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Use of TSHβ:EGFP transgenic zebrafish as a rapid *in vivo* model for assessing thyroid-disrupting chemicals

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ABSTRACT

Accumulating evidence indicates that a wide range of chemicals have the ability to interfere with the hypothalamic–pituitary–thyroid (HPT) axis. Novel endpoints should be evaluated in addition to existing methods in order to effectively assess the effects of these chemicals on the HPT axis. Thyroid-stimulating hormone subunit β (TSHβ) plays central regulatory roles in the HPT system. We identified the regulatory region that determines the expression level of zebrafish *TSHβ* in the anterior pituitary. In the transgenic zebrafish with EGFP driven by the TSHβ promoter, the similar responsive patterns between the expression levels of TSHβ:EGFP and endogenous *TSHβ* mRNA in the pituitary are observed following treatments with goitrogen chemicals and exogenous thyroid hormones (THs). These results suggest that the TSHβ:EGFP transgenic reporter zebrafish may be a useful alternative *in vivo* model for the assessment of chemicals interfering with the HPT system.

Introduction

The thyroid gland produces thyroid hormones (THs), and the two most important THs are tetraiodothyronine (thyroxine or T4) and triiodothyronine (T3). These hormones are essential for life and have many effects on body metabolism, growth, and development. The thyroid gland participates with the hypothalamus and pituitary in classical feedback control loops and is influenced by hormones produced by the pituitary and hypothalamus (Kratzsch and Pulzer, 2008). When the hypothalamus and the pituitary detect low levels of THs in the blood, thyrotropin-releasing hormone (TRH) is secreted to stimulate the pituitary to release TSH. In turn, increased level of TSH stimulates the thyroid to produce more THs, returns the TH levels to baseline. In healthy individuals, the HPT axis maintains TH production at a finely controlled level and enables the thyroid to respond to conditions requiring more or less TH production (Howdeshell, 2002; Kratzsch and Pulzer, 2008).

The activity of the thyroid gland is predominantly regulated by the concentration of the glycoprotein hormone TSH (also known as thyrotropin), which is synthesized and secreted by thyrotrope cells located in the anterior pituitary gland. TSH is a glycoprotein complex that consists of two subunits, α and β. The α subunit is nearly identical to that of several other hormones, including luteinizing hormone (LH) and follicle-stimulating hormone (FSH). The β subunit (TSHβ), however, is unique to TSH and determines the specificity to its receptor (Kratzsch and Pulzer, 2008). TSH stimulates the thyroid gland to secrete the hormones thyroxine (T_4) and triiodothyronine (T_3). TSH levels are controlled by TRH which is produced in the hypothalamus. The levels of THs in the blood also influence the production of TSHβ from the pituitary *via* the negative feedback loops. Therefore, the measurement of *TSHβ* expression in the anterior pituitary is potentially a useful biomarker for the function of HPT systems (Schmutzler et al., 2007; Kratzsch and Pulzer, 2008).

Bodies of water receive significant inputs of natural and synthetic chemicals that act as endocrine disrupting chemicals (EDCs). Initially, attention was given to threats on the reproductive health of fish populations (Carr and Patino, 2011). It has been demonstrated that EDCs may also have multiple targets on the complex regulatory network of endocrine systems. In addition to the reproductive effects, increased evidence suggests that the HPT is a target of endocrine disruption by EDCs as well (Howdeshell, 2002; Schmutzler et al., 2007). Classical tests for the assessment of these thyroid-disrupting chemicals (TDCs) include the quantification of serum TH concentration, measurement of thyroid weight and/or assessment of histopathology in mammals. The amphibian metamorphosis assay is designed as a screen for thyroid activity in amphibians (Howdeshell, 2002; Schmutzler et al., 2007; Kratzsch and Pulzer, 2008). Recently, a T4 immunofluorescence quantitative disruption test (TIQDT) was developed to measure thyroid function using a zebrafish model (Raldua and Babin, 2009). Although this method is relatively simple, rapid and cost-effective for TDC screening, it still requires a laborious whole-mount immunohistochemistry procedure. Additionally, it is important to note that there are various types of thyroid disruption,

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resulting in differential effects on the various endpoints assessed in the screens.

Zebrafish, *Danio rerio*, is a vertebrate model ideal for studying the expression patterns of specific genes during development. To monitor the dynamic expression of *TSHβ in vivo*, we identified its regulatory region within the zebrafish genome. We generated transgenic zebrafish expressing enhanced green fluorescent protein (EGFP) under control of the regulatory elements of the *TSHβ* gene. EGFP expression within the pituitary gland for each transgenic line recapitulated the expression patterns of endogenous pituitary *TSHβ* mRNA and was specifically targeted to the thyrotrope cells located in the anterior pituitary gland. Our results also demonstrated that TSHβ:EGFP expression and endogenous *TSHβ* mRNA expression in the pituitary exhibited similar responses to treatments with various goitrogens and exogenous THs. Despite some temporal and spatial differences, the zebrafish HPT axis shares a conserved basic mechanism with higher vertebrates. Thus, the TSHβ:EGFP reporter zebrafish generated in our studies provide a potentially useful *in vivo* model for the assessment of chemicals that may interfere with the activities of animal HPT systems.

Materials and methods

Zebrafish embryo and larva maintenance. Zebrafish (*D. rerio*) embryos were maintained and raised according to the protocol described by Westerfield (1995). Zebrafish were kept at 28.5 °C with a light–dark cycle of 14:10 h, and about 5% of the system water was replaced daily with filtered tap water. Eggs were collected and kept at 28.5 °C in a clean Petri dish in egg water containing 60 mg/L of "Instant Ocean" salt.

