Nuclear Hormone Retinoid X Receptor (RXR) Negatively Regulates the Glucose-Stimulated Insulin Secretion of Pancreatic β-Cells

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OBJECTIVE—Retinoid X receptors (RXRs) are members of the nuclear hormone receptor superfamily and are thought to be key regulators in differentiation, cellular growth, and gene expression. Although several experiments using pancreatic β-cell lines have shown that the ligands of nuclear hormone receptors modulate insulin secretion, it is not clear whether RXRs have any role in insulin secretion.

RESEARCH DESIGN AND METHODS—To elucidate the function of RXRs in pancreatic β-cells, we generated a double-transgenic mouse in which a dominant-negative form of RXRβ was inducibly expressed in pancreatic β-cells using the Tet-On system. We also established a pancreatic β-cell line from an insulinoma caused by the β-cell–specific expression of simian virus 40 T antigen in the above transgenic mouse.

RESULTS—In the transgenic mouse, expression of the dominant-negative RXR enhanced the insulin secretion with high glucose stimulation. In the pancreatic β-cell line, the suppression of RXRs also enhanced glucose-stimulated insulin secretion at a high glucose concentration, while 9-*cis*-retinoic acid, an RXR agonist, repressed it. High-density oligonucleotide microarray analysis showed that expression of the dominant-negative RXR affected the expression levels of a number of genes, some of which have been implicated in the function and/or differentiation of β-cells.

CONCLUSIONS—These results suggest that endogenous RXR negatively regulates the glucose-stimulated insulin secretion. Given these findings, we propose that the modulation of endogenous RXR in β-cells may be a new therapeutic approach for improving impaired insulin secretion in type 2 diabetes. ***Diabetes*** **59:2854–2861, 2010**

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The nuclear hormone receptor superfamily plays essential roles in various aspects of biological regulation, such as differentiation, cellular growth, and metabolism. The retinoid X receptors (RXRs) play a unique and central role in the activity of many members of this superfamily. They function as an obligate heterodimeric partner for retinoic acid receptors (RARs), thyroid hormone receptor, vitamin D receptor, peroxisome proliferator–activated receptors (PPARs), liver X receptors (LXRs), farnesoid X receptor, and others (1). Because of this unique position within the superfamily, the modulation of the activity of RXRs is considered to cause a broad spectrum of effects (2,3).

In pancreatic β-cells, there are several lines of evidence from in vitro studies that nuclear receptors are involved in the insulin secretory mechanism. Retinoic acid affects the expression of the glucokinase and preproinsulin genes and promotes insulin secretion in RIN-m5F cells (4,5) and in isolated islets (6). In INS-1 cells, both 9-*cis*-retinoic acid (9cRA), a ligand of RXRs, and all-*trans*-retinoic acid (ATRA), a ligand of RARs, increase insulin secretion, and ATRA raises GLUT2 mRNA (7). In contrast, 9cRA and a high concentration of rosiglitazone, a ligand of PPAR-γ, are reported to inhibit glucose-stimulated insulin secretion (GSIS) in INS-1 cells (7), suggesting that the PPAR/RXR heterodimer is inhibitory to insulin secretion in β-cells. Recent reports showed that the co-overexpression of PPAR-α and RXR-α in INS-1 cells potentiated glucose-induced insulin secretion, whereas the co-overexpression of PPAR-γ and RXR-α in INS-1 cells attenuated it (8). Although these results suggest that RXR plays a role in regulating the insulin secretory machinery of β-cells, it is still controversial whether RXR has a direct effect on insulin secretion.

RXR is composed of three family members—RXR-α, -β, and -γ—that have redundant roles (8–10). Therefore, it is necessary to disrupt the genes for all three RXR members to inactivate RXR function. Considering the practical difficulty of a β-cell–specific knockout of all three genes, we took another approach using a dominant-negative form of RXR. RXRβΔC2, which lacks the ligand-binding domain, is known to act as a dominant-negative receptor and to inhibit the ligand-dependent transcriptional activation of target genes by homodimerized and heterodimerized RXRs (11). To investigate the in vivo function of RXR in β-cells, we generated a double-transgenic (Tg) mouse: one transgene expresses reverse tetracycline-regulated transactivator (rtTA) under the insulin promoter, and the other expresses RXRβΔC2 under the tetracycline-responsive TetO promoter. In this Tg mouse line, the addition of

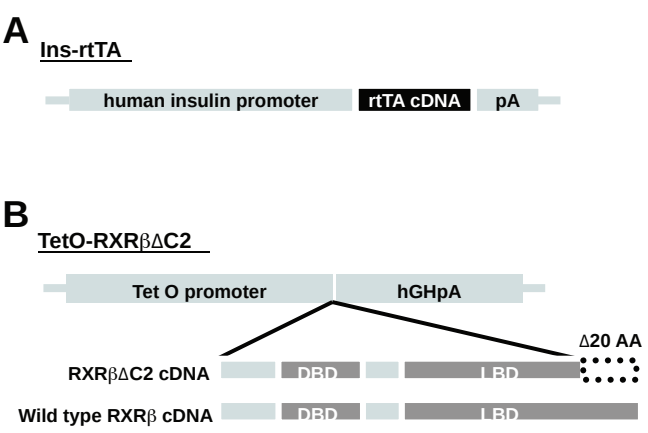

FIG. 1. DNA constructs used to generate transgenic mice. Two transgenes were constructed to enable the Tet-On system to work: the transgene termed Ins-rtTA (*A*) carried the reverse tetracycline-regulated transactivator (rtTA) gene under the human insulin promoter, and the other transgene, termed TetO-RXRβΔC2 (*B*), carried the RXRβΔC2 cDNA, which encodes a dominant-negative form of mouse RXRβ lacking 20 conserved COOH-terminal amino residues of the ligand-binding and dimerization domain, following the TetO promoter. DBD, DNA-binding domain; LBD, ligand-binding domain. (A high-quality color representation of this figure is available in the online issue.)

tetracycline to the drinking water led to the expression of RXRβΔC2 in the β-cells. The tetracycline-treated Tg mice showed elevated glucose tolerance, and islets isolated from these mice also showed enhanced GSIS.

