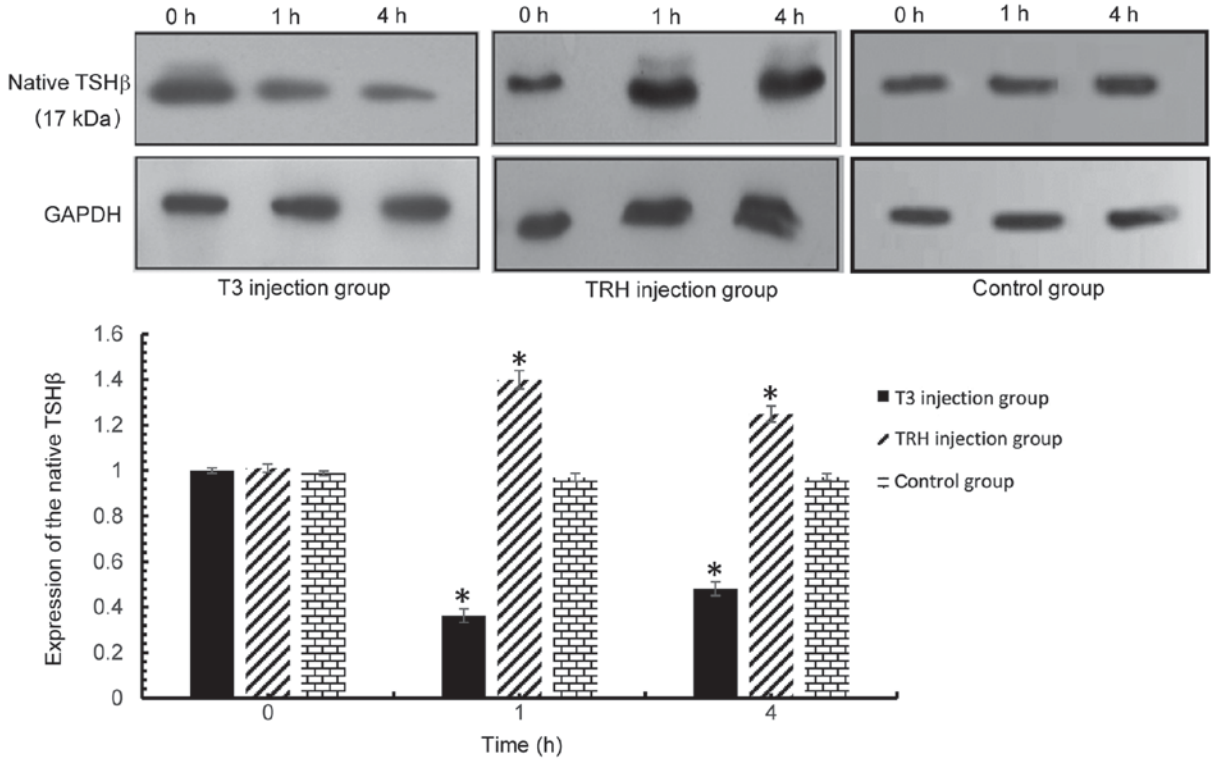

Figure 5. Native TSHβ expression in the pituitary was detected via western blot analysis at 0, 1 and 4 h following injection with T3 or TRH. T3 injection group vs. control group: 0 h, P=0.379; 1 h, P<0.001; and 4 h, P<0.001. TRH injection group vs. control group: 0 h, P=0.107; 1 h, P<0.001; and 4 h, P<0.001. *P<0.001 vs. respective control. TSHβ, thyroid-stimulating hormone β; T3, tri-iodothyronine; TRH, thyroid-releasing hormone.

T3 stimulation in the pituitary (Fig. 5). However, T3 and TRH demonstrated no effect on the expression of the TSHβ splice variant protein in the pituitary, thyroid, spleen and PBLs.