Characterization of the TSHβ promoter region. Initially, a 2.5 kb DNA fragment of the 5′ region of zebrafish *TSHβ* was amplified by PCR from genomic DNA using the following pair of primers: TSHβ-F (SacI restriction site was underlined), 5′-GAGCTC GTGCTGTTTAT-CTGACGCAGC-3′; and TSHβ-R (ApaI restriction site was underlined), 5′-GGGCCCGGTGGCAGTGTATGAGGATGTC-3′. The PCR fragment was digested with SacI and ApaI and then ligated to the multiple cloning sites (MCS) of a EGFP reporter vector (pEGFP-N1-CMV^{-} vector, Clontech) to generate the TSHβ:EGFP reporter construct.

Serial deletions containing 0.3 to 2.2 kb of the original 2.561 kb regulatory region fragment were directionally subcloned into the promoterless pEGFP-N1 reporter vector to analyze the promoter region of the *TSHβ* gene. Additionally, a fragment containing a TAA site deletion between positions −1650 to −1648 was also cloned into the reporter vector for *in vivo* transcriptional activity assessment. Each reporter construct was micro-injected into zebrafish embryos. Their transcriptional activities were evaluated by analyzing EGFP expression *in vivo* in at least 50 F0 transgenic larvae at 4.5 days post fertilization (dpf) *via* fluorescence microscopy.

Generation of TSHβ:EGFP transgenic zebrafish. The TSHβ:EGFP reporter construct containing the 2.5 kb as TSHβ regulatory fragment was linearized with the restriction enzyme SacI and injected into one- or two-cell stage embryos at a concentration of 100 μg/ml. Expression of EGFP was observed and photographed under a Leica fluorescence stereomicroscope. The injected embryos showing EGFP expression were raised to adulthood. Individual founder fish were crossed with wild-type fish, and their progenies were observed under a fluorescence stereomicroscope to examine EGFP expression in the pituitary gland. Founder fish that produced EGFP-positive embryos were bred to generate F1 heterozygotes and F2 homozygotes.

Chemical treatments. Potassium perchlorate ($KClO_4$), 6-propyl-2-thiouracil (PTuracil), triiodothyronine (T3) and thyroxine (T4) were purchased from Sigma-Aldrich (St. Louis, MO). Additional information regarding the selected chemicals is described in the Supplementary material. All dilutions are reported as nominal concentrations. Stock solutions (1000×) of 1.3 M PTuracil, 3.96 M $KClO_4$, 50 μM T3, and 30 μM T4 were prepared in DMSO on the day of the experiment. Embryos exposed to 0.1% DMSO were used as a vehicle control. At the selected dilution, DMSO did not have any visible morphological effects on zebrafish development. Working solutions were freshly prepared by diluting the stock solutions with filtered egg water. All concentrations tested for goitrogens ($KClO_4$, PTuracil) and TH reagents (T3, T4) were below the aqueous solubility values provided in the SRC PhysProp Database (www.syrres.com/esc/physprop.htm). Embryos at 36 h post fertilization (hpf) stage were exposed for 3 days to freshly prepared test solutions under semi-static conditions. A minimum of 15 TSHβ:EGFP or wild-type embryos were randomly selected for each tested experimental condition. Observations for each condition were repeated at least two times. All the reporter larvae were examined, and the fluorescence images of the pituitary glands were captured with a Leica fluorescence stereomicroscope.

Whole-mount in situ hybridization (WISH). The zebrafish *nr2e3* fragment was amplified from an embryonic zebrafish cDNA pool with the following primers: *nr2es*-F(5′-AAATCAGTGCCAGGCCTGTCGC-3′) and *nr2es*-R (5′-CTTGCAAGGGCTTGAACCGGCT-3′). The 704 bp fragment was used as a template to synthesize the antisense probe. The zebrafish *TSHβ* probe was described previously (Li et al., 2009). The procedure for probe labeling and WISH using digoxigenin (DIG)-labeled riboprobes was carried out as previously described (Li et al., 2009).

Fluorescence signal analysis. Quantitative analysis of fluorescence signals was performed with free-processing ImageJ software (NCBI) as previously described (Raldua and Babin, 2009), with modifications described in the Supplementary material.

Statistical analysis. Statistical Product and Service Solutions (SPSS) software was used to determine significant differences between treatments at the 5% level. The data were analyzed by Least Significant Difference (LSD) test following Analysis of Variance (ANOVA) and Student t-tests. A total of 15 samples for each group were assessed. Treatment means and standard errors were calculated.

Results

Based on genomic DNA information provided by the NCBI GenBank (accession no. NC_007117.5), the zebrafish *TSHβ* gene contains three exons and two introns (Fig. 1A). The exon1 contains a 21 bp untranslated region. The 188 bp exon2 contains a 29 bp fragment which encodes a portion of the 5′ untranslated region of zebrafish *TSHβ* mRNA. Most *TSHβ* coding sequences are located within exon3 (Fig. 1A). To search for the potential regulatory DNA sequences in zebrafish, a 2561 bp fragment of the 5′ flanking region containing the region between the sites −2565 and −5 was amplified with a pair of primers, TSHβ-F and TSHβ-R. This fragment contains the first 24 bp of exon2, 1602 bp of the intron1, 21 bp of exon1 and an additional 910 bp of the 5′ flanking region of the *TSHβ* gene (Fig. 1A). Based on sequence analysis of this 2561 bp fragment, many putative response elements important for mammalian *TSHβ* expressions were found in this region, particularly in the region containing exon1 and intron1 of the *TSHβ* gene. These putative response elements included the pineal expression-promoting elements (PEPE) (Alon et al., 2009), negative T3-responsive elements (nTRE) (Sohn et al., 1999), thyrotroph site or GATA-2 responsive site (Alexander et al., 1989; Kashiwabara et al., 2009), Pit-1 responsive site (Pit-1, Kashiwabara et al., 2009) and LIM homeodomain transcription factor (Lhx) responsive site (Kim et al., 2007) (Fig. 1B).

