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NOTE

Conditional deletion of insulin receptor in thyrocytes does not affect thyroid structure and function

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Abstract. Thyroid-stimulating hormone (TSH) is the primary regulator of thyroid growth and function acting *via* cyclic AMP signaling cascades. In cultured thyrocytes, insulin and/or insulin-like growth factor-1 (IGF-1) are required for mediating thyrocyte proliferation in concert with TSH. To determine the role of insulin signaling in thyroid, growth *in vivo*, mice with thyrocyte-selective ablation of the insulin receptor (IR) were generated by crossing mice homozygous for a floxed IR allele with transgenic mice in which thyrocyte-specific expression of *Cre* recombinase was driven by the human thyroid peroxidase (TPO) gene promoter. Immunohistochemistry and Western blot analysis confirmed near complete loss of IR expression in the thyroid of thyrocyte IR knockout mice. These mice are viable and have no obvious thyroid dysfunction and macro- and microscopic thyroid morphology was normal. Thus, insulin signaling in thyrocytes does not play an essential role in the architecture and function of the thyroid *in vivo*.

Key words: Insulin receptor, Mice, Thyroid

THE THYROID gland consists of follicles of varying size composed of follicular cells, called thyrocytes that surround colloid, which contains thyroglobulin (Tg), the protein precursor to thyroid hormones such as thyroxine (T_4) and triiodothyronine (T_3). Thyroid hormones regulate body temperature and metabolic homeostasis [1]. Thyroid-stimulating hormone (TSH) secreted by the anterior pituitary is the primary regulator of the thyroid growth and hormone secretion. Binding of TSH to the G protein-coupled TSHR on thyrocytes, activates adenylate cyclase, which increases cAMP and regulates iodide uptake, transcription of Tg and the activities of thyroid peroxidase (TPO), and the sodium-iodide symporter (NIS) [2, 3]. Moreover, TSH induces cell cycle progression and the expression of differentiation markers [4]. Indeed, TSHR knockout mice show severe hypothyroidism and reduced thyroid size [5].

Multiple lines of *in vitro* evidence suggest that insulin or insulin-like growth factor-1 (IGF-1) may be involved in the thyroid growth and function. Both TSH and insulin/IGF-I are required for cell growth and uptake and organification of iodide in cultured sheep thyroid cells suggesting cooperative interactions between growth factors and TSH in regulating both thyroid growth and function [6]. Likewise, TSH increases insulin receptor (IR) expression and IR and IGF-1 receptor (IGF-1R) autophosphorylation in cultured thyrocytes [7, 8, 9]. Moreover, overexpression of IGF-1 and IGF-1R in the thyroid led to goiter, with increased thyroidal iodine uptake, further supporting an important role for the insulin and IGF-1 pathways in thyroid archi-

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tecture and function [10]. Clinical studies have shown that higher serum insulin or IGF-1 levels are associated with increased thyroid volume or goiter, measured by ultrasonography [11, 12]. Thus, understanding the relationship between insulin signaling and thyroid growth and function may have important clinical implications. In the present study, we generated thyrocyte-selective IR deficient mice to determine the impact of insulin signaling on thyroid growth and function.

Materials and Methods

Generation of $IR^{loxP/loxP}$;TPO-Cre mice

Mice with thyrocyte-selective ablation of the IR ($IR^{loxP/loxP}$;TPO-Cre) were generated by crossing mice that were homozygous for a floxed insulin receptor allele in which *loxP* sites flank exon 4 of IR gene [13] with transgenic mice in which thyrocyte-specific expression of *Cre* recombinase was driven by the human TPO gene promoter [14]. Animals were fed standard chow and housed in temperature-controlled, pathogen-free facilities with a 12 h light/dark cycle. Mice were maintained on a mixed C57BL6J/129Sv/FVB background, and littermate controls were used for experiments. All animal experiments were conducted in accordance with guidelines approved by the institutional animal care and use committee of the Chung-Ang University.

