Synthetic gene network restoring endogenous pituitary–thyroid feedback control in experimental Graves' disease

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Graves' disease is an autoimmune disorder that causes hyperthyroidism because of autoantibodies that bind to the thyroid-stimulating hormone receptor (TSHR) on the thyroid gland, triggering thyroid hormone release. The physiological control of thyroid hormone homeostasis by the feedback loops involving the hypothalamus–pituitary–thyroid axis is disrupted by these stimulating autoantibodies. To reset the endogenous thyrotrophic feedback control, we designed a synthetic mammalian gene circuit that maintains thyroid hormone homeostasis by monitoring thyroid hormone levels and coordinating the expression of a thyroid-stimulating hormone receptor antagonist (TSH_{Antag}), which competitively inhibits the binding of thyroid-stimulating hormone or the human autoantibody to TSHR. This synthetic control device consists of a synthetic thyroid-sensing receptor (TSR), a yeast Gal4 protein/human thyroid receptor-α fusion, which reversibly triggers expression of the TSH_{Antag} gene from TSR-dependent promoters. In hyperthyroid mice, this synthetic circuit sensed pathological thyroid hormone levels and restored the thyrotrophic feedback control of the hypothalamus–pituitary–thyroid axis to euthyroid hormone levels. Therapeutic plug and play gene circuits that restore physiological feedback control in metabolic disorders foster advanced gene- and cell-based therapies.

synthetic biology | cell engineering | designer cells | gene switch | prosthetic networks

Thyroid hormones impact the function of all tissues and affect essentially every major pathway, including thermogenesis, carbohydrate metabolism, and lipid homeostasis (1). The thyroid gland releases a mixture of the thyroid hormones triiodothyronine (T3; 20%) and thyroxine (T4; 80%), which is converted to the more potent agonist T3 by type II deiodinase (DIO2) in the CNS (hypothalamus and the pituitary gland) or DIO1/2 in the peripheral tissues (e.g., liver, muscle, and heart) (2, 3). DIO1 and DIO2 sensitize target cells to T4 by triggering its deiodination to the more potent hormone T3 (3). The thyroid hormones, particularly T3, exert their action by nuclear thyroid hormone receptors, TRα and TRβ, which are differentially expressed in tissues and have distinct roles in thyroid hormone control of various target genes, such as *uncoupling protein 1*, *HMG-CoA reductase*, and *phosphoenolpyruvate carboxy kinase* (1). The TRα and TRβ can activate or repress gene expression depending on the target–promoter context in the target cells (4). The concentration of thyroid hormones in the body is tightly regulated by the combination of classical activation loops initiated at low thyroid hormone levels and negative feedback loops initiated by high thyroid hormone levels operating along the hypothalamus–pituitary–thyroid axis (2, 3) (Fig. 1*A*). At low thyroid hormone (T3 and T4) levels, the hypothalamus releases the thyroid-stimulating hormone (TSH) -releasing hormone, which binds and activates the TSH-releasing hormone receptor to stimulate the release of the TSH by the pituitary gland (activation loop) (5). TSH binding to the thyroid-stimulating hormone receptor (TSHR) in the thyroid gland stimulates the production and release of the T3/T4 mixture, which completes the activation loops along the hypothalamus–pituitary–thyroid axis (2, 3). To maintain the thyroid hormones at a homeostasis level and prevent hyperthyroidism by ill-controlled activation of the hypothalamus–pituitary–thyroid axis, the circulating thyroid hormones trigger a negative feedback loop by binding to the TRs in the hypothalamus and the pituitary gland and repress TSH-releasing hormone and TSH release, respectively (6) (Fig. 1*A*).

In humans, hyperthyroidism is mostly the result of an overactive thyroid gland caused by thyroid-stimulating autoantibodies (Graves' disease) or autonomous TSH-secreting pituitary and thyroid hormone-secreting thyroid adenomas, resulting in elevated blood thyroid hormone levels (7). Hyperthyroid symptoms include nervousness, increased sweating, weight loss, tachycardia, palpitations, hyperactivity, and tremor and are associated with serious complications, including life-threatening cardiac arrhythmia or psychosis if left untreated (8). Graves' disease, the most common cause of hyperthyroidism, is an autoimmune disorder characterized by the production of thyroid-stimulating hormone receptor-stimulating antibodies (TSAbs) that trigger constitutive thyroid hormone production and release from the thyroid gland (9–11). The TSHR is also expressed in ocular connective tissue and represents a candidate autoantigen for development of Graves' orbitopathy, an ocular manifestation of the disease that, in rare cases, may result in sight-impeding expansion of orbital tissue with inflammation,

Significance

Graves' disease is caused by autoantibodies that activate the thyroid-stimulating hormone (TSH) receptor and trigger a chronic increase in thyroid hormone levels. Current treatment options remain unchanged for decades and include antithyroid drugs as well as radioiodine treatment and thyroidectomy, which result in the destruction of the thyroid gland and require lifelong prescription of thyroid hormones. We designed a closed-loop gene circuit that enables engineered cells to monitor increased thyroid hormone levels and drive the expression of a validated TSH receptor antagonist that competes with TSH as well as TSH receptor autoantibodies, thereby overcoming the pathophysiological activation of the thyroid gland. Hyperthyroid animals implanted with these designer cells had thyroid hormone levels corrected by restoring the feedback control system of the hypothalamus–pituitary–thyroid axis.

