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Targeted Foxe1 overexpression in mouse thyroid causes the development of multinodular goiter but does not promote carcinogenesis

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Abstract

Recent genome-wide association studies have identified several single nucleotide polymorphisms in theFOXEl locus, which are strongly associated with the risk for thyroid cancer. In addition, our recent work has demonstrated FOXEl overexpression in papillary thyroid carcinomas. To assess possible contribution of Foxe1 to thyroid carcinogenesis, transgenic mice overexpressing Foxe1 in their thyroids under thyroglobulin promoter (Tg-Foxe1) were generated. Additionally, Tg-Foxe1 mice were exposed to X-rays at the age of 5 weeks or crossed with Pten+/− mice to examine the combined effect of Foxe1 overexpression with radiation or activated PI3K-Akt pathway, respectively. In 5–8 weeks old Tg-Foxe1 mice, severe hypothyroidism was observed, and mouse thyroids exhibited hypoplasia of the parenchyma. Adult 48-week-old mice were almost recovered from hypothyroidism, their thyroids were enlarged and featured colloid microcysts and multiple benign nodules of macrofollicular-papilloid growth pattern, but no malignancy was found. Exposure of transgenic mice to 1 Gy or 8 Gy of X-rays and Pten haploinsufficiency promoted hyperplastic nodule formation also without carcinogenic effect. These results indicate that Foxe1 overexpression is not directly involved in the development of thyroid cancer, and that proper Foxe1 dosage is essential for achieving normal structure and function of the thyroid.
FOXE1 is a thyroid-specific forkhead transcription factor crucial for craniopharyngeal embryogenesis and for the maintenance of differentiated state of thyroid. Germline loss-of-function FOXE1 mutations in humans are the basis for the rare autosomal-recessive Bamforth-Lazarus syndrome characterized by cleft palate, spiky hair, choanal atresia, bifid epiglottis and congenital hypothyroidism due to thyroid dysgenesis (1). Foxe1 deficiency in mice also leads to developmental abnormalities such as thyroid ectopy or loss of thyroid follicular cell (TFC) progenitors. Interestingly, the initiation of thyroid primordium formation at early stages of embryogenesis and functional differentiation of the TFC precursors are not impaired in Foxe1-null mice (2).

In functionally differentiated human TFC, FOXE1 regulates several thyroid-specific genes such as TG, TPO, NIS and DUOX2 (3-4), acting as a classical pioneer transcription factor (5-6). FOXE1 is a useful marker of differentiated state of normal or neoplastic thyroid tissues, and its expression correlates with differentiation level of thyroid cancer cells. Previous studies showed that FOXE1 expression is significantly down-regulated in poorly differentiated thyroid carcinoma and is absent in anaplastic thyroid cancer (7-8). On the other hand, our recent work has demonstrated FOXE1 overexpression and cytoplasmic translocation in human papillary thyroid carcinoma (PTC) (9). FOXE1 expression is not only elevated in PTC but also correlates with some clinicopathological features such as extra-capsular invasion, tumor stage and lymph node metastasis (10). Moreover, recent genome-wide and target gene association studies have identified two single-nucleotide polymorphisms (SNPs), rs965513 located 60 kb upstream of FOXE1 and rs1867277 in the 5’UTR of the same gene, which confer risk for thyroid cancer (11-12). These SNPs may be involved in transcriptional regulation of FOXE1. For instance, the risk allele of rs1867277 (A) enhances the activation of FOXE1 promoter in Hela cells through the recruitment of UCF transcription factors (13). Nevertheless, the precise role of FOXE1 in thyroid tumorigenesis is not fully understood so far.

To assess possible contribution of Foxe1 to thyroid carcinogenesis, we generated transgenic mice overexpressing Foxe1 under thyroglobulin promoter (Tg-Foxe1). Additionally, Tg-Foxe1 mice
were exposed to X-rays at the age of 5 weeks or crossed with Pten+/− mice to address the combined effect of Foxe1 overexpression with radiation or activated PI3K-Akt pathway, respectively. Surprisingly, we found that Tg-Foxe1 mice developed thyroid hypoplasia and overt hypothyroidism shortly after birth, but at older age had multinodular goiter. Congenital hypothyroidism (CH) is observed in 1:2000 to 1:4000 of neonates (14-15). The vast majority (up to 85%) of primary CH cases are caused by thyroid dysgenesis associated with loss-of-function mutations in TSHR, PAX8, NKX2-1, FOXE1 and NKX2-5, while dyshormonogenesis accounts for 10-15% of cases due to mutations in SLC5A5, TPO, DUOX2, DUOXA2, SLC26A4, TG and IYD/DEHALI (16-17). It should be noted that follicular (18-22) and papillary thyroid carcinoma (23-26) may arise from dyshormonogenetic goiter. No data on FOXE1 overexpression in CH or its effect on either human or murine thyroid is available, and comprehensive understanding of CH with subsequent goiter or thyroid carcinogenesis is impeded by the lack of adequate animal models.

Here we introduce the first mouse model of thyroid-specific overexpression of Foxe1 and provide a detailed histopathological characterization of Foxe1-associated hypothyroidism followed by the development of multinodular goiter. The combined effect of Foxe1 overexpression with X-ray irradiation or activated PI3K-Akt pathway is also presented.

