Studies on the Physiological Role of Fatty Acid Receptor GPR40/$FFAR_1$ with Genetically Modified Animals

A Dissertation Submitted to
the Graduate School of Life and Environmental Sciences,
the University of Tsukuba
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in Biological Science
(Doctoral Program in Biological Sciences)

Kae MATSUDA

Table of Contents

Abstract

Type 2 diabetes mellitus is characterized by elevated plasma glucose levels arising from increased peripheral insulin resistance and impaired insulin secretion.

Free fatty acids (FFAs) serve not only as nutrients but also as cell signaling mediators, and they are implicated in several metabolic disorders including diabetes. Elevated circulating FFAs cause insulin resistance and impair glucose metabolism in the liver, muscle, adipose tissue, and pancreatic β-cells. GPR40/$FFAR_1$ was identified as a receptor for medium- and long-chain FFAs and was preferentially expressed at high levels in the pancreatic β-cells. GPR40/$FFAR_1$ leads to glucose dependent augmentation of insulin secretion *in vitro*, whereas the function of GPR40/$FFAR_1$ *in vivo* was not clarified. In this study, I performed studies on the physiological role of GPR40/$FFAR_1$ with genetically modified animals to whether or not loss and overexpression of GPR40/$FFAR_1$ exacerbates diabetes, that is, whether GPR40 has a significant pathophysiological role in the development of diabetes or not.

In the first chapter, I describe the phenotypic analysis of GPR40/$FFAR_1$ knockout mice (KO). GPR40/$FFAR_1$ KO mice developed glucose intolerance to a similar degree as GPR40/$FFAR_1$ wild-type (WT) mice on a high-fat diet condition and diabetogenic KK gene background, so the lack of GPR40/$FFAR_1$ does not exacerbate glucose intolerance and insulin resistance. Glucose-induced insulin secretion under high palmitate concentration was significantly lower in the pancreatic islets of KO mice than those of WT mice, so GPR40/$FFAR_1$ has a major role in regulating fatty-acid-mediated insulin secretion. These findings indicate that loss of GPR40/$FFAR_1$ function does not contribute to induce or exacerbate diabetes, and especially GPR40/$FFAR_1$ is selectively involved in fatty-acid-augmented insulin secretion.

In the second chapter, I describe the phenotypic analysis of GPR40/$FFAR_1$ overexpressed mice, named GPR40/$FFAR_1$ transgenic (Tg) mice. In comparison with nontransgenic (NonTg) littermates, GPR40/$FFAR_1$-Tg mice resulted in improved glucose tolerance with augmented insulin secretion on a high-fat diet condition and diabetogenic KK gene background. Isolated islets from hGPR40/$FFAR_1$-Tg mice enhanced insulin secretion in response to high glucose than those from NonTg mice with unchanged low glucose stimulated insulin secretion. In addition, insulin secretion in hGPR40/$FFAR_1$-Tg islets significantly increased insulin secretion against palmitate in the presence of glucose. These results indicate that GPR40/$FFAR_1$ has a role in regulating glucose-stimulated insulin secretion *in vitro* and plasma glucose levels *in vivo*.

Taken together, I conclude that GPR40/$FFAR_1$ is concerned with overt increase in insulin secretion in the presence of elevated glucose such as during the postprandial period, and there data supported the concept that GPR40/$FFAR_1$ agonists might be effective insulin secretagogues for the treatment of type 2 diabetes with low risk of hypoglycemia.

Abbreviations

AUC	area under the curve
BSA	bovine serum albumin
DAG	diacylglycerol
DPP-IV	dipeptidyl peptidase-4
ELISA	enzyme-linked immunosorbent assay
ES	embryonic stem
FBS	fetal bovine serum
FFAs	free fatty acids
GIP	glucose-dependent insulinotropic polypeptide
GLP-1	glucagon-like peptide-1
GPR40/$FFAR_1$	G-protein coupled receptor 40/free fatty acid receptor 1
GSIS	glucose stimulated insulin secretion
HbA1c	hemoglobin a1c
HBSS	Hanks' Balanced Salt Solution
HE	heterozygote
HFD	high-fat diet
HOMA-IR	homeostatic model assessment of insulin ressitance
IP_3	inositol 1, 4, 5-triphosphate
KO	knockout
KRBH	Krebs-Ringer biocarbonate buffer
NEFA	non-esterified fatty acids
NonTg	nontransgenic
PCR	polymerase chain reaction
PKC	protein kinase C

PLC	phosholipase C
PPAR-γ	peroxisome proliferator-activated receptor-γ
SGLT2	sodium dependent-glucose cotransporter 2
Tg	transgenic
WT	wild-type

General Introduction

Why the treatments for diabetes are needed?

There is nothing irreplaceable other than health for us at any age. One of the things that support our health is medicine to care disease. Today, the number of patients in infectious diseases such as tuberculosis and dysentery decreased, so mortality rate of infants was significantly reduced. In addition to the fact that nutritional status and hygiene environment was dramatically improved, it is likely that good medicine has been developed recently and medical technology has advanced rapidly.

Diabetes is one of the diseases that plague many people even in the modern disease. By recent report of the International Diabetes Federation, 382 million people have diabetes (1) and the number of people with diabetes is increasing in every country year by year, and this will rise to 592 million people by 2035 (Figure 1) (1). The World Health Organization (WHO) reported that diabetes mellitus is the eight-leading cause of death worldwide (Figure 2).

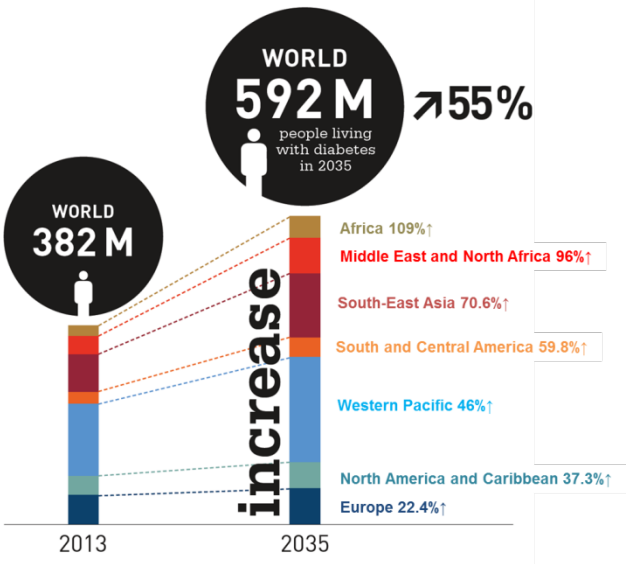


Figure 1. Diabetes is a huge and growing problem in the worldwide.

The data was cited from the reference (1).

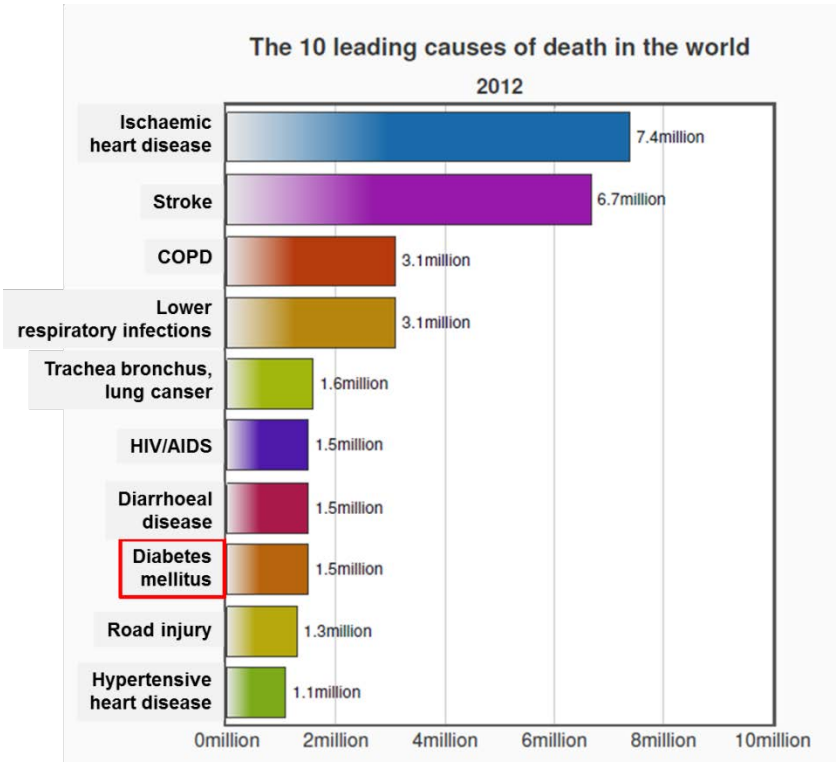


Figure 2. The top 10 causes of death worldwide in 2012.

The data on the 10 leading causes of death in the world was cited from WHO website (http://www.who.int/mediacentre/factsheets/fs310/en/)

Type 2 diabetes is the most common type of diabetes. It usually occurs in adults, but the number of patients is increasingly seen in children and adolescents. Type 2 diabetes mellitus is characterized by elevated plasma glucose levels arising from increased peripheral insulin resistance and impaired insulin secretion (2). Many people are able to manage their condition through a healthy diet, increased physical activity, and oral medication. The number of type 2 diabetes patients is growing rapidly worldwide. This rise is associated with economic development, aging populations, increasing urbanisation, dietary changes, reduced physical activity, and changes in other lifestyle patterns (3). Chronic hyperglycemia contributes to the complications associated

with type 2 diabetes (4), such as cardiovascular disease, kidney disease (diabetic nephropathy), and eye disease (diabetic retinopathy) (Figure 3). Especially, ischemic heart disease was the leading cause of death worldwide by the World Health Organization (WHO) (Figure 2). Patients with diabetes had greater risk of dying from cardiovascular disease and ischemic heart disease compared with people without diabetes. Risk of death increased through the range of HbA1c concentrations, with lowest rates in those with HbA1c concentrations less than 5% and a gradient of increasing rates through the whole distribution, so HbA1c levels correlate with cardiovascular mortality (5).

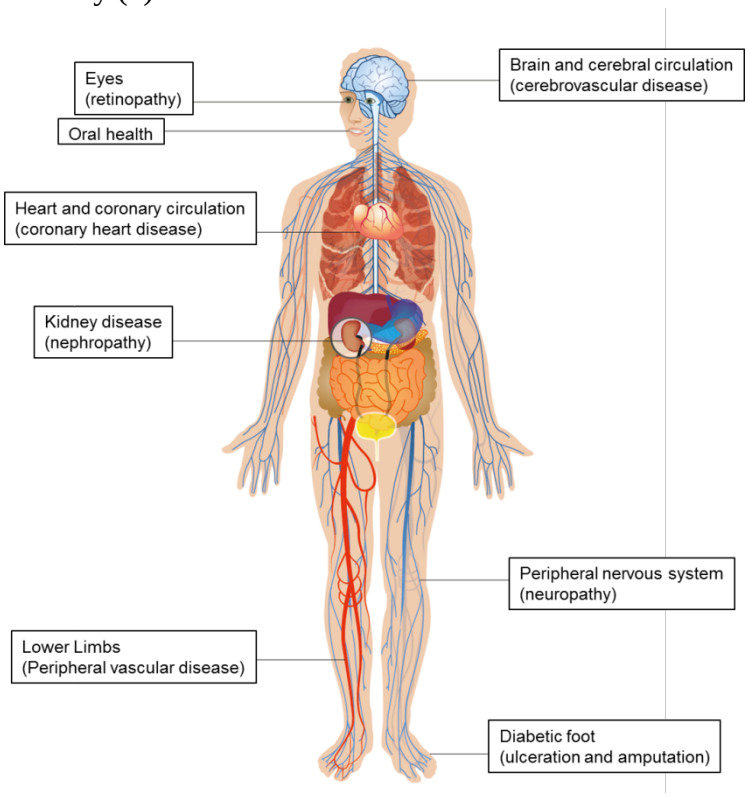


Figure 3. Major diabetes complications.

The data was cited from the reference (1).

Current status of therapeutics for type 2 diabetes

Conventional treatments of type 2 diabetes are primarily directed at reducing the demand for insulin and decreasing peripheral resistance by dietary management and physical exercise. In case the patients discontinue the lifestyle modification, medical therapy for type 2 diabetes is needed.

Biguanides, known as metformin, decreases glucose production in the liver and stimulates glucose uptake into peripheral tissues (6), and is a first line therapy for type 2 diabetes.Thiazolidinedione derivative is an insulin sensitizer agent that selectively binds to peroxisome proliferator-activated receptor-γ (PPAR-γ) and activates it. Thiazolidinedione derivative decreases plasma glucose by increasing insulin sensitivity in the liver, the muscle, and the adipose tissue, and decreases hepatic glucose production (7). Alpha-glucosidase inhibitors are used for the treatment of diabetes in Japan mainly. They reversibly inhibit enzymatic cleavage of complex carbohydrates to simple absorbable sugars, and slow the absorption of carbohydrate from the small intestine and lower postprandial hyperglycemia (8). They are frequently applied to patients with mild diabetes mellitus and impaired glucose tolerance (9-11). Dipeptidyl peptidase-4 (DPP-IV) inhibitor (12-14), has been demonstrated to improve glucose control in patients with type 2 diabetes (15, 16). The serine protease DPP-IV presents as the integral membrane form in a variety of organs and a soluble form in the circulation, and converts active forms of insulin-enhancing peptides glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) to inactive forms (17, 18). Thus, DPP-IV inhibition is associated with increased plasma levels of active forms of GLP-1 and GIP, thereby enhancing insulin secretion from pancreatic β-cells. Sodium

dependent-glucose cotransporter 2 (SGLT2) inhibitors are a new class of oral drugs for the treatment of type 2 diabetes mellitus. They inhibit glucose re-absorption in the proximal renal tubules in the kidney providing insulin independent mechanism to lower plasma glucose levels (19).

Drugs that enhance insulin secretion, such as sulfonylureas and nateglinides, are commonly used for the treatment of type 2 diabetes (20). However, these drugs enhance insulin secretion by direct closure of the K_{ATP} channel independent of plasma glucose levels, thereby causing hypoglycemia (21).

There are many medicines for the treatment for type 2 diabetes, but each of them have specific adverse effects. Therefore, patients with diabetes would benefit from the development of a novel anti-diabetic drug that has a low hypoglycemia risk and improves plasma glucose levels.