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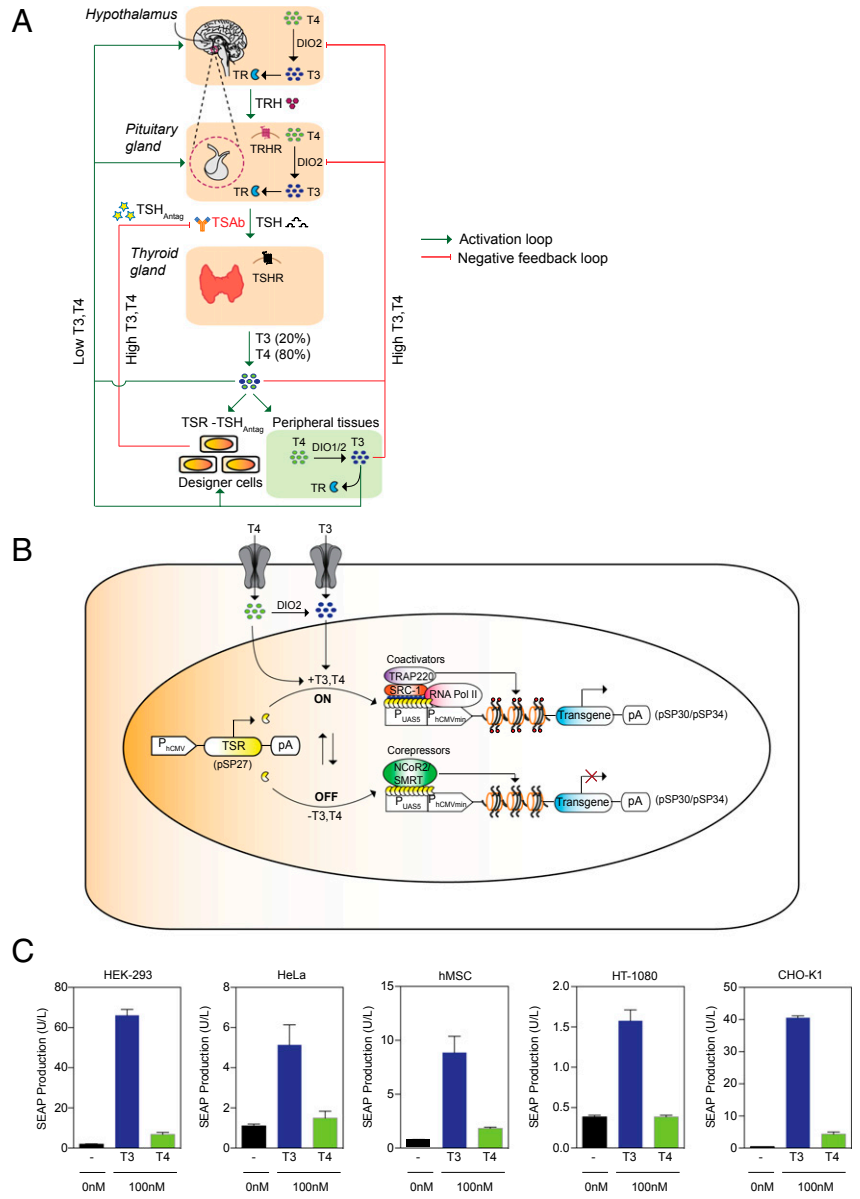

Fig. 1. A synthetic thyroid hormone-responsive mammalian gene switch interfacing with the hypothalamus–pituitary–thyroid axis. (*A*) At low thyroid hormone (T3 and T4), the hypothalamus stimulates thyrotrophic cells in the pituitary gland to release the TSH (activation loop). Binding of TSH to the TSHR in the thyroid gland (activation loop) triggers production and release of a mixture of the thyroid hormones T3 (20%) and T4 (80%), which is converted by DIO2 in the CNS (hypothalamus and the pituitary gland) and DIO1/2 in the peripheral tissues, such as the liver, muscle, and heart, to the more potent agonist T3. On reaching a specific threshold, the homeostasis of circulating thyroid hormone levels is maintained by negative feedback loops repressing thyroid-stimulating hormone-releasing hormone (TRH) and TSH production and release by the hypothalamus and the pituitary gland, respectively. In Graves' disease, autoantibodies, such as M22, constitutively activate T3 and T4 release by the thyroid gland, thereby disrupting the physiological negative feedback loops maintaining TRH and TSH homeostasis and resulting in chronically increased thyroid hormone levels. The synthetic sensor–effector TSR-TSH$_{Antag}$ circuit, which constantly monitors systemic T3 levels and coordinates corresponding production of the TSH$_{Antag}$ to neutralize excessive activation of TSHR by the autoantibody, overrules antibody-triggered hyperthyroidism and restores thyroid hormone homeostasis by a synthetic negative feedback loop. TRHR, thyroid-stimulating hormone-releasing hormone receptor. (*B*) The synthetic thyroid hormone-responsive gene switch (TSR) consists of a fusion protein, combining the ligand-binding domain of the TR with the DNA-binding domain of Gal4 (yeast Gal4 protein), which binds to a Gal4-specific operator sequence (upstream activating sequence) linked to a minimal promoter (P$_{hCMVmin}$) to control transgene expression. In the absence of the thyroid hormones T3 and T4, TSR a priori associates with corepressors, such as silencing mediator for retinoid or thyroid hormone receptors (SMRT)/nuclear receptor corepressor 2 (NcoR2), triggers histone deacetylation, and inhibits gene repression. In the presence of T3 and T4 (converted to T3 by DIO2), TSR is known to interact with coactivators, such as steroid receptor coactivator-1 (SRC-1) and thyroid hormone receptor-associated protein complex 220-kDa component (TRAP 220), that trigger histone acetylation and mediate gene expression. (*C*) T3- and T4-induced SEAP expression in different cell lines. Mammalian cells were cotransfected with TSR-encoding expression vector (pSP27; P$_{hCMV}$-TSR-pA) and P$_{UAS5}$-driven SEAP expression vector (pSP30; P$_{UAS5}$-SEAP-pA) grown in presence of 0 and 100 nM T3 or T4, and SEAP expression in the supernatant was profiled after 48 h. Data are the means ± SD of three independent experiments done in triplicate.