**Materials and Methods**

**Mice**

A mouse model of targeted expression of Foxe1 driven by the bovine thyroglobulin promoter was generated. Fragment of the bovine thyroglobulin promoter (2045 bp), the murine Foxe1 gene (1116 bp) and the SV-40 polyadenylation signal (228 bp) were cloned into the pBlue-script-II SK+ vector (Stratagene, CA, USA). For transgenesis, purified construct DNA was microinjected into zygotes and transferred into pseudopregnant C57BL/6J females at the UNITECH facility (Chiba,
Japan). Transgene integration into the genome of founders was confirmed by Southern blotting. Two independent lines were established. Founder mice were mated with wild-type C57BL/6J partners, and the progeny was screened for the presence of transgene by PCR as described below. Heterozygous Pten-knockout mice (B6.129-Pten<tm1Rps>, hereafter designated as Pten<+/-> mice) were obtained from National Cancer Institute at Frederick, USA. Double transgenic mice were obtained by cross-mating of Tg-Foxe1 mice with Pten<+/-> mice.

Mice were bred in a specific pathogen-free facility and fed with a standardized regular diet. Animal care and all experimental procedures were performed in accordance with the Guidelines for Animal Experimentation of Nagasaki University with the approval of the Institutional Animal Care and Use Committee.

PCR genotyping

Genotyping was performed at the age of 4 weeks by PCR using tail-extracted DNA (REDExtract-N-Amp Tissue PCR KIT; Sigma, USA) or amnion-derived DNA for embryos. The primers used to detect the Tg-Foxe1 transgene were: 5’-CTACAGCCTCCACAGATTCTCA-3’ and 5’-TGAGTTTGGACAAACCACAACTA-3’ yielding a 1552-bp PCR product. The primers for Pten<+/-> mice were: P012 (5’-TTGCACAGTATCCTTTTGAAG-3’) and P013 (5’-GTCTCTGGTCCTTACTTCC-3’) yielding a 240-bp product for wild-type Pten; and P012 and P014 (5’-ACGAGACTAGTGAGACGTGC -3’) yielding a 320-bp product for Pten.

X-ray irradiation

Wild-type and Tg-Foxe1 littermates were exposed to 1 Gy or 8 Gy of X-rays at the age of 5 weeks. Mice were anesthetized by intraperitoneal injection of Nembutal (Sodium Pentobarbital) into the lower left quadrant of abdomen at a dose of 40 mg/kg and immobilized. Unshielded front neck area was exposed to X-rays at a dose rate of 0.5531 Gy/min using a Toshiba ISOVOLT TITAN 320.
Animal groups, and tissue and serum sampling

In the present study, mice were divided into four main groups according to the genetic background: C57BL/6J wild-type mice (WT), Tg-Foxe1, Pten+/- and double transgenic Tg-Foxe1/Pten+/- mice. Not exposed to X-ray WT and Tg-Foxe1 mice were subdivided into four age groups: 5–8, 24–48 weeks; mice exposed to X-ray were sacrificed at the age of 8, 24 and 48 weeks. Pten+/− and Tg-Foxe1/Pten+/− mice were examined at the age of 5–8 and 24 weeks.

At the indicated time points, mice were anesthetized by intraperitoneal injection of Nembutal at the dose of 50 mg/kg. Blood was collected by cardiac puncture, and the animals were euthanized by cervical dislocation. Thyroid lobes were dissected and weighted. One lobe was snap-frozen in liquid nitrogen and stored at -80°C until use, and the other was put in 10% neutral-buffered formalin. After 24 h fixation in formalin at 4°C, tissue samples were rinsed in water and embedded into paraffin. Five-micrometer-thick serial sections were prepared for further hematoxylin-eosin or immunohistochemical staining. For cryosectioning, fresh tissue samples were washed in ice-cold PBS and frozen in Tissue-Tek O.C.T. compound (Sakura Finetek, USA). Sections were taken in a cryostat Leica CM3050 S (Leica Biosystems).

Brown adipose tissue staining

Cryosections were fixed in 10% formalin for 15 min at 4°C. After intensive washing in distilled water, slides were incubated in propylene glycol 2 x 5 min and stained with 150 nM solution of Sudan Black B in propylene glycol for 7 min with agitation. After washing for 3 min in 85% propylene glycol and rinsing in distilled water, sections were counterstained with Nuclear Fast Red (Sigma, USA) for 5 min and mounted with aqueous mounting media.


**Immunohistochemistry (IHC)**

Formalin-fixed paraffin-embedded (FFPE) 4 μm serial sections were deparaffinized and subjected to antigen retrieval in a microwave in Tris-EDTA buffer, pH 9.0 at 95°C for 25 min (for Foxe1 antigen unmasking) or in citrate buffer, pH 6.0 at 95°C for 25 min (for Ttf-1, Thyroglobulin, Calcitonin and Ki-67 antigens unmasking). Blocking reagent (Dako, Denmark) was applied at room temperature (RT) for 1 hr. After blocking, the sections were incubated with primary antibodies diluted in Antibody Diluent (Dako, Denmark) solution: rabbit anti-TTF1 (1:750; Biopat, Italy), rabbit anti-TTF2 (1:750; Biopat, Italy), rabbit anti-Thyroglobulin (1:1000; Dako, Denmark), rat anti-Ki-67 (1:100; Dako, Denmark), rabbit anti-PTEN (1:400; Abcam, UK) and anti-Calcitonin (prediluted; Dako, USA) overnight at 4°C. After washing, HRP-conjugated secondary antibodies anti-Rabbit (1:100, Dako, Denmark) or anti-Rat (1:100, Dako, Denmark) were applied for 1 hour at RT. Detailed information about antibodies used in this study is presented in Supplemental Table 1. Visualization was performed with DAB Enhanced Liquid Substrate System tetrahydrochloride (Sigma, USA). Nuclei were counterstained with hematoxylin.