Discussion

TSH, secreted by the anterior pituitary, is known to induce the

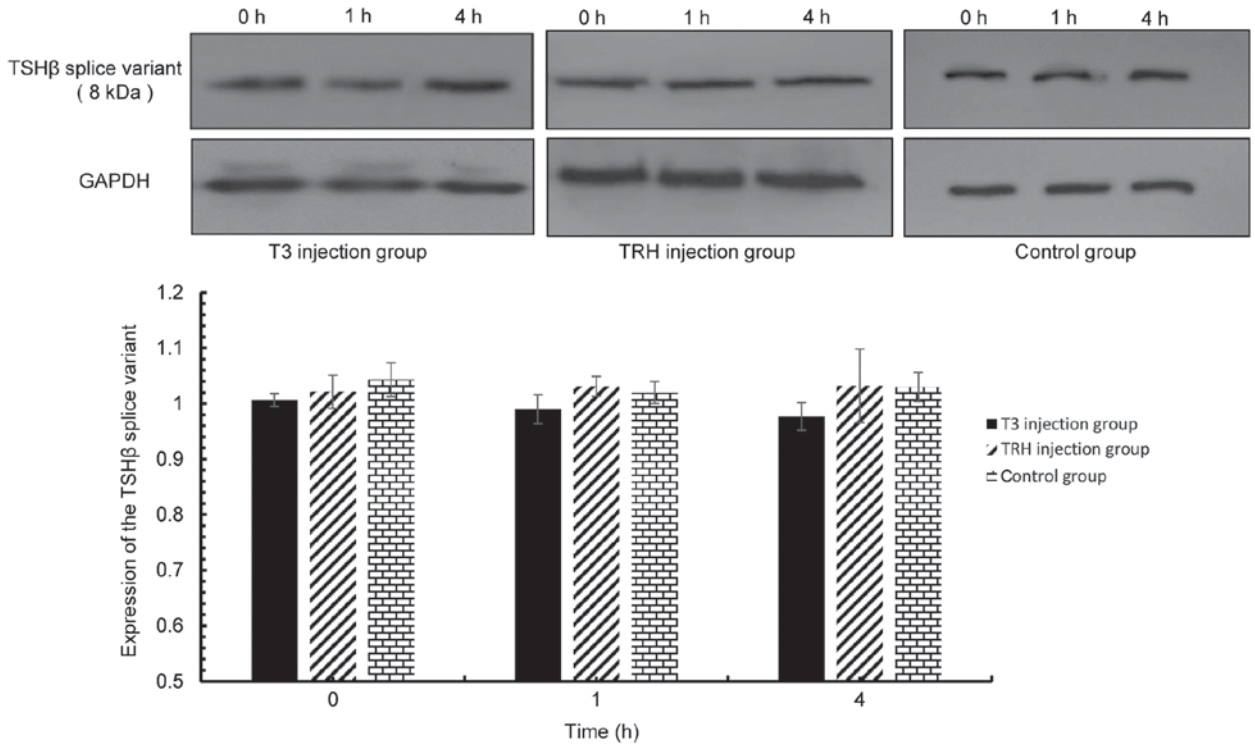

Figure 6. TSHβ splice variant expression in the pituitary was detected via western blot analysis at 0, 1 and 4 h following injection with T3 and TRH. T3 injection group vs. control group: 0 h, P=0.123; 1 h, P=0.804; 4 h, P=0.110. TRH injection group vs. control group: 0 h, P=0.073; 1 h, P=0.754; and 4 h, P=0.650. TSHβ, thyroid-stimulating hormone β; T3, tri-iodothyronine; TRH, thyroid-releasing hormone.