exophthalmos, and optic nerve compression (9). Current treatments for Graves' disease are based on the suppression of thyroid hormone production with antithyroid drugs (thionamides) and the destruction of the thyroid gland with ^{131}I-radiotherapy or surgical removal of the thyroid gland (10, 12, 13). The relapse rate of antithyroid drugs is very high (50–60%); therefore, most patients in the United States are treated by radioiodine or less frequently, surgical removal of the thyroid gland, with the necessity for lifelong thyroid hormone replacement in both cases (10, 11). An ideal treatment for hyperthyroidism would restore the highly effective control of the thyroid gland's function by the pituitary gland and thus, reestablish the physiological feedback control mechanism of the hypothalamus–pituitary–thyroid axis without destruction of the thyroid gland (14–17). One such treatment would involve blocking TSAb by a soluble TSH variant; however, determining the precise dosage and optimal administration time point of these modified TSH variants remains challenging (18). Here, we present a synthetic biology-inspired gene circuit that can dynamically coordinate the therapeutic expression of a thyroid-stimulating hormone receptor antagonist (TSH$_{Antag}$) that competes with endogenous TSH or TSAb in the case of increased thyroid hormone levels, restore the feedback control mechanism along the hypothalamus–pituitary axis, and reset the homeostasis levels of the thyroid hormones.

Design and Validation of a Synthetic Thyroid Hormone-Responsive Gene Switch

TRα is a nuclear receptor that heterodimerizes with the retinoic acid receptor and binds to the target promoters regulating the growth, metabolism, and differentiation of a wide variety of cell types and tissues (19). To precisely monitor thyroid hormone levels, a synthetic thyroid hormone-sensing receptor (TSR) was designed by fusing the DNA-binding domain of yeast Gal4 protein (20) (amino acids 1–147) to the ligand-binding domain of human TRα (amino acids 122–410). In the absence of the thyroid hormones T3 and T4, TSR is supposed to bind to its cognate promoter [promoter with upstream activating sequences (P$_{UAS}$)] and repress gene expression by recruiting endogenous corepressors, such as

silencing mediator for retinoid or thyroid hormone receptors (SMRT)/nuclear receptor corepressor 2, that subsequently trigger histone deacetylation (21) (Fig. 1*B*). In the presence of thyroid hormones, TSR predominantly interacts with T3 and a priori recruits coactivators, such as steroid receptor coactivator-1 and TR-associated protein, to trigger histone acetylation and gene expression (21, 22) (Fig. 1*B*). Cotransfection of several rodent and human cell lines with plasmids encoding TSR (pSP27, P_{hCMV}-TSR-pA) and a TSR-dependent human placental secreted alkaline phosphatase (SEAP) expression vector (pSP30, P_{UAS5}-SEAP-pA) corroborated the induction of SEAP in response to T3 and T4 (Fig. 1*C*). Diversity in the cellular repertoires of coactivators and corepressors, variability in nuclear receptor isoforms, and differences in transfection efficiency as well as SEAP production and secretion capacities may explain the differences in the induction profiles among different cell lines (21). CHO-K1 cells showed the best transgene induction profile in response to T3 and were chosen for additional experiments. Control experiments showed that CHO-K1 cell viability was not affected by the transfection of circuit components and that the activation required the presence of the TSR (Figs. S1, S2, and S3).