Tissue preparation and staining

For the analysis of histology, dissected thyroid glands were fixed in 4% paraformaldehyde, embedded in paraffin, and sliced at a thickness of 6 μm. Sections were stained with hematoxylin and eosin. The slides were examined using an Olympus BX51 microscope and the following parameters were measured using AxioVison 3.1 software as previously described [15]. Briefly, from each slide, 15 randomly selected thyroid follicles were analyzed for colloid-containing area, whole follicle area, thyrocyte area (whole follicle area minus colloid area), number of visible nuclei, and average thyrocyte size (thyrocyte area divided by number of visible nuclei).

Tissue sections were mounted on poly-L-lysine coated slides for immunohistochemical analysis using the EnVision system Kit (Dako, DK-2600 Glostrup, Denmark). The primary antibodies used in this study included anti-IR antibody (1:50, Thermo Scientific, Fremont, CA, USA) and anti-TPO antibody (1:50, Abbiotec, San Diego, CA, USA). Briefly, after standard de-paraffinization, hydration and blocking of the endogenous peroxidase, the sections were subjected to microwave antigen retrieval followed by incubation at 121°C for 10 min. After rinsing with normal goat serum for 10 min, all the prediluted primary antibodies were applied for 60 min at room temperature. The sections were then allowed to react with peroxidase-conjugated streptavidin for 45 min, followed by color development with diaminobenzidine, and counterstained with hematoxylin.

Western blot analysis

Total protein lysates (18 μg) were extracted from frozen thyroids. Proteins were resolved by SDS-PAGE and electrotransferred to nitrocellulose membranes (GE Healthcare, Piscataway, NJ, USA). The antibodies used were IR and IGF-1R (Cell Signaling Technology, Danvers, MA, USA). Immunoblotting was detected by SuperSignal West Femto Maximum Sensitivity Substrate (ThermoFisher Scientific, Pittsburgh, PA, USA).

RNA isolation and quantitative RT-PCR analysis

Total RNA was obtained from thyroid tissues using the RNA-STAT 60 reagent (AMS Biotechnology, Abingdon, UK). To quantify IGF-1 transcripts, the Light Cycler System (Roche Molecular Biochemicals, Indianapolis, IN, USA) was used. PCRs were performed using SYBR Green I master mix and the following primers; IGF-1 (5′- CACCAGCTC CACCACAGC -3’ and 5’- GGGCATGTCAGTGTGGCG -3’), NIS (5’- GTGGGCCAGTTGCTCAATTC -3′ and 5’- GTGCGTAGATCACGATGCCA -3’), and Tg (5’-GCCCACCATCTGTGGACTTC -3’ and 5’-CATTCCCCTTTCACATCCCA -3’). To assess the specificity of the amplified PCR products, a postamplification melting curve analysis was performed and the reaction end-products were subjected to electrophoresis in 1.5% agarose gels, followed by staining with ethidium bromide.

T_4 and TSH measurements

Serum total T_4 concentrations were measured by coated tube RIA (Diagnostic Products) using 25 μL mouse serum. TSH was measured in 50 μL serum using a sensitive, heterologous, disequilibrium, double-antibody precipitation RIA [16, 17].