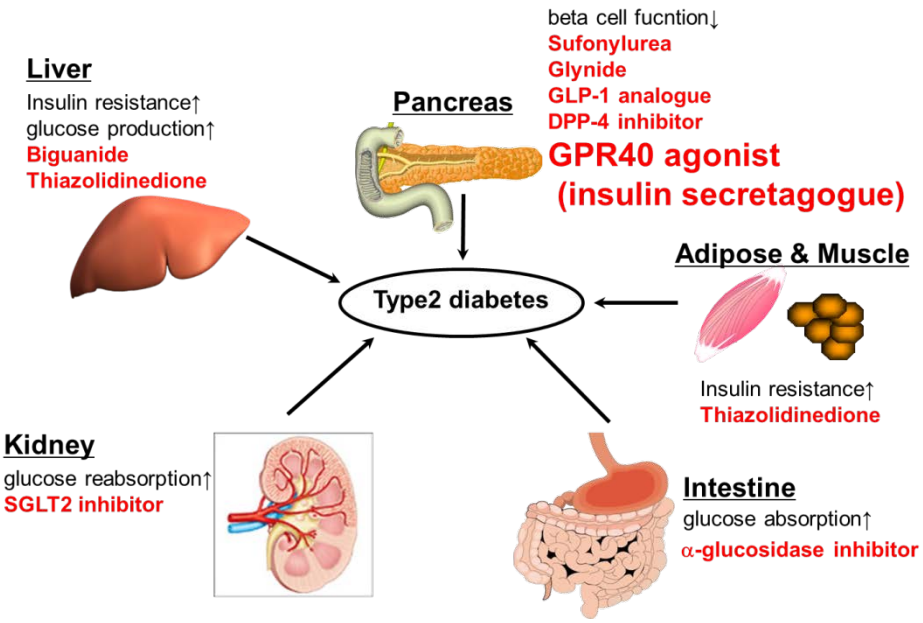


Figure 4. Pathology of anti-diabetic medicine

The function of free fatty acids

Free fatty acids (FFAs) are not only energy sources, but also have pleiotropic effects in various tissues. In pancreatic β-cells, FFAs are well known to enhance insulin secretion in the presence of elevated plasma glucose levels (22, 23). Plasma concentrations of FFAs are elevated in the fasted state, and they play a role in the enhancement of the postprandial insulin response *in vivo* (24, 25)

GPR40/FFAR$_1$ as a novel drug target for type 2 diabetes

G-protein coupled receptor 40/free fatty acid receptor 1 (GPR40/FFAR$_1$) was identified as a receptor for medium- and long-chain FFAs and was preferentially expressed at high levels in pancreatic β-cells (26-31). Several reports have shown that GPR40/FFAR$_1$ is mainly coupled with Gα_q/Gα_{11}, which activates phosholipase C (PLC) resulting in the formation of inositol 1, 4, 5-triphosphate (IP_3) and induction of calcium release from endoplasmic reticulum (29, 32-34). In fact, FFAs increase intracellular calcium concentration via GPR40/FFAR$_1$ and lead to glucose dependent augmentation of insulin secretion (21-24, 28, 30). Therefore, GPR40/FFAR$_1$ agonist is a promissing candidate of new drug for the treatment of type 2 diabetes. Although several insulinotoropic drugs are used in clinical, such as sulfonylureas, DPP-IV inhibitors, and GLP-1 analogues, the signaling pathway activated by them in pancreatic β-cells is different from that induced by stimulation of GPR40/FFAR1. In the process of drug discovery of GPR40/FFAR$_1$ agonist, I needed to clarify the function of GPR40/FFAR$_1$ *in vivo*, so I generated transgenic mice overexpressing the human GPR40/FFAR$_1$ gene

and knockout mice to delete GPR40/FFAR$_1$ gene function. One reason of generating transgenic and knockout mice was to examine the function of GPR40/FFAR$_1$ in the whole body, not *in vitro*. Another reason of it was to examine the specific action of GPR40/FFAR$_1$ *in vivo*, because the drug might have nonspecific action *in vivo*.

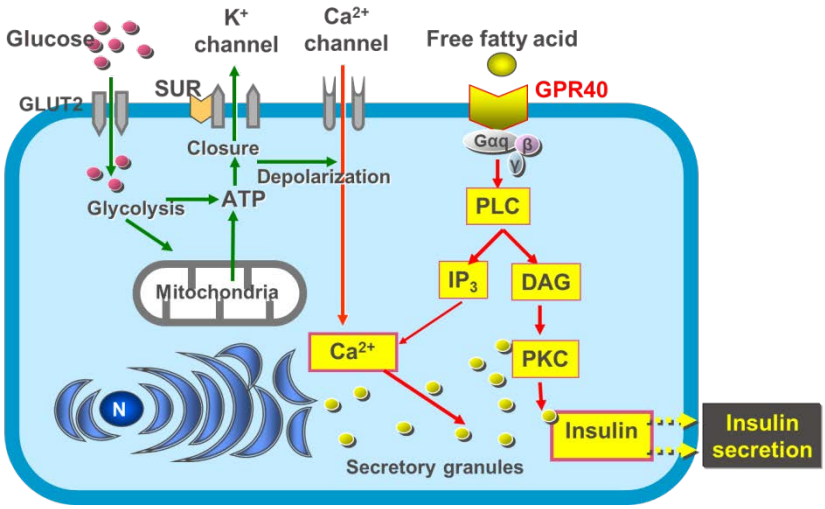


Figure 5. Presumed mechanism of insulin secretion via GPR40 in pancreatic beta cell

PLC, phospholipase C; IP$_3$, inositol triphosphate; DAG, diacylglycerol; PKC, protein kinase C

Objective of this research

To clarify whether loss of function of GPR40/FFAR$_1$ exacerbates diabetes or not, I have generated GPR40/FFAR$_1$ knockout mice and examined their phenotypes *in vitro* and *in vivo* under high-fat diet feeding in chapter 1. Moreover, to clarify the function of GPR40/FFAR$_1$ in pancreatic β-cells more extensively, I generated transgenic

mice, hGPR40/$FFAR_1$-Tg, overexpressing the human GPR40/$FFAR_1$ gene under control of the insulin II promoter and examined the role of GPR40/$FFAR_1$ in the regulation of insulin secretion and glucose homeostasis in chapter 2.

Chapter 1:

Lack of GPR40/$FFAR_1$ Does not Induce Diabetes even under Insulin Resistance Condition

Abstract

Background: G protein-coupled receptor/free fatty acid receptor 1 (GPR40/$FFAR_1$) regulates free fatty acid-induced insulin secretion. This study has been performed to clarify whether or not loss of GPR40/$FFAR_1$ function exacerbates diabetes, that is, whether GPR40 has an essential physiological role in the development of diabetes or not.

Methods: I generated GPR40/$FFAR_1$-knockout (KO) mice and analyzed their phenotypes *in vitro* and *in vivo* under the condition of dietary or genetically-induced insulin resistance.

Results: GPR40/$FFAR_1$ KO mice kept on a high-fat diet became obese, developed glucose intolerance to a similar degree as GPR40/$FFAR_1$ wild-type (WT) mice. In addition, the phenotype of KO mice harboring diabetogenic KK background genes showed glucose intolerance at a level similar to level for control KK mice. In both mouse models with insulin resistance, insulin secretion after oral glucose load and HOMA-IR did not change between GPR40/$FFAR_1$ KO and WT mice. Although glucose-induced insulin secretion under high palmitate concentration was significantly lower in KO than in WT islets, pancreatic insulin content and insulin secretion stimulated with glucose alone were not different between KO and WT mice.

Conclusions: GPR40/$FFAR_1$ has a major role in regulating fatty-acid-mediated insulin secretion, but the lack of GPR40/$FFAR_1$ does not exacerbate glucose intolerance or insulin resistance induced by high-fat diet or diabetogenic KK gene. These findings indicate that loss of GPR40/$FFAR_1$ function does not play an important role in inducing or exacerbating diabetes.

Introduction

Free fatty acids (FFAs) are not only energy sources, but also have pleiotropic effects in various tissues. In pancreatic β-cells, FFAs are well known to enhance insulin secretion in the presence of elevated glucose (22, 23). GPR40/$FFAR_1$ was identified as a receptor for medium- and long-chain FFAs, highly expressed in rodent primary β-cells, β-cell lines (26, 27, 29, 35), and human islets (30, 31). Several reports have shown that GPR40/$FFAR_1$ is mainly coupled with $G\alpha_q$, and the ligands stimulate phospholipase C (PLC), resulting in the formation of inositol 1, 4, 5-triphosphate (IP_3) and induction of calcium release from endoplasmic reticulum (29, 32-34). In pancreatic β-cells, these signal cascades enhance intracellular calcium concentrations and augment glucose-stimulated insulin secretion (GSIS) (26, 27, 29, 33, 35, 36). Recent studies have shown that acute treatment by small-molecule agonists of GPR40/$FFAR_1$ enhanced GSIS and improved postprandial glucose tolerance (37-39). Together with these findings, my previous results have confirmed that transgenic mice overexpressing human GPR40/$FFAR_1$ in pancreatic β-cells exhibit improved glucose tolerance and enhanced GSIS (40). Furthermore, Tsujihata *et al* also have reported that TAK-875, an orally available GPR40/$FFAR_1$ agonist, improves not only postprandial but also fasting hyperglycemia, without affecting normoglycemia (41). These studies indicate that pharmacological activation of GPR40/$FFAR_1$ would have great potential as a novel treatment of type 2 diabetes.

On the other hand, many preclinical studies suggest that prolonged exposure to elevated levels of fatty acids cause insulin resistance and pancreatic β-cell dysfunction, both of which promote type 2 diabetes (2, 42-44). Although one group has reported that

GPR40/$FFAR_1$ knockout mice fed with a high-fat diet protected hyperinsulinemia, hepatic steatosis, and impaired glucose homeostasis (45), the studies in the independent strains of GPR40/$FFAR_1$ knockout mice did not reproduce a role for GPR40/$FFAR_1$ in high-fat-diet-induced insulin resistance or hepatic steatosis (46-49).

To clarify whether loss of function of GPR40/$FFAR_1$ exacerbates diabetes or not, I have generated GPR40/$FFAR_1$ knockout mice and examined their phenotypes *in vitro* and *in vivo* under high-fat-diet feeding. I also investigated their phenotypes by introducing the diabetogenic polygene, KK (50-52), into the knockout mice. These findings indicate that GPR40/$FFAR_1$ has a major role in regulating fatty-acid-mediated insulin secretion, but does not exacerbate high-fat-diet and diabetogenic KK-induced glucose intolerance and insulin resistance.

Materials and Methods

Animals

GPR40/$FFAR_1$ knockout mice were generated by homologous recombination in embryonic stem (ES) cells as described below. The mouse GPR40/$FFAR_1$ gene was derived from the mouse 129SvJBAC genomic library (Invitrogen, Carlsbad, CA, USA), and construction of mouse GPR40/$FFAR_1$ gene targeting vector was carried out by inserting 11.1 kbp of the 5' downstream arm and 3.5 kbp of the 3' downstream arm into the vector harboring neomycin-resistant gene expression unit (Figure 6A). I transfected targeting vectors into ES cells (AB2.2 prime, Lexicon Genetics Incorporated, Texas, USA). The homologous recombinant was identified by Southern blot analyses (Figure 6B). The cells were injected into the eight-cell stage embryos from C57BL/6 mice and transferred into pseudopregnant ICR mouse females to generate chimeric mice. Male chimeric mice with agouti coat color were mated with C57BL/6 female mice to generate heterozygote mice. After crossing with heterozygote mice, the homozygotes were identified by PCR of tail DNAs using the mouse GPR40/$FFAR_1$ gene specific primers (5'-CAGCCAGTCCCTTCCCGCTTCA-3'and 5'-GCAGGTCCGAAATGGTCAGGTTTAGCA-3') (Figure 6C). After confirmation of the lack of mouse GPR40/$FFAR_1$ gene expression (Figure 6D), the obtained GPR40/$FFAR_1$ KO mice were maintained by backcrossing with C57BL/6J mice more than five generations, and the GPR40/$FFAR_1$ KO mice were identified by PCR analyses described as above. When examining the GPR40/$FFAR_1$ KO mice in genetically diabetic condition, the backcrossed GPR40/$FFAR_1$ KO mice were mated with mice harboring a KK background. The resulted heterozygote littermates harboring KK background were

further inter-crossed, and the genotype of the obtained pups were determined by PCR of tail DNAs. Then, the resultant mice were used for further experiments as wild-type mice (WT x KK) and GPR40/$FFAR_1$ KO mice (KO x KK) contained almost 50% KK background. The almost age- and sex-matched wild-type littermates were used as control mice throughout the study, and all experiments were conducted using male mice. The mice were fed with a regular diet CE-2 containing 11.5 kcal% fat (CLEA Japan Inc.), and were housed in colony cages and maintained on a 12-hr/12-hr light-dark cycle with freely access to water and food. For examination of the effects of a high-fat diet, mice were fed with a high-fat diet containing 60 kcal% fat (D12492, Research Diets Inc., New Brunswick, NJ, USA) or a low-fat diet containing 10 kcal% fat (D12450B, Research Diets Inc.) from 8 weeks of age. Plasma glucose levels were automatically analyzed by Dri-Chem (FujiFilm Medical, Tokyo, Japan), and plasma nonesterified fatty acids (NEFAs) were determined using a Wako test (Wako Pure Chemical Industries, Osaka, Japan). Plasma insulin levels were measured by enzyme-linked immunosorbent assay (Morinaga Institute of Biological Science, Tokyo, Japan). Plasma leptin levels were measured by ELISA (Mouse Leptin Quantikine$^{(R)}$ ELISA Kit, R&D systems Inc., Minneapolis, USA**).** To evaluate the degree of insulin resistance, we calculated the homeostasis model assessment-insulin resistance (HOMA-IR, fasted insulin (μU/ml)×fasted glucose (mmol/l)/22.5) (53). All procedures were conducted according to the Experimental Animal Care Use Committee of Takeda Pharmaceutical Company Ltd.

Oral glucose tolerance test

Mice were orally administered glucose at a dose of 1 g/kg body weight after an

overnight fast. Blood samples were obtained at time 0 (just before glucose load), 7.5, 15, 30, 60, and 120 min after glucose administration, followed by plasma preparation by centrifugation. Plasma glucose and insulin levels were measured as described above.

Preparation of islets

Islets were isolated from pancreas by collagenase digestion (54). The solution of 183 units/mg/kg collagenase (Wako Pure Chemical Industries Ltd.) dissolved in Hanks' Balanced Salt Solution (HBSS; Invitrogen, Carlsbad, CA, USA) containing 10 μg/ml DNase I (Roche Diagnostics, Indianapolis, IN, USA) and 0.2% bovine serum albumin (BSA; Wako Pure Chemical Industries Ltd.), was injected into the common bile duct. The pancreas was removed and incubated at 37°C for 20 min, and washed 3 times with HBSS, and islets were hand-collected under a microscope. For insulin secretion experiments, purified islets were used after overnight culture in RPMI 1640 medium containing 5.5 mmol/l glucose and 10% fetal bovine serum (FBS) in a humidified atmosphere containing 5% CO_2/95% air at 37°C.

Insulin secretion assay

A group of five islets was preincubated in Krebs-Ringer biocarbonate buffer (KRBH) containing 0.2% BSA and 1 mmol/l glucose for 30 min at 37°C, and then incubated for another 1 hr in KRBH containing glucose. For examination of the effect of fatty acids, sodium palmitate (CHEM SERVICE Inc., West Chester, PA, USA) was conjugated with FFA-free BSA (SIGMA ALDRICH Inc., St. Louis, MO, USA) at final palmitate and BSA concentrations of 1 mmol/l and 1.3%, respectively. The conjugated palmitate was added to KRBH containing 11 mmol/l glucose, and incubated for 1hr. At

the end of the incubation period, the amount of insulin in the culture supernatant was determined with ELISA as described above. Residual islets were sonicated and used to determine the DNA content (Quant-iT Picogreen dsDNA Assay Kit, Invitrogen).