To examine the role of RXR in β-cells, we established β-cell lines (dnRXR-MIN6 cells) from an insulinoma that developed in a triple-Tg mouse harboring the SV40 T antigen gene under the insulin promoter in addition to the two transgenes for RXRβΔC2 expression. Using this cell line, we confirmed that the suppression of RXR enhanced the glucose-stimulated insulin secretion at a high glucose concentration in vitro. High-density oligonucleotide microarray analysis showed that the expression of a number of genes was affected by the suppression of RXR. Our findings suggest that modulating the activity of the endogenous RXRs in β-cells may provide a novel therapeutic approach for improving impaired insulin secretion in type 2 diabetes.

RESEARCH DESIGN AND METHODS

Generation of double-Tg mice. To generate Tg mice in which the expression of a dominant-negative form of RXRβ could be induced in β-cells, we designed two transgenes to use the Tet-On system (12–14). The first transgene, Ins-rtTA, carried the reverse tetracycline-regulated transactivator (rtTA) gene under the human insulin promoter (Fig. 1*A*). The second transgene, TetO-RXRβΔC2, carried the RXRβΔC2 cDNA, which encodes a dominant-negative form of mouse RXRβ, lacking 20 conserved COOH-terminal amino residues of the ligand-binding and dimerization domain (11), and the human growth hormone gene, including the polyadenylation signal, following the TetO promoter (Fig. 1*B*). Tg mice were generated by DNA microinjection into the pronuclei of fertilized eggs of C57BL/6J. Tg founder mice were identified by PCR analyses of the genomic DNA obtained from tail tips. In the Ins-rtTA/TetO-RXRβΔC2 double-Tg mice, the islet-specific expression of rtTA and inducible expression of RXRβΔC2 were confirmed by RT-PCR and immunohistochemical analyses. For doxycycline (Dox) treatment, doxycycline hydrochloride (Sigma-Aldrich, St. Louis, MO) was dissolved in water at a concentration of 1 mg/ml, and this solution was used as the drinking water for the double-Tg mice.

Oral glucose tolerance test. An oral glucose tolerance test (OGTT) was performed twice on the same groups of the double-Tg mice: after 2 weeks of Dox treatment (Dox On) and 2 weeks after the withdrawal of Dox treatment (Dox Off). The Tg mice were fasted overnight for 16 h followed by oral administration of glucose (2 g/kg body wt). Blood samples were collected by retro-orbital sinus puncture before (0 min) and 10, 30, 60, and 120 min after glucose administration. Plasma samples were obtained by centrifugation at 4°C and were stored at −80°C until analysis. The plasma glucose and insulin concentrations were measured using a blood glucose test kit (Glucose CII-test; Wako Pure Chemicals, Osaka, Japan) and an enzyme-linked immunosorbent assay (ELISA) kit (Morinaga, Yokohama, Japan), respectively.

Measurement of insulin secretion and insulin content. Double-Tg mice with or without 2 weeks of Dox treatment were killed, and their islets were isolated according to a previously described method (15). The isolated islets were cultured in RPMI medium with 11 mmol/l glucose, 10% FBS, and antibiotics. For the islets from Dox-treated Tg mice, 5 μg/ml Dox was added to the medium. After cultivation for 24 h, 10 μmol/l 9cRA was added to the culture of half of the islets from Dox-treated Tg mice and that from Dox-untreated Tg mice. After further cultivation for 20 h, the islets of a similar size were washed with Krebs-Ringer bicarbonate buffer (KRBB) containing 0.2% BSA and transferred to microcentrifuge tubes (10 islets per tube; $n = 5–6$ for each group). They were then cultured in KRBB containing 0.2% BSA with 3, 9, or 27 mmol/l glucose for 60 min, and the supernatant was collected and assayed for insulin using an ELISA kit (Mercodia, Uppsala, Sweden). For measurement of insulin content, double-Tg mice with or without 2 weeks of Dox treatment were killed and their pancreases were isolated. Insulin was extracted from them with acid ethanol and measured by immunoassay as described above.

Establishment of the dnRXR-MIN6 β-cell lines. To establish β-cell lines with inducible RXRβΔC2 expression, Ins-rtTA/TetO-RXRβΔC2 double-Tg mice were mated with IT-6 Tg mice. IT-6 Tg mice bear the SV40 T antigen gene under the human insulin promoter and were originally used to establish MIN6 cells, a β-cell line that retains GSIS (16). The resulting triple-Tg mice were identified by the PCR analysis of genomic DNA obtained from the tail tips. Pancreatic β-cell lines were generated from 22 insulinomas isolated from the triple-Tg mice at the age of 9 weeks. Finally, two clones with good GSIS and stable induction of RXRβΔC2 by Dox were selected (named dnRXR-MIN6) and used for experiments.

Western blotting and immunocytochemistry. The total protein was extracted from dnRXR-MIN6 cells after cultivation with or without Dox for 4 days and subjected to Western blotting using a mouse anti-mouse RXRβ monoclonal antibody (MA3-812 [clone MOK13.17]; Affinity Bioreagents, Golden, CO) that detects RXRβΔC2 (11) and a horseradish peroxidase–conjugated second antibody. Detection was done by enhanced chemiluminescence (ECL kit; Amersham, Arlington Heights, IL). Immunocytochemistry was performed with dnRXR-MIN6 cells. The cultured cells were washed with PBS and fixed in 4% paraformaldehyde for 10 min. After fixation, the cells were rinsed with PBS, incubated for 5 min in 1% Triton X-100, and, after a second wash, incubated in a blocking reagent. The samples were incubated with the first antibody for 60 min at room temperature, washed with PBS, and then incubated with the second antibody for 60 min at room temperature. The primary antibody was a mouse anti-mouse RXRβ antibody (MOK13.17); the secondary antibody was Alexa Fluor 488–conjugated anti-mouse IgG_1 (Molecular Probes, Eugene, OR).