The intensity score of nuclear Foxe1 staining was categorized as negative (0), weak (1), mild (2) or strong (3). The proportion score was determined as a percentage of positively stained nuclei of thyroid epithelial cells within the intensity category. The total Foxe1 immunohistochemistry score (IHC-score) was calculated as a sum of products of staining intensity scores and corresponding proportion scores. Ki-67 labeling index was calculated as a percentage of positively stained nuclei of thyroid epithelial cells. At least 1000 thyroid epithelial cells were counted in 5 random fields at ×400 magnification for evaluation of the Foxe1 IHC-score and Ki-67 labeling index.

**Dual-labeled immunofluorescence analysis**

Formalin-fixed paraffin-embedded (FFPE) 4 μm sections were deparaffinized and subjected to antigen retrieval in a microwave in Tris-EDTA buffer, pH 9.0 at 95°C for 20 min. Sections were
blocked for 1 hour in 5% BSA in PBS, and incubated with primary antibodies diluted in 5% skim milk in TBST: rabbit anti-TTF2 (1:250; Biopat, Italy) and rat anti-Ki67 (1:50; Dako, Denmark) overnight at 4°C. Sections were then incubated with 4’, 6-diamidino-2-phenolindole (1:1000; DAPI; Dojindo, Japan) and secondary antibodies diluted in 5% skim milk in TBST: anti-rabbit Alexa Fluor 546 and anti-rat Alexa Fluor 647 (1:1000, Invitrogen, USA) for 1 hour at RT. Stained slides were imaged using a BZ-9000 microscope (Keyence, Osaka, Japan) and were recorded with a BZ-II analysis application (Keyence). Exposition time for 450 nm, 546 nm and 647 signals were optimized to obtain the widest dynamic range of recorded fluorescence intensity.

Quantitative real-time reverse transcription-PCR (qRT-PCR)

Total RNA was extracted from homogenized fresh-frozen thyroid tissues with ISOGEN reagent (Nippon Gene, Tokyo, Japan). Two hundred nanograms of total RNA were transcribed with ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, Japan). Quantitative PCR was carried out in a Thermal Cycler Dice Real-time system (Takara Bio Inc., Otsu, Shiga, Japan) using SYBR Premix Ex Taq II reagent (Takara Bio Inc., Otsu, Shiga, Japan). The profile of thermal cycle was as follows: 95°C for 2 min, 40 cycles of 95°C for 5 sec and 60°C for 30 sec, followed by dissociation curve analysis for all primer pairs. The average of the relative quantity of replicates was calculated with Q-Gene software (27) using Actb (β-actin) or Pax8 data for normalization. Sequences of the primers are listed in Supplemental Table 2.

Serum Free T4, T3 and TSH measurement

FT4 and FT3 were measured using standard laboratory assay (SRL Inc.). Mouse serum TSH was measured using in-house radioimmunoassay as described previously (28).
Statistical analysis

Statistical comparison of categorical variables was performed using the 3x2 or 4x2 extensions of Fisher’s exact test (http://in-silico.net/tools/statistics/fisher_exact_test/2x3). Continuous data were analyzed by applying non-parametrical Mann-Whitney U-test for comparison of two groups or Kruskal-Wallis test for multiple group comparisons as appropriate. Analysis was performed with IBM SPSS Statistics Version 21 and GraphPad 4.1 Prism (GraphPad Software) software packages. All p-values were 2-sided and considered significant if <0.05.

Results

Generation of Tg-Foxe1 mice

For thyroid-specific overexpression of Foxe1, a 3.4 kb genetic construct combining the bovine thyroglobulin promoter, the murine Foxe1 and the SV-40 polyadenylation signal was created (Figure 1A). Two independent founder lines bearing 12 (line A) and 2 (line B) copies of the transgene were established. Both lines developed similar thyroid phenotype within 48 weeks of life span (Supplemental Figure 1A). Transgenic Foxe1 expression was verified by qRT-PCR with transgene-specific primers (Supplemental Figure 1B). The line A bearing a greater number of transgene copies was chosen for the detailed investigation.

qRT-PCR assessment of transgenic Foxe1 expression demonstrated its age-dependent decline (Figure 1B). Total Foxe1 expression (i.e., endogenous and transgenic Foxe1 combined) did not change with age in wild-type (WT) mice but was decreasing in Tg-Foxe1 animals (Figure 1C). By the age of 48 weeks no difference in total Foxe1 expression was observed between transgenic and WT animals. The decrease in total Foxe1 expression in Tg-Foxe1 mice with age is thus likely to be fully attributed to the decline in the expression of the Foxe1 transgene.
Foxe1 overexpression in the thyroids of Tg-Foxe1 mice was confirmed by IHC (Figure 1D). The proportion of cells showing the highest score (3, “strong”) of immunoreactivity to Foxe1 remained significantly higher in Tg-Foxe1 mice compared to WT at all age groups (Figure 1E), but the drastic difference at 5–8 weeks declined at 24–48 weeks. Similar observations were made for the total Foxe1 IHC score (Figure 1F). The results of IHC corresponded well with qRT-PCR data.