Germline transgenic zebrafish were generated with the 2561 bp fragment amplified as the regulatory unit for the EGFP reporter construct (Fig. 2A). The identical ectopic EGFP expression pattern was observed in the progenies from 3 independent transmission germlines. The EGFP-

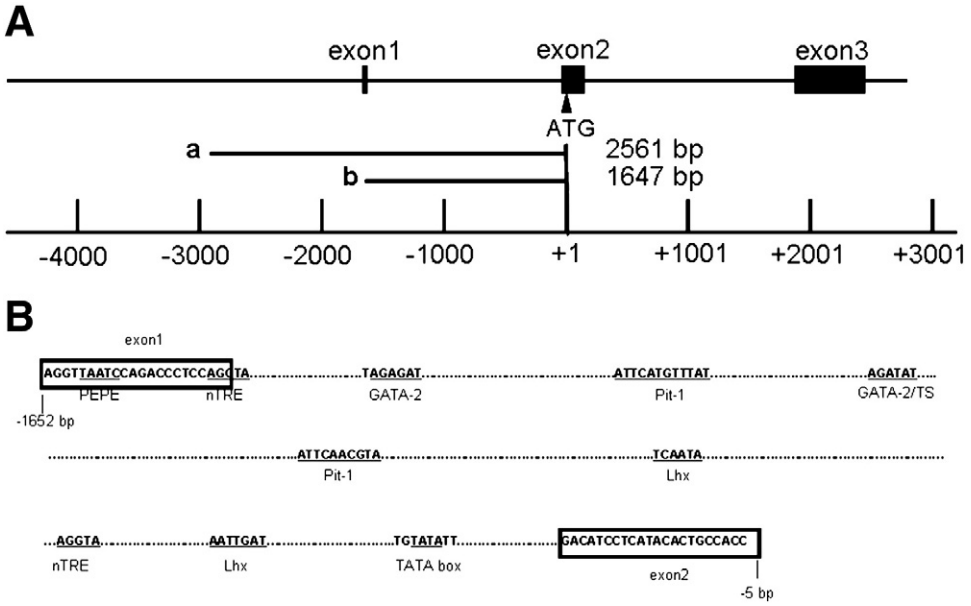

Fig. 1. Genomic structure of the zebrafish TSHβ gene. (A) Schematic representation of the genomic structure of the zebrafish TSHβ gene. Exons1–3 of the zebrafish TSHβ gene are shown with filled boxes. The 5′ flanking noncoding region and introns are depicted as a straight line. a, 2561 bp promoter fragment of the TSHβ gene; b, 1647 bp promoter fragment of the TSHβ gene. (B) Putative regulatory elements identified in the minimal proximal 5′ flanking regulation region (1647 bp fragment from −1652 to −5) in the zebrafish TSHβ gene. The following sequences were used for consensus sequences: PEPE, pineal expression-promoting elements [5′-TAAT(C/T)-3′, Alon et al., 2009], nTRE, negative T3-responsive elements (5′-AGGTA-3′ and 5′-GGGTCA-3′, Sohn et al., 1999); GATA-2, [5′-(A/T)GATA(A/G)-3′, Kashiwabara et al., 2009]; TS, thyrotroph site, (5′-AGATAT-3′, Alexander et al., 1989); Pit-1, [5′-ATTC(N)$_{2-6}$AT-3′, Kashiwabara et al., 2009]; Lhx, [5′-(G/T)CAAT(T/A)-3′, Kim et al., 2007].