Measurement of pancreatic insulin content

The pancreas was isolated and homogenized in acid–ethanol containing 74% ethanol with 0.15 mol/l HCl for the determination of insulin concentrations. The homogenized tissues were extracted overnight at 4°C and centrifuged at 12,000 *g* for 10 min. The resultant supernatants were then diluted with phosphate-buffered saline containing 0.1% (wt/vol) bovine serum albumin, and the insulin levels in the supernatants were determined with ELISA as described above.

Immunohistochemistry of pancreas

Formalin-fixed, paraffin-embedded tissue sections (thickness of 4 μm) of the pancreas were immunostained using the avidin-biotin detection system (Ventana Medical Systems, Tucson, AZ, USA) and M.O.M. Immunodetection Kit (VECTOR Laboratories, Inc., Burlingame, CA, USA), according to the manufacturer's instructions, and counterstained with haematoxylin. Anti-insulin antibody (1:200, MP Biomedicals Inc., Irvine, CA, USA), anti-glucagon antibody (1:2, ZYMED, South San Francisco, CA, USA), anti-glucose transporter 2 (GLUT2) antibody (1:200, CHEMICON International Inc., Temecula, CA, USA), and anti-proinsulin antibody (ready-to-use, Lab Vision, Fremont, CA, USA) were used as primary antibodies. For examining the β-cell and islet area, the area of the islets were traced manually and analyzed with Win ROOF software (Mitani Co., Fukui, Japan). More than 20 islets per pancreas were analyzed for the

average calculation.

Statistical analysis

Statistical analysis was performed using SAS system Version 8.2 (SAS Institute Inc., Cary, NC, USA). All values were expressed as means and SD. Statistical significance between groups was analyzed by the Student's *t*-test.

Results

Phenotypic characterization of GPR40/FFAR$_1$ KO mice

To generate GPR40/FFAR$_1$ KO mice, a segment of the gene encoding the GPR40/FFAR$_1$ protein was targeted by homologous recombination (Figure 6A). Obtained targeted ES cells were identified by Southern blotting using probes that hybridize outside of and adjacent to the targeting construct arms (Figure 6B), and the targeted clone was further used for creation of GPR40/FFAR$_1$ KO mice. The genotypes of the obtained mice were determined to be wild-type (WT), heterozygote (HE), and homozygote (KO) by PCR (Figure 6C). The lack of mouse GPR40/FFAR$_1$ gene expression was confirmed by quantitative real-time PCR analyses using pancreas tissues (Figure 6D). The GPR40/FFAR$_1$ KO mice appeared generally normal, reproduced successfully, and showed Mendelian inheritance characteristics.

Effects of high fat diet feeding on glucose homeostasis in GPR40/FFAR$_1$ KO mice

I explored the function of GPR40/FFAR$_1$ *in vivo* under high-fat-diet conditions. GPR40/FFAR$_1$ WT and KO mice were exposed to a high fat diet (containing 60 kcal% fat) for 8-11 weeks from 8 weeks of age. When WT mice were exposed to high-fat diet, plasma glucose, insulin, leptin, and NEFA levels and body weight in the fed state were significantly increased in compared with low-fat diet group (Table1). There was no significant difference in body weight and plasma parameters in the fed state between WT and KO mice under high-fat-diet conditions (Table 1). To assess the glucose homeostasis in these mice more precisely, an oral glucose tolerance test was performed after overnight fasting and areas under the curve (AUC) for glucose and insulin were

evaluated. Fasting plasma glucose, glucose levels in each time period of blood collection, and insulin levels at 15 min increased in both WT and KO mice given the high-fat diet (Figure 7B, D) as compared with those given low fat diet (Figure 7A, C), so the high-fat diet induced insulin resistance. But no significant differences in plasma glucose and insulin were observed between WT and KO mice on a high-fat diet (AUC of plasma glucose: 2261.8 ± 130.8 vs. 2424.9 ± 216.7 mmol/l x min, AUC of plasma insulin: 26521.6 ± 5709.0 vs. 27661.6± 15382.7 pmol/l x min for WT vs. KO mice, n=8). To evaluate the degree of insulin resistance, I calculated HOMA-IR. But no significant difference in HOMA-IR was observed between WT and KO mice on a low- and high-fat diet (Figure 7E). These data indicate that deletion of the GPR40/$FFAR_1$ gene does not worsen high-fat-diet-induced glucose intolerance and insulin resistance.

Metabolic characterizations of GPR40/$FFAR_1$ KO mice harboring a diabetic genetic background

The effects of the deletion of GPR40/$FFAR_1$ were examined in genetically diabetic mice. Mice harboring KK background (50-52), were selected as mates, and the resultant mice containing almost 50% KK background (WT x KK and KO x KK) were used for further experiments. WT x KK mice showed the phenotype of apparent obesity and glucose intolerance almost similar to original KK mice, whereas KO x KK mice did not show significant differences in body weight, plasma glucose, or insulin levels in the fed state compared with WT x KK mice (Figure 8A-C). The oral glucose tolerance test revealed that glucose tolerance and insulin secretion in response to glucose remained identical in WT x KK and KO x KK mice (Figure 8D, E). To evaluate the degree of insulin resistance, I calculated HOMA-IR. But no significant difference in HOMA-IR

was observed between WT x KK and KO x KK mice (Figure 8F). These data indicate that deletion of GPR40/$FFAR_1$ gene does not affect the glucose intolerance and other phenotypes of insulin resistance induced by KK background.

Islet structure and β-cell function in vitro

I next examined whether GPR40/$FFAR_1$ deletion affected islet morphology. At 16 weeks of age, immunohistochemical analyses of pancreas sections with antibodies against insulin, glucagon, GLUT2, and proinsulin showed no apparent differences between WT and KO mice (Figure 9A). β-cell and islet areas of KO mice were almost the same as those of WT mice (Figure 9B). To further examine the effects of GPR40/$FFAR_1$ deletion on islet function *in vitro,* isolated islets from WT and KO mice at 19 weeks of age were stimulated with glucose or glucose plus palmitate, and secreted insulin was measured. As shown in Figure 9C, insulin secretory responses to high glucose (16 mmol/l) alone were similar in WT and KO mice islets. Stimulation with high glucose plus palmitate (1 mmol/l), an endogenous ligand of GPR40/$FFAR_1$, significantly increased insulin secretion in islets of WT mice, but the insulinotropic effects of palmitate were significantly attenuated in the islets derived from KO mice (Figure 9D). On the other hand, insulin content was not different between WT and KO mice pancreas (Figure 9E).

Discussion

The objectives of this study were to examine whether loss of function of GPR40/$FFAR_1$ exacerbates diabetes or not. I generated a strain of GPR40/$FFAR_1$ knockout mice and examined their phenotypes *in vitro* and *in vivo*. I found that GPR40/$FFAR_1$ KO mice showed similar insulin resistance and the related harmful phenotypes induced by a high-fat diet and by transfer into a diabetic background compared to WT mice. That is; GPR40/$FFAR_1$ KO mice on a high-fat diet became obese, developed glucose intolerance and insulin resistance at similar level as control mice; GPR40/$FFAR_1$ KO mice harboring KK background also exhibited obesity, glucose intolerance, and insulin resistance at almost similar level as control KK background mice. These findings indicate that GPR40/$FFAR_1$ does not contribute to worsen impaired glucose homeostasis under a high-fat diet and the KK genetic background. I observed that the insulinotropic effects of palmitate were selectively reduced in islets of the GPR40/$FFAR_1$ KO mice, while the response to glucose was maintained. The results of my experiments using islets isolated from GPR40/$FFAR_1$ KO mice are consistent with my *in vivo* results during the oral glucose tolerance test, and this is consistent with the results obtained by Latour *et al* (47). Meanwhile, insulin secretion after glucose load was not reduced in GPR40/$FFAR_1$ KO mice, during glucose tolerance test. In addition, the plasma insulin levels were not significantly different between low fat diet and high fat diet condition. Regarding this discrepancy between *ex vivo* and *in vivo*, plasma insulin level is known to be affected by several things such as extrapancreatic factors (55) and intracellular metabolism of fatty acids (56). Although further studies are needed, I speculate that extrapancreatic factors and/or non-

GPR40/$FFAR_1$ mediated action by fatty acids may affect plasma insulin *in vivo*. Furthermore, I observed no difference in islet structure and total insulin content between GPR40/$FFAR_1$ KO and WT mice pancreas. These results suggest that GPR40/$FFAR_1$ plays a selective role in regulating insulin secretory process triggered by FFAs.

Although several studies have shown the consistent results regarding the role of GPR40/$FFAR_1$ in FFA-induced insulin secretion, the involvement in FFA-induced lipotoxicity in β-cells remains undetermined. While GPR40/$FFAR_1$ has been proposed as a possible lipotoxicity mediator using a gene-engineered mouse (45), a number of experimental observations could not reproduce the results (40, 41, 46-49). This study also shows that there is no apparent difference between WT and KO mice in glucose-stimulated insulin secretion in islets *ex vivo*. Taken together, these results suggest that GPR40/$FFAR_1$ would not play an significant role in mediating lipotoxicity. Although further studies are needed more *in vivo* experiments, this is consistent with reports using selective and potent small-molecule GPR40 agonists (37, 41). In addition, this immunohistochemical analyses showed that deletion of GPR40/$FFAR_1$ did not alter the β-cell architecture, suggesting that GPR40/$FFAR_1$ does not have a direct role in islet function and development.

The correlation of the GPR40/$FFAR_1$ gene and insulin secretion has been poorly studied in human subjects (30). It is reported that the genotype and allele frequencies of Arg211His polymorphism did not differ between type 2 diabetic patients and healthy subjects in Danish Caucasians (57). On the other hand, in healthy Japanese men, Arg211His polymorphism might contribute to the variation of insulin secretory capacity, which might link β-cell dysfunction and type2 diabetes (58). This discrepancy in the relationship between the Arg211His polymorphism of the GPR40/$FFAR_1$ gene

and insulin secretory action might arise from different genetic backgrounds in nation or race. Furthermore, a newly identified GPR40/$FFAR_1$ variant, Gly180Ser, is associated with reduced β-cell ability to adequately sense lipids as an insulin secretory stimulus, due to impaired increase in intracellular Ca^{2+} concentration (59). Although further studies will be needed, these findings may suggest that functional modulation of GPR40/$FFAR_1$ gene, not GPR40/$FFAR_1$ null mutation, affects insulin secretion and/or metabolic regulation.

In conclusion, these data obtained from knockout mice suggest that GPR40/$FFAR_1$ is selectively involved in fatty-acid-augmented insulin secretion, and does not contribute to development of glucose intolerance and insulin resistance under diabetogenic condition such as a high-fat diet and transfer of KK background. These findings indicate that loss of GPR40/$FFAR_1$ function does not play an important role in inducing or exacerbating diabetes.

Tables and Figures

Table 1. Metabolic parameters in GPR40/$FFAR_1$ WT and KO mice fed on low-fat diet and high-fat diet for 8 weeks

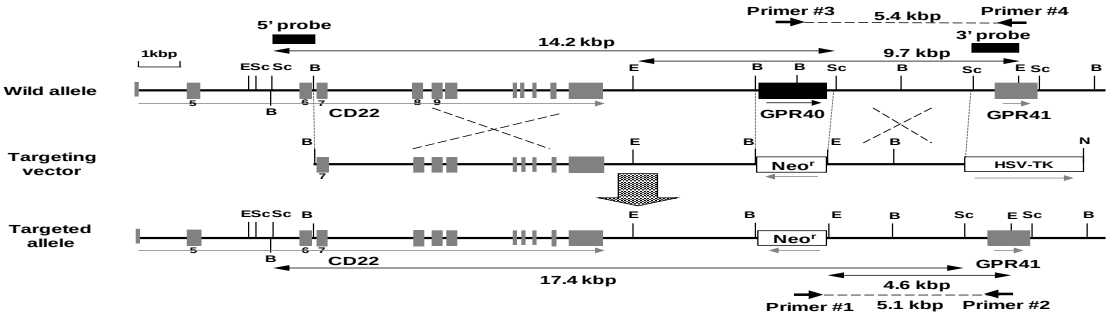
The GPR40/$FFAR_1$ WT and KO mice were fed a low-fat diet containing 10 kcal% fat or a high-fat diet containing 60 kcal% fat diet for 8 weeks from 8 weeks of age. All parameters were measured in the fed state at 16 weeks of age. All values are means ± SD. n = 8 per genotype.

	Low-fat diet		High-fat diet	
	WT	KO	WT	KO
Body weight (g)	28.9 ± 1.1	28.3 ± 3.2	36.8 ± 3.1	35.8 ± 3.4
Calorie intake (kcal/day)	10.0 ± 1.0	11.5 ± 1.4	12.9 ± 1.3	14.0 ± 1.9
Plasma glucose (mmol/l)	9.8 ± 1.0	9.9 ± 1.7	11.8 ± 1.3	11.7 ± 1.2
Plasma insulin (pmol/l)	261.2 ± 101.3	105.7 ± 59.3	415.3 ± 160.0	412.5 ± 243.4
Plasma leptin (ng/ml)	15.11 ± 8.11	11.84 ± 7.75	95.87 ± 25.03	68.33 ± 28.53
Plasma NEFA (mmol/l)	0.33 ± 0.12	0.44 ± 0.21	0.65 ± 0.15	0.63 ± 0.07

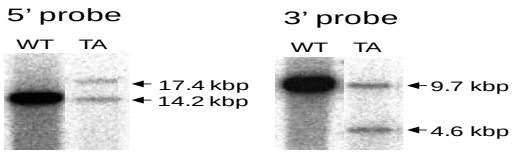
Figure 6. Generation and characterization of GPR40/FFAR$_1$ KO mice.

(A) Structure of the targeting vector. (B) Southern blotting of ES cell clones. Shown are DNA samples from the targeted clones digested by SacI and probed with a 5' probe, and by EcoRI and probed with a 3' probe, respectively. WT: original ES cell, TA: targeted ES cell. (C) Representative PCR genotyping of DNA samples from tail clips of wild-type (WT), heterozygote (HE), and knock-out (KO) mice.WT:4, 6, 8, HE:1, 7, 9, 10, 11, KO:2, 3, 5. (D) Expression of GPR40/FFAR$_1$ mRNA in pancreas of WT, HE, and KO mice by quantitative real-time PCR analyses. mRNA levels of Actin were used as an internal control. All values are means ± SD.