**Systemic characterization of the Tg-Foxe1 mice**

No obvious differences between newborn Tg-Foxe1 mice and their WT siblings were observed. However, the signs of growth retardation became apparent 2–3 weeks after birth. The Tg-Foxe1 mice exhibited cretinous body habitus (Figure 2A) and significantly lower body weight in both males and females until the age of 8 weeks (Figure 2B). The thyroid weights of 5- and 8-week-old Tg-Foxe1 mice were comparable to those of WT mice, but became significantly greater at 24 and 48 weeks (Figure 2C). The thyroid-weight-to-body-weight ratio was significantly higher in Tg-Foxe1 than in WT mice at 8, 24 and 48 weeks, but not at 5 weeks (Figure 2D). Gross anatomy of transgenic animals was normal except thyroid. As representatively shown for the 48-week-old mice (Figure 2E), Tg-Foxe1 animals had enlarged thyroids with irregular surface and visible nodules.

Taking into consideration the essential role of Foxe1 in thyroid primordium migration and TFC precursors survival, mouse embryos were examined histologically. Thyroid bud formation and migration of TFC precursors towards the front neck area was not altered. The thyroid reached its conventional position at E14.5. The appearance of isolated TFC highly positive for Foxe1 in E14.5 transgenic mice (Supplemental Figure 2A) coincided with the onset of thyroglobulin expression (29). The ultimobranchial bodies were successfully enclosed by thyroid tissue. As a result, widely disseminated calcitonin-positive cells were detected in the thyroids of postnatal transgenic animals (Supplemental Figure 2B).


**Tg-Foxe1 mice developed hypothyroidism**

Because of the pronounced growth retardation in transgenic mice, serum TSH, FT4 and FT3 were measured. TSH levels were significantly elevated and FT4 diminished in 5 and 8 weeks old Tg-Foxe1 mice (Figure 3). Despite there was no difference in TSH levels between Tg-Foxe1 and WT mice in 24–48-week-old animals, serum FT4 was gradually increased but not fully recovered. We also measured serum FT3 in Tg-Foxe1 and WT mice, and surprisingly they were not different in all age group (Figure 3). We therefore examined the expression of Dio1 (type I deiodinase) and Dio2 (type II deiodinase) in the extracted thyroid lobes. Both Dio1 and Dio2 expression in Tg-Foxe1 mice were robustly up-regulated in young animals and then declined but still remained higher than in WT mice even at the older age (Supplemental Figure 3), which may be the reason for imbalance between FT4 and FT3.

We also measured transcriptional levels of thyroid-specific genes Slc5a5 (Nis, sodium/iodide symporter), Tpo (thyroid peroxidase), Duox2 (dual oxidase 2) and Slc26a4 (Pds, Pendrin), which could be regulated by Foxe1 and are involved in thyroid hormone biosynthesis. Compared to WT mice, all except Duox2 were age-dependently up-regulated, presumably due to corresponding Foxe1 overexpression, but none was suppressed (Supplemental Figure 3). Therefore, hypothyroidism in young Tg-Foxe1 mice was not caused by the disruption of thyroid hormone biosynthesis and was mainly due to thyroid hypoplasia (see histological description below). On the whole, our observations indicate that Tg-Foxe1 mice exhibited severe hypothyroidism in young age and a gradual recovery until 48 weeks.

**Histological features of the thyroid in young (5–8 weeks old) mice**

At the age of 5–8 weeks, thyroids of WT animals showed predominantly normo-microfollicular structure without pathological abnormalities. In contrast, thyroids of Tg-Foxe1 mice displayed the abnormal irregular architecture with dominant micro-normofollicular, minor macrofollicular, solid and papilloid areas (Figures 4, A and B). Thyroid epithelium in the papilloid
areas showed some oxyphilic changes. The number of thyroid follicles in the young transgenic animals was decreased in comparison to the control littermates; normal parenchyma was abundantly substituted by brown adipose tissue (BAT) (Figure 4A) as confirmed by staining of thyroid cryosections with Sudan Black B and qRT-PCR for *Ucp1* (Supplemental Figures 4A and B). In some animals, BAT occupied more than 40% of the thyroid volume (Supplemental Figure 4C). In *Tg-Foxel* mice, thyroid follicles were predominantly filled with pale colloid; some follicles contained heterogeneous, foamed or depleted colloid (Figure 4B, b).

Thyroid follicles in WT mice were predominantly lined by a single uniform layer of cuboidal epithelium and a small fraction of flattened epithelial cells at the periphery of the gland. Besides of conventional epithelium, thyroids of *Tg-Foxel* mice featured tall cuboidal and columnar follicular cells (Figure 4B, b). Thyrocytes of young Foxe1 overexpressing mice also displayed prominent nuclear pleomorphism and hyperchromatosis, especially in solid clusters; giant hyperchromatic/bizarre nuclei were also revealed.

Functional differentiation of thyroid follicular cells was confirmed by IHC for thyroglobulin, Ttf-1 and Foxe1 (Figure 5A). Interestingly, some thyrocytes in transgenic animals showed stronger cytoplasmic thyroglobulin staining than in control mice. The intensity and proportion of Ttf-1 staining was similar between *Tg-Foxel* and WT littermates. The intensity of Foxe1 immunoreactivity was heterogeneous in thyrocytes in both transgenic and WT mice. Nevertheless, the total Foxe1 IHC-score was significantly higher in 5–8 weeks old transgenic mice in comparison to WT animals (see also Figure 1F). Small immature follicles contained thyrocytes with the highest intensity of Foxe1 staining were commonly seen (Figure 5A, arrow in the Foxe1 panel), while in mature follicles and areas of focal hyperplasia such cells were less frequent.

