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HYPERTHYROIDISM AND PAPILLARY THYROID CARCINOMA IN THYROTROPIN RECEPTOR D633H MUTANT MICE

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The purpose of this study is the characterization of a TSHR D633H mutation *in vivo* mouse model. Mutation in TSHR D633 is constitutively active mutation of the thyrotropin receptor and it was previously identified in patients with toxic thyroid nodules with the clinical diagnosis of non-autoimmune hyperthyroidism and in one patient with thyroid cancer.

To understand the pathophysiology of non-autoimmune hyperthyroidism we created a mouse model with constitutively active mutation. For this study, we used three mouse groups of 2, 6 and 12 months. Each group was also divided by sex and genotype. Histology, hormonal analysis, gene expression, Western blots and mouse primary cell culture experiments were done.

TSHR D633H mice developed recurrent hyperthyroidism. Both subclinical and overt hyperthyroidism was observed depending of the sex, age and genotype. At 2 months of age homozygous mice showed overt hyperthyroidism compared to wild type littermates. Heterozygous mice showed only decreased thyrotropin level in serum. At age 6 months, there was no difference in serum thyroid hormone concentrations in hetero- and homozygous mice despite suppressed thyrotropin levels in homozygous mice of both sexes. At age 12 months hyperthyroidism was again present in homozygous female mice. Also at 12 months age nearly all homozygous mice presented large papillary thyroid carcinomas. Our conclusions are that non-autoimmune hyperthyroidism is age-, sex- and genotype-dependent and that constitutively active TSHR mutations can trigger malignant transformation of thyrocytes.

Hyperthyroidism and papillary thyroid carcinoma in thyrotropin receptor D633H mutant mice

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1

ABSTRACT

Background: Constitutively active thyrotropin receptor (TSHR) mutations are the most common etiology of non-autoimmune hyperthyroidism (NAH). Thus far, the functionality of these mutations has been tested *in vitro,* but the *in vivo* models are lacking.

Methods: To understand the pathophysiology of NAH, we introduced the patient-derived constitutively active TSHR D633H mutation into the murine *Tshr* by homologous recombination.

Results: In this model, we observed both subclinical and overt hyperthyroidism depending on the age, sex and copy number of the mutated allele. Homozygous mice presented hyperthyroidism at 2 months of age, while heterozygous animals showed only suppressed TSH. Interestingly, at 6 months of age, thyroid hormone concentrations in all mutant mice were analogous to wildtypes, and they showed colloid goiter with flattened thyrocytes. Strikingly, at one-year of age nearly all homozygous mice presented large papillary thyroid carcinomas (PTC). Mechanistically, this PTC phenotype was associated with an overactive thyroid and strongly increased stainings of proliferation, pERK, and NKX2-1 markers, but no mutations in the "hot-spot" areas of common oncogenes (*Braf, Nras, Kras*) were found.

Conclusions: this is the first study to reveal the dynamic age-, sex- and gene dosage-dependent development of NAH. Furthermore, we show that a constitutively active TSHR can trigger a malignant transformation of thyrocytes.

INTRODUCTION

Hyperthyroidism is defined as an excess of thyroid hormone (TH) production. When unopposed by defects in thyroid hormone action, it is typically characterized by tachycardia, an increased metabolic rate and weight loss (1-3). An overactive thyroid function may affect virtually any organ system, can lead to excess comorbidity and mortality, and is potentially lethal, if not treated (4). Hyperthyroidism has an approximate lifetime risk of 3% and is 5-10 times more common in women than men (5). Most commonly, it is caused via activation of the thyrotropin receptor (TSHR), either by TSHR autoantibodies in the case of Graves' disease (6), or via constitutively activating mutations (CAM) in the TSHR or very rarely in G_s protein α subunit leading to non-autoimmune hyperthyroidism (NAH) (7-9). Unlike Graves' disease, in which remission after antithyroid drug treatment may occur in up to 50% of patients, NAH is usually permanent and commonly treated by primary ablative treatment (10). However, the constitutive activity of the TSHR mutations has so far been studied exclusively *in vitro* (11). Thus, there is still a lack of precise knowledge concerning the molecular events leading to hyperthyroidism and long-term consequences of NAH.