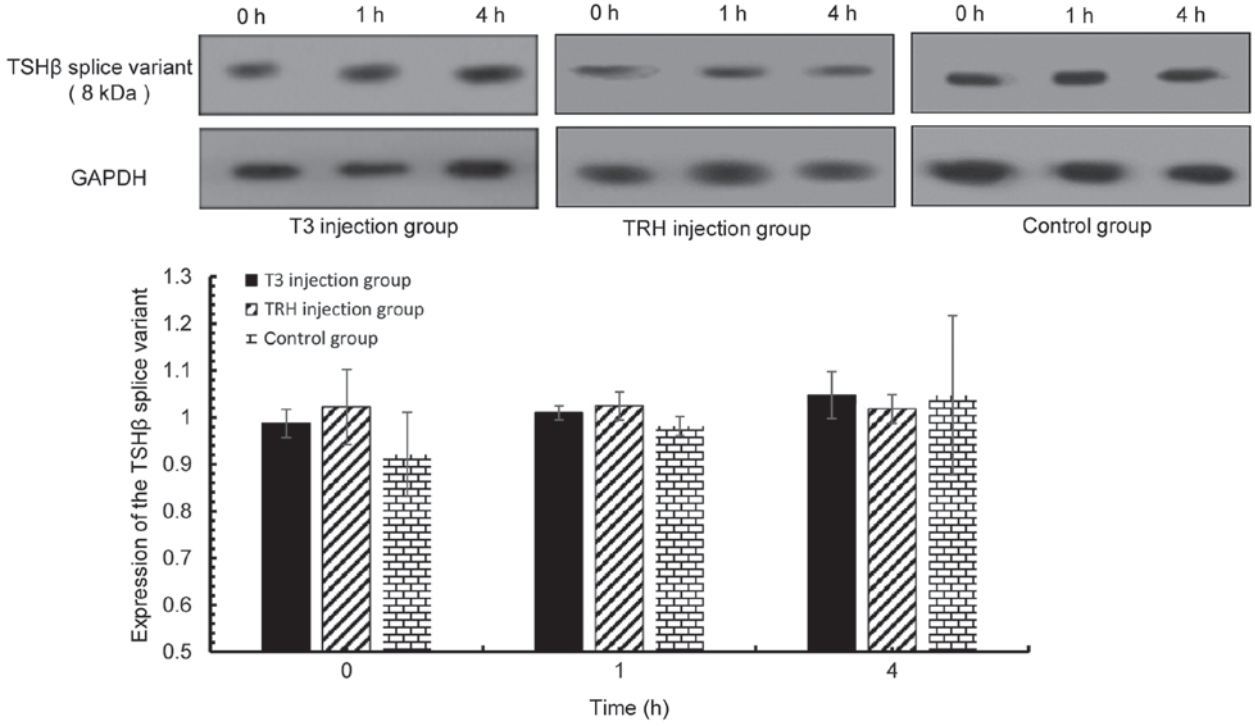

Figure 7. TSHβ splice variant expression in PBLs was detected via western blot analysis at 0, 1 and 4 h following T3 and TRH injection. T3 injection group vs. control group: 0 h, P=0.323; 1 h, P=0.189; and 4 h, P=0.997. TRH injection group vs. control group: 0 h, P=0.152; 1 h, P=0.064; and 4 h, P=0.740. TSHβ, thyroid-stimulating hormone β; PBLs, peripheral blood leukocytes; T3, tri-iodothyronine; TRH, thyroid-releasing hormone.

production and secretion of thyroid hormones, T4 and T3 (1). It has since been established that there are extra-pituitary sources of TSH, including the TSHβ splice variant produced by cells of the immune system (8). Baliram *et al* (14) demonstrated that bone marrow-derived macrophages preferentially produce the TSHβ splice variant. An additional study indicated that the