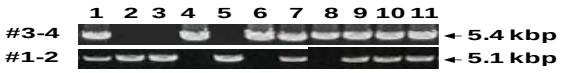
A


5' probe
Primer #3
5.4 kbp
Primer #4
3' probe
14.2 kbp
9.7 kbp
1kbp
Wild allele
CD22
GPR40
GPR41
Targeting vector
Neo^r
HSV-TK
Targeted allele
17.4 kbp
4.6 kbp
Primer #1
5.1 kbp
Primer #2

B


5' probe
WT
TA
17.4 kbp
14.2 kbp
3' probe
WT
TA
9.7 kbp
4.6 kbp

C


1 2 3 4 5 6 7 8 9 10 11
#3-4
#1-2
5.4 kbp
5.1 kbp

D

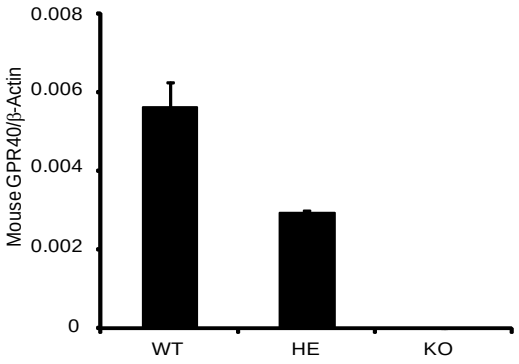
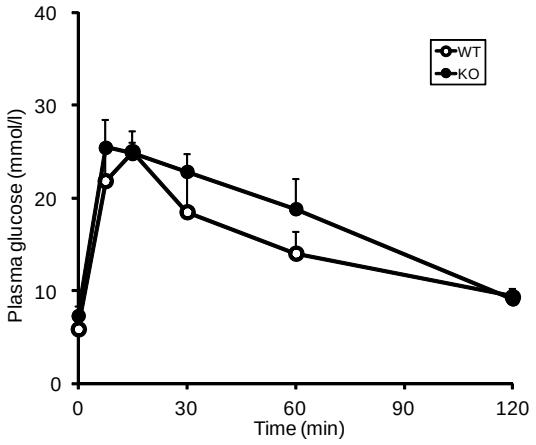

0.008
0.006
0.004
0.002
0
Mouse GPR40/β-Actin
WT
HE
KO

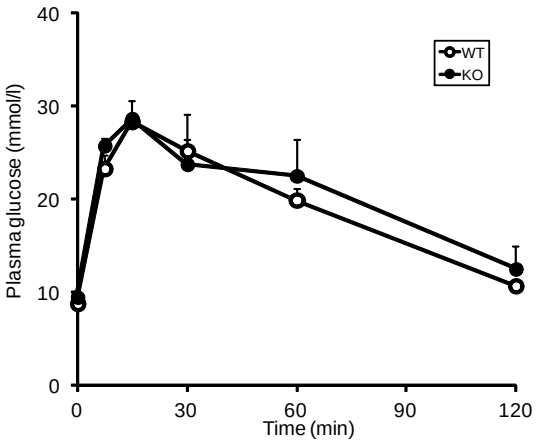
Figure 7. Effects of high-fat diet on glucose homeostasis in GPR40/FFAR$_1$ WT and KO mice.

The WT (open circle) and KO(closed circle) mice were fed on low-fat (10 kcal%) diet and high-fat (60 kcal%) diet from 8 weeks of age; oral glucose tolerance test was performed at 19 weeks of age. Glucose was administered orally at 1 g/kg body weight. Plasma glucose and plasma insulin in low-fat diet (A, C) and high-fat diet (B, D) were measured and used to calculate HOMA-IR (E). All values are means ± SD. n = 8 per genotype.

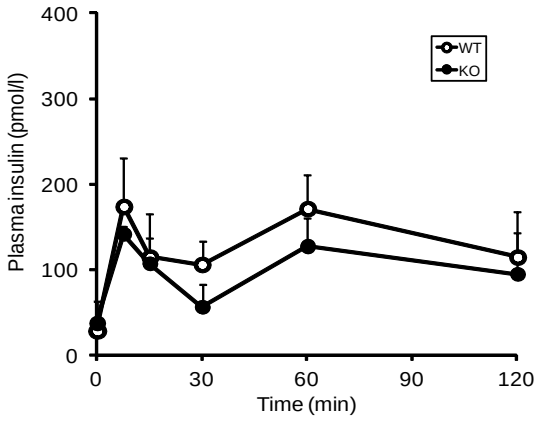
A


Plasma glucose (mmol/l)
Time (min)
WT
KO
0
10
20
30
40
0
30
60
90
120

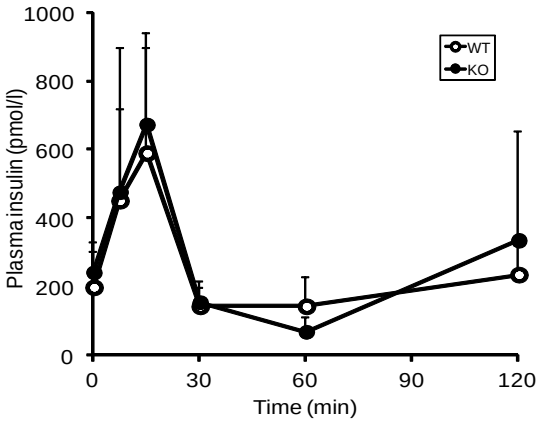
B


Plasma glucose (mmol/l)
Time (min)
WT
KO
0
10
20
30
40
0
30
60
90
120

C


Plasma insulin (pmol/l)
Time (min)
WT
KO
0
100
200
300
400
0
30
60
90
120

D


Plasma insulin (pmol/l)
Time (min)
WT
KO
0
200
400
600
800
1000
0
30
60
90
120

E

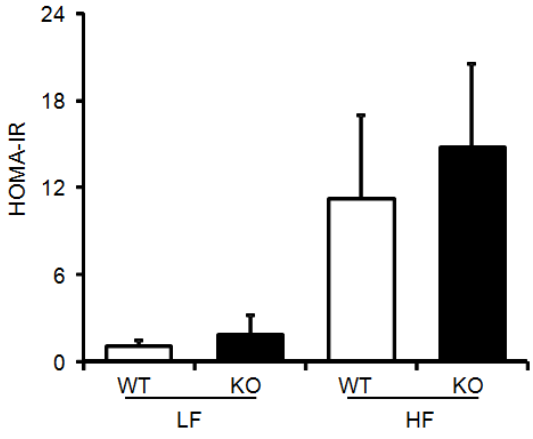
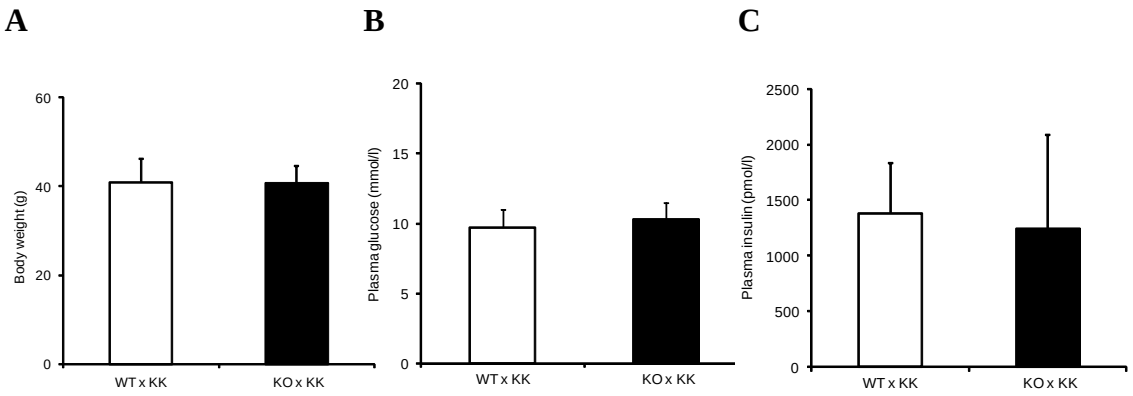
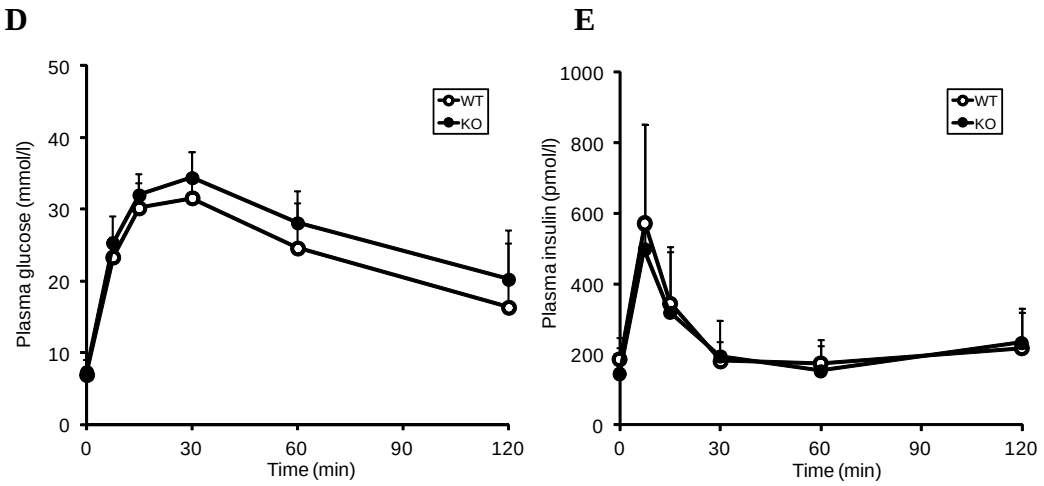

HOMA-IR
0
6
12
18
24
WT
KO
WT
KO
LF
HF

Figure 8. Metabolic characterizations of GPR40/FFAR$_1$ WT(open circles) and KO(closed circles) mice harboring diabetic KK background on regular diet.

Body weight (A), plasma glucose (B), and plasma insulin (C) at 18 weeks of age on regular diet. Oral glucose tolerance test for WT mice and KO mice harboring KK background. Glucose was administered orally at 1 g/kg body weight. Plasma glucose (D) and plasma insulin (E) at 24 weeks of age on regular diet were measured and used to calculate HOMA-IR (F). WT x KK (open circle) and KO x KK (closed circle) mean WT and KO mice containing almost 50% KK background, respectively. All values are means ± SD. n = 8 per genotype.


A
B
C
Body weight (g)
60
40
20
0
WT x KK
KO x KK
Plasma glucose (mmol/l)
20
15
10
5
0
WT x KK
KO x KK
Plasma insulin (pmol/l)
2500
2000
1500
1000
500
0
WT x KK
KO x KK


D
E
Plasma glucose (mmol/l)
50
40
30
20
10
0
0
30
60
90
120
Time (min)
WT
KO
Plasma insulin (pmol/l)
1000
800
600
400
200
0
0
30
60
90
120
Time (min)
WT
KO

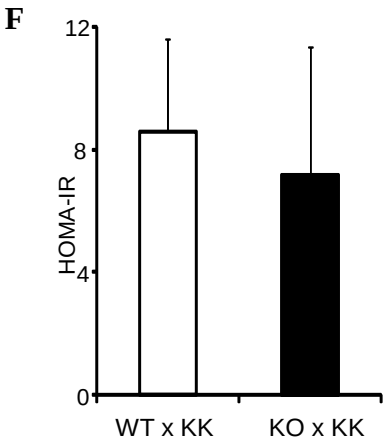
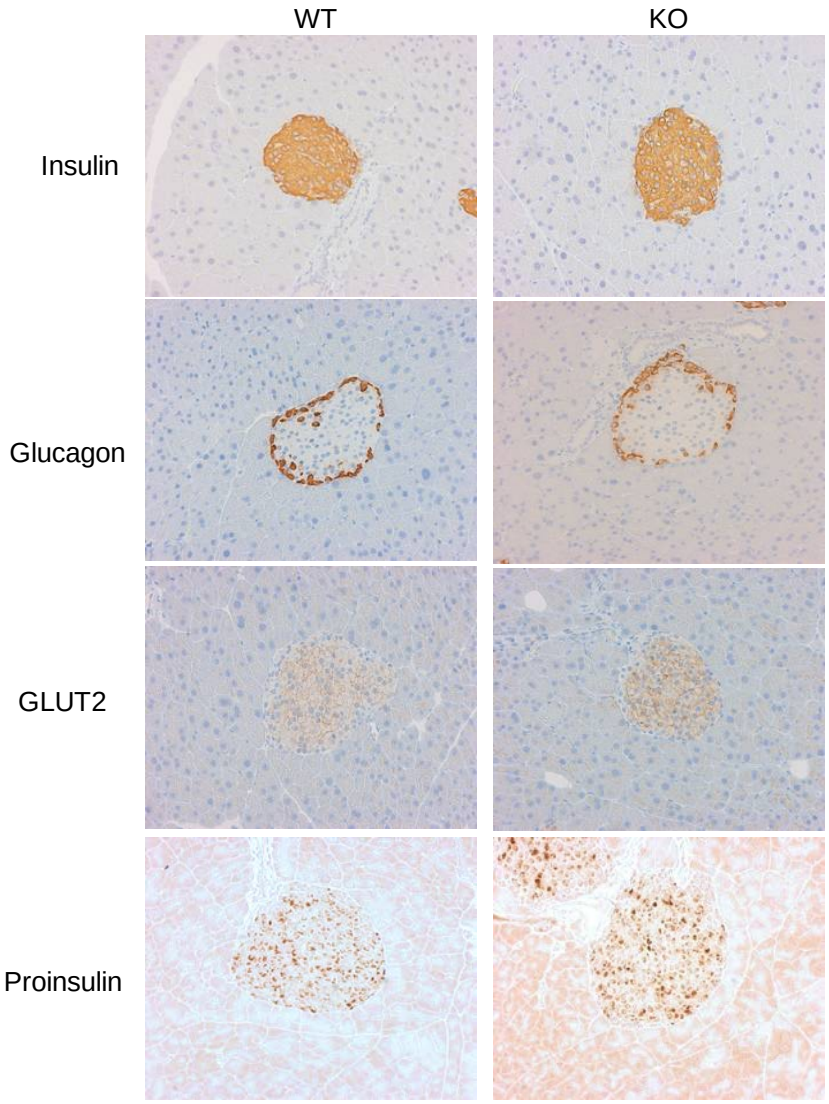

F
HOMA-IR
12
8
4
0
WT x KK
KO x KK

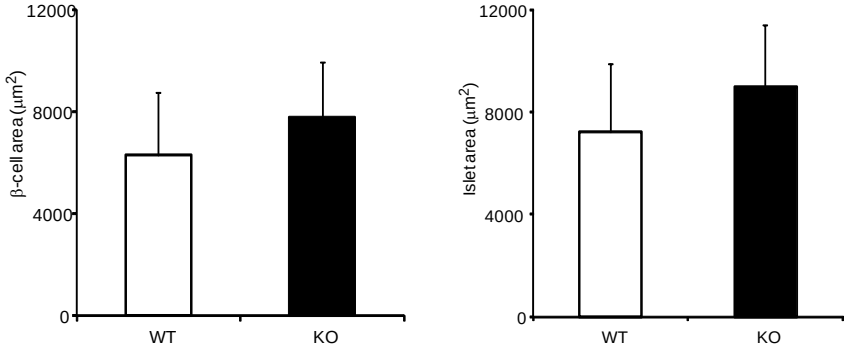
Figure 9. Islet structure and β-cell function of GPR40/FFAR$_1$ WT and KO mice.