The activation of the TSHR via thyrotropin (TSH), TSHR autoantibodies or an activating TSHR mutation induces an increase of iodide uptake, thyroid hormone synthesis and release via G_s- and $G_{q/11}$-mediated pathways. While the G_s pathway is the main regulator of TH synthesis, secretion, iodide uptake and thyrocyte proliferation (12), also $G_{q/11}$ signaling has been shown to be important for iodine organification, TH release and goiter growth in a mouse model (13). This diversity in signaling has been suggested to play a distinct role in the pathogenesis of thyroid diseases. TSHR autoantibodies (14, 15) or receptor mutations can alter G protein signaling differently, resulting in different phenotypes. All known constitutively active mutations of the TSHR activate G_s and rarely also $G_{q/11}$ signaling. However, there is no clear correlation between the severity of the phenotype and the signaling of the mutant TSHR receptors *in vitro* (7, 16). In mice, transgenic overexpression of a

constitutively active G_s mutant or an ectopic G_s-coupled receptor in thyrocytes leads to thyroid adenomas and hyperthyroidism (17, 18). Furthermore, the G_s- or other TSHR-mediated signaling pathways have been reported to play an important role in the etiology of BRAF-induced papillary thyroid cancer models in mice and humans (19, 20), although thyroid cancer in hot nodules is rare (21).

To understand the role of TSHR signaling in the development of hyperthyroidism and thyroid growth, we generated a knock-in (KI) mouse model harboring a patient-derived TSHR D633H mutation. This mutation is located in the TSHR "hot-spot" area in transmembrane helix 6, and has been identified in patients with hot thyroid nodules (22) and in one thyroid insular carcinoma (23). It leads to a simultaneous increase of basal G_s and $G_{q/11}$ activation *in vitro* (24).

For the first time, we report that a constitutive TSHR activity leads to a variable development of subclinical to overt hyperthyroidism in an age-, sex-, and genotype-dependent manner. Furthermore, development of papillary thyroid carcinoma was observed in older TSHR D633H KI animals.

1

METHODS

Generation of the TSHR mutants and functional characterization in vitro

The TSHR D633H variant was generated by PCR mutagenesis as previously described using human or mouse wild type *TSHR*-pSVL constructs as templates (25). Mutated *TSHR* sequence was verified by sequencing (ABI Advanced Biotechnologies, Inc., Columbia, MD). Briefly, COS-7 cells grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco Life technologies, Paisley, UK) at 37 °C in a humidified 5% CO_2 incubator were transiently transfected in 12-well plates (1 x 10^5 cells per well) or 48-well plates (0.25 x 10^5 cells per well) with 1 µg and 0.25 µg DNA per well, respectively, using the GeneJammer® Transfection Reagent (Stratagene, Amsterdam, NL). The determination of TSHR cell surface expression, TSH (recombinant human TSH, Thyrogen, USA) stimulated intracellular cAMP and inositol phosphate (IP) levels were performed as previously described (25, 26).

Generation of TSHR D633H KI mice, animal husbandry and genotyping

To introduce the D633H mutation into the murine *Tshr* locus, the nucleotide sequence GAC coding for codon 633 was replaced with the sequence CAC by homologous recombination (Figure 1). In detail, BAC clones containing the murine *Tshr* gene (ENSMUSG00000020963) were obtained from BACPAC Resources Center (Children's Hospital Oakland Research Institute, USA). An 8100 bp genomic DNA fragment spanning Exon 9 and 10, intron 9 and 3' UTR of *Tshr* gene was cloned into the pACYCY177 vector (New England Biolabs, USA) by Red/ET recombination according to the manufacturer's instructions (Gene Bridges GmbH, Germany). Site directed mutagenesis was performed with following sense 5'-CTGTGTTGATCTTCACTCACTTCATGTGCATGGCGC-3' and antisense 5'-GCGCCATGCACATGAAGTGAGTGAAGATCAACACAG-3' primers to generate point mutation in exon 10 by using the QuikChange Site-Directed Mutagenesis kit (Stratagene,