Characterization of Thyroid Hormone-Triggered Transgene Expression

Detailed characterization of CHO-K1 cells transgenic for T3-inducible TSR-dependent SEAP expression (pSP27/pSP30) revealed that the thyroid sensor circuit was reversible and responded efficiently to a wide range of thyroid hormone (T3) concentrations (Fig. 2 *A* and *B*). Additionally, the set point and the response dynamics of the thyroid hormone sensor circuit could be modulated using different promoter variants, which may enable the fine-tuning of the circuit's negative feedback control to the physiological requirements of different cases of hyperthyroidism (Figs. 1*A* and 2*C*). Early forms of hyperthyroidism are characterized by an increase of T4 levels, because the thyroid gland releases more T4 (80%) than T3 (20%) (2, 3). However, because T4 is a weak agonist for the TSR, we tested whether overexpression of DIO2 (23, 24), an enzyme that converts the weak TR/TSR agonist T4 into the strong agonist T3, can improve TSR's response to T4 (Fig. 1*B*). After transfection of a DIO2 expression vector (pDIO2, P_{SV40}-DIO2-pA) CHO-K1 cells transgenic for T3-inducible TSR-dependent SEAP expression (pSP27/pSP30) were, indeed, sensitized to T4 (Fig. 2*D*) (23). The rapid induction kinetics of the TSR device were determined in real time using the yellow fluorescent protein variant Citrine (pSP35, P_{UAS5}-Citrine-pA) and SEAP (pSP30, P_{UAS5}-SEAP-pA) as reporter proteins (Fig. 2 *E* and *F*).

Closed-Loop Control of TSH_{Antag} Secretion by the Thyroid Hormone Sensor Device

To autonomously control thyroid hormone homeostasis in a self-sufficient manner, we coupled the TSR sensor device with the expression of an engineered high-affinity TSH_{Antag} that competes with endogenous TSH for the TSHR-binding site (25). TSH_{Antag} is an engineered variant of human TSH containing a human chorionic gonadotrophin-β C-terminal peptide linker between the β- and α-chains as well as two mutated glycosylation sites (18). The secretion of TSH_{Antag} (pSP32, P_{hCMV}-TSH_{Antag}-pA) was confirmed by ELISA in several mammalian cell lines. CHO-K1 cells, which showed the best transgene induction profile, also efficiently secreted TSH_{Antag} into the culture medium (Fig. 3*A*). RT-PCR and ELISA confirmed the rapid T3-triggered production and release of TSH_{Antag} by CHO-K1 cells cotransfected with pSP27 (P_{hCMV}-TSR-pA) and pSP34 (P_{UAS5}-TSH_{Antag}-pA) (Fig. 3 *B* and *C*). The functionality of TSH_{Antag} was validated by a standard cell-based assay (26) that confirmed the inhibition of TSHR activation when stimulated by bovine thyroid-stimulating hormone (bTSH) (27) or human thyroid-stimulating autoantibody (M22) (28) (Fig. 3*D*).

Restoration of Euthyroid Hormone Levels in a Mouse Model of Experimental Graves' Disease

To validate thyroid hormone-induced gene expression in vivo, we used an established animal model of experimental Graves' disease (29–31). Therefore, CHO-K1 cells transgenic for TSR-driven SEAP expression (pSP27/pSP30) were encapsulated in alginate-poly-(L-lysine)-alginate beads (32) and implanted i.p. into mice. The TSR device was insensitive to physiological T3 levels but triggered SEAP expression in response to hyperthyroid hormone levels (Fig. 4 *A* and *B*). To confirm the designer circuit's capacity to control thyroid hormone homeostasis in mice, we implanted microencapsulated CHO-K1 cells transgenic for TSR-driven TSH_{Antag} expression (pSP27/pSP34) i.p. into hyperthyroid mice. Hyperthyroid control mice implanted with TSR-SEAP (pSP27/pSP30) -transgenic CHO-K1 cells showed significantly increased thyroid hormone T4 levels compared with mice containing the TSR-TSH_{Antag} implant (Fig. 4 *C* and *D*). Placebo phosphate-buffered saline (PBS) injection into hyperthyroid mice with or without the implant had no discernible effect on T4 levels (Fig. 4*E* and Fig. S4). Thus, the TSR-TSH_{Antag} implant suppressed bTSH and M22 antibody-mediated TSHR activation, which confirmed that the designer circuit coordinated the serum TSH_{Antag} levels in response to elevated thyroid hormone concentration and restored thyroid hormone homeostasis of the hypothalamus–pituitary–thyroid axis in animals with experimental Graves' disease.