Immunohistochemical analyses. Immunohistochemistry was performed with frozen sections or paraffin sections of pancreatic tissue. The 8-μm-thick frozen sections were placed on slides and fixed in cold acetone for 10 min. Pancreatic tissue was also fixed in 4% paraformaldehyde overnight and processed for paraffin embedding. Sections of paraffin-embedded pancreatic tissue (3–5 μm thick) were deparaffinized and dehydrated. The frozen and paraffin sections were incubated with 3% normal goat serum in PBS containing 10% Blocking One (Nacalai Tesque, Kyoto, Japan) for 60 min at room temperature. The sections were then incubated with the first antibody at 4°C overnight and with a fluorescein-conjugated second antibody for 60 min at room temperature. After each antibody incubation, the sections were washed in PBS for 5 min with three changes. The first antibodies were guinea pig anti-insulin antibody (Dako, Carpentaria, CA), rabbit anti-VP16 antibody for the detection of rtTA, and mouse anti-mouse RXRβ antibody. The secondary antibodies were Alexa Fluor 594–conjugated anti-guinea pig IgG, Alexa Fluor 488–conjugated anti-rabbit IgG, and Alexa Fluor 488–conjugated anti-mouse IgG_1 (Molecular Probes). The sections were observed by fluorescence microscopy (Olympus, Tokyo, Japan).

RT-PCR and real-time PCR analyses. The total RNA was extracted from isolated islets and dnRXR-MIN6 cells after they were cultivated with or without Dox for 4 days by the acid guanidinium-phenol-chloroform method and was subjected to cDNA synthesis using ReverTra Ace α (Toyobo, Tokyo, Japan) (17). The cDNA was subjected to PCR with the following primers: rtTA forward primer, 5′-CGCATTAGAGCTGCTTAAT-3′ and backward primer, 5′-TGACTTAG TAAAGCACATCT-3′; RXR forward primer, 5′-GCTGCTGTTACGTCTTCCTG-3′ and backward primer 5′-AGCGTTGTCAAAAAGCCTGG-3′ that binds to the sequence within the second exon of the human growth hormone gene in the TetO-RXRβΔC2 transgene; and HPRT forward primer, 5′-CTCGAAGTGTTG GATACAGG-3′ and backward primer, 5′-TGGCCTATAGGCTCATAGTG-3′. These

primers were designed to encompass intronic sequences to distinguish the appropriate PCR products from products amplified from contaminating genomic DNA. Real-time PCR was performed on an ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA), using the SYBR Green PCR core reagents detection system (Applied Biosystems). The primer sequences are shown in supplementary Table 1. PCR was performed with an initial step of 10 s at 95°C followed by 40 cycles of 5 s at 95°C and 31 s at 60°C. The levels of targeted gene expression were normalized to that of β-actin. Statistical analysis was performed by a Student *t* test.

Glucose-stimulated insulin secretion in vitro. dnRXR-MIN6 cells were cultured in 24-well plates with Dulbecco's modified Eagle's medium in the presence or absence of 2 μg/ml Dox for 4 days. In some experiments, 5 μmol/l 9cRA was added for the last 2 days of culture. Cells were starved in KRBB containing 0.1% BSA and 3 mmol/l glucose for 1 h, and wells were replenished with the same buffer twice. Then, cells were stimulated with 3, 10, or 25 mmol/l glucose in 500 μl KRBB containing 0.1% BSA for 1 h. The supernatants were collected and assayed for insulin using an ELISA kit. The values were normalized to the protein amount of each well. Statistical analysis was performed by a Student *t* test.

Microarray analysis. Total RNA was extracted by the acid guanidinium-phenol-chloroform method from dnRXR-MIN6 cells, after they were cultivated with or without Dox for 4 days. The quality of the purified total RNA was examined with an Agilent 2100 Bioanalyzer. We used 500 ng verified RNA to obtain cyanine 3 (Cy3)- or cyanine 5 (Cy5)-labeled cRNA using a Low RNA Input Fluorescent Linear Amplification Kit (Agilent), following the protocols recommended by the manufacturer. The cRNA was purified with RNeasy mini spin columns (Qiagen), and then 0.75 μg each of the Cy3- and Cy5-labeled cRNAs were combined, fragmented, and used for hybridization to a high-density oligonucleotide microarray (Affymetrix). For the first hybridization, we used RNA from dnRXR-MIN6 Dox(+) (Cy5) and dnRXR-MIN6 Dox(−) (Cy3), and for the second, we used RNA from dnRXR-MIN6 Dox(+) (Cy3) and dnRXR-MIN6 Dox(−) (Cy5). We quantified the image using Agilent Feature Extraction Software.

RESULTS

Islet-specific overexpression of RXRβΔC2 in Ins-rtTA/TetO-RXRβΔC2 double-Tg mice. To achieve tetracycline-inducible expression of the RXRβΔC2 gene in vivo, two types of Tg mouse were generated. The first Tg mouse, Ins-rtTA, expressed rtTA under the human insulin promoter (Fig. 1*A*). One line was obtained, and the specific expression of rtTA in the pancreas of these mice was confirmed by RT-PCR analysis (Fig. 2*A*). Immunohistochemical staining of the pancreas using an anti-VP16 antibody for the detection of rtTA confirmed the islet-specific expression of rtTA, while the pancreas of non-Tg control mice did not show specific staining (Fig. 2*B*). The second Tg mouse was termed TetO-RXRβΔC2 (Fig. 1*B*). To select one line out of the five obtained, an adenoviral vector AdV-rtTA, which expressed the rtTA gene under the control of the ubiquitous promoter (18), was injected into one mouse of each TetO-RXRβΔC2 Tg line via the tail vein. After 1 week of Dox treatment, the isolated livers were subjected to Western blotting analysis with an anti-RXRβ antibody. One line in which RXRβΔC2 was properly induced in the liver was selected. Finally, the Ins-rtTA/TetO-RXRβΔC2 double-Tg mice were generated as described in RESEARCH DESIGN AND METHODS. These mice are called dominant-negative RXR (dnRXR) Tg mice in the following study.