Statistical analysis

Data are presented as the mean ± S.E. Statistical

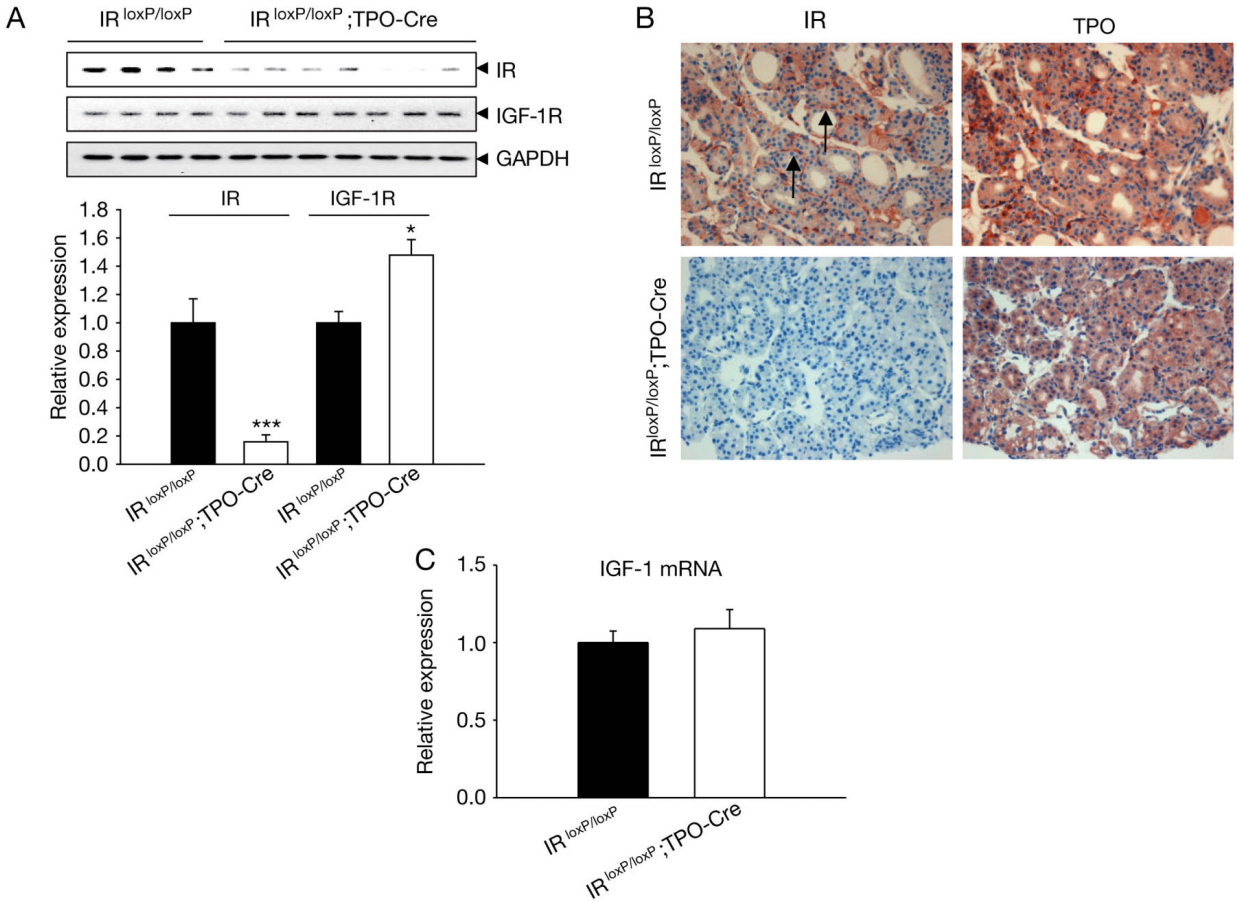

Fig. 1 Thyrocyte deletion of the IR. (A) Western blot analysis of IR or IGF-1R protein obtained from thyroids of 9-week-old littermate $IR^{loxP/loxP}$ and $IR^{loxP/loxP}$;TPO-Cre mice. Upper panels are representative immunoblots, and lower panel is densitometry of results from four to seven thyroids per group. GAPDH is the loading control. Data are presented as the mean ± S.E. ***, $P < 0.001$ *; $P < 0.05$ versus $IR^{loxP/loxP}$. (B) Thyroid serial sections from 7-week-old littermate $IR^{loxP/loxP}$ and $IR^{loxP/loxP}$;TPO-Cre mice were subjected to IR and TPO immunostaining. IR is highly expressed (representative is shown by *arrow*) in $IR^{loxP/loxP}$ thyroid but not expressed in $IR^{loxP/loxP}$;TPO-Cre thyroid. TPO is highly expressed in both $IR^{loxP/loxP}$ and $IR^{loxP/loxP}$;TPO-Cre thyroids (magnification, x400). (C) IGF-1 mRNA expression in the thyroids of 9-week-old littermate $IR^{loxP/loxP}$ and $IR^{loxP/loxP}$;TPO-Cre mice measured by quantitative RT-PCR. Results were normalized to actin signals and mRNA level of $IR^{loxP/loxP}$ was arbitrarily set as 1. n = 5 per group. Data are presented as the mean ± S.E.

analyses were performed using Student's *t*-test. A two-tailed $P < 0.05$ was considered significant.