USA). Neo resistance gene flanked by two loxP sites was introduced into intron 9 by Red/ET recombination. The DNA fragment containing exon 10 with point mutation and Neo cassette was replaced with wild type exon 10 in pACYC 177 backbone by Red/ET recombination. Restriction enzyme digestion and sequencing confirmed validity of the final targeting construct. G4 embryonic stem cells (derived from mouse 129S6/C57bl/6Ncr) were cultured on neomycin-resistant primary embryonic fibroblast feeder cells. Ten million cells were electroporated with 30 µg of linearized targeting construct and cultured in presence of 300 µg/ml G418 (Sigma-Aldrich, USA), and 96 colonies were picked after 7-9 days selection for further processing. For Neo cassette deletion, the targeted ES cells were electroporated with pCAGGS-Cre plasmid and cultured for 3-5 days, screened for the correct homologous recombination by PCR, and confirmed by sequencing. The targeted ES cells were injected into C57bl/N6 mouse blastocysts (Charles River Laboratories, Willmington, USA) to generate chimeric mice. The presence of the D633H mutation was investigated by PCR using genomic DNA with primers P1 and P2 at 63°C annealing temperature and analyzed via agarose gel electrophoresis (Figure 1A, Supplemental Table 1). Experiments were performed with mice of mixed background. Mice were housed under controlled conditions (IVC units, 12 h light / 12 h dark, 21 ± 1 °C) at the Central Animal Laboratory, University of Turku. Animals were provided ad libitum access to pelleted chow (SDS RM-3 (P); Special Diet Service, UK) and water. For hormone analysis, blood from the lateral saphenous vein or via cardiac puncture was collected. Animals were sacrificed with CO_2.

Histology, immunohistochemistry and morphometric analysis

Formalin-fixed (10% formalin in PBS), paraffin-embedded tissue samples were cut into 4 µm thick sections and stained with hematoxylin and eosin for histological analysis using standard methods. Stained sections were imaged with a Pannoramic Slidescanner (3D HISTECH, Hungary). Immunohistochemistry was performed as described previously (27). The following

antibodies were used in the given concentrations: mouse anti-TTF-1 (anti-NKX2-1) (8G7G3/1, #M3575, Dako, USA) 1.68 µg/ml, rat anti-mouse Ki67 0.5 µg/ml (SolA15, #14-5698-82, eBioscience, USA), rabbit anti-mouse p44/42 MAPK (ERK1/2) 0.4 µg/ml (137F5, #4695, Cell Signaling, USA) and rabbit anti-mouse phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) 0.25 µg/ml (D13.14.4E9, #4370, Cell Signaling, USA). Corresponding secondary horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies and rat-on-mouse HRP polymer (#RT517, Biocare Medical, USA) with Dako EnVision detection kits (#K500711-2, Dako, USA) were used. From 3 animals per genotype and sex, the thyrocyte thickness of 2 neighboring thyrocyte layers was measured for 20 randomly selected follicle-follicle borders using Pannoramic Viewer (Version 5.14.4, 3D HISTECH, Hungary). In addition, this software was used to determine the intrafollicular area of 20 randomly chosen follicles; for 12-month-old animals the follicles from PTC areas were excluded. The thyrocyte proliferation rate was determined using Fiji software (28). The number of Ki67 positive cells and total number of cells was counted in three to six not overlapping, randomly selected areas per thyroid section of three mice per age, sex and genotype. The ratio of Ki67 positive cells to total cell number was expressed as proliferation index in percent.

Hormone measurements, cAMP determination, primary cell culture

Free T4 (fT4) serum concentrations were determined using a commercially available ELISA (Novatec, Germany). Serum TSH levels were analyzed with the Mouse Pituitary Magnetic Bead Panel (Merck Millipore, Germany) according to the manufacturer's instructions. Intracellular cAMP was measured using a radio-immunoassay (RIA) as previously described (29). Primary cell culture of dissected thyroids was performed according to Jeker et al. (30). For cAMP accumulation, cells were incubated at 37°C and 5% CO_2 with DMEM/F12 in the presence of 0.6 mM IBMX for 2 h and with or without 10 mU/ml bTSH (Sigma-Aldrich, USA). Next, cells were incubated with 0.1 M HCl for 30 min on ice. HCl was evaporated and

the cAMP was resuspended in PBS with 0.1% BSA. For normalization, protein concentrations were determined from three wells per genotype using the Pierce BCA protein assay (Thermo Scientific, USA).