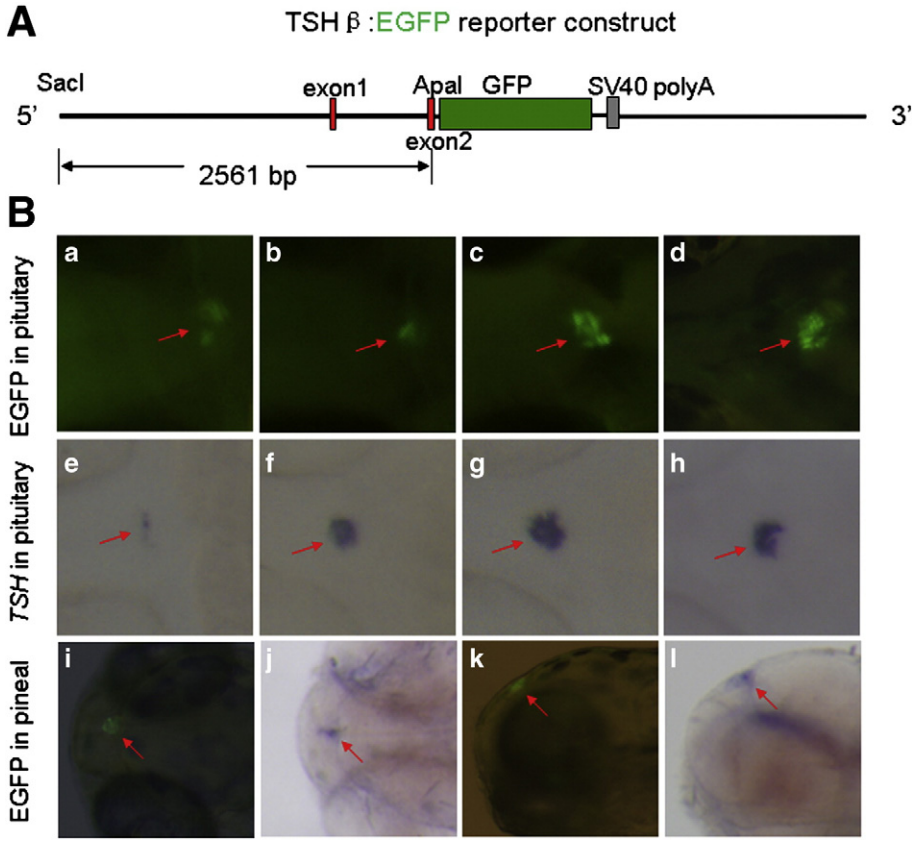

Fig. 2. Generation of TSHβ:EGFP transgenic zebrafish. (A) Schematic representation of the EGFP reporter construct used for generating transgenic zebrafish. The DNA fragment containing a 2561 bp fragment, including the 5′ flanking region, entire exon1, intron1 and the first 29 bp in exon2 of the zebrafish *TSHβ* gene, was ligated to the multiple cloning sites (MCS) of an EGFP reporter gene. (B) EGFP expression patterns in F1 TSHβ:EGFP transgenic fish. a–d, EGFP expression in pituitary of the TSHβ:EGFP transgenic larva at 33 hpf (a), 36 hpf (b), 48 hpf (c) and 4.5 dpf (d). e–h, *TSHβ* mRNA visualized by WISH in pituitary of wild-type larva at 33 hpf (e), 36 hpf (f), 48 hpf (g), 4.5 dpf (h). i–l. EGFP (i, k) and WISH of *nr2e3* expression (j, l) in the pineal in F1 TSHβ:EGFP transgenic fish at 4.5 dpf. a–j, Ventral view with the anterior to the left, k–l. Lateral view with the anterior to the left.

positive cells began to become evident in the pituitary gland as early as 33 hpf (Fig. 2Ba). At this stage, the appearance of EGFP-positive cells was symmetrical, revealing two single-cell masses. Next, two single-cell masses converged into a single-cell cluster at 36 hpf (Fig. 2Bb). The pattern of TSHβ promoter-driven EGFP expression in the pituitary primordia was similar to that observed for the pituitary *TSHβ* mRNA expression in wild-type zebrafish embryos, as shown by *in situ* hybridization (Figs. 2B.e–h). An additional EGFP expression domain, however, was unexpectedly observed in the pineal gland of all fish from the 3 independent TSHβ:EGFP transgenic lines. This pineal domain of the TSHβ:EGFP in the transgenic fish was co-localized with the expression domain of *nr2e3*, a pineal-specific molecular marker (Figs. 2B.i–l).

To define the region within the 2561 bp of the *TSHβ* 5′ flanking sequence necessary for EGFP transactivation, a series of the fragments used as regulatory units for the EGFP reporter constructs was prepared and micro-injected into zebrafish embryos (Fig. 3A). Based on our