Results

Generation of $IR^{loxP/loxP}$;TPO-Cre mice

Both IR and IGF-1R are expressed in the mouse thyroid (Fig. 1A). In $IR^{loxP/loxP}$;TPO-Cre mice, thyroid IR protein expression was reduced to 15.8% of the level of controls ($IR^{loxP/loxP}$) ($P < 0.001$). Immunohistochemical images demonstrated that almost all thyrocytes of $IR^{loxP/loxP}$ mice were positive for IR, while essentially none were positive for $IR^{loxP/loxP}$;TPO-Cre thyrocytes (Fig. 1B). Thus, complete or nearly complete thyrocyte-specific IR knockout mice were obtained. As insulin can also bind and activate IGF-1R, although with reduced affinity [18], IGF-1R expression was measured in thyroids. There was a 1.5-fold compensatory increase of IGF-1R protein in 9-week-old $IR^{loxP/loxP}$;TPO-Cre thyroid ($P < 0.05$) (Fig. 1A), that occurred despite normal IGF-1 mRNA expression (Fig. 1C).

Normal thyroid architecture and function in $IR^{loxP/loxP}$;TPO-Cre mice

There was no difference in the ratio of thyroid weight (TW) to body weight (BW) between $IR^{loxP/}$

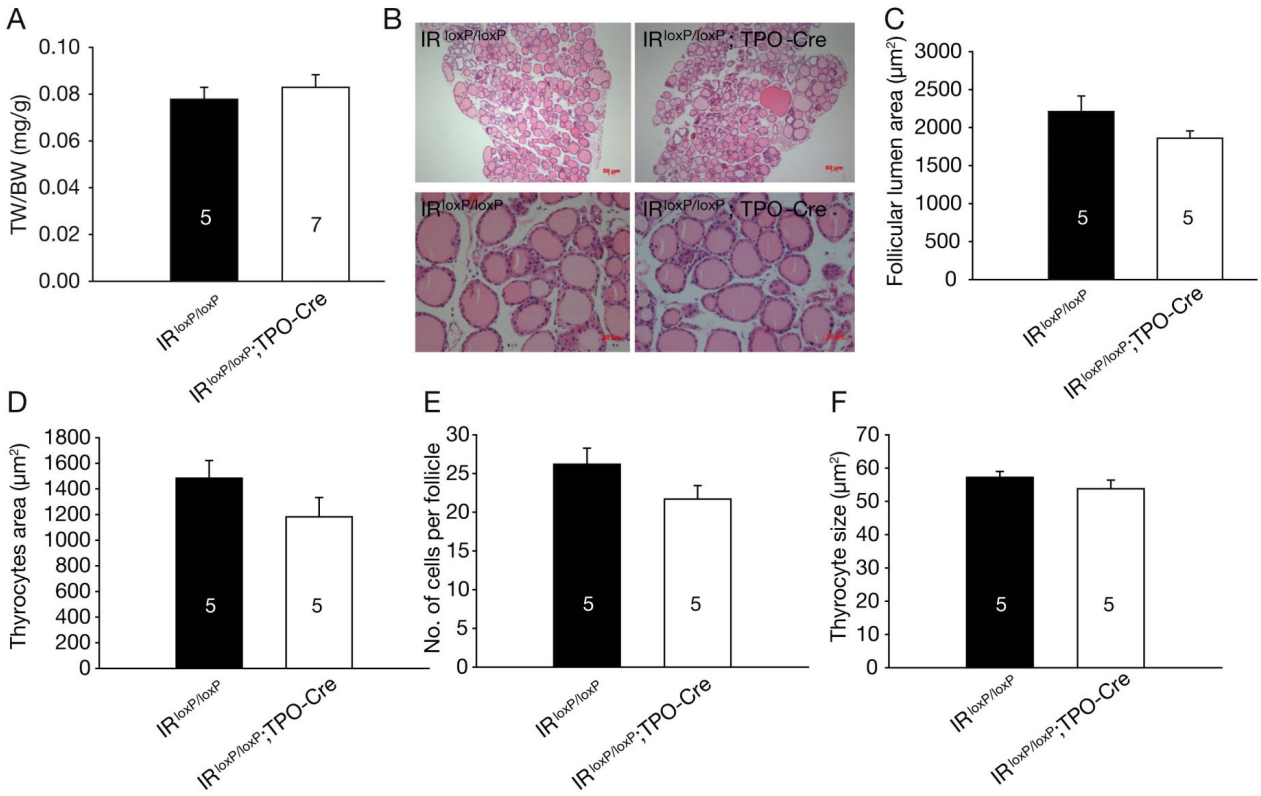

Fig. 2 Normal thyroid growth in IR$^{loxP/loxP}$;TPO-Cre mice. (A) Thyroid weight/body weight (TW/BW) ratio in 8-week-old littermate IR$^{loxP/loxP}$ and IR$^{loxP/loxP}$;TPO-Cre mice. (B) Hematoxylin and eosin staining of transverse sections of thyroids from 8-week-old littermate IR$^{loxP/loxP}$ and IR$^{loxP/loxP}$;TPO-Cre mice (magnification, x100 upper panels; x400 lower panels). (C-F) Morphological parameters in thyroids. (C) Follicular lumen area. (D) Thyrocyte area. (E) Number of cells per follicle. (F) Average thyrocyte size. See *material and methods* for details. Numbers of thyroids are indicated on the bars. Data are presented as the mean ± S.E. No statistical differences were found in any parameters.