Discussion

Synthetic gene networks have reached a level of sophistication where they can precisely program cellular behavior in response to endogenous or exogenous signals (33–42). Designer cell implants with embedded synthetic gene networks have been used for the treatment of gout, metabolic syndrome, and obesity in mice (36, 37, 43). In this study, we designed a thyroid hormone homeostasis circuit that enables the restoration of the physiological hypothalamus–pituitary–thyroid axis in a mouse model of experimental Graves' disease. The control dynamics of this circuit mimic essential characteristics of thyrotrophic cells in the pituitary gland, such as continuous monitoring of thyroid hormone concentrations and processing of excessive levels (3). Additionally, taking advantage of different promoter variants, the set point of the sensor circuit can be adjusted to the physiological requirements of different cases of hyperthyroidism, which may enable patient-specific fine-tuning of the circuit's response dynamics in future clinical applications. The pituitary gland has the ability to increase the secretion of TSH and therefore, stimulate thyroid hormone release by the thyroid gland whenever low thyroid hormone levels are detected (e.g., during the recovery phase of a transient thyroiditis), thereby resetting the thyroid hormone expression levels of the thyroid gland. However, the pituitary gland has no capacity to protect the organism from hyperthyroidism (8). Therefore, designer cell-based gene circuits dynamically controlling in situ production of TSH_{Antag} in response to pathological thyroid hormone levels may complement the capacity of the pituitary gland during hyperthyroidism. Graves' disease-induced hyperthyroidism is the most common cause for hyperthyroidism and affects 0.5% of the human population (10). The treatment options for this disease remained unchanged for decades and include antithyroid drugs, ^{131}I-radiotherapy, and thyroidectomy, which are often associated with high relapse rate or lifelong hypothyroidism (10). A treatment option that corrects TSAb-associated hyperthyroidism in a dose-dependent manner may restore the physiological thyrotrophic feedback loop of the hypothalamus–pituitary–thyroid axis and ultimately, reestablish the function of the thyroid gland as well as reduce the risk of Graves' ophthalmopathy (14). Self-sufficient designer cell implants capable of maintaining thyroid homeostasis under disease conditions are not anticipated to have the disadvantages of current hyperthyroidism treatments and may lead to an improvement in thyroid care.