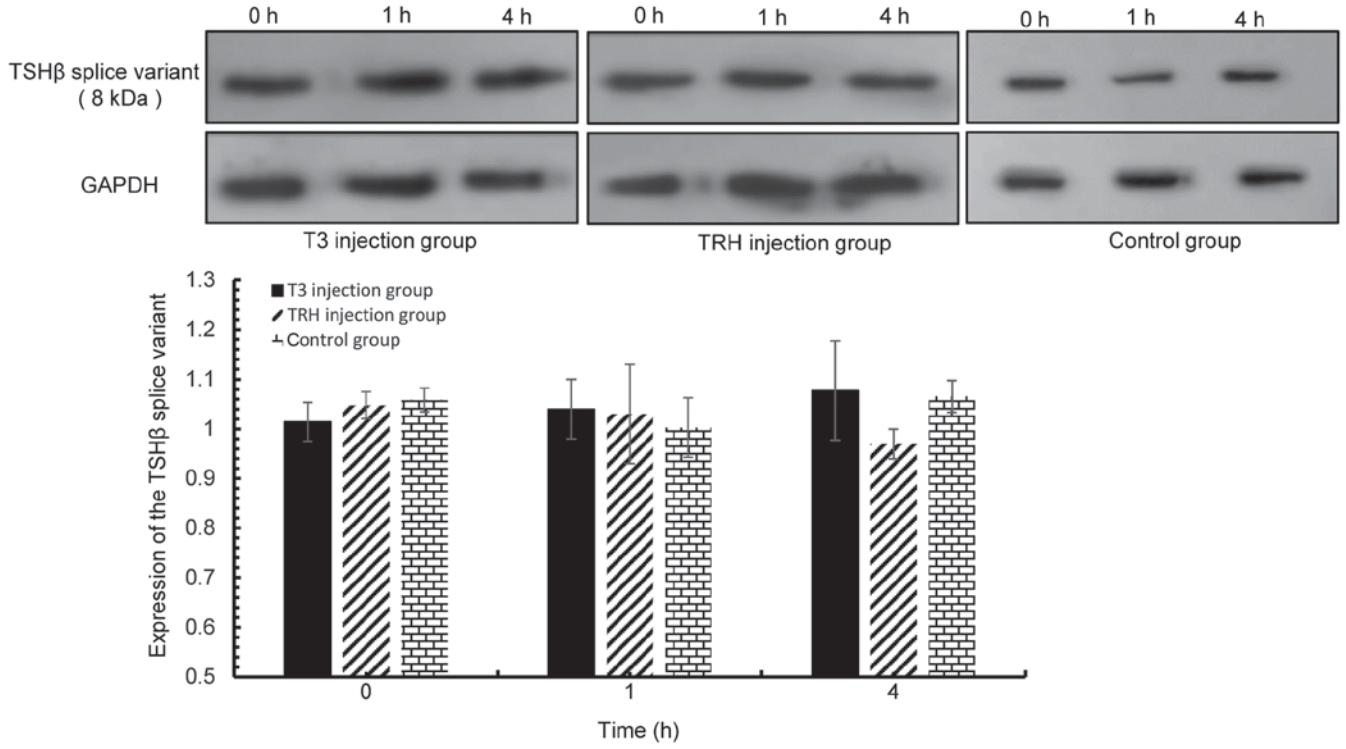

Figure 8. TSHβ splice variant expression in the spleen was detected via western blot analysis at 0, 1 and 4 h following T3 and TRH injection. T3 injection group vs. control group: 0 h, P=0.187; 1 h, P=0.603; and 4 h, P=0.829. TRH injection group vs. control group: 0 h, P=0.692; 1 h, P=0.702; and 4 h, P=0.114. TSHβ, thyroid-stimulating hormone β; T3, tri-iodothyronine; TRH, thyroid-releasing hormone.