^{loxP} and IR$^{loxP/loxP}$;TPO-Cre thyroids of 8-week-old male mice (Fig. 2A). Hematoxylin and eosin staining of transverse sections of thyroids from 8-week-old IR$^{loxP/loxP}$ and IR$^{loxP/loxP}$;TPO-Cre male mice showed no gross differences (Fig. 2B). The follicular lumen area, thyrocyte area, number of cells per follicle, and average thyrocyte size were not significantly different between thyroids of IR$^{loxP/loxP}$ and IR$^{loxP/loxP}$;TPO-Cre (Fig. 2C, D, E, and F). Taken together, these results suggest that loss of IR in thyrocytes did not cause morphological changes in thyroids. To investigate whether insulin signaling regulates thyroid function, serum T_4 and TSH levels were measured. Serum T_4 and TSH levels were not significantly different between IR$^{loxP/loxP}$ and IR$^{loxP/loxP}$;TPO-Cre mice (Fig. 3A and B). We next examined the expression of genes that are regulated by TSH. No differences were found in NIS and Tg mRNA levels (Fig. 3C). These results document normal thyroid function in IR$^{loxP/loxP}$;TPO-Cre mice.

Discussion

In addition to its classic role in regulating metabolism in liver, skeletal muscle, and adipose tissue, insulin may have non-metabolic actions in other tissues that express the IR [19]. This is supported by several lines of evidence. Kulkarni *et al*. [20] reported that mice with a specific deletion of the IR in β cells exhibit decreased islet size and insulin content as well as a selective loss of acute phase secretion in response to glucose. We previously demonstrated that cardiomyocyte-selective IR knockout mice show reduced heart size and mildly impaired contractile function [21]. Furthermore, podocyte-specific IR knockout mice developed albuminuria and cytoskeletal remodeling in the absence of hyperglycemia [22]. Both IR and IGF-1R are expressed in normal human thyroid specimens [23-25]. We confirmed IR expression in mouse thyroid tissues. It is possible that insulin signaling may have non-metabolic actions in

