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
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\textsuperscript{loxP/loxP;TPO-Cre} mice
Mice with thyrocyte-selective ablation of the IR (IR\textsuperscript{loxP/loxP;TPO-Cre}) were generated by crossing mice that were homozygous for a floxed insulin receptor allele in which \textit{loxP} sites flank exon 4 of IR gene [13] with transgenic mice in which thyrocyte-specific expression of \textit{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’-GCCGCCATCGTGGAACCTTCCACCCCA -3’ and 5’- GCCCCATTGTTGGAACCTTCC -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.

\textit{T}_4 and TSH measurements
Serum total \textit{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
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\(^{loxP/loxP}\); TPO-Cre mice and controls. Therefore, thyrocyte-specific IR knockout mice with IGF-1R compensation were normal in thyroid architecture and function.

*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.
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.

Fig. 2 Normal thyroid growth in IR<sup>loxP/loxP</sup>;TPO-Cre mice. (A) Thyroid weight/body weight (TW/BW) ratio in 8-week-old littermate IR<sup>loxP/loxP</sup> and IR<sup>loxP/loxP;TPO-Cre</sup> mice. (B) Hematoxylin and eosin staining of transverse sections of thyroids from 8-week-old littermate IR<sup>loxP/loxP</sup> and IR<sup>loxP/loxP;TPO-Cre</sup> 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.

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Discussion

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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 T4 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 IRloxP/loxP;TPO-Cre thyroid.

There are some possible explanations for the absence of a phenotype in IRloxP/loxP;TPO-Cre mice. First, it was reported that serum TSH and T4 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 T4 levels were reported to be decreased independent of feedback by T4 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 IRloxP/loxP;TPO-Cre thyroid. The use of IGF-1RloxP/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 IRloxP/loxP;TPO-Cre mice and/or IRloxP/loxP;IGF-1RloxP/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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References