(A) Islet morphology of WT and KO mice at 16 weeks of age fed with a regular diet. The sections were stained with anti-insulin, anti-glucagon, anti-GLUT2, and anti-proinsulin antibodies respectively. (B) β-cell and islet area was measured as the stained area for anti-insulin antibody. (C, D) Glucose and palmitate stimulated insulin secretion in isolated islets from WT (white bar) and KO (black bar) mice. Islets were isolated from WT and KO mice fed with a regular diet at 19 weeks. Five islets with similar sizes from each group (four batches in each group) were used. (E) Pancreatic insulin content. Pancreas was isolated from WT and KO mice fed with a regular diet at 28 weeks and extracted by the acid-ethanol method. All values are means ± SD. * $p \leq 0.05$ vs. WT mice by Student's *t*-test.

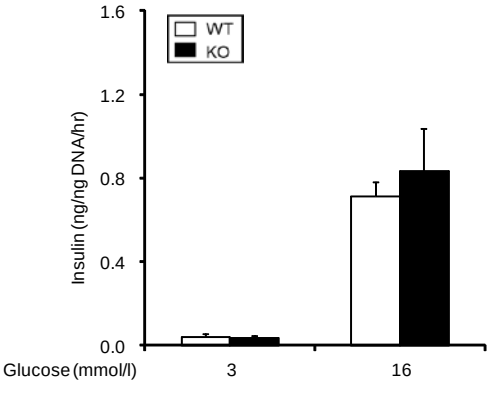
A



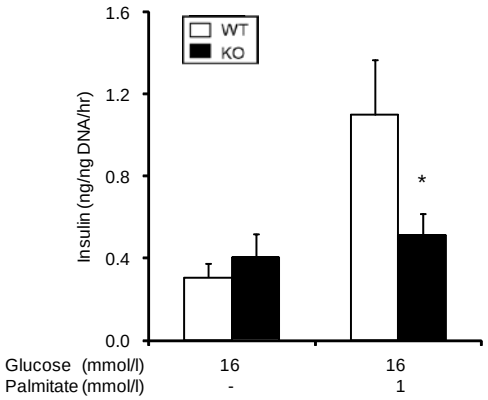
B


β-cell area (μm2)
12000
8000
4000
0
WT
KO
Islet area (μm2)
12000
8000
4000
0
WT
KO

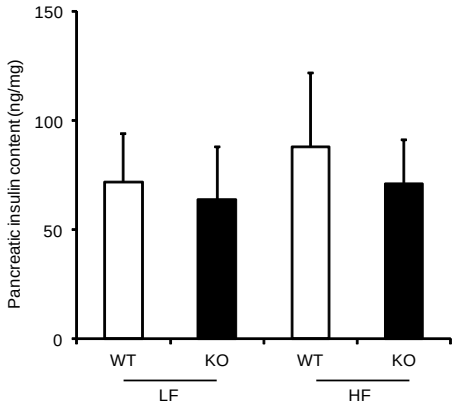
C


WT
KO
Insulin (ng/ng DNA/hr)
1.6
1.2
0.8
0.4
0.0
Glucose (mmol/l)
3
16

D


WT
KO
Insulin (ng/ng DNA/hr)
1.6
1.2
0.8
0.4
0.0
*
Glucose (mmol/l)
16
16
Palmitate (mmol/l)
-
1

E


Pancreatic insulin content (ng/mg)
150
100
50
0
WT
KO
WT
KO
LF
HF

Chapter 2

Overexpression of GPR40/FFAR1 in Pancreatic β-Cells Augments Glucose Stimulated Insulin Secretion and Improves Glucose Tolerance in Normal and Diabetic Mice

Abstract

Backgrounds: GPR40/$FFAR_1$ is a G protein-coupled receptor regulating free fatty acid-induced insulin secretion. I have generated transgenic mice overexpressing the human GPR40/$FFAR_1$ gene (hGPR40/$FFAR_1$-Tg) under control of the mouse insulin II promoter and have used them to examine the role of GPR40/$FFAR_1$ in the regulation of insulin secretion and glucose homeostasis.

Methods: Normal (C57BL/6J) and diabetic (KK) mice overexpressing the human GPR40/$FFAR_1$ gene under control of the insulin II promoter were generated, and their glucose metabolism and islet function were analyzed.

Results: In comparison with nontransgenic littermates, hGPR40/$FFAR_1$-Tg mice exhibited improved oral glucose tolerance with an increase in insulin secretion. Although islet morphological analysis showed no obvious differences between hGPR40/$FFAR_1$-Tg and nontransgenic (NonTg) mice, isolated islets from hGPR40/$FFAR_1$-Tg mice enhanced insulin secretion in response to high glucose (16 mM) than those from NonTg mice with unchanged low glucose (3 mM)-stimulated insulin secretion. In addition, hGPR40/$FFAR_1$-Tg islets significantly increased insulin secretion against a naturally occurring agonist palmitate in the presence of 11 mM glucose. hGPR40/$FFAR_1$-Tg mice were also found to be resistant to high fat diet-induced glucose intolerance, and hGPR40/$FFAR_1$-Tg harboring KK mice showed augmented insulin secretion and improved oral glucose tolerance compared to nontransgenic littermates.

Conclusions: These results suggest that GPR40/$FFAR_1$ have a role in regulating glucose-stimulated insulin secretion and plasma glucose levels *in vivo*, and that

pharmacological activation of GPR40/$FFAR_1$ may provide a novel insulin secretagogue beneficial for the treatment of type 2 diabetes.

Introduction

Free fatty acids (FFAs) serve not only as nutrients but also as cell signaling mediators (60), and they are implicated in several metabolic disorders including diabetes. Elevated circulating FFAs cause insulin resistance and impair glucose metabolism in the liver, the muscle, the adipose tissue, and the pancreatic β-cells (61). In pancreatic β-cells, prolonged exposure to elevated levels of fatty acids together with high levels of glucose impairs β-cell function (43, 44) and induces cell death (42). In contrast to the toxic effects that accompany with chronic exposure, in acute treatment FFAs play an essential role to amplify glucose stimulated insulin secretion (GSIS) (22, 23).

GPR40/$FFAR_1$ was identified as a receptor for medium- and long-chain FFAs and is preferentially expressed at high levels in rodent primary β-cells, β-cell lines (26-29), and human islets (30, 31). Several reports have shown that GPR40/$FFAR_1$ is mainly coupled with $G\alpha_q/G\alpha_{11}$, which activates phosholipase C (PLC) resulting in the formation of inositol 1, 4, 5-triphosphate (IP_3) and induction of calcium release from endoplasmic reticulum (29, 32-34). In fact, FFAs increase intracellular calcium concentration via GPR40/$FFAR_1$ and lead to glucose dependent augmentation of insulin secretion (26-29, 33, 36).

Although several studies have shown the important role of GPR40/$FFAR_1$ in FFA-induced insulin secretion, the involvement of GPR40/$FFAR_1$ in FFA-induced lipotoxicity in β-cells remains controversial. Steneberg *et al.* reported that overexpression of GPR40/$FFAR_1$ in β-cells under the control of the Ipf1/Pdx1 promoter lead to β-cell dysfunction, hypoinsulinemia and diabetes (45). In contrast, studies of

GPR40/$FFAR_1$ knockout mice showed that GPR40/$FFAR_1$ did not play a role in the mechanism by which chronic treatment with fatty acids impaired insulin secretion (37, 47). Furthermore, both acute and chronic treatment with small molecule agonists of GPR40/$FFAR_1$ caused enhancement of glucose stimulated insulin secretion and improved glucose tolerance (37-39). Together, these reports suggested that GPR40/$FFAR_1$ agonist is not harmful to β-cells, but in fact may prove beneficial for the treatment of type 2 diabetes.

To clarify the function of GPR40/$FFAR_1$ in pancreatic β-cells more extensively, I generated transgenic mice overexpressing the human GPR40/$FFAR_1$ gene under control of the insulin II promoter (hGPR40/$FFAR_1$-Tg) and examined the role of GPR40/$FFAR_1$ in the regulation of insulin secretion and glucose homeostasis. I found that hGPR40/$FFAR_1$-Tg mice displayed improved glucose tolerance with augmented insulin secretion both in regular and high fat diet feeding conditions. Moreover, even when the insulin resistance was reinforced in diabetic KK mice, overexpression of hGPR40 in this background also improved glucose tolerance with increasing insulin secretion. Thus, these findings indicated that GPR40/$FFAR_1$ has a role in regulating glucose-stimulated insulin secretion and plasma glucose levels *in vivo,* and supported the concept that GPR40/$FFAR_1$ agonists might be effective insulin secretagogues for the treatment of type 2 diabetes.

Materials and Methods

Generation of hGPR40/FFAR$_1$-Tg mice

The transgene consisted of 0.7 kbp of mouse insulin II gene promoter, followed by 2.2 kbp of the human GPR40/FFAR$_1$ cDNA including the 3'-noncoding region (8) and the complete 2.9 kbp fragment was purified and microinjected into the fertilized eggs of C57BL/6J mouse (CLEA Japan Inc., Tokyo, Japan) (62). The obtained transgenic mice were maintained by crossing with C57BL/6J mice, and the transgenic founder mice were identified by polymerase chain reaction (PCR) analyses of tail DNAs using the human GPR40/FFAR$_1$ gene specific primers (5'-GGAGTGTGGTGCTTAATCCGCTGGT-3' and 5'-AGACTGCCTCCTCCTTCCCGTAAGTACAA-3'). When examining the hGPR40/FFAR$_1$-Tg mice harboring KK hybrid background, hGPR40/FFAR$_1$-Tg mice were crossed with mice harboring KK background and obtained mice containing almost 50% of KK background (hGPR40/FFAR$_1$-Tg x KK). The transgene was identified by PCR analyses of tail DNAs using the human GPR40/FFAR$_1$ specific primers (described as above). Age and sex matched littermates were used as control mice throughout the study, and all experiments were conducted using male mice unless otherwise stated. The mice were fed with a regular diet CE-2 containing 11.5 kcal% fat (CLEA Japan Inc.), and were housed in colony cages and maintained on a 12-hr/12-hr light-dark cycle with free access to water and food. When examining the effects of high fat diet feeding, mice were fed with a high fat diet containing 60 kcal% fat (D12492, Research Diets Inc, New Brunswick, NJ, USA) from 8 weeks of age. Plasma glucose levels were automatically analyzed by DRI-CHEM (FujiFilm Medical, Tokyo, Japan) and plasma non-esterified

fatty acids (NEFA) were determined using Wako Test (Wako Pure Chemical Industries Ltd, Osaka, Japan). Plasma insulin levels were measured by enzyme-linked immunosorbent assay (ELISA) (Morinaga Institute of Biological Science Inc., Tokyo, Japan). All procedures were conducted according to the Experimental Animal Care Use Committee of Takeda Pharmaceutical Company Ltd.

Oral glucose tolerance test

As described in Chapter 1, mice were orally administered with glucose at a dose of 1 g/kg body weight in the case of C57BL/6J or 2 g/kg body weight in the case of KK hybrid mice after an overnight fast. In the case of C57BL/6J mice, blood samples were obtained at time 0 (just before glucose load), 7.5, 15, 30, 60, and 120 min after glucose administration, followed by plasma preparation by centrifugation. In the case of KK hybrid mice, blood samples were collected at 10 min instead of 7.5 and 15 min. Plasma glucose and insulin levels were measured as described above.

Insulin tolerance test

Insulin was injected intraperitoneally at 0.5 units/kg body weight (Novo Nordisk, Bagsværd, Denmark) after an overnight fast. Blood samples were obtained at time 0 (just before insulin injection), 30, 60, and 120 min after insulin injection, followed by plasma preparation by centrifugation. Plasma glucose level was measured as described above.

Preparation of islets

As described in Chapter 1, Islets were isolated from the pancreas by collagenase digestion (54). The solution of 183 units/mg/kg collagenase (Wako Pure Chemical Industries Ltd.) dissolved in Hanks' Balanced Salt Solutions (HBSS; Invitrogen, Carlsbad, CA, USA) containing 10 μg/ml DNase I (Roche Diagnostics, Indianapolis, IN, USA) and 0.2% bovine serum albumin (BSA; Wako Pure Chemical Industries Ltd.), was injected into the common bile duct. The pancreas was removed and incubated at 37°C for 20 min, washed 3 times with HBSS, and islets were hand-collected under a microscope. Freshly purified islets were used for gene expression experiments. For insulin secretion experiments, purified islets were used after overnight culture in RPMI 1640 medium containing 5.5 mM glucose, 10% fetal bovine serum (FBS) in a humidified atmosphere containing 5% CO_2/95% air at 37°C.

RNA extraction and gene expression analysis

Total RNA was extracted from the isolated islets, the liver, the subcutaneous adipose tissue, the muscle, the brain, and the kidney using RNeasy Mini kit (QIAGEN, Tokyo, Japan), and reverse transcription was performed with random hexamer and reverse transcriptase (GE Healthcare UK Ltd., Chalfont St. Giles, UK). Quantitative real-time PCR analyses were performed using a Prism 7700 sequence detector (Applied Biosystems, Foster City, CA, USA), following the manufacturer’s instructions. The primers were as follows: 5'-GCCCGCTTCAGCCTCTCT-3' and 5'-GAGGCAGCCCACGTAGCA-3' with 5'-FAM-TCTGCCCTTGGCCATCACAGCCT-TAMRA-3' TaqMan probe for the human GPR40/$FFAR_1$ gene, 5'-GGGCCTCTGGAAAGGGACT-3' and

5'-ACCAGCTCAGCACAGTTGACA-3' with 5'-FAM-TCCCAATGTTGCCCGTAATGCCA-TAMRA-3' TaqMan probe for the mouse UCP2 gene, and 5'-CGTGAAAAGATGACCCAGATCA-3' and 5'-CACAGCCTGGATGGCTACGT-3' with 5'-FAM-TGAGACCTTCAACACCCCAGCCATG-TAMRA-3' TaqMan probe for the mouse actin gene. The mouse GPR40/$FFAR_1$, insulin II, GLUT2, and glucokinase genes were quantified by Gene Expression Assays (Mm00809442_m1, Mm00731595_gH, Mm00446224_m1, and Mm00439129_m1, respectively, Applied Biosystems, Foster City, CA, USA)

Insulin secretion assay

Groups of five islets was preincubated in Krebs-Ringer biocarbonate buffer (KRBH) containing 0.2% BSA and 1 mM glucose for 30 min at 37°C, and then incubated for another 1 hr in KRBH containing glucose. When examining the effect of the fatty acids, sodium palmitate (CHEM SERVICE, Inc, West Chester, PA, USA) was conjugated with FFA-free BSA (SIGMA ALDRICH Inc., St.Louis, MO, USA) at final palmitate and BSA concentrations of 1 mM and 0.2%, respectively. The conjugated palmitate was added to KRBH containing 11 mM glucose, and incubated for 1hr. At the end of the incubation period, the amount of insulin in the culture supernatant was determined with ELISA as described above. Residual islets were sonicated and used to determine the DNA content (Quant-iT^{TM} Picogreen dsDNA Assay Kit, Invitrogen, Carlsbad, CA, USA).