Laser-capture-microdissection (LCM)

Formalin-fixed (10% formalin in PBS), paraffin-embedded thyroid tissue from 12 months old homozygous animals (7 females, 5 males) were cut into 4 µm thick sections, placed on MembraneSlides 1.0 PEN (Carl Zeiss, Germany) and stained with hematoxylin and eosin (HE). An image for each thyroid section was taken at low magnification for orientation and for labelling the areas of interest. LCM was performed using a Zeiss laser microdissection platform PALM MicroBeam (Carl Zeiss, Germany). DNA extraction from micro-dissected thyroid tissue was performed with a commercial kit (AllPrep DNA/RNA FFPE kit, Qiagen, Germany) according to the manufacturer's instructions. PCR with genomic DNA was then used to amplify the following gene mutations in *Braf* (exon 15, codon 600/601), *Kras* (exon 2, codon 12/13) and *Nras* (exon 3, codon 61). Following primers used are given in Table S2. The purified PCR products were then send for sequencing (GATC Biotech AG, Germany).

Quantitative RT-PCR

RNA isolation from snap frozen thyroids and quantitative RT-PCR was performed as described previously (31) for the following genes: *Ctsb*, *Ctsl*, *Arrb2*, *Nkx2-1*, *Pax8*, *Nis*, *Tg*, Thyroid Peroxidase (*Tpo*) and *Tshr* and normalized to peptidylprolyl isomerase A (*Ppia*) and receptor like protein 19 (*Rlp19*) with primers given in Supplemental Table 2.

Study approval

All experiments were authorized by the National Animal Experiment Board of Finland (License number: 10266).

Statistics

GraphPad Prism 7 software (GraphPad Software, Inc., La Jolla, USA) was used for statistical analysis. Unpaired t-test, one-way ANOVA with Dunnett post-hoc test and non-parametric Kruskal-Wallis test were used to determine statistical significances. $P<0.05$ was set as the limit of statistical significance, where * = $P<0.05$, ** = $P<0.01$ and *** = $P<0.001$.

RESULTS

Mouse and human TSHR D633H mutations have a similar constitutive activity *in vitro*

The TSHR D633H mutation has been identified in several patients with hyperthyroidism (22, 23) (http://tsh-receptor-mutation-database.org). To test if the human and mouse TSHR D633H mutation lead to a comparable increase in the constitutive activity, basal and TSH-stimulated cAMP and inositol phosphate accumulation in a cell culture system was measured (Supplemental Figure 1). In general, there was no difference in the tests between human and mouse receptors, which also showed a similar cell surface expression (mouse and human TSHR D633H were expressed 80-90% of the WT controls, (Supplemental Figure 1A). The D633H mutation in both species showed a strong, approximately 5-fold increase of the basal cAMP activity *in vitro* compared to the WT receptor (Supplemental Figure 1B). Similarly, a weaker, but significant 2-fold increase in basal inositol phosphate accumulation was detected for both, the human and mouse mutants in comparison to WT receptors *in vitro* (Supplemental Figure 1C). The stimulation with increasing concentrations of recombinant human TSH showed a comparable dose response in both human and mouse receptor mutants with a maximal response similar to the WT receptors (Supplemental Figure 1B, C). These results are consistent with previous results showing no difference *in vitro* between human and mouse TSHR CAMs I486F and V509A (32).