In transgenic mice, tall cuboidal and columnar thyrocytes had eosinophilic cytoplasm likely due to a high level of TSH stimulation. Concordantly, a proliferative index estimated by Ki-67 IHC (Figure 5B) was significantly higher as compared to that in WT animals both in 5–8-week old males and females (Figure 6A). Histologically, the high level of follicular cell proliferation activity was
represented by numerous papilloid structures inside follicular lumens and initial signs of hyperplastic nodule formation (as was demonstrated in Figures 4A and B). Interestingly, Ki-67-positive follicular cells had moderate to low levels of Foxe1 (Figure 5C), strongly suggesting that cells overexpressing Foxe1 were unlikely to be involved in the active proliferation upon TSH stimulation.

Histological features of the thyroid in mature/adult (24–48 weeks old) mice

The thyroids of WT mice at 24–48 weeks displayed normo-macrofollicular structure with normal age-associated histopathological changes. In transgenic mice, from the age of 24 weeks, hyperplastic areas of diffuse macrofollicular structure and hyperplastic micronodules were observed. The number of cells with nuclear pleomorphism and hyperchromatosis were drastically decreased in adult Tg-Foxel mice in comparison to 5–8-week-old ones. Marked accumulation of the colloid resulted in dilatation of follicles and formation of colloid microcysts. (Figure 4C and D). Gradual decrease of BAT content was also noted (Supplemental Figure 4C).

At 24–48 weeks, follicular epithelium of WT mice was predominantly cuboid and, to a less extent, flattened. In Tg-Foxel mice, macrofollicular thyroid structures contained somewhat flattened cuboid or fully flattened cells (Figure 4D, a). At the age of 48 weeks, well-formed hyperplastic, predominantly macrofollicular-papilloid micronodules in transgenic mice were seen (Figure 4D). Enlarged follicles contained papilloid projections of cuboid or columnar eosinophilic cells with pleomorphic nuclei (Figure 4D, b). Hyperplastic papilloid micronodules in Tg-Foxel mice did not show any specific features of papillary thyroid carcinoma such as capsular/lymphovascular invasion or nuclear grooves, pseudo-inclusions and optical clearing. Small hyperplastic follicles protruding into the lumen of larger follicles, so called Sanderson’s polysters, were also found.

At the age of 24–48 weeks, transgenic mice, both males and females, showed lower Ki-67 labeling indexes compared to 5–8 weeks old mice. Nevertheless, it remained significantly higher as compared to that in WT animals (Figure 6). Thus, by the age of 48 weeks Tg-Foxel mice did not
develop thyroid cancer, but the gland was affected by a diffuse macrofollicular hyperplastic process with multiple macro-normo-papilloid hyperplastic micronodules and colloid microcysts.

Effect of X-ray exposure

Irradiation of thyroids of WT mice with 1 Gy or 8 Gy of X-rays at the age of 5 weeks resulted in prominently flattened follicular epithelium and dilatation of the follicular lumen at the age of 48 weeks in comparison to non-exposed mice (Figure 7A). Exposure of Tg-Foxe1 mice significantly promoted hyperplastic micronodule formation (Figure 7B). After exposure to 1Gy, well-formed hyperplastic micronodules were found from 8 weeks of age, and from 24 weeks after 8 Gy. Despite the delay in micronodule formation (as compared to 1 Gy exposure), a significantly higher frequency of micronodules was observed in the latter group at 48 weeks of age (p<0.01). Histopathological features of thyroid micronodules in exposed Tg-Foxe1 animals were similar to those in unexposed transgenic mice of the same age. Thus, exposure of Foxe1-overexpressing animals to ionizing radiation stimulated the formation of hyperplastic nodules in a dose-related manner without carcinogenic effect.

Double transgenic Tg-Foxe1/Pten+/− mice

Double transgenic Tg-Foxe1/Pten+/− animals developed severe hypothyroidism at the age of 5 weeks similarly to Tg-Foxe1 mice. Congenital hypothyroidism was characterized by significant growth retardation, significantly elevated serum TSH and diminished FT4 (data not shown). Thyroid follicular epithelium was profoundly substituted by BAT. Colloid in normo-, micro- and macrofollicles was heterogeneous: pale, depleted, foamed and sometimes with mucinous content. Cellular areas showing pleomorphism of follicular cells with nuclear enlargement and hyperchromasia were observed.
Activation of the follicular epithelium in 5–8 weeks old Tg-Foxe1/Pten+/- mice was observed: cuboidal thyrocytes had increased eosinophilic cytoplasm with small clear vacuoles. Hyperplastic changes such as papilloid projections into the follicular lumen, nuclear crowding and foci of columnar cells, were more frequent in 5–8-week-old double transgenic mice in comparison to age-matched Tg-Foxe1 and Pten+/- mice. The proliferation rate of thyroid epithelial cells in Tg-Foxe1/Pten+/- mice was significantly higher in comparison to Pten+/- animals at 5 and 8 weeks of age (Figure 6). Immunohistochemical staining showed that there was no loss of the remaining Pten allele in any age group (Supplemental Figure 5). Ki-67 labeling indexes did not differ significantly between Tg-Foxe1/Pten+/- and Tg-Foxe1 mice in all age groups (Figure 6), indicating that heterozygous Pten deletion added a minor effect on the proliferative phenotype of Tg-Foxe1 mice thyroids. On the other hand, the labeling indexes in Tg-Foxe1/Pten+/- and Tg-Foxe1 mice were significantly higher than in age-matched WT animals (Figure 6).