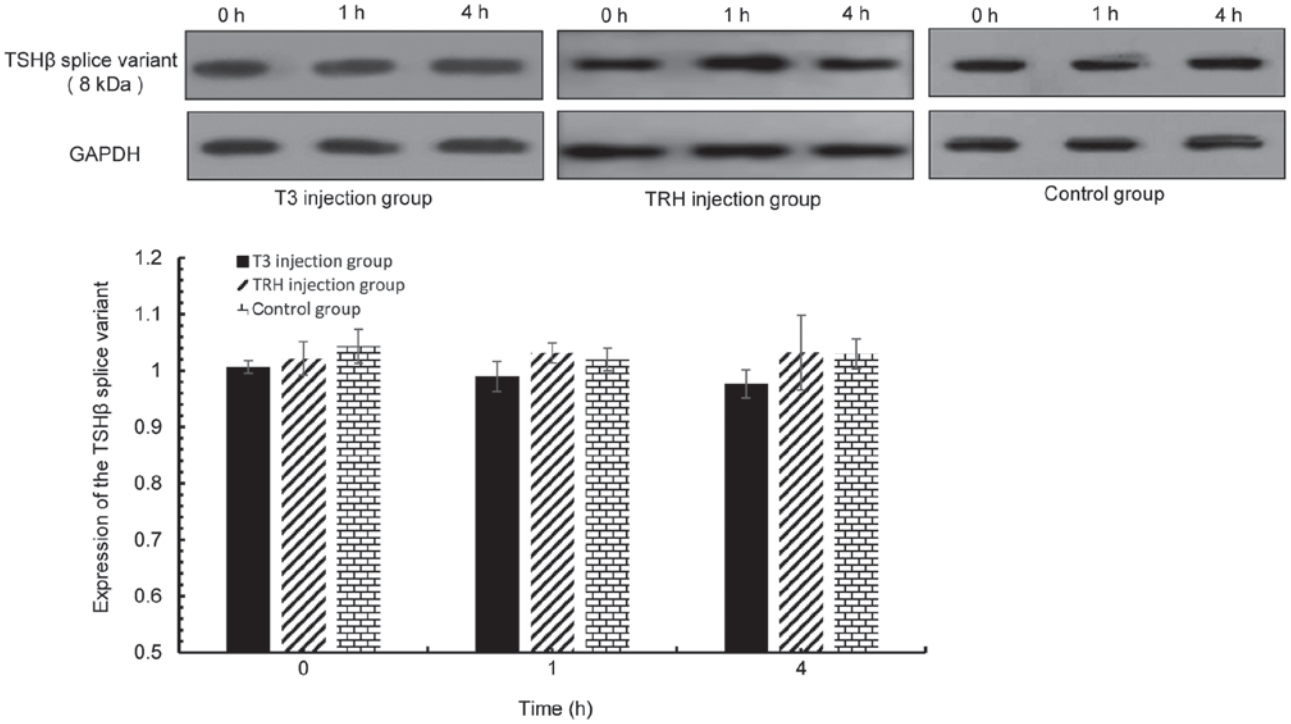

Figure 9. TSHβ splice variant expression in the thyroid was detected via western blot analysis at 0, 1 and 4 h following T3 or TRH injection. T3 injection group vs. control group: 0 h, P=0.12; 1 h, P=0.141; and 4 h, P=0.185. TRH injection group vs. control group: 0 h, P=0.322; 1 h, P=0.553; and 4 h, P=0.956. TSHβ, thyroid-stimulating hormone β; T3, tri-iodothyronine; TRH, thyroid-releasing hormone.

TSHβ splice variant was also expressed in plasma cells of the thyroid of a patient with Hashimoto's thyroiditis (15). However, whether the production of extrapituitary TSHβ splice variant promotes alterations in thyroid hormone synthesis, similar to pituitary TSH, is yet to be elucidated. Previously published *in vivo* and *in vitro* stimulation experiments have been performed

using isolated thyroid follicular epithelial cells. Upon incubation of these cells in media containing the native form of TSHβ, increased iodide transport activity, TG iodination, protein synthesis and phospholipid synthesis were observed (16). The results of the *in vivo* and *in vitro* experiments performed in the present study confirm that the TSHβ splice variant may also contribute to the synthesis and secretion of thyroid hormones, which is similar to the function of the native form of TSHβ. Therefore, the immune-derived TSHβ splice variant may microregulate thyroid hormone output via a paracrine pathway. This local regulatory circuit is likely to serve as a physiologically efficient modulator that conserves the energy-generating processes during and following immune responses. In Hashimoto's thyroiditis, thyroid follicles are destroyed by autoimmune attack induced by autoantibodies targeted against thyroid peroxidase and/or thyroglobulin, resulting in decreased T3 and T4 levels (17). TSHβ-sensing (18-20) and TSHβ-producing (5,21) leukocytes are trafficked to the thyroid and promote the synthesis and secretion of thyroid hormones under immune stress conditions, a defense response to maintain the energy-generating balance within the body. As there are known TSHβ splice variants in human serum (9), the TSHβ splice variant may exert regulatory effects via telecrine signaling, in addition to its paracrine signaling functions.

TSH synthesis in the anterior pituitary is stimulated by TRH and inhibited by thyroid hormone in the HPT axis (1). The HPT axis is a classical neuroendocrine feedback system that was considered to be functionally autonomous; however, an increasing amount of evidence indicates that immune and neuroendocrine feedback systems may interact (22). In addition to the pituitary-thyroid circuit, there are other TSH-associated circuits that regulate the immune-derived TSHβ splice variant, which may function in extrathyroidal sites within the immune system, as indicated by the ability of immune cells to produce this variant (5). Present research is focused on the mechanisms by which the expression of the TSHβ splice variant is regulated by the immune system in disease states (8,9,15,23). However, it is known that the TSHβ splice variant, besides being expressed in the pituitary, is also produced by immune cells, including bone marrow-derived macrophages, plasma cells in the thyroid and splenic leukocytes (10,14,15,23). Native TSHβ is regulated by the HPT axis. However, to the best of our knowledge, limited research has reported whether the HPT axis is involved in the expression of the TSHβ splice variant. Therefore, the HPT axis was selected as a research focus in the present study in order to provide further insights into the physiological regulation of the TSHβ splice variant, and to further explore its potential pathological effects.