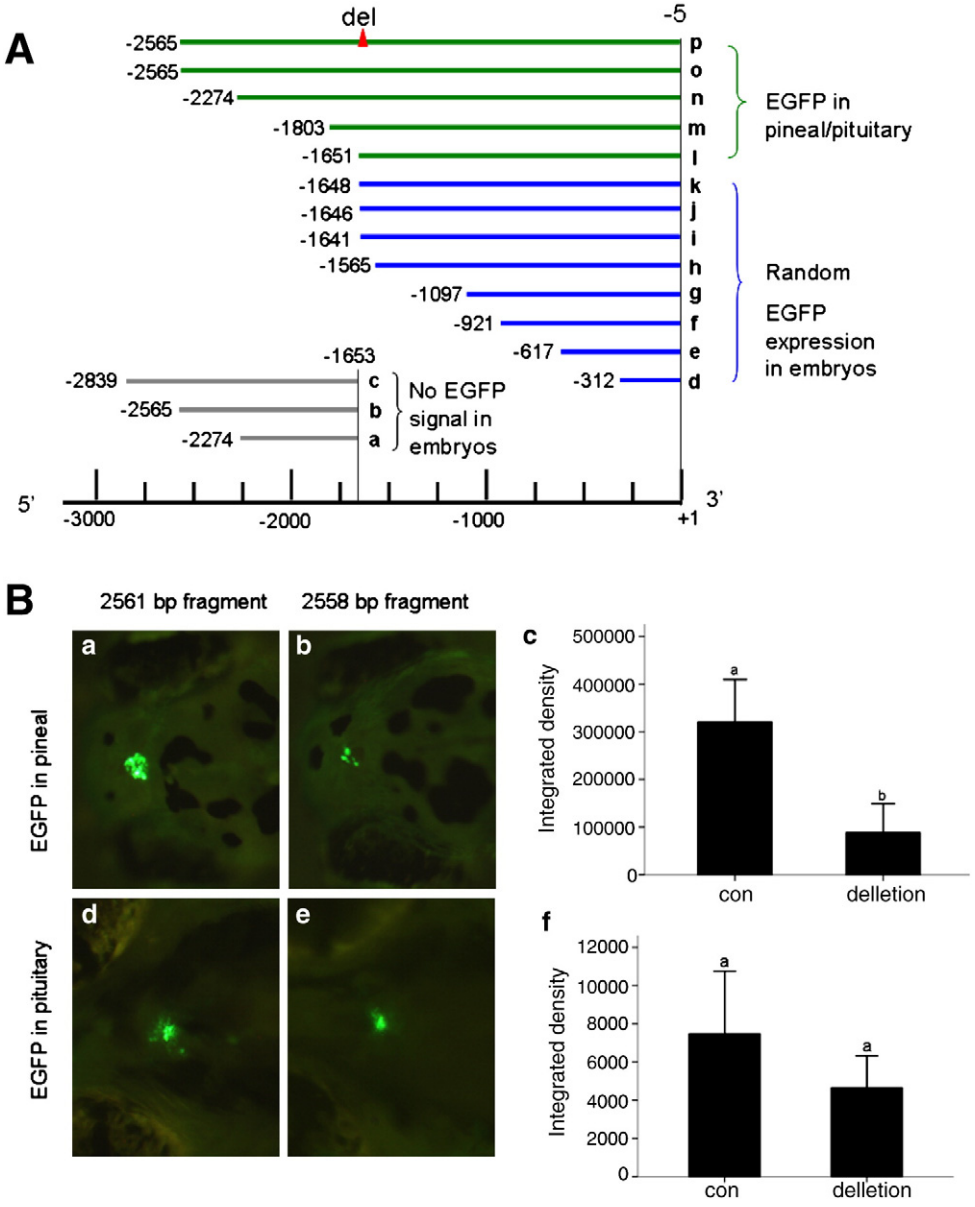

Fig. 3. Characterization of the promoter region of the zebrafish TSHβ gene. (A) Relationships between different 5′ upstream regions of the TSHβ functioning as the promoter fragments for transgenic constructs and the EGFP expression patterns observed in the zebrafish embryos at 4.5 dpf. Fragments a–c, the fragments from the −1653 to −2839 region could drive EGFP expression in embryos. Fragments d–k, the fragments from the −5 to −1648 region show random EGFP expression throughout the entire body, but not in the pituitary or pineal gland. Fragments i–o, the fragments from the −5 to −1651, −5 to −1803, −5 to −2274, or −5 to −2565 regions show specific EGFP signal expression in pituitary and pineal glands. Fragment p, the fragment spanning from the −5 to −2565 region with a deletion of a TAA at −1650 to −1648 demonstrates slightly decreased EGFP expression in the pituitary and dramatically decreased EGFP expression in the pineal gland. (B) Comparison of the EGFP expression patterns between promoter regions adapted from the fragment containing the original 2561 bp of the 5′ flanking region of the TSHβ gene or from the deletion mutant fragment with a 3 bp-deletion of the TAA site at −1650 to −1648. a, Strong EGFP expression in the pineal gland with a 2561 bp fragment as the promoter. b, Dramatically decreased EGFP expression in the pineal gland with a 3-bp deletion mutant fragment as the promoter. c, Comparison of the quantitative analysis of EGFP integrated densities (IDs) in the pineal glands from the 2561 bp fragment or the TAA deletion mutant 2558 bp as the promoter. A significant decrease ($P<0.0001$) in EGFP ID in the pineal glands was observed in the 3 bp mutant zebrafish at 3.5 dpf. d, Strong EGFP expression in the pituitary gland in fish with the 2561 bp fragment as the promoter. e, Slightly decreased EGFP expression in the pituitary gland in fish with the 3-bp deletion mutant fragment as the promoter. f, Comparison of the quantitative analysis of EGFP IDs in the domains of the pituitary glands from the 2561 bp fragment or the TAA deletion mutant 2558 bp as the promoter. No significant decrease ($P<0.05$) in EGFP ID in the pituitary was observed due to the 3 bp mutation in zebrafish at 3.5 dpf. a, b, Dorsal views. d, e, Ventral views. The values in c and f are represented the means ± SE. n = 15. Mean values with different letters are significantly different ($P<0.0001$).

observations, the regions between positions −1653 and −2839 were not sufficient to induce EGFP signal expression in zebrafish (Figs. 3A.d–k). The fragment containing the regions between positions −5 and −1648 demonstrates random EGFP expression throughout the entire body, but not in the pituitary or pineal gland (Figs. 3A.d–k). A minimal proximal region spanning from −5 to −1651 of the *TSHβ* 5′ flanking sequence was found to be essential for the regulation of EGFP expression in the pituitary and pineal glands (Figs. 3A.i–o). Interestingly, deletion of the TAA site (−1650 to −1648) within the 2561 bp 5′ flanking region of *TSHβ* resulted in 3/4 of the EGFP integrated density (ID) loss in the pineal gland of zebrafish at 3.5 dpf stage (Figs. 3Ba–Bc) but did not cause a statistically significant loss in the pituitary (Figs. 3Bd–Bf, $P>0.05$). These results indicate that this PEPE site found at −1650 to −1646 in the *TSHβ* 5′ flank region is more important for the regulation of the unexpected EGFP expression within the pineal gland of our transgenic fish than its role for the EGFP expression presence in pituitary.

Certain goitrogen chemicals (*e.g.*, $KClO_4$, PTuracil) and THs (*i.e.*, T3 and T4) that are known to affect *TSHβ* expression were selected for the initial development of this test procedure. Live TSHβ:EGFP transgenic fish were exposed to the selected goitrogens for 3 days beginning at 36 hpf. As depicted in our figures (Figs. 4a, d), enhanced EGFP expression patterns were observed in the pituitary gland of the TSHβ:EGFP transgenic zebrafish following 3 days of treatment with 1.3 mM PTuracil or 3.96 mM $KClO_4$. When TSHβ:EGFP transgenic zebrafish were treated with 50 nM T3 or 30 nM T4 starting from 1.5 dpf to 4.5 dpf, significant decreases in EGFP expression patterns were observed in the pituitary gland (Figs. 4g, j). The EGFP expression patterns in response to the goitrogens and TH reagents were also reflected in the *TSHβ* mRNA expression patterns observed in wild-type zebrafish following treatment with corresponding goitrogens and TH reagents assayed with WISH (Figs. 4c, f, i, l). Distinct differences were not observed, however, for EGFP expression patterns in the pineal gland in response to the chemical treatments (Figs. 4b, e, h, k). Our observations of EGFP expression in the pituitary gland following treatments with the goitrogens and TH reagents demonstrated that a 3-day exposure period for TSHβ:EGFP transgenic zebrafish larvae was long enough to cause a significant difference in EGFP expression patterns in the anterior pituitary. In addition, significant decreases in EGFP activity in the pituitary gland were observed following treatment with increasing T3 and T4 concentrations. As shown in our figures (Fig. 5), the no observed effect concentration (NOEC) for T3 was 2.5 nM with a relative ID of 106.74 ± 8.33% (Fig. 5A). Alternatively, treatments with T3 at concentrations of 50 nM and 20 nM resulted in significantly decreased EGFP expression, demonstrating Relative IDs of 28.65 ± 4.64% and 33.08 ± 3.74%, respectively ($P<0.0001$) (Fig. 5A). Our results also indicate that EGFP expression in the pituitary gland of fish treated with T4 at concentrations of 90 and 135 nM was significantly lower ($P<0.0001$) than the control group in terms of the Relative ID, with mean values of 43.82 ± 8.18% and 26.92 ± 4.73%, respectively (Fig. 5B). The NOEC for T4 treatment was 10 nM, with a Relative ID of 105.05 ± 11.19% (Fig. 5B).