In contrast to Tg-Foxe1 and Pten+/- mice, double transgenic animals developed multiple hyperplastic thyroid micronodules from the age of 8 weeks (Figure 7 C and D). The frequency of micronodules in Tg-Foxe1/Pten+/- mice was significantly higher in comparison to Pten+/- animals. Note that Pten+/- mice had predominantly adenomatous nodules with normo-microfollicular-solid or normofollicular-solid structure, prominent oxyphilic changes of follicular cells and areas of nuclear pleomorphism. Micronodules in double transgenic mice showed mixed features of hyperplastic nodules found in Tg-Foxe1 mice and adenomatous nodules of Pten+/- animals (Figure 7C). Thus, Foxe1 overexpression in thyroids of Pten+/- mice caused acceleration of hyperplastic processes, showing features of both Pten+/- and Tg-Foxe1 phenotypes, but no cancerous nodules were seen.

Discussion

To evaluate the role of high level of Foxe1 as a possible etiological factor in thyroid carcinogenesis, transgenic mice overexpressing Foxe1 under the thyroglobulin promoter were
generated. The transgenic animals were viable and showed no apparent gross developmental abnormalities. However, in the postnatal period, *Tg-Foxe1* mice at the age 5–8 weeks displayed congenital hypothyroidism manifesting as significant growth retardation, diminished level of FT4 and elevated TSH. In those mice, normal follicular organization in the thyroid gland was compromised, and thyroid parenchyma was replaced with BAT to a large extent.

Under the TSH stimulation, tall cuboidal and columnar thyrocytes with augmented eosinophilic cytoplasm appeared in the thyroids of transgenic mice. TSH-induced enhancement of thyroid hormone synthesis was accompanied by the activation of endocytosis in thyrocytes seen as colloid depletion in some follicles. High TSH levels also switched on the growth of thyroid parenchyma. The thyroids of 5-week-old transgenic mice showed a high (>10%) Ki-67 labeling index. It is worth noting, however, that follicular cells overexpressing Foxe1 were unlikely to be the primary responders to TSH stimulation. Several facts concordantly support this notion: 1) in the areas of evident proliferation, the majority of cells displayed moderate levels of Foxe1 on IHC or immunofluorescence; 2) the proportion of cells with strong Foxe1 staining intensity was declining with the increase of thyroid weight; 3) small immature follicles highly immunoreactive for Foxe1 persisted in the thyroids of 5–8-week-old *Tg-Foxe1* mice; and 4) no Ki-67 signals were seen in the cells overexpressing Foxe1. It is likely that Foxe1 overexpression may prevent the proliferative cellular reaction on TSH stimulation. Under this scenario, thyroid parenchyma regeneration and hyperplastic changes seen in older mice would be achieved through the propagation of epithelial cells with lower Foxe1 level. The inability of cells with high Foxe1 levels to proliferate is also consistent with and may explain thyroid hypoplasia observed during the first month of postnatal life of *Tg-Foxe1* mice. Molecular mechanisms of interference between the proliferative signals and Foxe1 overexpression as well as age-dependent down-regulation of transgene expression require further investigation.

TSH-induced activation and proliferation of follicular cells led to the gradual increase of FT4 level. However, surprisingly, the FT3 level in the *Tg-Foxe1* mice was not different from WT mice in all age groups. This may be due to the increased level of *Dio1* and *Dio2* in the thyroids of the
transgenic animals. Dio1 and Dio2 were highly up-regulated in the 5–8-week-old Tg-Foxe1 mice, in which BAT occupied a large part of thyroid tissue. It should be mentioned that TSH receptors are expressed in BAT cells and TSH stimulates Dio2 expression in these cells (30).

Exposure of Tg-Foxe1 mice thyroids to 1 Gy or 8 Gy of X-rays at the age of 5 weeks accelerated hyperplastic nodule formation in a dose-dependent manner. The changes were observed from 8-24 weeks of age, while irradiated WT mice did not develop any thyroid lesions. We speculate that high TSH may promote metaplastic changes in the thyroid follicular epithelium exposed to X-ray irradiation. A similar effect of TSH could be proposed with regard to Foxe1 overexpression combined with the activated PI3-Akt pathway. We found that hypothyroid 5 weeks old Tg-Foxe1/Pten+/− mice exhibited a remarkable increase in the thyrocyte proliferation rate as compared to age-matched Pten+/− mice. Moreover, double transgenic mice displayed an accelerated formation of hyperplastic and adenomatous nodules detectable from the age of 8 weeks, whose development was not due to the loss of the remaining Pten allele. More detailed investigation is needed to establish exact pathogenetic and molecular basis of these hyperplastic and neoplastic processes.

The model described in our study has some limitations. The overexpression of Foxe1 caused hypothyroidism, thus corresponding TSH elevation in young mice, and the transgene expression was then declined with age. This created a complicated situation, which made it difficult to assess the effect of Foxe1 overexpression only (i.e., without the hormone imbalance) on thyroid tumorigenesis. On the other hand, all transgenic mice displayed thyroid-related phenotype, and therefore the model may be useful for in vivo studies of the mechanisms of TSH-dependent proliferation of the thyrocytes or BAT cells under the condition of CH, and of pathogenesis of multinodular goiter.