RT-PCR analysis showed that RXRβΔC2 was expressed only in the islets of the Dox-treated double-Tg mice (Fig. 2*C*). Immunohistochemical analysis showed that the nuclei of the islet β-cells of Dox-treated dnRXR Tg mice [Dox(+)] were more strongly stained with an anti-RXRβ antibody than those of the Dox-untreated mice [Dox(−)] (Fig. 2*D*). The weak staining of the latter sample was probably due to the expression of endogenous RXRβ, because the pancreas samples from non-Tg mice were similarly stained. These findings indicated that islet-specific expression of RXRβΔC2 was inducible by Dox in the dnRXR Tg mice.

Suppression of RXRs increases the GSIS in vivo. To investigate the effect of dnRXR expression in β-cells on glucose homeostasis, OGTTs were performed after 2 weeks of Dox treatment (Dox On) and 2 weeks after the withdrawal of Dox treatment (Dox Off) (Fig. 3*A*). Interestingly, the dnRXR Tg mice showed significantly improved glucose tolerance under the expression of the dnRXRβ (Dox On) compared with after the withdrawal of the Dox treatment (Dox Off) (Fig. 3*B*). Plasma insulin levels at 10 min after glucose administration were significantly higher under the Dox treatment (Dox On) compared with after the withdrawal of the Dox treatment (Dox Off) (Fig. 3*C*). The non-Tg control mice showed no significant change in the glucose tolerance with Dox treatment (data not shown). In a separate experiment, we examined whether the Dox treatment affected the body weight of the dnRXR Tg mice. Eight-week-old dnRXR Tg mice were maintained with or without Dox treatment ($n = 5$ each) for 2 weeks, and their body weights were measured. The result showed that there were no significant changes in the body weight between Dox-treated and Dox-untreated mice. Therefore, the improved glucose tolerance seen under the expression of the dnRXRβ was mostly due to the improved response of β-cells to glucose.

We then examined the GSIS of isolated islets. In this analysis, the effect of 9cRA, an RXR agonist, was also examined. At 9 and 27 mmol/l glucose, the insulin secretion of the Dox(+) islets was significantly higher (~1.3-fold) than that of the Dox(−) islets (Fig. 4*A*). Interestingly, 9cRA treatment significantly reduced the insulin secretion at the high glucose concentration both from the Dox(+) and Dox(−) islets. This effect of 9cRA was not observed at 3 or 9 mmol/l glucose.

To examine whether expression of the dnRXRβ affected the transcription of insulin genes, the mRNA for the two insulin genes was quantified. The total RNA of islets isolated from Dox-treated and Dox-untreated mice was subjected to semi-quantitative RT-PCR analyses for the insulin 1 and insulin 2 genes. The results showed no significant differences in the expression level of these genes between the Dox(+) and Dox(−) islets (data not shown). We further examined whether expression of dnRXRβ affected the insulin content of the pancreas. Pancreases were isolated from Dox-treated and Dox-untreated mice, and insulin content was measured. No significant differences were observed in the insulin content between the Dox(+) and Dox(−) pancreases (Fig. 4*B*). These results suggested that the expression of the dnRXRβ affected insulin secretion through a mechanism occurring after insulin production.

dnRXRβ increases the GSIS in a β-cell line. To examine the molecular mechanism underlying the elevated GSIS of islets cells by the expression of the dnRXRβ, we sought to establish β-cell lines with Tet-inducible RXRβΔC2 expression using a triple-Tg mouse strategy involving the IT-6 Tg mouse expressing the SV40 T antigen gene under the human insulin promoter (16). A total of 22 β-cell lines were established from each insulinoma developed in the pancreases of the triple-Tg mice and were tested for GSIS. Most lines exhibited appropriate GSIS. Two lines (19 and 21) were ultimately selected, termed dnRXR-MIN6 cells, and subjected to further analyses. Both clones showed similar results, and the data with clone 19 were presented. In dnRXR-MIN6 cells, the stable expres-