Discussion

Exposure to numerous man-made and natural environmental agents poses a significant threat to human health. For many of these dangerous toxic agents, aquatic environments serve as the major route of distribution, and their sediments represent the ultimate sink. Fish accumulate environmental contaminants *via* absorption across the gill epithelium and, primarily, by bioconcentration up the food chain. Due to this accumulation, fish are often used as a biomonitor to indicate the potential for human exposure to environmental agents (Segner, 2009; Carr and Patino, 2011). We are developing a model system that uses transgenic zebrafish with an easily assayable reporter gene under the control of specific inducible DNA response elements. The monitoring system is both convenient and cheap. This assay does not require euthanizing the fish. Additionally, effects of *in vivo* bioaccumulation in fish can also be assessed during the evaluation procedure with our transgenic reporter fish model.

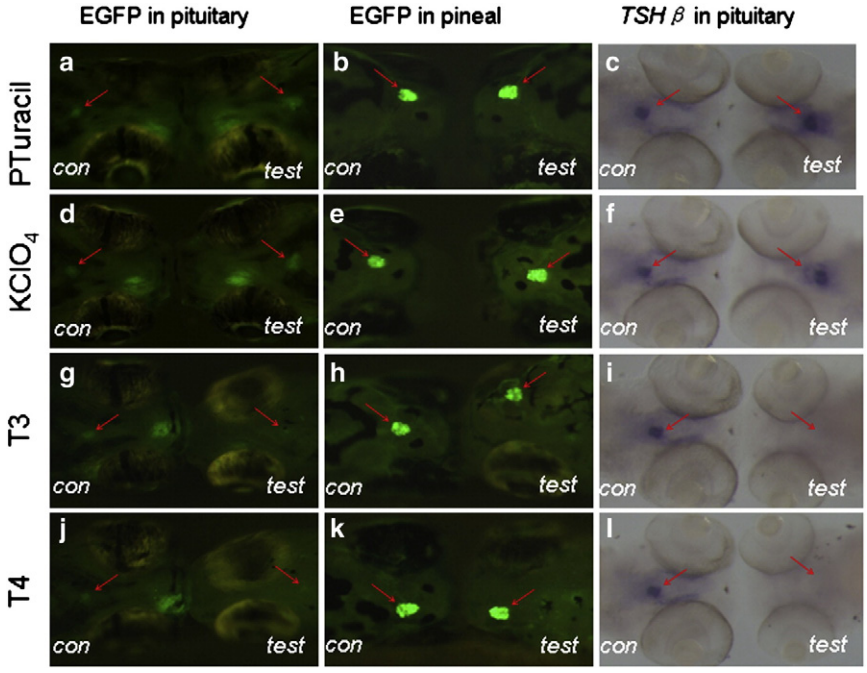

Fig. 4. Comparison of the expression of TSHβ:EGFP to endogenous *TSHβ* mRNA in response to known goitrogens and THs. a, b, c, Fish treated with 1.3 mM 6-n-propyl-2-thiouracil (PTuracil). d, e, f, Fish treated with 3.96 mM potassium perchlorate ($KClO_4$). g, h, i, Fish treated with 50 nM triiodothyronine (T3). j, k, l, Fish treated with 30 nM thyroxine (T4). a, d, g, j, EGFP expression in the pituitary gland of TSHβ:EGFP transgenic fish following the treatments (ventral views). b, e, h, k, EGFP expression in the pineal gland of TSHβ:EGFP transgenic fish following the treatments (dorsal views). c, f, i, l, Endogenous *TSHβ* mRNA expression in the pituitary of wild-type zebrafish following the treatments (ventral views). The control larvae are shown on the left, and the goitrogen/TH-treated larvae are shown on the right for each picture. A solution of 0.1% DMSO was used as a vehicle control. The fish were treated with a 3-day exposure beginning at 1.5 dpf to 4.5 dpf. Expression domains in the pituitary and pineal glands are indicated with red arrows.