In summary, our mouse model of thyroid-specific overexpression of Foxe1 allowed us to make several important observations. By the age of 5 weeks, transgenic mice displayed thyroid hypoplasia accompanied by the extensive replacement of thyroid parenchyma with BAT and the development of overt hypothyroidism. Likely due to the prolonged TSH stimulation at young age, the reactive proliferation of TFC took place and resulted in the nearly full compensation of
hypothyroidism by the age of 24 weeks and the development of hyperplastic changes representative of multinodular goiter. No direct evidence of thyroid carcinogenesis due to Foxe1 overexpression during the course of 48 week-long observation was found either in Tg-Foxe1 mice, Tg-Foxe1 mice exposed to 1–8 Gy of X-rays or in 24-week-old Tg-Foxe1/Pten+/− mice. We conclude that proper Foxe1 dosage is essential for thyroid development and functioning, and excessive Foxe1 in the thyroid does not induce carcinogenesis in our model.

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449 References


Figure legends

**Figure 1.** Generation and analysis of Tg-Foxe1 mice. A, The genetic construct used to generate the Tg-Foxe1 mice. The bovine thyroglobulin promoter (bTg, 2045 bp), murine Foxe1 gene (Foxe1, 1116 bp) and the SV-40 polyadenylation signal (pA, 228 bp) are indicated by the rectangles. For Southern blotting, the 2770 bp Sph I/BamH I restriction fragment was hybridized with a probe located in the bTg area. For PCR screening of the Foxe1 transgene, primers were designed to amplify the 1552 bp region spanning the bTg and pA sequences. B, Relative cDNA levels of transgenic Foxe1 in the thyroid of Tg-Foxe1 line A determined by qRT-PCR and normalized for Pax8 expression. For qRT-PCR assessment of transgenic Foxe1 expression, primers located in the 3' end of Foxe1 and in pA sequences were used. Data are presented as a mean±SE of triplicates averaged for 8 mice for each group. C, Relative cDNA levels of total Foxe1 in the thyroids of WT and Tg-Foxe1 line A determined by qRT-PCR and normalized for Pax8 expression. For qRT-PCR assessment of Foxe1 expression, primers located in the coding region of Foxe1 were used. Data are presented as a mean±SE of triplicates averaged for 8 mice for each group. D, Representative images of thyroid histology and Foxe1 immunoreactivity in WT and Tg-Foxe1 mice of different age. H&E and IHC for Foxe1. E and F, The proportion of cells with the highest intensity score (3, “strong”) in Foxe1 IHC. F, The total Foxe1 IHC score. In E and F, the boxes include 50% of the values; lines inside the boxes represent median values; whiskers indicate the 10–90% range; *p<0.01, **p<0.001, ***p<0.0001.

**Figure 2.** Systemic characterization of Tg-Foxe1 mice. A, Body habitus of representative female WT and Tg-Foxe1 mice at the age of 5 weeks. B, Body weight (males n=7–24 mice/group, females n=8–
38 mice/group); C, Thyroid weight (males n=5–16 mice/group, females n=8–38 mice/group) and D, Thyroid-to-body-weight ratio (males n=5–16 mice/group, females n=8–38 mice/group) in WT and Tg-Foxe1 animals of different age. Boxes include 50% of the values; lines inside the boxes represent median values; whiskers indicate the 10–90% range; *p<0.01, **p<0.001, ***p<0.0001. E, Gross anatomy of WT and Tg-Foxe1 mouse thyroids at the age of 48 weeks. Arrow points at the irregular surface of the thyroid.

Figure 3. The hypothyroid status of Tg-Foxe1 mice. A, C and E, Relative TSH (A), FT4 (C) and FT3 (E) levels in WT and Tg-Foxe1 mice of different age. The median value is represented by the solid line. Horizontal dashed lines represent the first (Q1) and the third quartile (Q3) of the relative TSH or FT4 values in WT mice estimated for each sex separately (see below). TSH (B), FT4 (D) and FT3 (F) level category in WT (n=6–11 mice/group) and Tg-Foxe1 (n=6–9 mice/group) animals of different age combined for both sexes (see below).

Because of limitations in the in-house produced reagent availability and small sample volumes, statistical analysis of raw TSH, FT4 and FT3 concentrations in separate subgroups of male and female animals was impeded. We therefore determined the normal ranges of relative sex-specific TSH and FT4 levels as intervals between the first (Q1) and the third (Q3) quartiles calculated from the integrated data across all age groups of WT mice (distributions between which did not differ significantly, p>0.05, Kruskal-Wallis test). The defined normal ranges of relative TSH level in WT mice were 0.85–1.06 ng/ml (n=15) and 0.52–0.78 ng/ml (n=14) for males and females, respectively; 0.70–0.92 ng/dL (n=17) and 0.63–1.02 ng/dL (n=16) for FT4; and 1.16–1.38 pg/mL (n=23) and 1.18–1.35 pg/mL (n=24) for FT3. Then each raw value was categorized as diminished (<Q1), normal (Q1-Q3) or elevated (>Q3) for either TSH, FT4 or FT3. This approach allowed merging data for two sexes to increase statistical power. Differences between WT (n=6-11 mice/group) and Tg-Foxe1 (n=6–11 mice/group) animals were evaluated using the 3x2 Fisher’s exact test extension.
**Figure 4.** Histopathology of the *Tg-Foxe1* thyroid at different age. A, Representative microphotographs of *Tg-Foxe1* and WT mice thyroids at the age of 5 weeks, H&E staining. BAT denotes brown adipose tissue, arrows point at foci of hyperplastic micronodules. B, The representative image of 8-week-old *Tg-Foxe1* thyroid with a colloid microcyst (Mc) and featuring (a) abnormal solid/papilloid structures, and (b) colloid heterogeneity and columnar follicular epithelium (arrow). C, The representative image of 24-week-old *Tg-Foxe1* thyroid. D, The representative image of 48-week-old *Tg-Foxe1* thyroid; (a) area with flattened thyroid epithelium and (b) a nodule with papilloid structures.