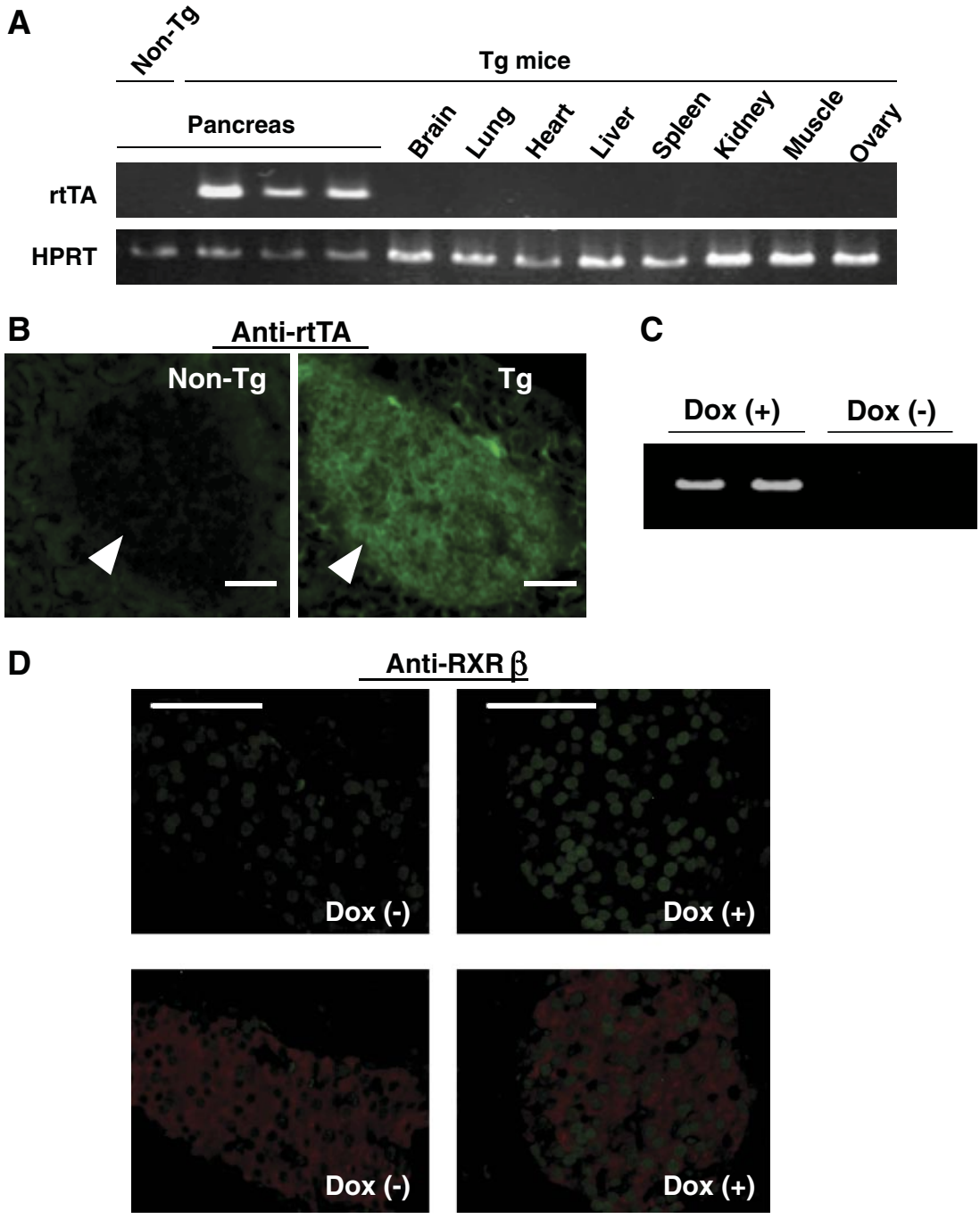

FIG. 2. Islet-specific induction of RXRβΔC2 in the Ins-rtTA/TetO-RXRβΔC2 double-Tg mice. The total RNA of the pancreas and other organs was subjected to RT-PCR analyses. The rtTA transgene expression was detected only in the pancreas samples from three different Tg mice and not in the other organs or in the non-Tg pancreas (*A*, *upper panel*). Hypoxanthine-guanine phosphoribosyltransferase (HPRT) was used as an internal control (*A*, *lower panel*). Immunohistochemistry for rtTA in the pancreas of the Ins-rtTA Tg mouse (*B*, *right panel*) or a non-Tg mouse (*B*, *left panel*) is shown. The islets in the pancreas (*arrowhead*) of the Tg mouse were stained by an anti-rtTA antibody. The scale bars represent 100 μm. By RT-PCR, expression of the RXRβΔC2 transgene was detected in the islets from two different double-Tg mice treated with Dox, but not in those from two untreated mice (*C*). *D*: Immunohistochemistry of the pancreas of an Ins-rtTA/TetO-RXRβΔC2 double-Tg mouse using an anti-RXRβ antibody. Magnified views of the islets before (*left panel*) and after (*right panel*) Dox treatment are shown. *Lower panels* show double staining of the same islets with anti-RXRβ (*green*) and anti-insulin (*red*) antibodies. Dox treatment induced a high-level expression of the dnRXRβ in the islet β-cells of the double-Tg mouse. Scale bars represent 100 μm. (A high-quality digital representation of this figure is available in the online issue.)

sion of rtTA and the Dox-mediated induction of RXRβΔC2 were confirmed by RT-PCR analysis, Western blotting, and immunocytochemistry (Fig. 5*A*–*C*). We also confirmed that Dox treatment affected neither the proliferation nor apoptosis of dnRXR-MIN6 cells (supplementary Figs. 1 and 2).

After incubation with or without Dox for 4 days, insulin secretion was measured at different glucose concentrations. In this analysis, the effect of 9cRA was also examined. At 10 and 25 mmol/l glucose, the insulin secretion of the Dox-treated cells was significantly higher (1.5-fold) than that of the Dox-untreated cells (Fig. 5*D*). Interestingly, 9cRA treatment significantly reduced the insulin secretion at the high glucose concentration from both Dox-treated and untreated dnRXR-

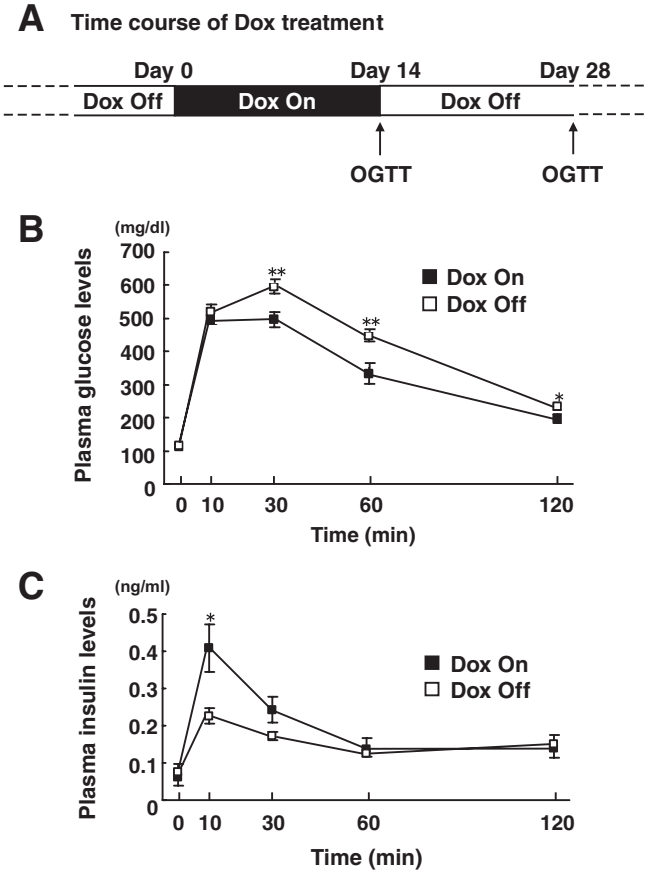

FIG. 3. Experimental protocols for the Dox treatment (*A*) and OGTT in the dnRXR Tg mice (*B* and *C*). The Dox-containing water was given for 2 weeks and renewed every 4 days. The OGTTs were performed after 2 weeks of the Dox treatment (Dox On) and 2 weeks after the withdrawal of the Dox treatment (Dox Off). Compared with the Dox On state, the withdrawal of Dox (Dox Off) deteriorated the glucose tolerance. Plasma insulin levels were also measured at each time point (*C*). Values are expressed as the mean ± SE. Statistical analyses were carried out by the Student *t* test. *$P < 0.05$, **$P < 0.01$. $n = 6$.