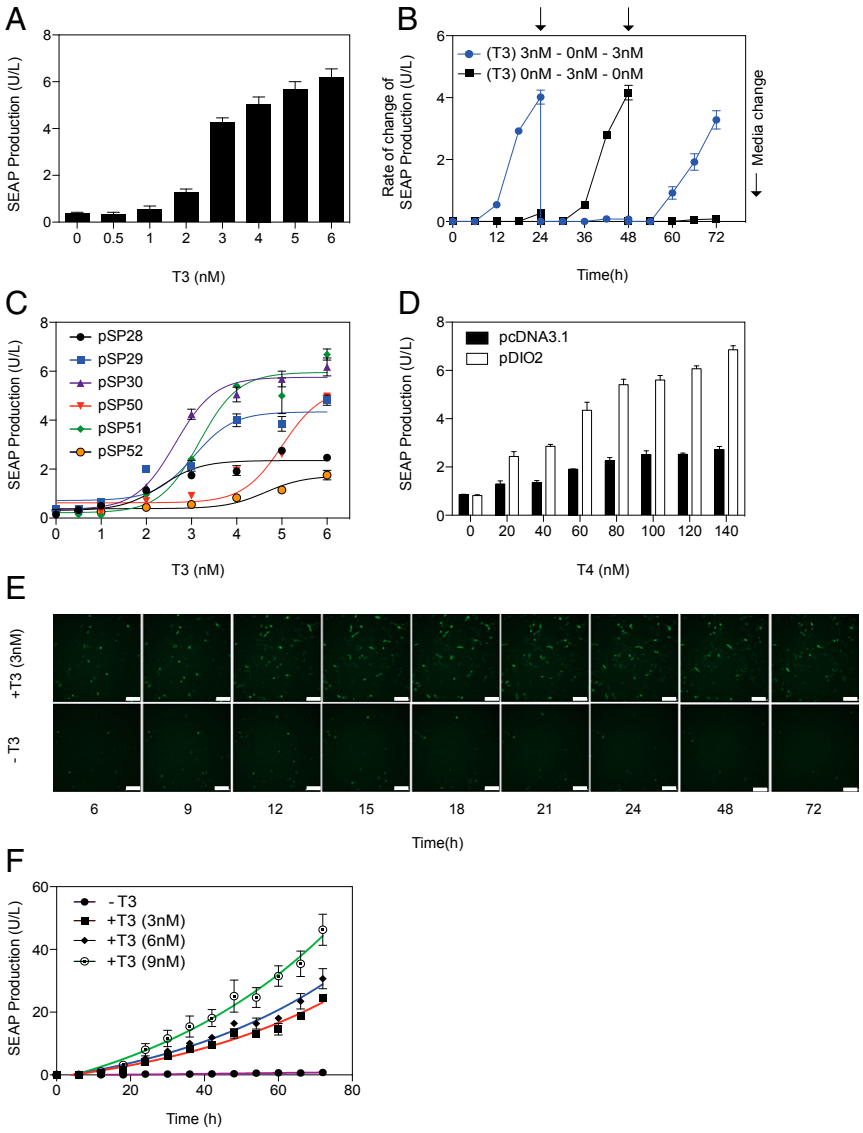

Fig. 2. Characterization of thyroid hormone-triggered transgene expression. (*A*) Dose-dependent SEAP expression profile for TSR-SEAP–transgenic (pSP27/pSP30) CHO-K1 cells in the presence of increasing T3 concentrations and after 24 h. (*B*) Reversibility of thyroid hormone T3-responsive SEAP expression was assessed by cultivating TSR-SEAP–transgenic (pSP27/pSP30) CHO-K1 cells for 72 h while alternating the T3 levels (3 nM) every 24 h. (*C*) Fine-tuning of the TSR sensor circuit for CHO-K1 cells cotransfected with pSP27 (P_{hCMV}-TSR-pA) and pSP28 (P_{UAS1}-SEAP-pA), pSP29 (P_{UAS2}-SEAP-pA), pSP30 (P_{UAS5}-SEAP-pA), pSP50 ($P_{UAS2.1}$-SEAP-pA), pSP51 ($P_{UAS5.1}$-SEAP-pA), or pSP52 (P_{UAS3}-SEAP-pA) in the presence of increasing T3 concentrations and after 24 h. For the purpose of comparison, the data from *A* were replicated. (*D*) Dose-dependent SEAP expression profile for TSR-SEAP–transgenic (pSP27/pSP30) CHO-K1 cells cotransfected with DIO2 (pDIO2) or pcDNA3.1 in the presence of increasing T4 concentrations and after 24 h. Induction kinetics of (*E*) TSR-Citrine–transgenic (pSP27/pSP35) CHO-K1 cells and (*F*) TSR-SEAP–transgenic (pSP27/pSP30) CHO-K1 cells exposed to 3 nM T3. Data are the means ± SD of three independent experiments done in triplicate. (Scale bar: 100 μm.)

Materials and Methods

Thyroid Sensor–Effector Components. Comprehensive design and construction details for all expression vectors are provided in Table S1. Key plasmids are as follows: pSP27 encodes a constitutively expressed hybrid thyroid sensor (P_{hCMV}-TSR-pA_{bGH}), pSP30 encodes a thyroid hormone-responsive SEAP expression unit (P_{UAS5}-SEAP-pA_{SV40}), and pSP34 encodes a thyroid hormone-responsive TSH_{Antag} expression unit (P_{UAS5}-TSH_{Antag}-pA_{SV40}).

Cell Culture and Transfection. Human mesenchymal stem cells transgenic for the human telomerase [hMSC-TERT (44)] catalytic subunit, HEKs (HEK-293T; ATCC: CRL-11268), human cervical adenocarcinoma cells (HeLa; ATCC: CCL-2), and human fibrosarcoma cells (HT-1080; ATCC: CCL-121) were cultivated in DMEM (Invitrogen) supplemented with 10% (vol/vol) FCS (lot no. PE01026P; Bioconcept) and 1% (vol/vol) penicillin/streptomycin solution (Sigma-Aldrich) at 37 °C in a humidified atmosphere containing 5% CO_2. CHO cells (CHO-K1; ATCC: CCL-61) were cultured in ChoMaster high-throughput screening (HTS) media (Cell Culture Technologies GmbH) containing 5% (vol/vol) FCS and 1% penicillin/streptomycin. For the transfection of hMSC-TERT, HEK-293T, HeLa, HT-1080, and CHO-K1, 2×10^5 cells were seeded per well of a six-well plate 24 h before transfection and incubated for 12 h with 400 μL DNA-PEI (polyethylenimine) mixture that was produced by incubating 6 μL PEI for hMSC-TERT and HEK-293T and 12 μL PEI for HT-1080, HeLa, and CHO-K1 (PEI < 20,000 molecular weight; Polysciences; stock solution: 1 mg/mL double-distilled H_2O, pH 7.2) with 3 μg total DNA, vortexing for 5 s, and incubating for 15 min at 22 °C. The ratio of the plasmids encoding thyroid hormone-responsive designer circuit pSP27:pSP30 or pSP27:pSP34 was 1:1. The transfection efficiency was determined by FACS analysis using pEGFP-N1–transfected cells (Fig. S3). After transfection, the medium was changed to ChoMaster HTS supplemented with charcoal stripped serum [5% (vol/vol); catalog no. F6765; Sigma-Aldrich] and 1% (vol/vol) penicillin/streptomycin. After the addition of the inducers T3 (catalog no. T2877; Sigma-Aldrich) and T4 (catalog no. T2376; Sigma-Aldrich), the reporter expression level was quantified at defined time points.

Analytical Assays.

SEAP. Human placental SEAP was profiled in cell culture supernatants using a *p*-nitrophenylphosphate–based light absorbance time course assay. In brief, 80-μL cell culture supernatant was heat-inactivated for 30 min at 65 °C. Next, 100 μL 2× SEAP buffer [20 mM Homoarginine, 1 mM $MgCl_2$, 21% (vol/vol) diethanolamine, pH 9.8; Sigma-Aldrich] was mixed with 20-μL substrate solution (120 mM paranitrophenyl phosphate) and added to each sample. The absorbance time course was measured using an Envision Reader (Perkin-Elmer). For blood serum samples, SEAP was quantified using a chemiluminescence-based assay (Roche Diagnostics GmbH).

TSH_{Antag}. TSH_{Antag} was profiled in the blood samples and cell culture supernatants using a human TSH ELISA (catalog no. TS227T; Calbiotech Inc.).

T3. Total T3 levels were quantified in the blood samples and cell culture supernatant using a T3 ELISA kit (catalog no. T3225T; Calbiotech Inc.).

T4. Total T4 levels were quantified in the blood samples and cell culture supernatant using a T4 ELISA kit (catalog no. T4224T; Calbiotech Inc.).