The TSHR D633H mutation leads to an increased cAMP concentration in mouse primary thyrocytes

To study the physiological consequences of TSHR D633H mutation, we generated a knock-in mouse line harboring the TSHR D633H mutation in the corresponding mouse *Tshr* locus. Classical gene-targeting in mouse embryonic stem cells with homologous recombination technique was used to exchange the nucleotide sequence GAG coding for aspartic acid (D) at codon 633 with the sequence CAC coding for histidine (H) (Figure 1A). Chimeric mice were

obtained from two different embryonic stem cell clones. The presence of *Tshr* mutant allele was screened by PCR (Figure 1B), and confirmed by direct sequencing of the targeted region using genomic DNA from WT, HET and HOM mice (Figure 1C). Heterozygous breeding showed a normal Mendelian distribution of the genotype (WT:HET:HOM = 1:1.8:1.2), sex (female:male = 1:0.9) and normal litter size (6.1 ± 2.4 pups/litter).

To test if the TSHR D633H mutation leads to an increased constitutive activity *in vivo*, cAMP levels were measured in primary thyroid cell cultures from 2 and 6 months old mice (Figure 1D). In 2- month-old mice, cAMP levels were slightly but significantly upregulated in HOM mice compared to the WT thyrocytes. A similar trend of increased basal cAMP production was seen in 6-month-old HOM mice versus controls. Furthermore, thyrocytes from 2- and 6-month-old WT, HET and HOM mice responded similarly to the TSH stimulation, indicating a normal TSHR expression and function in WT and TSHR mutants.

TSHR D633H mice develop age-, sex- and mutant allele frequency-dependent hyperthyroidism

As hyperthyroidism may lead to weight loss and accelerated growth, these parameters were monitored over 12 months. In overall phenotypic analysis, no obvious differences in body or tail lengths were noticed over the investigated period of 12 months (Table 1, Supplemental Figure 2). However, after the first 6 months HOM males and females did not gain as much weight as did the WT littermates (Supplemental Figure 2C). Thus, at 12 months of age the bodyweights of HOM males and females were over 25% lower than in their WT littermates (Table 1, Supplemental Figure 2C).

To understand the impact of the TSHR D633H on the thyroid physiology, we measured serum TSH and TH concentrations at different time points during the lifespan of 12 months. Interestingly, a dynamic age, sex and mutant allele dependent development of hyperthyroidism was detected. Despite the embryonic onset of TSHR expression (starting

embryonic day E15) (33) and the initiation of TH synthesis thereafter, no obvious signs of congenital hyperthyroidism were evident in TSHR D633H mice at any age group analyzed (Supplemental Figure 2D). However, the HOM females had significantly elevated serum TH concentrations already at 1 and 2 months of age, both HOM females and males showed overt hyperthyroidism indicated by a 2.3 and 1.7-fold increase in fT4 and suppressed TSH values compared to control littermates (Figure 2, upper panel). Heterozygous females and males displayed decreased serum TSH, but unaltered TH levels (Figure 2, upper panel). Unexpectedly, no difference in serum fT4, total T4 or T3 levels in HET or HOM females and males was observed at 6 months of age (Figure 2, middle panel, and data not shown) despite the suppressed serum TSH levels in HOM animals of both sexes (Figure 2, middle panel). Interestingly, overt hyperthyroidism was again present in one year old HOM female mice (Figure 2 lower panel). Together, these data show that a constitutively active TSHR leads to hyperthyroidism, but it can be compensated for in males, HET animals and also temporarily in HOM females.

TSHR D633H mutant mice develop colloid goiter associated with thin thyroid epithelia

Based on the identification of the TSHR D633H from patients with thyroid adenomas, the thyroid growth and histology of TSHR D633H mice was carefully analyzed. In general, thyroids were larger in HET and HOM animals of both sexes at all time points compared to their WT littermates. Already at 2 months of age, HOM mice presented with a significantly increased thyroid weight (2.8- and 2.3-fold increase in female and male HOM mice compared with WT littermates) (Figure 3A). The thyroid weight increased progressively with age in TSHR D633H KI mice. At the age of 6 months, the thyroid weight in HET animals was 2.0 and 2.2-fold, and in HOM mice 3.7- and 2.5-fold higher than in WT in female and male mice, respectively (Figure 3A). In the histological analysis of the thyroid at 2 months of age, HET mice did not show clear histological alterations, while HOM animals presented areas with

increased follicle size and flattened thyrocytes (Figure 3B upper panel, 3C and Table 1). However, at 6 months of age, especially in HOM mice of both sexes nearly all follicles had very thin, flattened thyrocyte epithelium (Table 1) and nuclei (Figure 3B, middle panel). In line with this, the morphometric analysis showed a significantly increased colloid amount per follicle and a reduced thyrocyte thickness also in HET animals compared to controls (Figure 3C, Table 1). In detail, HOM females and males have 2.9- and 2.7-fold increased follicle area at 6 months of age, and 37% and 45% thinner thyrocyte epithelium in males and females, respectively, compared with the WT (Table 1 and Figure 3).