Immunohistochemistry of pancreas

Formalin-fixed, paraffin-embedded tissue sections (thickness of 4 μm) of the pancreas were immunostained using the avidin-biotin detection system (Ventana Medical Systems, Tucson, AZ, USA) and M.O.M. immunodettection kit (VECTOR Laboratories, Inc., Burlingame, CA, USA), according to the manufacturer's instructions. Anti-insulin antibody (1:200, MP Biomedicals Inc, Morgan Irvine, CA, USA), anti-glucagon antibody (1:2, ZYMED, South San Francisco, CA, USA), anti-GLUT2 antibody (1:200, CHEMICON International Inc, Temecula, CA, USA), and anti-proinsulin antibody (ready-to-use, Lab Vision, Fremont, CA, USA) were used as primary antibodies. When examining the β-cells area, the area of the islets were traced manually and analyzed with Win ROOF software (Mitani Co., Fukui, Japan). More than 20 islets per pancreas were analyzed for the average calculation.

Statistical analysis

Statistical analysis was performed using SAS system Version 8.2 (SAS institute Inc., Cary, NC, USA). All values were expressed as means and S.E. Statistical significance between groups was analyzed by the Student's *t*-test.

Results

Improved glucose tolerance in human GPR40/FFAR$_1$ transgenic mouse harboring normal background

To investigate the role of the GPR40/FFAR$_1$ gene in pancreatic β-cells, I generated mice overexpressing human GPR40/FFAR$_1$ cDNA under control of the mouse insulin II promoter (Figure 10A). Two transgene expressing lines, named hGPR40/FFAR$_1$-Tg 47M and 23F, were established by microinjection of the construct into C57BL/6J oocytes. High expression levels of human GPR40/FFAR$_1$ gene were confirmed in isolated islets from both male transgenic lines at 9 weeks of age (Figure 10B). The expression levels of the human GPR40/FFAR$_1$ mRNA were more than 10 times higher than those of endogenous mouse GPR40/FFAR$_1$ gene in both of the two lines. The 47M lines showed 2.8 times higher expression levels of the human GPR40/FFAR$_1$ mRNA in islets than the 23F lines. Expression of human GPR40/FFAR$_1$ gene was extremely low in the other tissues, but slight expression was found in the kidney in 47M line, but the expression level in kidney in 23F line is extremely low. These transgenic lines appeared generally normal, reproduced successfully, and showed Mendelian inheritance characteristics.

Both of hGPR40/FFAR$_1$-Tg lines (47M and 23F) did not show significant differences in body weight, plasma glucose, plasma insulin, and NEFA levels compared to nontransgenic mice (NonTg) in the fed state on a regular diet (Table 2). However, fasting plasma glucose levels were lower in hGPR40/FFAR$_1$-Tg than in NonTg mice. To assess the glucose homeostasis in these mice more precisely, oral glucose tolerance test and insulin tolerance test were performed and area under the curve (AUC) for glucose

and insulin were calculated. $AUC_{0\text{-}120min}$ of plasma glucose after glucose load in both lines of hGPR40/$FFAR_1$-Tg mice were significantly lower than those in NonTg mice (Figure 11A, B, E). In parallel with improvement of glucose tolerance, insulin responses to glucose at the early-phase were higher in both hGPR40/$FFAR_1$-Tg than NonTg mice (Figure 11C, D), and $AUC_{0\text{-}30min}$ of plasma insulin was significantly increased in both line of hGPR40/$FFAR_1$-Tg mice compared with NonTg mice (Figure 11F). Whole body insulin sensitivity was assessed by the insulin tolerance test, and no apparent differences were observed between hGPR40/$FFAR_1$-Tg and NonTg mice (Figure 11G, H).

Islet structure and β-cell function in vitro

I next examined whether the human GPR40/$FFAR_1$ overexpression affects islet structure. Immunohistochemical analysis of pancreas sections with antibodies against insulin, glucagon, GLUT2, and proinsulin showed no apparent differences between hGPR40/$FFAR_1$-Tg (47M and 23F) and NonTg mice at 16 weeks of age (Figure 12A). β-cell area of 47M and 23F lines were almost the same as that of NonTg mice (Figure 12B). To further examine the effects of the human GPR40/$FFAR_1$ overexpression on islet function, isolated islets from hGPR40/$FFAR_1$-Tg (47M, 23F) mice and NonTg mice at 9 weeks of age were stimulated with glucose or glucose plus palmitate *in vitro*, and secreted insulin was measured. Basal insulin secretion at low glucose concentration (3 mM) was similar between hGPR40/$FFAR_1$-Tg and NonTg islets, but isolated islets from hGPR40/$FFAR_1$-Tg mice showed enhanced insulin secretion in response to high glucose (16 mM) than those from NonTg mice (Figure 12C). Similar results were obtained when using islets isolated from 23F line (Figure 12D). Stimulation with palmitate significantly increased insulin secretion in islets of hGPR40/$FFAR_1$-Tg mice

than those of NonTg mice at 11 mM glucose, indicating that the expressed human GPR40/$FFAR_1$ might be functional in β-cells of hGPR40/$FFAR_1$-Tg mice (Figure 12E). According to these results, it was speculated that the quantity of insulin secretion was more enhanced than that of insulin synthesis in hGPR40/$FFAR_1$-Tg mice islets.

Effects of high fat diet feeding on glucose homeostasis in hGPR40/$FFAR_1$-Tg mice

Since GPR40/$FFAR_1$ is the receptor for medium- and long-chain FFAs (26-28) and a previous report suggested the involvement of GPR40/$FFAR_1$ in FFA-induced lipotoxicity in β-cell (45), I next explored the function of GPR40/$FFAR_1$ *in vivo* under high fat diet (HFD) conditions. hGPR40/$FFAR_1$-Tg mice (47M) were exposed to HFD for 9-12 weeks from 8 weeks of age. When NonTg mice were exposed to HFD, body weight, plasma glucose, insulin, and NEFA levels in the fed state were increased in compared with regular diet (Table 2). There were no obvious differences in body weight and plasma parameters in the fed state between hGPR40/$FFAR_1$-Tg and NonTg mice, but fasting plasma glucose levels were significantly decreased in hGPR40/$FFAR_1$-Tg mice in HFD condition, compared with those observed in regular diet condition (Table 2). Oral glucose tolerance test showed improved glucose tolerance (Figure 13A, C) and augmented insulin secretion in response to glucose (Figure 13B, D) in hGPR40/$FFAR_1$-Tg mice compared to NonTg mice. No apparent difference in insulin sensitivity or in the epididymal adipose tissue weight was observed between hGPR40/$FFAR_1$-Tg and NonTg mice (Figure 13E, F). These results indicated that improved glucose tolerance with increased insulin secretion in hGPR40/$FFAR_1$-Tg mice was maintained under conditions of HFD-induced insulin resistance.

Improved glucose tolerance in hGPR40/FFAR$_1$-Tg mice harboring diabetic genetic background

The effects of overexpression of GPR40/FFAR$_1$ in pancreatic β-cells were examined in genetically diabetic mice. Mice harboring KK background, exhibiting obese, glucose intolerance and insulin resistance (51), were selected as mates, and male hGPR40/FFAR$_1$-Tg (47M) and Non-Tg mice were crossed to female harboring KK background mice. Although the obtained mice had a hybrid genetic background harboring approximate 50% of KK and C57BL/6J mice, the obtained nontransgenic mice (NonTg x KK mice) showed heavier body weight and higher plasma insulin levels than NonTg C57BL/6J mice at 16 weeks of age fed with regular chow (body weight 28.8 ± 0.4 v.s. 38.9 ± 0.57 g, plasma insulin 1.54 ± 0.30 v.s. 5.79 ± 1.83 ng/ml, NonTg C57BL/6J v.s. NonTg x KK, respectively; n=7-10). Human GPR40/FFAR$_1$ gene expression level in islets of hGPR40/FFAR$_1$-Tg x KK mice was more than 10 times higher than that of the mouse gene (Figure 14A). The hGPR40/FFAR$_1$-Tg x KK mice did not show significant differences in body weight, plasma glucose, and insulin levels from NonTg x KK mice at 10 weeks of age (Table 3). Oral glucose tolerance test at 12 weeks of age revealed improved glucose tolerance and increased insulin secretion in hGPR40/FFAR$_1$-Tg x KK mice compared to NonTg x KK mice (Figure 14B-E). We next examined the insulin secretion in response to glucose *in vitro* using islets from hGPR40/FFAR$_1$-Tg x KK and NonTg x KK mice at 12 weeks of age. Although basal insulin secretion from islets in response to low glucose concentration (3 mM) was closely similar between hGPR40/FFAR$_1$-Tg x KK and NonTg x KK mice, insulin secretion against high glucose stimulation (16 mM) was 3.5-fold higher in hGPR40/FFAR$_1$-Tg x KK islets compared to NonTg x KK islets (Figure 14F).

Stimulation with 1 mM palmitate enhanced insulin secretion in islets of hGPR40/FFAR$_1$-Tg x KK mice more than those of NonTg x KK mice at 11 mM glucose (Figure 14G).

Gene expression of factors regulating insulin secretion in islets isolated from hGPR40/FFAR$_1$-Tg mice

To investigate the molecular mechanisms for enhanced insulin secretion in response to high glucose stimulation in hGPR40/FFAR$_1$-Tg mice *in vitro* and *in vivo*, gene expression levels of factors regulating insulin secretion were compared between islets from hGPR40/FFAR$_1$-Tg (47M) and NonTg mice after HFD feeding for 9 weeks. The gene expression levels of insulin II, GLUT2, and glucokinase were found to be almost the same between hGPR40/FFAR$_1$-Tg and NonTg mice islets (Figure 15). I also examined the gene expression levels of UCP2, which negatively regulates insulin secretion in β-cells (26-28), and the expression level of UCP2 did not change between hGPR40/FFAR$_1$-Tg and NonTg mice (Figure 15).

Discussion

In this study, I have shown that overexpression of the human GPR40/$FFAR_1$ gene by using insulin II promoter resulted in improved glucose tolerance with augmented insulin secretion, and the phenotype of hGPR40/$FFAR_1$-Tg mice was not altered by HFD feeding or by gene transfer into a diabetic background. I found the extremely high expression in pancreatic islets in two independent lines, but slight expression was detected in the kidney in 47M line. Both 47M and 23F h GPR40/$FFAR_1$-Tg mice showed the same phenotype, and therefore it is unlikely that the expression in kidney affects the phenotype of hGPR40/$FFAR_1$-Tg mice. The hGPR40/$FFAR_1$-Tg mice showed slightly lower plasma glucose levels than NonTg mice in fasted state but not in fed state. These results might reflect the differential activation of GPR40/$FFAR_1$ between the fasted and the fed state, since natural ligands of GPR40/$FFAR_1$ were FFAs (26-28), and FFA levels were significantly increased after an overnight fast in mice. In the fasted state, GPR40/$FFAR_1$ may be more strongly activated than in the fed state, resulting in enhanced insulin secretion and reduced plasma glucose levels. In fact, insulin levels were tended to increase in hGPR40/$FFAR_1$-Tg mice than NonTg mice in the fasted state.

Isolated islets from hGPR40/$FFAR_1$-Tg mice secreted insulin significantly when stimulated with palmitate and glucose *per se.* Although I do not have direct evidence for GPR40/$FFAR_1$ protein expression level, it was proposed that GPR40/$FFAR_1$ activation by fatty acids stimulates the $G\alpha_q$-PLC signaling pathway, involving activation of PLC and production of IP_3, which leads to release of calcium from the endoplasmic reticulum (29, 32-34). Glucose and fatty acids could also

augment insulin secretion through pathways involving protein kinase C (PKC) (63, 64). The mechanism of enhanced GSIS observed in hGPR40/$FFAR_1$-Tg mice islets remains unclear so far, so further studies will be needed to clarify the precise mechanism.

Steneberg *et al.* reported that the GPR40/$FFAR_1$ transgenic mice driven by the Ipf1/Pdx1 promoter leads to impaired β-cells function, hypoinsulinemia, and glucose intolerance (45), suggesting the involvement of GPR40/$FFAR_1$ in FFA-induced toxicity in β-cells. In contrast, hGPR40/$FFAR_1$-Tg mice did not develop diabetes even after HFD feeding for 8-12 weeks. The reason for the discrepancy remains unknown. Although I may need further examination, the possible explanations are below. One is the different promoters used for the production of transgenic mice. The temporal pattern of expression of Ipf1/Pdx1 during development was different from that of insulin II (65). Transgene of GPR40/$FFAR_1$ regulated by Ipf1/Pdx1 promoter might be expressed in pancreatic progenitors in the early embryonic stage and these expression patterns might influenced the phenotype. Second, it may be caused by the difference of genetic background of each transgenic mouse. Third, the difference of GPR40/$FFAR_1$ gene levels between my transgenic mouse and Steneberg's mouse would be possible. In addition, my preliminary results showed that the hGPR40/$FFAR_1$-Tg mice fed with HFD for long term (more than 50 weeks) maintained improved glucose tolerance with increased insulin secretion, without changes in body weight and plasma parameters including plasma insulin levels. Therefore, from these results, it was suggested that activation of GPR40/$FFAR_1$ function might not cause lipotoxicity. Latour *et al.* (47) and Tan *et al.* (37) demonstrated that islets from GPR40/$FFAR_1$ knockout mice were as sensitive to fatty acid inhibition of insulin secretion upon prolonged exposure as islets from wild-type animals, and they concluded that GPR40/$FFAR_1$ does not play a role in

the mechanisms by which fatty acids chronically impair insulin secretion. Moreover, Kebede *et al.* reported that GPR40/$FFAR_1$ knockout mice showed fasting hyperglycemia and were not protected from HFD-induced insulin resistance (48). Although further studies will be needed to clarify the relation between GPR40/$FFAR_1$ and lipotoxicity, these observations are consistent with my findings. Furthermore, small molecule agonists of GPR40/$FFAR_1$ have been reported (37-39), and they could enhance glucose stimulated insulin secretion and improved glucose tolerance in both acutely and chronically. Results from these reports indicated that activation of GPR40/$FFAR_1$ might be beneficial for glucose control in type 2 diabetes mellitus without lipotoxicity and the phenotypes of hGPR40/$FFAR_1$-Tg mice strongly support this conclusion.

Obesity commonly induces insulin resistance and causes an increase in the requirement of insulin secretion from the pancreas, and the enhancement of this phenomenon further exacerbates obesity and insulin resistance. The molecular mechanism of this phenomenon is unclear, but may be related to increased production and secretion of non-esterified fatty acids and metabolically harmful adipokines by insulin-resistant adipocytes during obesity (66-68). In these results, hGPR40/$FFAR_1$-Tg mice fed on HFD did not show significant differences in body weight, adipose tissue weight, and plasma insulin level. Moreover, when insulin resistance was reinforced by crossing with KK background harboring mice, hGPR40/$FFAR_1$-Tg x KK mice did not show significant differences in body weight and plasma insulin level from NonTg x KK mice. Therefore, activation of GPR40/$FFAR_1$ may not cause sustained hyperinsulinemia but may enhance glucose-stimulated insulin secretion essentially in the prandial period.

The M3 muscarinic acetylcholine receptor subtype is coupled with $G\alpha_q$ in

pancreatic β-cells (69, 70). It has been reported that overexpressed M3 muscarinic receptors in pancreatic β-cells showed almost the same phenotype with the hGPR40/FFAR$_1$-Tg mice (71), and the potentiation of Gα_q signaling in pancreatic β-cells may maintain proper insulin release and glucose homeostasis *in vivo*.