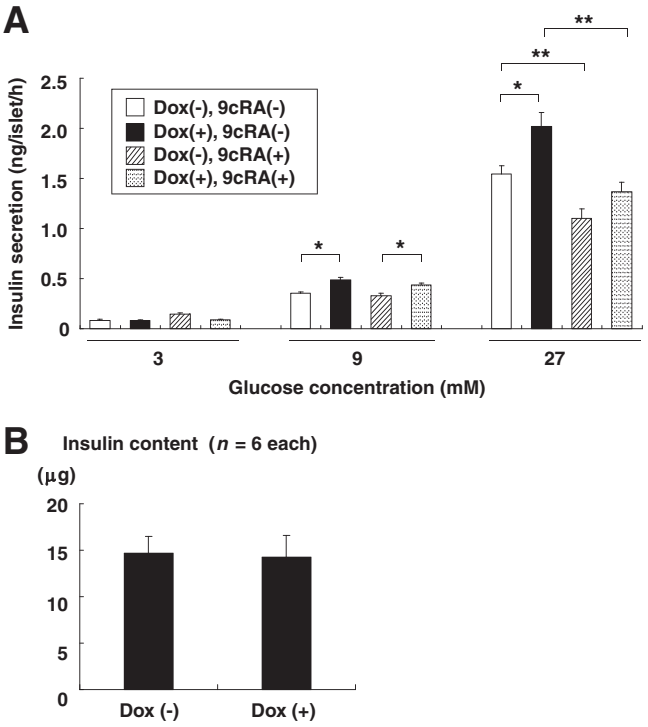

FIG. 4. Analysis of insulin secretion in the dnRXR Tg mice. Islets were isolated from Dox-treated and -untreated dnRXR Tg mice and cultured in the presence and absence of Dox, respectively (*A*). Insulin secretion from isolated islets in response to different concentrations of glucose is shown ($n = 5–6$ each). The effect of 9cRA treatment on the GSIS was also examined (*A*). For measurement of insulin content, dnRXR Tg mice with or without 2 weeks of Dox treatment were killed, and their pancreases were isolated ($n = 6$ each) (*B*). Values are expressed as the mean ± SE. Statistical analyses were carried out by the Student *t* test. *$P < 0.05$, **$P < 0.01$.

MIN6 cells. This effect of 9cRA was not observed at 3 or 10 mmol/l glucose.

cDNA microarray analysis. RXRs function as transcription factors in a homodimeric or heterodimeric form. Therefore, the induced expression of the dnRXRβ must influence the transcriptional levels of various target genes, some of which are assumed to be related to the regulation of insulin secretion in β-cells. We used high-density (Affymetrix) oligonucleotide microarrays to identify genes that were differentially expressed by the Dox-treated and untreated dnRXR-MIN6 cells. To correct for dye bias and exclude false-positive genes, dye swapping and subsequent normalization were performed. Supplementary Tables 2 and 3 list the genes for which a mean twofold or greater change in transcript level was demonstrated in the dye-swap experiment. We noted that the list included genes that might be related to the regulation of insulin secretion. We selected some of these genes to analyze by real-time PCR (Fig. 6). The results showed that these genes were clearly down- or upregulated by the Dox treatment, consistent with the microarray analysis.

DISCUSSION

Several studies have shown that RXR agonists have a beneficial effect on glucose tolerance in type 2 diabetes. The RXR agonist LG100268 was reported to protect the insulin secretory capacity in the *db/db* mouse, a model for this disease (19,20). A recent report also showed that LG101506, a heterodimer-selective RXR stimulator, also has insulin-sensitizing activity in the *fa/fa* rat (21). Although these data suggest that RXR agonists are beneficial for glucose tolerance, there has been no direct evidence for an effect of RXR on the insulin secretory mechanism in β-cells. Thus, to elucidate the function of RXRs in β-cells, we produced a Tg mouse and established β-cell lines in which conditionally inducible dnRXR gene expression was achieved.

The dnRXR Tg mice under the Dox treatment showed improved glucose tolerance compared with the same mice after the removal of Dox (Fig. 3*B*). Consistently, plasma insulin levels after glucose administration were significantly higher under the Dox treatment compared with after the removal of Dox (Fig. 3*C*). The insulin secretion at high glucose concentration was significantly increased in the isolated islets of dnRXR Tg mice by Dox treatment, suggesting that the suppression of RXR function led to the increased insulin secretory response by glucose stimulation (Fig. 4*A*). Moreover, we confirmed that the expression of a dnRXR enhanced the GSIS in a transgenic β-cell line, dnRXR-MIN6. Our findings suggest that RXR acts as a negative regulator of the high-glucose response in β-cells, which was further supported by the negative effect of 9cRA on the GSIS at high glucose in isolated islets and also in dnRXR-MIN6 cells (Figs. 4*A* and 5*D*). Such negative effect of 9cRA was seen, even under the dnRXRβ overex-