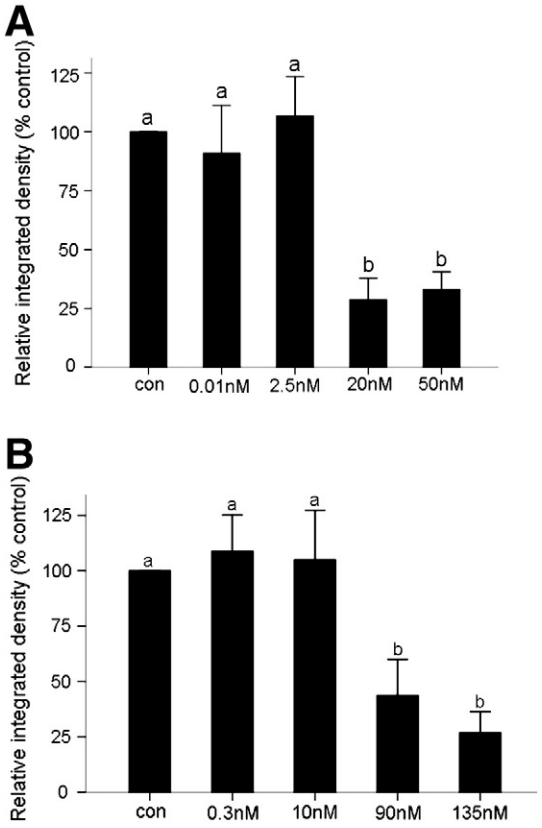

Fig. 5. Dose-dependent response of the ectopic TSHβ:EGFP in the pituitary to the TH treatments. (A) Decreasing EGFP expression patterns in the pituitary in response to treatments with increasing concentrations of T3. (B) Decreasing EGFP expression patterns in the pituitary gland in response to treatments with increasing concentrations of T4. Zebrafish at 1.5 to 4.5 dpf were treated with THs for 3 days. A solution of 0.1% DMSO was used as a control. Columns represent the arithmetic mean of relative integrated density (% control) and are shown as means ± SE. n = 15. Mean values with different letters are significantly different ($P<0.0001$).

Most of the our knowledge on the regulatory elements of animal *TSHβ* was derived from *in vitro* experiments conducted in various thyrotropic tumor or even kidney cell lines (Alexander et al., 1989; Wood et al., 1990; Haugen et al., 1996; Sohn et al., 1999; Kim et al., 2007; Alon et al., 2009; Kashiwabara et al., 2009). Transfection experiments in thyrotrope cells have demonstrated that cell-specific activity of the mouse *TSHβ* promoter is localized between −270 and −80 of the 5′ flanking region (Haugen et al., 1996). Prior to our present study, an *in vivo* test assessing the *TSHβ* promoter of any type of animal has not been reported. To explore possible mechanisms modulating the expression of the zebrafish *TSHβ* gene *in vivo*, we characterized the zebrafish *TSHβ* gene promoter. Sequence analyses revealed that the majority of the conserved regulatory elements described previously for the mammalian *TSHβ* promoter were located in the intron 1 region of the zebrafish *TSHβ* gene. Although the initial germline transgenic zebrafish were generated with an EGFP reporter construct containing a 2561-bp fragment, the fragment from −1651 to −5 containing the most putative response elements is a minimal regulation fragment sufficient for TSHβ:EGFP expression. These potential regulatory elements include putative binding sites for critical transcriptional factors, such as nTRE, Pit-1, GATA-2 and Lhx, which might be critical for the TH regulation and thyroid-specific expression of zebrafish *TSHβ* (Alexander et al., 1989; Kim et al., 2007; Lema et al., 2009). These data may indicate that the same regulatory system for *TSHβ* expression, involving Pit-1, GATA-2 and Lhx proteins, is conserved in a teleost model.

Our RT-PCR and whole-mount *in situ* hybridization results indicate that zebrafish *TSHβ* transcript is only expressed in the anterior pituitary during the early larvae stages. Additionally, endogenous *TSHβ* transcript has not been detected in adult zebrafish and common carp pineal gland tissue by RT-PCR (data not shown). A clear expression domain for EGFP driven by the regulatory region identified in the zebrafish TSHβ gene, however, has been observed in our transgenic fish (Figs. 2i–l). Several putative pineal expression-promoting elements (PEPEs) were observed in the 5′ flanking regulatory region of the zebrafish *TSHβ* gene as well (Alon et al., 2009). Moreover, a transactivation function of one PEPE located at the −1650 to −1646 region critical for EGFP expression in the pineal gland of our transgenic fish was also observed. It is still unknown how the existing PEPE in the minimal regulatory region of the pituitary gland induces *TSHβ* expression and the expression of EGFP in the pineal glands of our transgenic zebrafish. It may be a distal regulatory element for an unknown adjacent gene close to zebrafish *TSHβ*. Results from our chemical treatments, however, demonstrated that this pineal EGFP expression domain found in our transgenic fish is more resistant to the effects of the goitrogens or THs. This suggests that regulation of the unexpected pineal EGFP expression domain in these transgenic reporter fish is not involved in the HPT system.

Conservation observed in the gene and protein structure of TSH and its receptor suggests that their function has been conserved throughout the course of vertebrate evolution. Reduction in fish *TSHβ* expression following either T4 or T3 treatment has been documented by previous studies (Lema et al., 2009; MacKenzie et al., 2009; Raldua and Babin, 2009; Carr and Patino, 2011). These observations indicate that similar feedback regulation of *TSHβ* expression in the mammalian model also exists in teleosts. Additionally, our results demonstrate that the expression of EGFP within the pituitary primordia of TSHβ:EGFP transgenic zebrafish is similar to that of endogenous *TSHβ* levels in wild-type zebrafish (Fig. 4). Goitrogens are substances that cause an enlargement of the thyroid *via* various mechanisms. PTuracil can reduce TH synthesis by inhibiting thyroperoxidase activity. $KClO_4$ is also able to reduce TH synthesis *via* impairing iodide uptake (Raldua and Babin, 2009). In order to achieve an adequate secretion of THs, the adaptive processes are triggered and maintained by increased expression and secretion of TSH. The levels of EGFP expression in the transgenic reporter fish were highly associated with endogenous *TSHβ* transcript levels. Considering the signal cascade for hormone regulation within the HPT system (Fig. 6), the expression of anterior pituitary EGFP in our reporter zebrafish exhibited appropriate responsiveness to treatment with these goitrogens and THs. Based on these results, measuring anterior pituitary EGFP expression driven by the *TSHβ* promoter as a reflection of the endogenous *TSHβ* mRNA expression domain in anterior pituitary thyrotrope cells may be a useful and unique *in vivo* system for the detection of chemicals that may potentially affect critical signals along the HPT cascade.