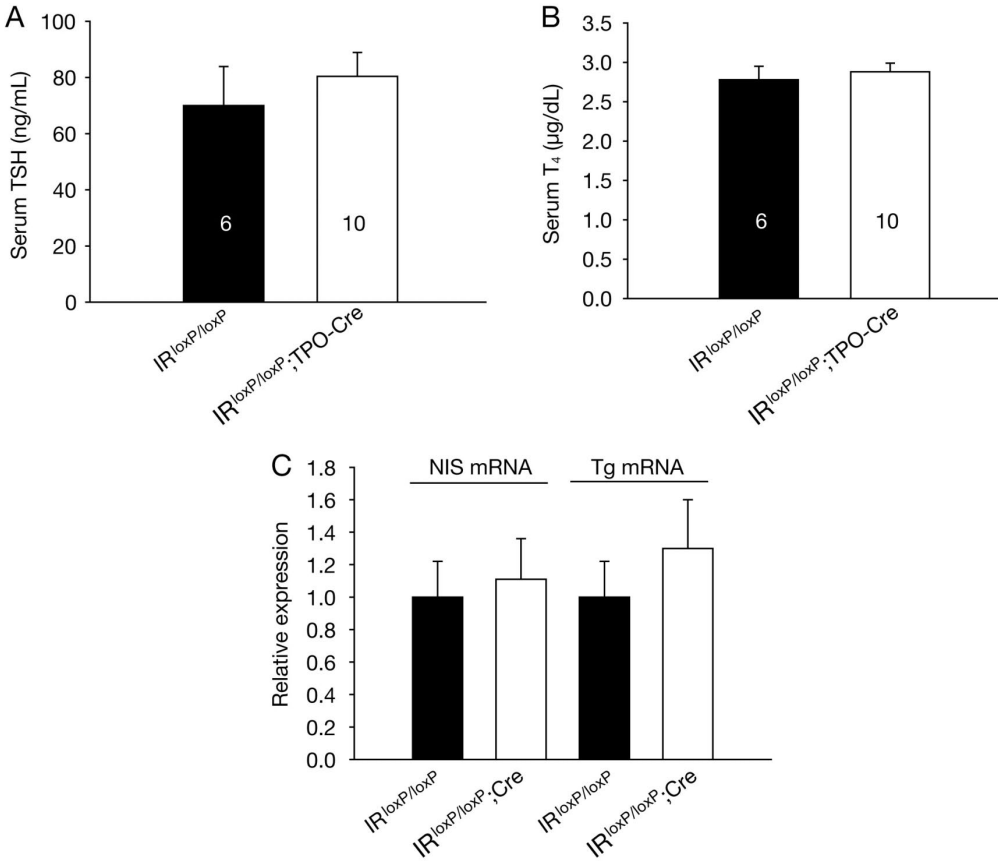

Fig. 3 Normal thyroid function in $IR^{loxP/loxP}$;TPO-Cre mice. Serum TSH (A) and T_4 (B) levels in littermate $IR^{loxP/loxP}$ and $IR^{loxP/loxP}$;TPO-Cre mice. Numbers of thyroids are indicated on the bars. Data are presented as the mean ± S.E. (C) NIS and Tg mRNA expression in the thyroids of 9-week-old littermate $IR^{loxP/loxP}$ and $IR^{loxP/loxP}$;TPO-Cre mice determined by quantitative RT-PCR. Results were normalized to actin signals and mRNA level of $IR^{loxP/loxP}$ was arbitrarily set as 1. n = 9 per group. Data are presented as the mean ± S.E. No statistical differences were found in any parameters.

thyrocytes. However, despite of extensive histological analysis, loss of insulin signaling did not affect thyroid architecture in mice. The normal microscopic structure correlated with normal thyroid function assessed by T_4 and TSH measurements. Future studies will be needed to identify the metabolic actions of insulin such as glucose transport, glycolysis, glucose oxidation, and glycogen synthesis in $IR^{loxP/loxP}$;TPO-Cre thyroid.

There are some possible explanations for the absence of a phenotype in $IR^{loxP/loxP}$;TPO-Cre mice. First, it was reported that serum TSH and T_4 concentrations were significantly different among mouse strains [16]. Our mice are on a mixed genetic background. Therefore subtle functional hormonal changes might not be identified. Second, our mice were 7-9 weeks old at the time of analysis. The effect of age is critical because serum T_4 levels were reported to be decreased independent of feedback by T_4 as mice age [26]. Finally, functional overlap between IR and IGF-1R should be considered. The possibility exists that normal IGF-1 signaling could compensate for the loss of IR because IGF-1R levels were significantly increased in $IR^{loxP/loxP}$;TPO-Cre thyroid. The use of IGF-$1R^{loxP/loxP}$;TPO-Cre mice will be required to address this question.

In conclusion, we have established to the best of our knowledge for the first time, mice with thyrocyte-restricted deletion of insulin receptors. Our study demonstrates that insulin signaling is not required for the maintenance of normal thyroid architecture and function. Additional studies will be required using congenic older $IR^{loxP/loxP}$;TPO-Cre mice and/or $IR^{loxP/loxP}$;IGF-$1R^{loxP/loxP}$;TPO-Cre mice to fully evaluate the role of IR and IGF-1 signaling in goitrogenesis or tumorigenesis in the thyroid gland.

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