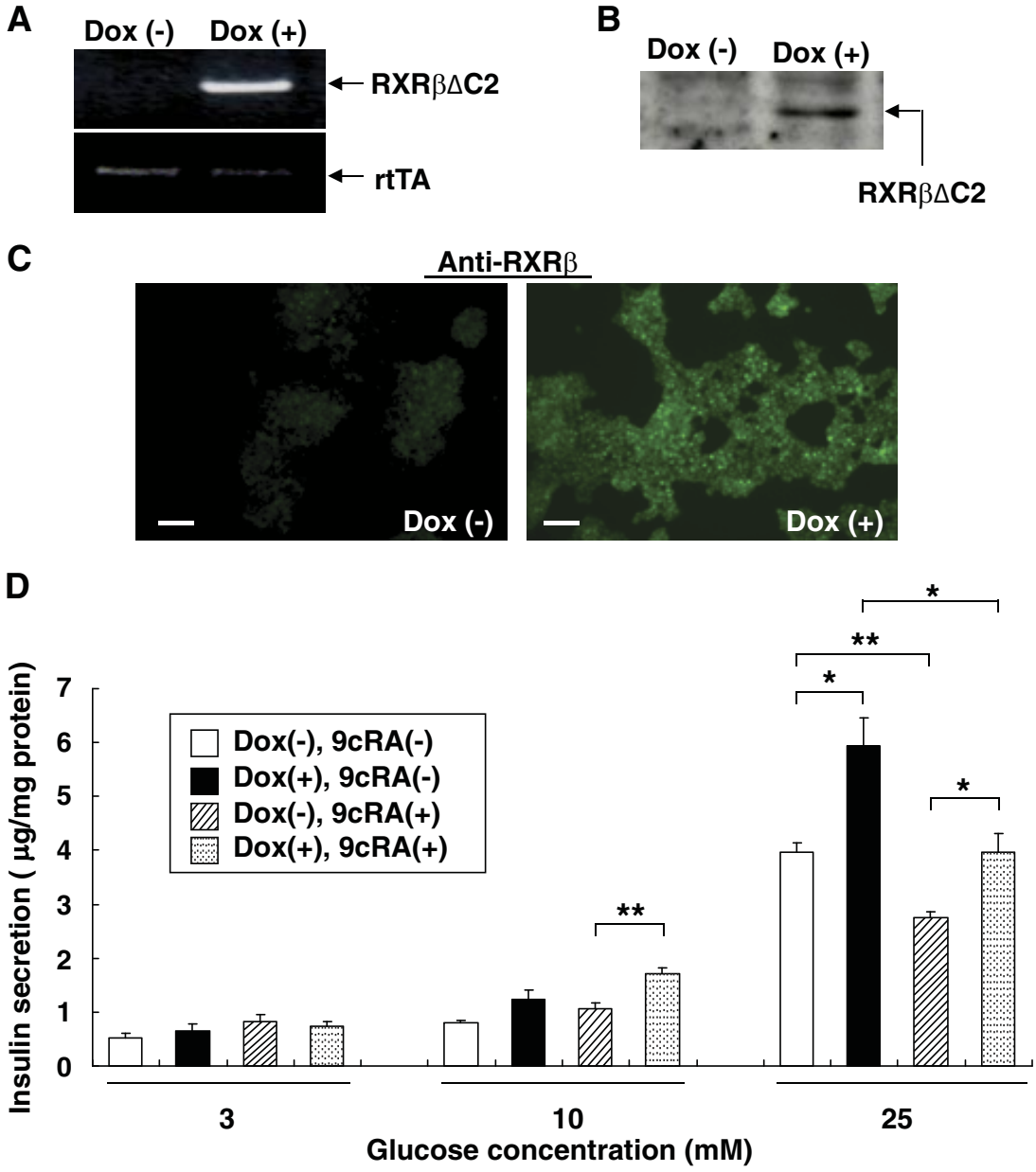

FIG. 5. Establishment of dnRXR-MIN6 cells and their GSIS. RT-PCR (*A*), Western blotting (*B*), and immunocytochemical analyses (*C*) were performed to detect the expression of rtTA and RXRβΔC2 in this cell line. Dox treatment of the dnRXR-MIN6 cells induced the expression of the RXRβΔC2 mRNA (*A*) and protein (*B*). Immunocytochemical analyses also showed the induction of the dnRXRβ by Dox treatment in the nuclei of these cells (*C*). The scale bars represent 100 μm. dnRXR-MIN6 cells were cultured with or without Dox for 4 days. 9cRA was added to the medium for the last 2 days of culture. Insulin secretion was measured at different glucose concentrations ($n = 4$ each) (*D*). Values are expressed as the mean ± SE. Statistical analyses were carried out by the Student *t* test. *$P < 0.05$, **$P < 0.01$. (A high-quality digital representation of this figure is available in the online issue.)

pression for both isolated islets and dnRXR-MIN6 cells (Figs. 4*A* and 5*D*). It was probably due to incomplete inhibition of the RXR function by the dominant-negative form of RXRβ.

In contrast, the basal insulin secretion at 3 mmol/l glucose from isolated islets or dnRXR-MIN6 cells was not significantly affected by the suppression of RXR (Fig. 4*B*). In addition, the suppression of insulin secretion by 9cRA treatment was seen only at high glucose for both isolated islets and dnRXR-MIN6 cells (Figs. 4*A* and 5*D*). These findings suggested that RXR might function to inhibit excessive insulin release under high-glucose conditions. These opposing results for insulin release between the high- and low-glucose responses suggest that the function of the RXR on β-cells may change, depending on the ambient glucose concentration. Thus, RXR may deeply participate in the regulatory mechanisms of GSIS.

Although the mechanism of the inhibitory effect of RXR is unknown, several reports suggest that RXR elicits diverse effects in β-cells by combining with different dimerization partners. PPAR-γ agonists are reported to prevent the impairment of the insulin secretory capacity induced by oxidative stress and lipotoxicity (22). A recent report showed that the combination of a PPAR-α agonist and an RXR agonist acted synergistically and led to protection against lipotoxicity in β-cells (23). Thus, the activation of PPAR/RXR signaling protects β-cells against oxidative stress and apoptosis. LXR is another het-