The long-term activation of TSHR leads to papillary thyroid cancer in TSHR D633H mutant mice

To determine the long-term impact of constitutive TSHR activity on thyroid growth, thyroid weight and histology were analyzed in one-year-old mice. Surprisingly, the 12-month-old HET and HOM mice developed thyroid neoplasia with thick thyrocyte epithelium and protrusions of the thyrocyte layer into the lumen of the follicle (Figure 3B, lower panel). Further histological analysis exhibited typical characteristics of papillary thyroid carcinoma (PTC) comprising papillae with distinct fibrovascular cores, nuclear clearance, nuclear grooves, and pseudo-inclusions as well as overlapping nuclei (Figure 4A and B). PTCs were detected in 88% of HOM females (7/8 mice) and in 80% of HOM males (4/5 mice) (Figure 5A), as well as in about 30% of HET females (2/7 mice) and in one HET male (1/5 mice). The proportion of the PTC area in comparison to the whole thyroid section was on average 50% in HOM males and females (Figure 5B and C), and clearly smaller in HET animals (Figure 5B). In line with the development of PTC, the thyroid weight increased strongly after 6 months in HOM animals, whereas the increase in thyroid weight gain before that age was only marginal (Figure 3A). Furthermore, PTCs of 12-month-old HOM mice presented a high proliferation rate as shown by strong Ki67-positive staining (Figure 5D). Quantification of

Ki67-positive cells showed that 12-month-old HOM animals have a significantly increased proliferation index compared to WT thyroids (14-17% in HOM animals vs. 2% in WTs) (Figure 5E). Lower but significantly increased proliferation was determined also in HET females at 12 months of age (Figure 5E). In general, the TSHR D633H KI mice had a higher thyrocyte proliferation rate, and the proliferation index in the mutant mice was already slightly increased at 2 months and 6 months of age (Figure 5E). Immunohistochemistry for NK2 homeobox 1 (NKX2-1) showed that all PTC-forming cells express NKX2-1, which indicates maintenance of thyrocyte differentiation also in neoplasia (Figure 5F). Furthermore, an activation of MAPK pathway in the PTCs was indicated by abundant staining of phosphorylated ERK1/2 in the majority PTC lesions of the TSHR D633H mice (Figure 5H).

In humans, mutations in *BRAF, KRAS or NRAS* can be found in up to 70% of PTCs (34). To test, if our KI model harbors any mutations in the "hot-spot" areas of these genes, genomic DNA from micro-dissected PTC lesions was isolated and sequenced. However, the DNA from the PTC areas of TSHR D633H mice revealed no mutations in 19 tumors tested for Braf mutations, 13 tumors tested for Kras mutations, and 9 tumors tested for Nras mutations.

Determination of expression levels of thyroid-specific genes

To reveal the possible compensatory mechanisms in the dynamic development of hyperthyroidism in TSHR D633H KI mice, the expression of thyroid-specific genes was analyzed by qPCR. Despite the constitutive activation of the mutant TSHR, the differences in serum TSH and hyperthyroidism in different sex and genotypes, no difference was detected between HET and HOM mice vs WTs for *Tshr* mRNA at any time point (Supplemental Figure 3). At 2 months of age, a differential expression of the analyzed thyroid-specific genes was only seen in HOM male thyroids, in which the expression of *paired box 8 (Pax8)* and sodium-iodide symporter *(Nis)* was significantly increased, compared to WT controls. At 6 months of age, when the TH concentrations in both sexes of HOM and HET TSHR D633H