Disclosure statement

The authors declare no conflict of interest.

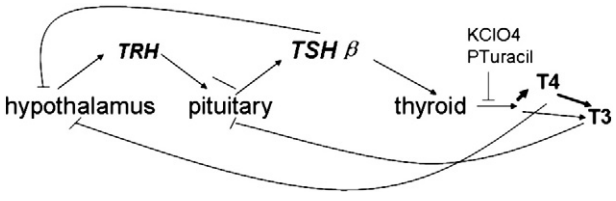

Fig. 6. Schematic diagram of the feedback loops within the HPT axis for regulation of pituitary *TSHβ* expression. *TSHβ* expression is influenced by TRH synthesized in the hypothalamus, whereas the presence of high levels of *TSHβ* suppresses *TRH* expression in the hypothalamus. TSHβ stimulates the thyroid to produce THs. Through an additional negative feedback mechanism, increased levels of free THs inhibit *TSHβ* expression in the pituitary, whereas decreased levels of THs cause an increase in *TSHβ* expression in the pituitary.

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Appendix A. Supplementary data

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References

Alexander, L.M., Gordon, D.F., Wood, W.M., Kao, M.Y., Ridgway, E.C., Gutierrez-Hartmann, A., 1989. Identification of thyrotroph-specific factors and cis-acting sequences of the murine thyrotropin beta subunit gene. Mol. Endocrinol. 3, 1037–1045.

Alon, S., Eisenberg, E., Jacob-Hirsch, J., Rechavi, G., Vatine, G., Toyama, R., Coon, S.L., Klein, D.C., Gothilf, Y., 2009. A new cis-acting regulatory element driving gene expression in the zebrafish pineal gland. Bioinformatics 25, 559–562.

Carr, J.A., Patino, R., 2011. The hypothalamus–pituitary–thyroid axis in teleosts and amphibians: endocrine disruption and its consequences to natural populations. Gen. Comp. Endocrinol. 170, 299–312.

Haugen, B.R., McDermott, M.T., Gordon, D.F., Rupp, C.L., Wood, W.M., Ridgway, E.C., 1996. Determinants of thyrotrope-specific thyrotropin beta promoter activation. Cooperation of Pit-1 with another factor. J. Biol. Chem. 271, 385–389.

Howdeshell, K.L., 2002. A model of the development of the brain as a construct of the thyroid system. Environ. Health Perspect. 110 (Suppl. 3), 337–348.

Kashiwabara, Y., Sasaki, S., Matsushita, A., Nagayama, K., Ohba, K., Iwaki, H., Matsunaga, H., Suzuki, S., Misawa, H., Ishizuka, K., Oki, Y., Nakamura, H., 2009. Functions of PIT1 in GATA2-dependent transactivation of the thyrotropin beta promoter. J. Mol. Endocrinol. 42, 225–237.

Kim, K.K., Song, S.B., Kang, K.I., Rhee, M., Kim, K.E., 2007. Activation of the thyroid-stimulating hormone beta-subunit gene by LIM homeodomain transcription factor Lhx2. Endocrinology 148, 3468–3476.

Kratzsch, J., Pulzer, F., 2008. Thyroid gland development and defects. Best Pract. Res. Clin. Endocrinol. Metab. 22, 57–75.

Lema, S.C., Dickey, J.T., Schultz, I.R., Swanson, P., 2009. Thyroid hormone regulation of mRNAs encoding thyrotropin beta-subunit, glycoprotein alpha-subunit, and thyroid hormone receptors alpha and beta in brain, pituitary gland, liver, and gonads of an adult teleost, *Pimephales promelas*. J. Endocrinol. 202, 43–54.

Li, X., He, J., Hu, W., Yin, Z., 2009. The essential role of endogenous ghrelin in growth hormone expression during zebrafish adenohypophysis development. Endocrinology 150, 2767–2774.

MacKenzie, D.S., Jones, R.A., Miller, T.C., 2009. Thyrotropin in teleost fish. Gen. Comp. Endocrinol. 161, 83–89.

Raldua, D., Babin, P.J., 2009. Simple, rapid zebrafish larva bioassay for assessing the potential of chemical pollutants and drugs to disrupt thyroid gland function. Environ. Sci. Technol. 43, 6844–6850.

Schmutzler, C., Gotthardt, I., Hofmann, P.J., Radovic, B., Kovacs, G., Stemmler, L., Nobis, I., Bacinski, A., Mentrup, B., Ambrugger, P., Gruters, A., Malendowicz, L.K., Christoffel, J., Jarry, H., Seidlova-Wuttke, D., Wuttke, W., Kohrle, J., 2007. Endocrine disruptors and the thyroid gland—a combined *in vitro* and *in vivo* analysis of potential new biomarkers. Environ. Health Perspect. 115 (Suppl. 1), 77–83.

Segner, H., 2009. Zebrafish (*Danio rerio*) as a model organism for investigating endocrine disruption. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 149, 187–195.

Sohn, Y.C., Yoshiura, Y., Suetake, H., Kobayashi, M., Aida, K., 1999. Isolation and characterization of the goldfish thyrotropin beta subunit gene including the 5′-flanking region. Gen. Comp. Endocrinol. 115, 463–473.

Westerfield, M., 1995. The Zebrafish Book, Third ed. University of Oregon Press, Eugene, OR.

Wood, W.M., Ocran, K.W., Kao, M.Y., Gordon, D.F., Alexander, L.M., Gutierrez-Hartmann, A., Ridgway, E.C., 1990. Protein factors in thyrotropic tumor nuclear extracts bind to a region of the mouse thyrotropin beta-subunit promoter essential for expression in thyrotropes. Mol. Endocrinol. 4, 1897–1904.