In conclusion, these results demonstrate that GPR40/FFAR$_1$ may play an important role to regulate glucose-stimulated insulin secretion and glucose homeostasis. My observation indicates that GPR40/FFAR$_1$ might be concerned with overt increase in insulin secretion in the presence of elevated glucose such as during the postprandial period, and that specific agonists may be useful as novel insulin secretagogues with low risk of hypoglycemia.

Table 2. Metabolic parameters in hGPR40/FFAR$_1$-Tg (47M, 23F) mice fed on regular diet and high fat diet

All parameters were measured in the fed and fasted state at 16 weeks of age on regular diet. The hGPR40/FFAR$_1$-Tg (47M) and NonTg mice were fed on high fat diet containing 60 kcal% fat for 12 weeks. All parameters were measured in the fed and fasted state at 20 weeks of age. All values are means ± S.E. n = 7-10 per genotype. $^{**}p \leq 0.01$ by Student's t-test v.s. NonTg mice.

			Body weight (g)	Plasma glucose (mg/dl)	Plasma insulin (ng/ml)	Plasma NEFA (mEq/l)
Regular diet	Fed	NonTg	28.8 ± 0.4	170.0 ± 4.1	1.54 ± 0.30	0.33 ± 0.08
		Tg (47M)	27.5 ± 0.6	153.6 ± 6.2	1.61 ± 0.43	0.24 ± 0.06
		NonTg	28.7 ± 0.4	187.6 ± 9.8	1.43 ± 0.38	0.37 ± 0.08
		Tg (23F)	28.1 ± 0.3	161.4 ± 8.1	1.46 ± 0.38	0.41 ±0.06
	Fasted	NonTg	24.3 ± 0.4	106.0 ± 4.8	0.19 ± 0.04	1.35 ± 0.06
		Tg (47M)	22.9 ± 0.6	84.5 ± 4.4**	0.23 ± 0.05	1.00 ± 0.07
		NonTg	24.0 ± 0.3	117.7 ± 5.0	0.21 ± 0.03	1.07 ± 0.07
		Tg (23F)	23.6 ± 0.3	89.9 ± 5.2**	0.24 ± 0.05	0.98 ± 0.07
High fat diet	Fed	NonTg	49.9 ± 0.7	215.6 ± 8.1	18.87 ± 3.52	0.52 ± 0.04
		Tg (47M)	49.6 ± 1.0	212.8 ± 9.9	16.17 ± 2.95	0.49 ± 0.03
	Fasted	NonTg	46.0 ± 0.6	173.8 ± 5.6	2.88 ± 0.31	0.80 ± 0.04
		Tg (47M)	45.4 ± 0.9	133.3 ± 4.5**	3.07 ± 0.39	0.80 ± 0.04

Table 3. Metabolic parameters in hGPR40/FFAR$_1$-Tg mice crossed with KK mice fed on regular diet

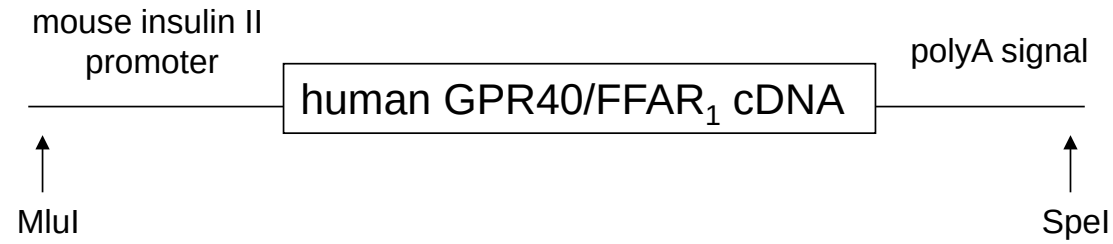
The hGPR40/FFAR$_1$-Tg (47M) mice harboring C57BL/6J and KK background were used for analyses. All parameters were measured in fed state at 10 weeks of age. All values are means ± S.E. n = 8-10 per genotype.

Regular diet Crossed with KK	Body weight (g)	Plasma glucose (mg/dl)	Plasma insulin (ng/ml)
NonTg	33.8 ± 0.7	200.1 ± 5.8	2.18 ± 0.58
Tg	33.3 ± 0.5	208.5 ± 5.9	3.65 ± 0.76

Figure 10. Generation and characterization of hGPR40/FFAR$_1$-Tg mice.

(A) A DNA construct for microinjection to generate transgenic mice for the mouse insulin II promoter and human GPR40/FFAR$_1$ gene. The box denotes the coding region of human GPR40/FFAR$_1$ cDNA. B: Human and mouse GPR40/FFAR$_1$ mRNA levels from hGPR40/FFAR$_1$-Tg (47M, 23F, n = 3) and NonTg mice (n = 3) by quantitative real-time PCR analyses. mRNA levels of Actin were used as an internal control. Is: islet, L: liver, A: subcutaneous adipose tissue, M: muscle, B: brain, K: kidney.

A


mouse insulin II promoter
human GPR40/FFAR1 cDNA
polyA signal
MluI
SpeI

B

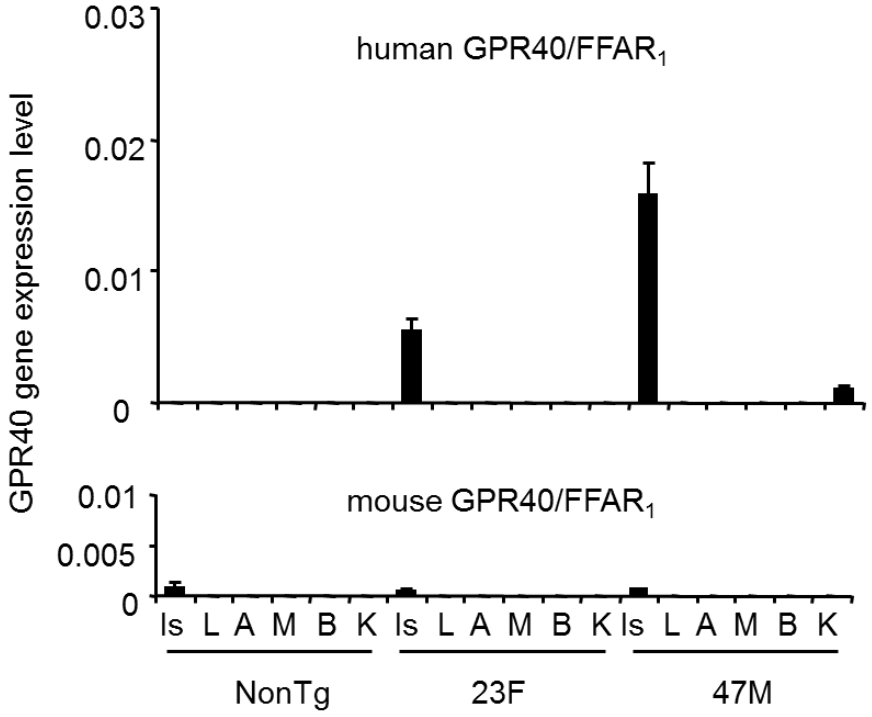

GPR40 gene expression level
human GPR40/FFAR1
mouse GPR40/FFAR1
0.03
0.02
0.01
0
0.01
0.005
0
Is L A M B K Is L A M B K Is L A M B K
NonTg
23F
47M

Figure 11. Improved glucose tolerance in human GPR40/FFAR$_1$ transgenic mice harboring C57BL/6J background.

Oral glucose tolerance test in NonTg (open circle) and hGPR40/FFAR$_1$-Tg (closed circle) on regular diet. Glucose was administered orally at 1 g/kg body weight. Plasma glucose and plasma insulin in 47M line at 16 weeks of age (A, C, n = 8) and in 23F line at 15 weeks of age (B, D, n = 10), respectively. Data in (E) represents the area under the curve of plasma glucose (0-120 min) shown in (A, B) and data in (F) represents the area under the curve of plasma insulin (0-30 min) shown in (C, D). (G, H) Insulin tolerance test for NonTg and hGPR40/FFAR$_1$-Tg mice. Insulin was injected intraperitonealy at 0.5 units/kg. Plasma glucose on regular diet at 14-15 weeks of 47M (G, n = 13-15) and 23F (H, n = 5-7), respectively. All values are means ± S.E. $^{**}p \leq 0.01$, $^{*}p \leq 0.05$ v.s. NonTg mice by Student's *t*-test.

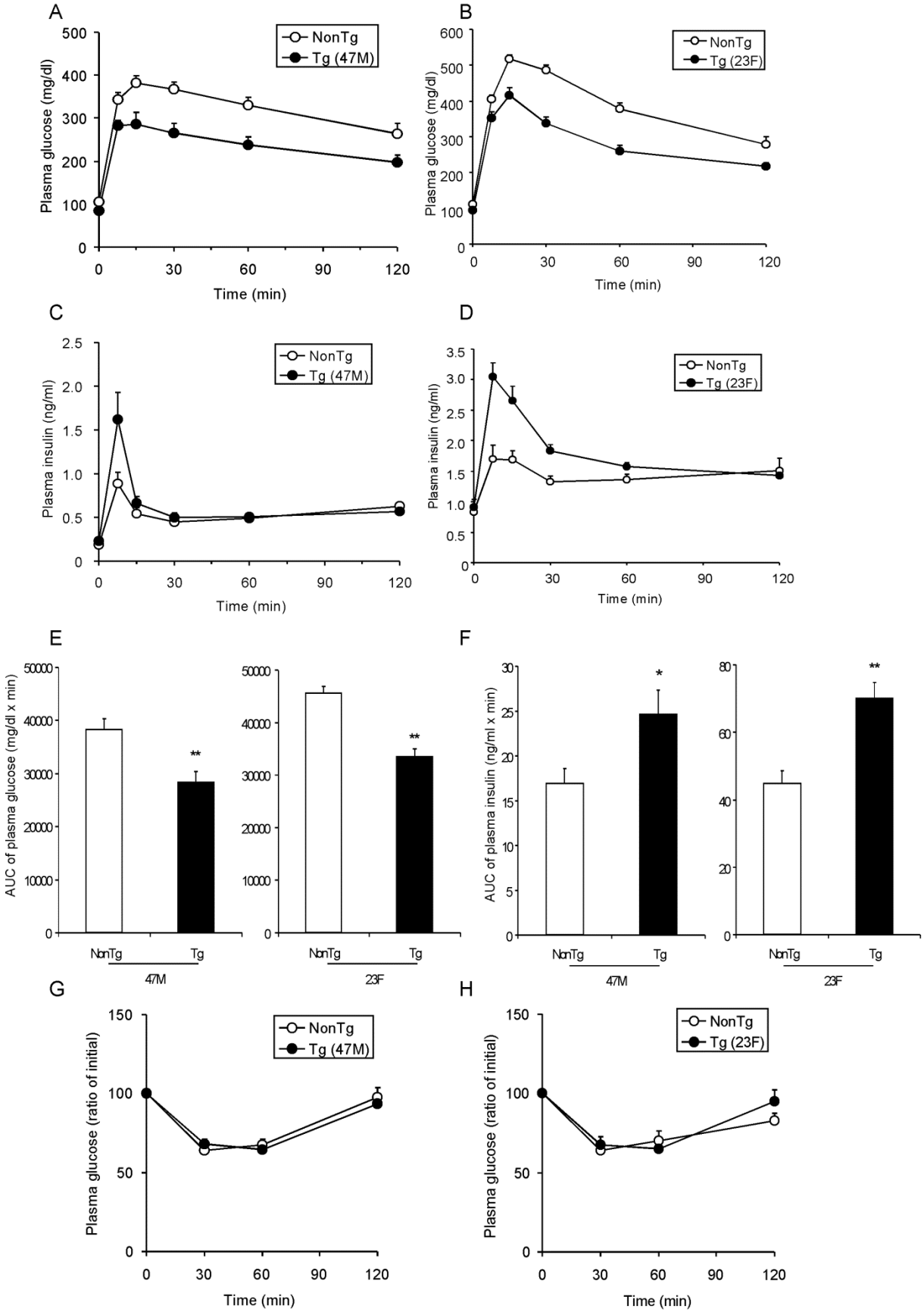
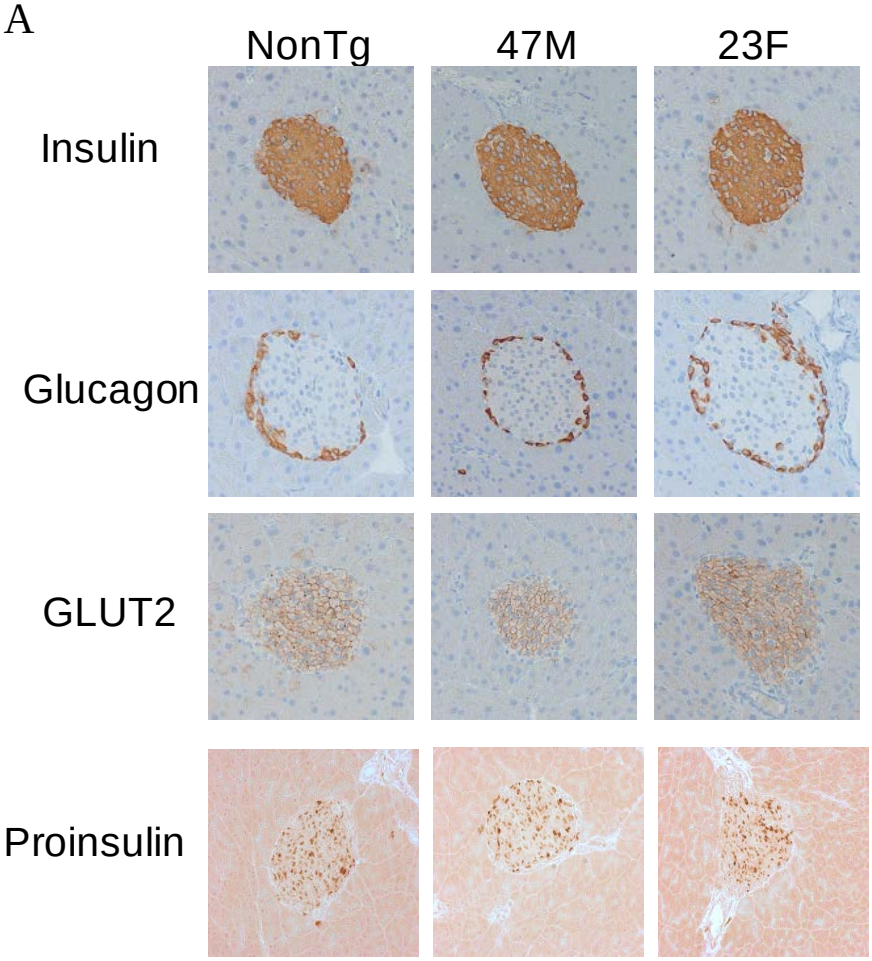

A
Plasma glucose (mg/dl)
NonTg
Tg (47M)
Time (min)
B
Plasma glucose (mg/dl)
NonTg
Tg (23F)
Time (min)
C
Plasma insulin (ng/ml)
NonTg
Tg (47M)
Time (min)
D
Plasma insulin (ng/ml)
NonTg
Tg (23F)
Time (min)
E
AUC of plasma glucose (mg/dl x min)
NonTg
Tg
47M
23F
F
AUC of plasma insulin (ng/ml x min)
NonTg
Tg
47M
23F
G
Plasma glucose (ratio of initial)
NonTg
Tg (47M)
Time (min)
H
Plasma glucose (ratio of initial)
NonTg
Tg (23F)
Time (min)

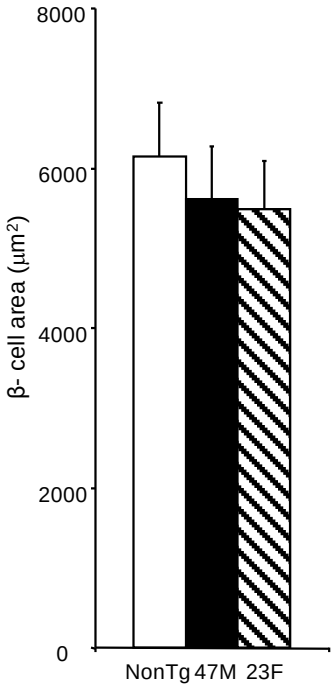
Figure 12. Islet structure and β-cell function of hGPR40/FFAR$_1$-Tg mice and NonTg mice.