KI animals were comparable to WT, no differences in the gene expression were seen, except for decreased thyroglobulin (*Tg*) mRNA expression in mutant females (Supplemental Figure 3). The 12-month-old animals revealed the most prominent changes in thyroid-specific gene expression, most likely reflecting the changes in the large PTC areas seen in HET and HOM TSHR D633H mice. Additionally, a clear sex difference was observed. In 12-month-old HET and HOM female mice, the mRNA levels for *Pax8, and Tg* were downregulated, while in HOM females beta-arrestin 2 (*Arrb2*) and cathepsin B (*Ctsb*) were up- and *Nis* was downregulated. In contrast, HOM males showed an upregulated expression for *Nkx2-1* and cathepsin L (*Ctsl*), whereas HET male mice did not show any changes (Supplemental Figure 3).

DISCUSSION

There is still a knowledge gap concerning the natural development and the long-term effects of NAH. Here, we generated a knock-in mouse model carrying the constitutively active TSHR mutation D633H. This mutation was first identified in patients with thyroid hormone producing hot thyroid nodules (22, 23). Our results reveal that the constitutively active TSHR leads to a dynamic age-, sex- and gene dosage-dependent development of NAH, and triggers the development of PTC in mice. Homozygous TSHR D633H female mice presented hyperthyroidism soon after birth, which then ameliorated to euthyroid TH levels at 6 months of age. However, concomitantly with the appearance of PTC, TH levels rose again at one year of age. The hyperthyroidism phenotype is clearly milder in HOM males than females with an only transient form of hyperthyroidism at an early age. The variability and compensation of hyperthyroidism in our model with the same genetic background suggests different thyroidal adaption mechanisms between males and females. In contrast to most human patients with long-standing NAH due to heterozygous TSHR activating mutations, our HET mice presented only suppressed TSH levels. In humans, subclinical hyperthyroidism has been rarely reported in family members with inherited NAH due to heterozygous TSHR germline mutations (35, 36). This prompts the question, whether there are some yet undiscovered mechanisms to prevent TSHR-mediated NAH in mice. However, it must be kept in mind that the NAH and PTC phenotypes described here could also result from the specific feature of the TSHR D633H mutation, which can constitutively activate both G_s and $G_{q/11}$ pathways (24).

The TSHR D633H mutation selected for our mouse model was found in adult patients with hot nodules and overt hyperthyroidism (22, 23) (http://tsh-receptor-mutation-database.org). In line with a previous publication (24), the TSHR D633H induces strong basal G_s-mediated adenylyl cyclase/cAMP activation. However, unlike most TSHR CAMs, it also activates the $G_{q/11}$/phospholipase C/Ca^{2+} signaling pathway. The basal cAMP activity of TSHR D633H was strongly elevated in the heterologous *in vitro* overexpression cell system;

whereas the elevation was remarkably lower in primary thyrocytes from HET and HOM mice. A similar discrepancy between TSHR characterization *in vitro* and the response detected in primary cells has been reported earlier in human hot thyroid nodules (37). This most likely reflects the low TSHR receptor number expressed in primary cells versus cell culture systems using receptor overexpression. In our study, we observed relatively slight elevation in basal cAMP production in thyrocytes of 2-month-old HOM TSHR D633H mice and only a marginally elevated basal cAMP in HET. This difference could explain the mild hyperthyroidism in HOM animals and the unaltered serum TH levels in HET animals.

The normalization of TH levels in HOM TSHR D633H mice at 6 months of age could be caused by several counteracting mechanisms leading to a reduced cAMP response, such as downregulation, desensitization and/or internalization of the TSHR. Specifically, G protein coupled receptor kinases (GRKs) and ARRB2 have been shown to lower the number of receptors on the cell surface and simultaneously reduce intracellular cAMP levels (38, 39). Furthermore, an increased phosphodiesterase activity has been described in human hot thyroid nodules (37). Accordingly, an increased expression of *Arrb2* mRNA was detected in our 6 and 12 months old HOM females (Figure S3). This is in line with an increased ARRB2 expression shown in human hot thyroid nodules and might reflect a counter-regulatory mechanism leading to partial desensitization of the TSHR (40).