As TSHβ splice variant expression was detected in the thyroid, spleen and PBLs, alterations in the expression of TSHβ splice variant in these tissues and the pituitary were determined in mice following injection with T3 or TRH. The results demonstrated that only the native form of TSHβ expression was altered in the pituitary. By contrast, the expression of TSHβ splice variant remained unaltered in all evaluated tissues. There is strong evidence to suggest that TSHβ splice variant expression may be altered under immune stress. Vincent *et al* (5) reported that TSHβ splice variant expression is increased in the thyroid following systemic virus infection. In addition, Baliram *et al* (10) reported that in hyperthyroidism, bone marrow resident macrophages exhibit the potential to induce osteoprotective effects by overexpressing human TSHβ splice variant, which may perform its local osteoprotective role via TSH receptors on osteoblasts and osteoclasts. These results suggest that the TSHβ splice variant may influence bone biology and serve as a local osteoprotective resource for bone remodeling in disease states and fracture repair.

Our previous study demonstrated that the TSHβ splice variant is expressed at significantly higher levels in thyroid tissues of patients with Hashimoto's thyroiditis compared with normal thyroid tissues (15). In addition, the expression of the TSHβ splice variant was positively associated with the degree of thyroid follicle damage in patients with Hashimoto's thyroiditis (15). Montufar-Solis and Klein (23) reported that splenic leukocytes in *Listeria monocytogenes*-infected mice migrate to the thyroid and produce the intrathyroidal TSHβ splice variant. These published observations and the results of the present study suggest that the TSHβ splice variant may participate in the regulation of thyroid hormone synthesis independently of the HPT axis. Moreover, there may be a unique regulatory mechanism of the expression of the TSHβ splice variant that may occur in certain disease states. The TSHβ splice variant may also serve as a critical immunological regulator during immune stress. The two putative nuclear factor-κB subunit binding sites in intron 4 of mouse TSHβ are hypothesized to regulate the TSHβ splice variant, and may be under the control of immunologically-mediated transcription signals (22). Elucidating the precise mechanisms underlying this regulation will require promoter activity and binding assays.

In conclusion, thyroid hormones serve a major role in metabolic function, and in response to stress and critical illness (9,10,15,24,25). The results of the present study suggest that the immune-derived TSHβ splice variant may contribute to the higher levels of serum thyroid hormones. The splice variant of TSHβ may contribute to this increase via paracrine microregulation of thyroidal follicular cells during advanced stages of infection or inflammatory-associated disorders, and is independent of regulation by the HPT axis; however, the mechanism of thyroid hormone secretion stimulated by TSHβ splice variant has not been clarified. Whether the TSHβ splice variant stimulates thyroid follicular cells to produce thyroid hormone via the TSHβ receptor or via any other pathways remains unknown. In the future, the authors of the present study intend to produce a specific antibody against TSHβ splice variant to answer these questions. The functional role of TSHβ splice variant synthesis in the immune system also remains to be determined. Nevertheless, the current study provides novel insights into the biological features and role of TSHβ splice variant, and these results may have implications in the current understanding of immune-neuroendocrine interactions.

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Availability of data and materials

The datasets used and/or analyzed in the present study are available from the corresponding author upon reasonable request.

Authors' contributions

JM and CL conceived and designed the study; XL, ZZ, TK, JL, RW and QD performed the experiments; CL and LL analyzed and interpreted the data; CL completed the draft. All authors read and approved the manuscript.

Ethics approval and consent to participate

All procedures used were in accordance with the Logistics University of Chinese People's Armed Police Force animal welfare guidelines. The animal protocols were approved by the Ethics Review Committee of the Logistics University of Chinese People's Armed Police Force (Tianjin, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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