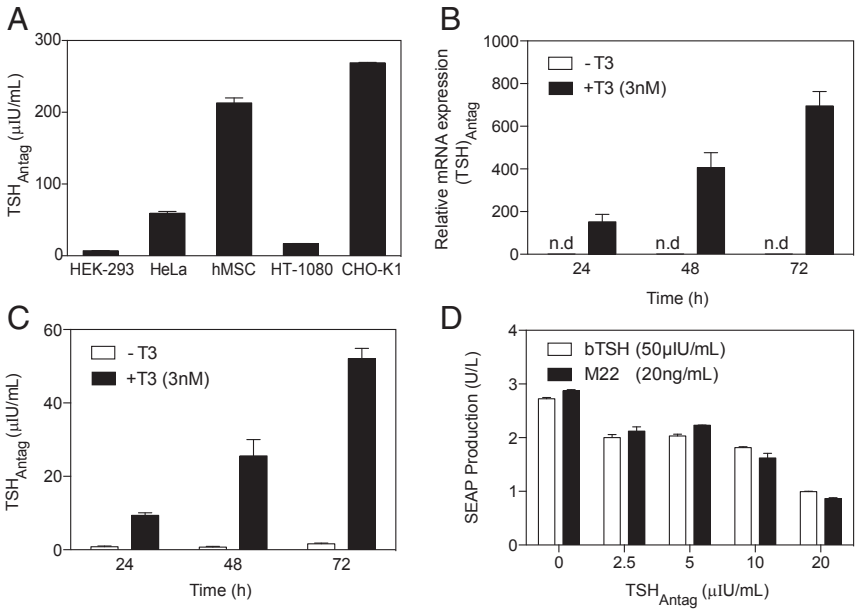

Fig. 3. Characterization of TSH_{Antag} in vitro. (*A*) TSH_{Antag} secretion profiling from different mammalian cell lines transfected with constitutive TSH_{Antag} expression vector pSP32 (P_{hCMV}-TSH_{Antag}-pA) and after 48 h using ELISA. (*B*) RT-PCR–based expression profiling of TSH_{Antag} from TSR-TSH_{Antag}–transgenic (pSP27/pSP34) CHO-K1 cells induced with 3 nM T3. (*C*) TSH_{Antag} secretion profiling of TSR-TSH_{Antag}–transgenic (pSP27/pSP34) CHO-K1 cells induced with 3 nM T3. (*D*) Stable TSHR-expressing CHO-K1 cells were transfected with pSP16 (P_{CREm}-SEAP-pA), incubated with conditioned medium containing different amounts of TSH_{Antag}, and challenged with 50 μIU/mL bTSH or 20 ng/mL M22 before SEAP levels were profiled in the culture supernatant. Data are the means ± SD of three independent experiments done in triplicate. n.d, not detectable.

Viability assay. Viability of transfected CHO-K1 cells was assessed using Calcein AM Dye (catalog no. 65–0853-39; Affymetrix Ebioscience). In brief, 1×10^6 pcDNA3.1/pSP27/pDIO2-cotransfected CHO-K1 cells were incubated for 30 min with 20 nM Calcein AM Dye at 22 °C. The samples were washed twice with PBS before FACS analysis.

Cell-based TSHR activation assay. CHO-K1 cells stably expressing TSHR were transfected with pSP16 (P_{CREm}-SEAP-pA) and incubated with conditioned medium (ChoMaster HTS with 1% penicillin/streptomycin) containing different amounts of TSH_{Antag} with bTSH or M22 antibody for 48 h, and SEAP expression was profiled in the supernatant as described above.

Quantitative RT-PCR. Total RNA was extracted from the pSP27/pSP34-transgenic CHO-K1 cells using the ZR RNA MiniPrep Kit (Zymo Research) and TURBO DNase (Invitrogen). Real-time RT-PCR was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and SYBR Green PCR Master Mix (Invitrogen) with custom-designed primers (forward: 5′-TTAATGGCAAGCTGTTCCTG-3′; reverse: 5′-ACTTGCATGACAGAGCGACT-3′). The Eppendorf Realplex Mastercycler (Eppendorf GmbH) was set to the following amplification parameters: 2 min at 50 °C, 20 s at 95 °C, and 40 cycles of 1 s at 95 °C followed by 1 min at 60 °C. The expression of endogenous GAPDH was used as an internal control.

FACS of Transgenic CHO-K1 Cells. For FACS analysis, the pEGFP-N1/pcDNA3.1/pSP27/pDIO2-transgenic CHO-K1 cells were detached using 0.5 mL StemPro Accutase Cell Dissociation Reagent (Invitrogen). The cell populations were analyzed using a Becton Dickinson LSRII Fortessa Flow Cytometer (Becton Dickinson) equipped for FITC/EGFP (488-nm laser, 505-nm long-pass filter, and 530/30 emission filter) detection and set to exclude dead cells and cell doublets. For the viability assay using Calcein AM Dye, all of the cells were included.