In general, the acute overall physiological responses to hyperthyroidism in the TSHR D633H KI mice were rather mild. Although HOM females had elevated serum TH between 1-4 months and males at least temporarily at 2 months of age, no obvious differences in weight gain or growth (body or tail length) were noticed during the first 6 months of age. This differs markedly from the symptoms observed in young hyperthyroid patients, in which weight loss and increased growth rate are classical symptoms (2, 41). However, after 6 months of age, HOM animals of both sexes did not gain weight as much as WT littermates. This diminished

weight gain suggests delayed tissue-specific responses to TH, or other metabolic adaptation processes, which require further investigation.

The thyroid histology of the TSHR D633H KI mice at 2 months of age showed only mild morphological changes. In contrast to that, 6-month-old HOM mice revealed severely altered thyroid histology with colloid goiter, and very thin and flattened thyrocyte epithelium. Thereafter, nearly all HOM and some HET of both sexes developed PTCs. In addition to the morphologic and functional remission of hyperthyroidism, the later development of PTC in TSHR D633H KI mice was surprising. The previous transgenic mouse models for hyperthyroidism led to papillary structures, but did not show typical features of PTCs (17, 18, 42). However, our model shares some similarities with transgenic mice expressing a mutant α1B-adrenergic receptor in the thyroid (43). In these mice, the overexpression of a α1B-adrenergic receptor mutant led to the malignant transformation of thyrocytes and activation of both G_s and $G_{q/11}$- signaling pathways. Therefore, it can be speculated that the simultaneous activation of $G_{q/11}$ is an important player in tumor formation in our model.

The majority of PTCs in humans are typically characterized by a permanently active MAPK signaling cascade due to mutations in *BRAF*, *KRAS* or *NRAS* (34, 44). Therefore, we screened possible mutations in the "hot-spot"- areas of *Braf*, *Kras* and *Nras* genes in our PTC lesions, but no mutations were found. Most likely, the chronic constitutive cAMP and Ca^{2+} signaling via increased proliferation triggers also other growth signals and finally transformation of the thyrocytes in our model. This was supported by phosphorylated ERK1/2 staining seen in the PTC areas of the TSHR D633H mutants, which indicated the activation of MAPK pathway in these lesions. TSH signaling can converge on MAPK growth signal e.g. via EPAC and/or RAP1B leading to RAS activation as shown previously in thyrocyte models (45, 46). However, together with the focal and rather late appearance of the PTC, probably other genetic or epigenetic events are required.

In contrast to 30% of HET TSHR D633H KI females and 20% HET males, patients with hot thyroid nodules rarely harbor thyroid cancer in the respective thyroid tissue. The published data regarding the association of thyroid cancer and hot nodules are mostly limited to case reports or series with a small number of patients. The reported probability of a hot thyroid nodule being associated with a thyroid carcinoma in or outside the hot nodule ranges between 1-10.3% (21). Interestingly, there are also several reports of thyroid carcinomas presenting as hot thyroid nodules (hot thyroid carcinomas). These reports identified the constitutively activating TSHR mutations M453T, I486F, L512R, A623V, F631I, T632A, T632I, D633H and D633Y (23, 47-52). A search for *RAS* and *BRAF* mutations, as well as *PAX8/PPARG* rearrangements has been thus far reported only for 4 hot thyroid carcinomas in adults out of 90 hot carcinomas reported during the last 28 years (23, 48, 53, 54). The increased malignancy rate of hot thyroid carcinomas of children does not appear to be associated with *RAS* and *BRAF* mutations or *PAX8/PPARG* and *RET/PTC* rearrangements (55). Other genetic factors could include the recently identified mutation in one fourth of hot nodules in the enhancer of zeste homolog 1 (*EZH1*), which is associated with the increased proliferation of thyrocytes (56).

Taken together, our results from the TSHR D633H KI model indicate that NAH is not as stable as expected but rather a dynamic condition involving age, sex and *Tshr* allele-dependent compensatory mechanisms. Furthermore, our data strongly suggests that a permanently active TSHR can lead to the transformation of thyrocytes into cancer cells.

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