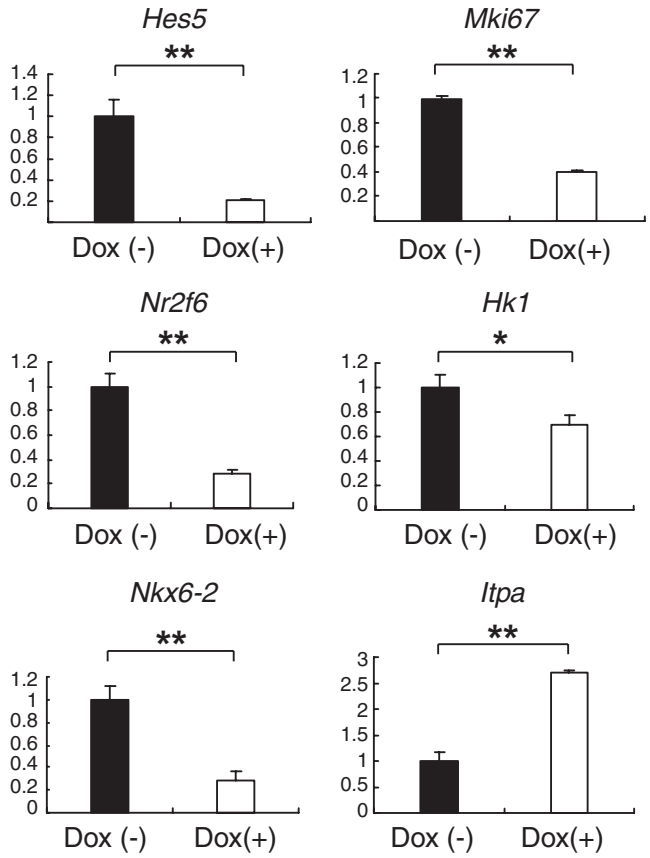

FIG. 6. Real-time PCR analysis of several candidate genes. Microarray analysis revealed a number of genes that were downregulated or upregulated by Dox. Some of these genes (downregulated: *Hes5*, *Mki67*, *Nr2f6*, *Hk1*, and *Nkx6-2*; upregulated: *Itpa*) were subjected to real-time PCR analysis. The relative expression levels (mean ± SE) are shown. Statistical analyses were carried out by Student *t* test. *$P < 0.05$, **$P < 0.01$.

erodimeric partner of RXR (24,25). Recent reports showed that, in contrast to PPAR/RXR, the activation of LXR/RXR signaling inhibits cell proliferation and induces apoptosis in β-cells (26,27). Efanov et al. (28) showed that the LXR agonist T0901317 enhances the glucose-induced insulin release from MIN6 cells, but the addition of 9cRA inhibits the effect of T0901317, especially on the insulin release response to high glucose. Thus, the function of RXR may be altered by its heterodimeric partner in β-cells.

RXR forms heterodimers with various partners of the nuclear hormone receptor family. To identify genes regulated by RXRs, we performed an oligonucleotide microarray analysis of dnRXR-MIN6 cells before and after induction of the dnRXRβ. The results revealed a number of genes that were upregulated or downregulated by the Dox treatment (supplementary Tables 2 and 3, respectively; Fig. 6). The downregulated genes included *Stra6*, *Hk1*, *Kcnk9*, *Nr2f6*, *Hes5*, *cMaf*, and *Mki67*. *Stra6* was first identified as a retinoic acid–stimulated gene, and it encodes a membrane receptor for retinol-binding protein that mediates the cellular uptake of vitamin A (29). The finding that this retinoic acid–stimulated gene was downregulated by dnRXR expression indicates that our dnRXR Tg mouse system worked as expected.

Hk1 encodes hexokinase, which has a lower K_m for glucose than glucokinase, the major isozyme in β-cells. The expression levels of *Hk1* might affect the insulin secretion from dnRXR-MIN6 cells (30). *Kcnk9* (*Task3*) encodes an acid-sensitive member of the two-pore-domain background K^+ (K_{2P}) channel family (31). K_{2P} channels contribute to the resting membrane potential by allowing K^+ to leak out of the cell. Thus, the downregulation of *Kcnk9* might influence the insulin secretion.

cMaf is one of the *large-Maf* transcription factor family genes, which also includes *MafA* and *MafB*. These factors are reported to be expressed in β-cells and to function as transcription factors for the insulin gene (32). *Nr2f6* (*Ear2*) encodes an orphan nuclear receptor, which is an RXR binding partner (33). However, the role of NR2F6 in β-cells has not been reported. *Hes5* is an effector protein in Notch signaling and belongs to the *Hes* family, as does *Hes1*, whose role in β-cells has been studied. The inhibition of *Hes1* expression using small hairpin RNA reduces the proliferation and dedifferentiation of β-cells (34,35). Another downregulated gene, *Nkx6-2*, encodes a transcription factor that is required for endocrine development in the pancreas (36).

Among the genes upregulated by the expression of the dnRXR were those involved in transcription (*Gtf2a2*, *Gtf2h3*, *Trfp*, *Lsm1*, *Lsm8*, *Polr3k*, and *Cdca4*), protein synthesis (*Rpl22*, *Rps27l*, *Rpl13Rps18*, *Rps25*, *Rpl15*, *Nat5*, and *Senp3*), signal transduction (Itpa), protein trafficking (*Sec61b*, *Vps22*, *Lztr1*, *Arfgap3*, *Rab37*, *Fkbp4*, and *Trappc3*), and mitochondrial function (*Ndufa4*, *Atp5j2*, *Cyb561d1*, *Mrpl44*, *Mrps5*, *Slc25a11*, and *Cyb5*). These genes could contribute to the elevation of the secretory capacity of β-cells.

The Tet-On system, which provides a tissue-specific and inducible expression of transgenes in animals, is suitable for investigating the functions of genes that have not been well characterized in vivo. Here, we successfully applied the Tet-On system to express a dnRXR specifically in β-cells. Our data suggest that RXRs inhibit insulin secretion from β-cells at high glucose levels. In addition, our DNA microarray analysis suggested several candidate genes that might be involved in this regulation. Although further studies on partners of RXRs and their target genes are needed, our finding that RXRs have multiple functions in the insulin secretion from β-cells may lead to novel therapeutic strategies for treating the impaired insulin secretion in type 2 diabetes.

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S.M. researched data and wrote the manuscript. H.T., Y.M., F.T., and T.Y. researched data. E.Y. and H.I. contributed to the discussion. K.O. researched data and reviewed/edited the manuscript. J.-i.M. researched data and wrote the manuscript.

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