Animal Experiments. To validate thyroid hormone T3-induced gene expression in vivo, we used an established animal model of experimental Graves' disease

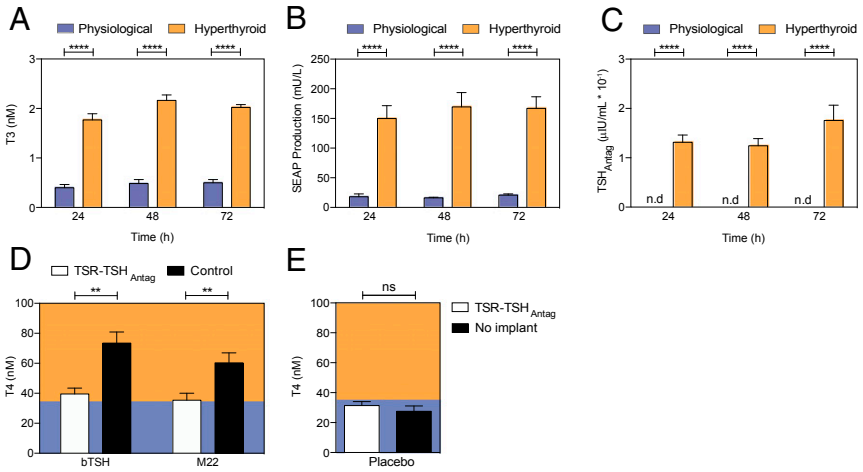

Fig. 4. Self-sufficient control of thyroid hormone homeostasis in mice. (*A*) WT mice were implanted with control TSR-SEAP–transgenic (pSP27/pSP30) CHO-K1 cells or TSR-TSH_{Antag}–transgenic (pSP27/pSP34) CHO-K1 cells and received daily injection of 5 μg T3 for 3 consecutive days to maintain hyperthyroid serum levels. (*B*) Resulting SEAP levels. (*C*) Resulting TSH_{Antag} levels. (*D*) WT mice were implanted with TSR-SEAP–transgenic (pSP27/pSP30) CHO-K1 cells (control) or TSR-TSH_{Antag}–transgenic (pSP27/pSP34) CHO-K1 cells and received daily injection of 5 μg T3 for 3 consecutive d to maintain hyperthyroid serum levels. Next, 48 h after injection of the implants, the mice were challenged with bTSH (10 mIU) or M22 antibody (0.5 μg), and total T4 serum levels were profiled. (*E*) Hyperthyroid mice implanted with TSR-TSH_{Antag}–transgenic (pSP27/pSP34) CHO-K1 cells or no implant were challenged with placebo (PBS), and total T4 serum levels were profiled. Data are the means ± SEM of eight mice. Statistics by Student's *t* test. Panel background colors indicate the physiological (light blue) and hyperthyroid (light brown) conditions as indicated. ns, Not significant; n.d, not detectable. $^{**}P < 0.005$; $^{****}P < 0.0001$.

(29–31). In brief, the animal model requires injection of T3 to inhibit endogenous TSH-mediated activation of thyroid gland by the negative feedback loops (Fig. 1*A* and Fig. S4). Subsequent injection of TSH or M22 antibody triggers the thyroid gland to produce and secrete primarily the thyroid hormone T4 (80%). Therefore, the T4 levels serve as a readout to evaluate the effect of a TSH_{Antag} on the thyroid hormone release by the thyroid gland. A stock solution of T3 (100 μg/mL) was prepared by dissolving 2.5 mg T3 in 1.25 mL 0.1 N NaOH and diluting to a final volume of 25 mL with distilled water. Then, a working solution of 10 μg/mL was prepared, and the pH was adjusted to 7.5 with 1 M HCl. Elevated serum levels of T3 hormone were induced in BALB/c mice (Charles River Laboratories) by i.p. injection of 5 μg T3 for 3 consecutive d; i.p. implants were prepared by encapsulating pSP27/pSP30- or pSP27/pSP34-transgenic CHO-K1 cells into alginate-poly-(L-lysine)-alginate beads (400 μm; 200 cells per capsule) using an Inotech Encapsulator IE-50R (Buchi Labortechnik AG) set to the following parameters: a 200-μm nozzle with a vibration frequency of 1,023 Hz and 900 V for bead dispersion and a 20-mL syringe operated at a flow rate of 403 U. Next, 1 mL serum-free HTS containing 5×10^6 encapsulated pSP27/pSP30- or pSP27/pSP34-transgenic CHO-K1 cells was injected i.p. into WT BALB/c male mice; 48 h after implantation, the mice were challenged with 10 mIU bTSH (catalog no. T8931; Sigma-Aldrich) or 0.5 μg M22 antibody (M22/FD/0.04; RSR Ltd.) for 5 h to elevate serum T4 hormone levels. Blood levels of SEAP were profiled at defined time points using a chemiluminescence based assay. Blood levels of TSH_{Antag} and thyroid hormones T3 and T4 (microtainer serum separator tubes; Becton Dickinson) were profiled at defined time points using ELISA. All experiments involving animals were performed according to the European Parliament and European Union Directive 2010/63/EU approved by the French Republic (no. 69266309) and conducted by G.C.-E.H. at the Institut Universitaire de Technology.

Statistical Analyses. In vitro data represent the means ± SD of three independent experiments with three samples per experiment. In vivo data represent the means ± SEM of eight animals. Group comparisons were analyzed by the Student's *t* test (cutoff of $P < 0.05$) using GraphPad Prism 6 software (GraphPad Software Inc.).

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