**Figure 5.** Functional differentiation and proliferative status of thyroid cells in young *Tg-Foxe1* and WT mice. A, H&E and IHC for thyroglobulin, Ttf-1 and Foxe1, serial sections. The arrow in the Foxe1 panel indicates immature follicle with high Foxe1 level. B, IHC for Ki-67. C, Double immunofluorescent staining for Ki-67 (green) and Foxe1 (red). Nuclei were counterstained with DAPI. PS, papillary structures.

**Figure 6.** Ki-67 labeling index in the thyroids of mice of different age. A, *Tg-Foxe1* (n=5–16 mice/group), *Pten*+/− (n=5–8 mice/group) and *Tg-Foxe1/Pten*+/− (n=5–9 mice/group) males. B, *Tg-Foxe1* (n=8–12 mice/group), *Pten*+/− (n=7–9 mice/group) and *Tg-Foxe1/Pten*+/− (n=4–11 mice/group) females. Boxes include 50% of the values; lines inside the boxes represent median values; whiskers indicate the 10-90% range; *p<0.05, **p<0.01, ***p<0.001.

**Figure 7.** Combination effect of Foxe1 overexpression with X-ray irradiation or activated PI3K-Akt signaling pathway. A, Representative microphotographs showing X-ray-associated histopathological changes in WT and *Tg-Foxe1* mice thyroids at the age of 48 weeks, H&E staining. Scale bar, 0.5mm, applies to all microphotographs. B, Frequencies of micronodule finding in thyroids of *Tg-Foxe1* mice of different age by X-ray dose. Differences between unexposed (n=14–28 mice/group), and exposed to 1 Gy (n=12–14 mice/group) or 8 Gy (n=13–14 mice/group) of X-rays mice were evaluated using
the 3x2 Fisher’s exact test extension: *p<0.01, **p<0.001, ***p<0.0001; ns: not significant. C, Representative images of histopathological features of thyroids in 24 weeks old Tg-Foxe1/Pten\(^{+/−}\) and Pten\(^{+/−}\) mice, H&E staining. Hyperplastic areas with adenomatous (Ad) and papillary (Pap) structures.

D, Frequencies of micronodules in thyroids of Tg-Foxe1/Pten\(^{+/−}\) (n=14–21 mice/group) and Pten\(^{+/−}\) (n=15–17 mice/group) mice of different age, *p<0.01.

Supplemental Figure 1. A, Histological structure of thyroids from two Tg-Foxe1 lines A and B at the age of 48 weeks showing diffuse goiter with micronodules, H&E staining. B, Relative cDNA levels of transgenic Foxe1 in the thyroids of two Tg-Foxe1 lines determined by qRT-PCR and normalized for Actb (β-actin) expression. Data are presented as a mean±SE of triplicates for 3 mice in each group.

Supplemental Figure 2. Normal thyroid development in Tg-Foxe1 mice. A, Representative microphotographs of the thyroid of Tg-Foxe1 and WT mice at E14.5, H&E and IHC staining. Ts: thymus, Th: thyroid, UB: ultimobranchial body. B, Representative images of Tg-Foxe1 thyroid lobe at the age of 5 weeks, frontal plane, H&E and IHC for Thyroglobulin, calcitonin (Ct) and Ttf-1, serial sections.

Supplemental Figure 3. Real-time PCR analysis of the relative expression of thyroid hormone biosynthesis-related Dio1 and Dio2 genes normalized for Actb (β-actin) or Pax8, and of Slc5a5 (Nis), Tpo, Duox2 and Slc26a4 (Pendrin) normalized for Pax8, and relative expression of Pax8 and Ucp1 normalized for Actb. Data are presented as a mean±SE of duplicates for 8 mice in each group.

Supplemental Figure 4. Brown adipose tissue (BAT) in Tg-Foxe1 and WT mice. A, Prominent BAT accumulation in the thyroid of a 8-week-old Tg-Foxe1 mouse in comparison to an age- and sex-
matched WT animal. Cryosections were stained with H&E or Sudan Black B, nuclei counterstained with Nuclear Fast Red. B, Relative $Ucp1$ expression in the thyroids of 8-week-old WT and Tg-Foxe1 mice determined by qRT-PCR and normalized for $Actb$ ($\beta$-actin). Data are presented as a mean±SE of triplicates (n=3 mice/group). BAT from the interscapular region of WT mice was used as a positive control (WT BAT). C, Relative amount of BAT in the thyroids of Tg-Foxe1 and WT mice of different age. Differences between Tg-Foxe1 (n=14–28 mice/group) and WT (n=13–19 mice/group) animals were evaluated using the 4x2 Fisher’s exact test extension, ***p<0.0001.

**Supplemental Figure 5.** Representative images of IHC for Pten in 24-week-old mice of different genetic backgrounds. Similar results were obtained for animals of any age.
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