(A) Islet morphology of hGPR40/FFAR$_1$-Tg (47M, 23F) and NonTg mice at 16 weeks of age. The sections were stained with anti-insulin, anti-glucagon, anti-GLUT2, and anti-proinsulin antibodies respectively. (B) β-cell area was measured as the stained area for anti-insulin antibody. (C, D) Glucose stimulated insulin secretion in isolated islets from hGPR40/FFAR$_1$-Tg and NonTg mice. (E) Palmitate stimulated insulin secretion in isolated islets from hGPR40/FFAR$_1$-Tg (47M) mice and NonTg mice. Islets were isolated from hGPR40/FFAR$_1$-Tg and NonTg mice fed on regular diet at 9 weeks. Five islets with similar sizes from each group (four batches in each group) were used. All values are means ± S.E. $^{**}p \leq 0.01$, $^{*}p \leq 0.05$ v.s. NonTg mice by Student's *t*-test.

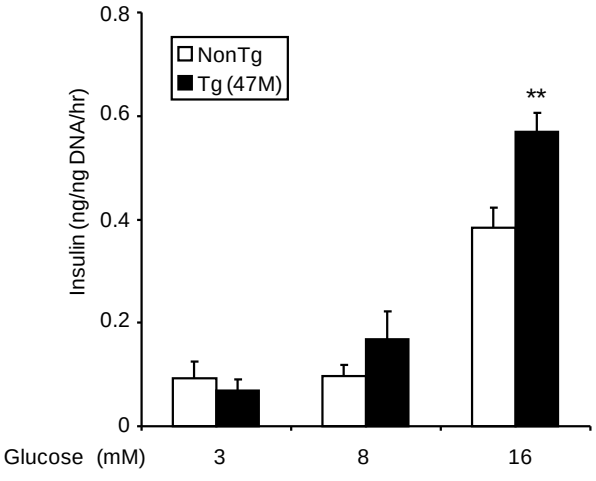
A


NonTg
47M
23F
Insulin
Glucagon
GLUT2
Proinsulin

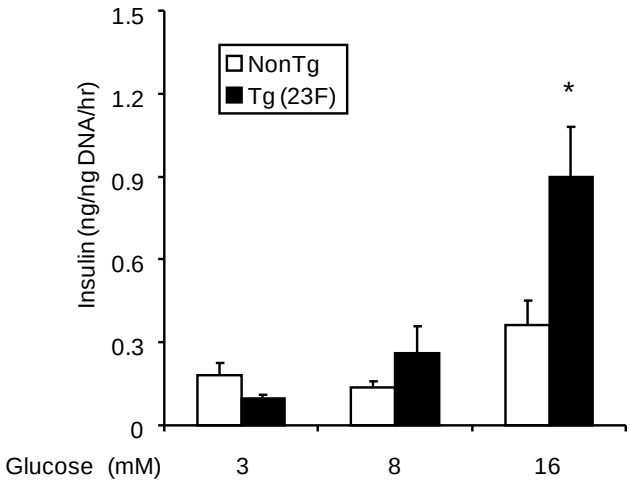
B


8000
6000
4000
2000
0
β- cell area (μm²)
NonTg 47M 23F

C


0.8
0.6
0.4
0.2
0
Insulin (ng/ng DNA/hr)
NonTg
Tg (47M)
**
Glucose (mM) 3 8 16

D


1.5
1.2
0.9
0.6
0.3
0
Insulin (ng/ng DNA/hr)
NonTg
Tg (23F)
*
Glucose (mM) 3 8 16

E

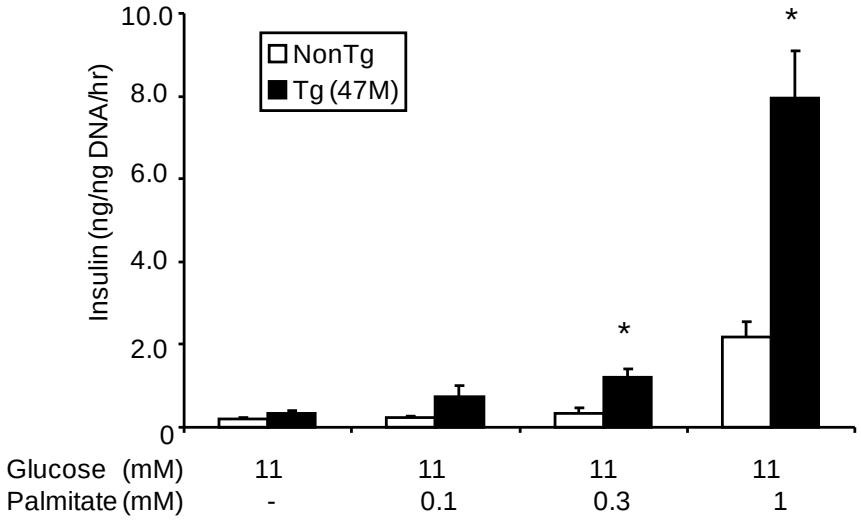

10.0
8.0
6.0
4.0
2.0
0
Insulin (ng/ng DNA/hr)
NonTg
Tg (47M)
*
*
Glucose (mM) 11 11 11 11
Palmitate (mM) - 0.1 0.3 1

Figure 13. Effects of high fat diet feeding on glucose homeostasis in hGPR40/FFAR$_1$-Tg mice.

The hGPR40/FFAR$_1$-Tg (47M) and NonTg mice were fed on 60 kcal% fat diet from 8 weeks of age. Oral glucose tolerance test for hGPR40/FFAR$_1$-Tg mice and NonTg mice. Glucose was administered orally at 1 g/kg body weight. Plasma glucose (A) and plasma insulin (B) at 17 weeks of age, respectively. Data in (C) represents the area under the curve of plasma glucose (0-120 min) shown in (A) and data in (D) represents the area under the curve of plasma insulin (0-30 min) shown in (B). (E) Insulin tolerance test for NonTg and hGPR40/FFAR$_1$-Tg mice. Insulin was injected intraperitonealy at 0.5 units/kg. Plasma glucose on high fat diet at 20 weeks of age. (F) Epididymal adipose tissue weight at 20 weeks of age. All values are means ± S.E. (n = 10). $^{**}p \leq 0.01$ v.s. NonTg mice by Student's *t*-test.

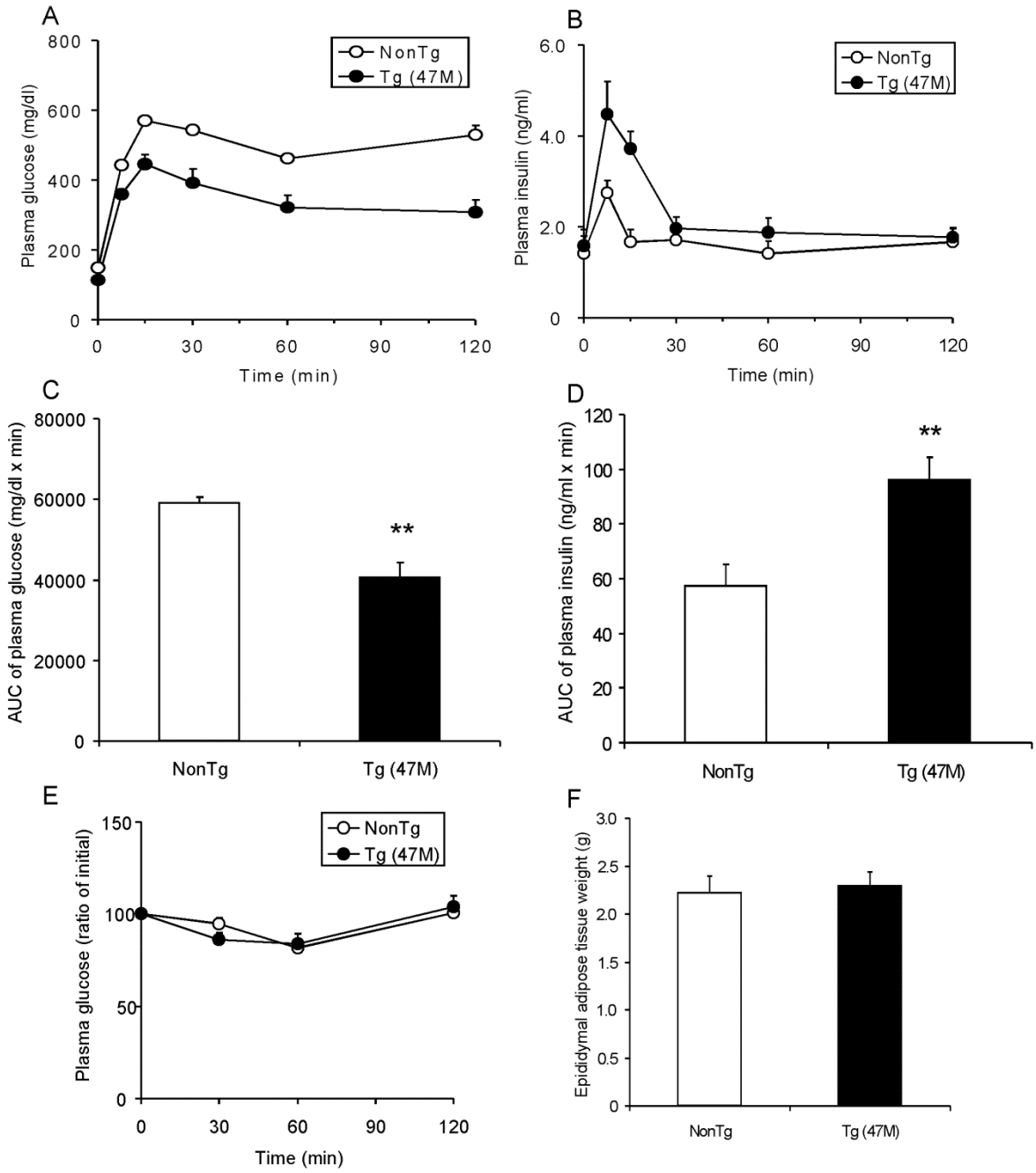
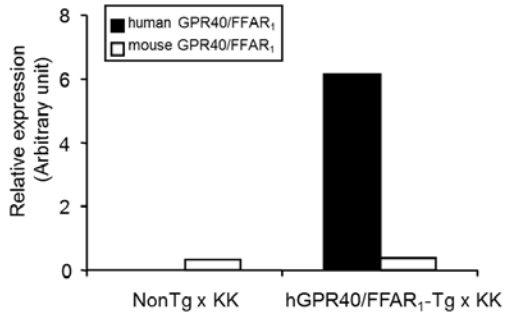

A
Plasma glucose (mg/dl)
800
600
400
200
0
NonTg
Tg (47M)
0
30
60
90
120
Time (min)
B
Plasma insulin (ng/ml)
6.0
4.0
2.0
0
NonTg
Tg (47M)
0
30
60
90
120
Time (min)
C
AUC of plasma glucose (mg/dl x min)
80000
60000
40000
20000
0
**
NonTg
Tg (47M)
D
AUC of plasma insulin (ng/ml x min)
120
100
80
60
40
20
0
**
NonTg
Tg (47M)
E
Plasma glucose (ratio of initial)
150
100
50
0
NonTg
Tg (47M)
0
30
60
90
120
Time (min)
F
Epididymal adipose tissue weight (g)
3.0
2.5
2.0
1.5
1.0
0.5
0
NonTg
Tg (47M)

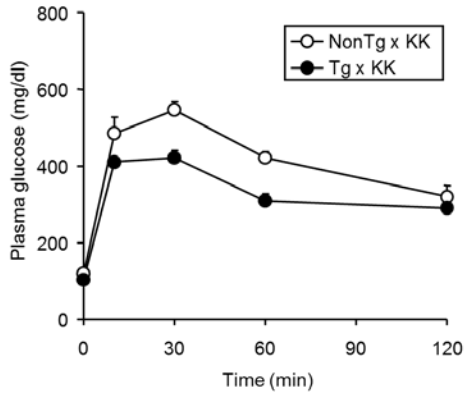
Figure 14. Improved glucose tolerance in hGPR40/FFAR$_1$-Tg mice harboring diabetic background of KK on regular diet.

(A) Human and mouse GPR40/FFAR$_1$ mRNA levels in islets from hGPR40/FFAR$_1$-Tg x KK and NonTg x KK mice (n = 3) by quantitative real-time PCR analyses. mRNA levels of Actin were used as an internal control. (B-E) Oral glucose tolerance test for hGPR40/FFAR$_1$-Tg mice and NonTg mice harboring hybrid background. Glucose was administered orally at 2 g/kg body weight. Plasma glucose (B) and plasma insulin (C) at 12 weeks of age on regular diet. Data in (D) represents the area under the curve of plasma glucose (0-120 min) shown in (B) and data in (E) represents the area under the curve of plasma insulin (0-30 min) shown in (C). (F, G) Glucose and palmitate stimulated insulin secretion in isolated islets from hGPR40/FFAR$_1$-Tg x KK and NonTg x KK mice. Islets were isolated from mice fed on regular diet at 12 weeks. Five islets with similar sizes from each group (four batches in each group) were used. All values are means ± S.E. (n = 8-10). $^{**}p \leq 0.01$, $^{*}p \leq 0.05$ v.s. NonTg mice by Student's *t*-test.

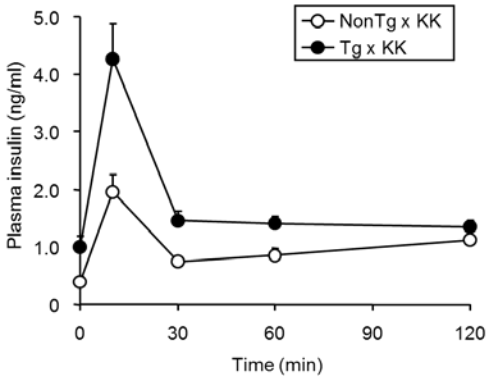
A


human GPR40/FFAR1
mouse GPR40/FFAR1
Relative expression (Arbitrary unit)
0
2
4
6
8
NonTg x KK
hGPR40/FFAR1-Tg x KK

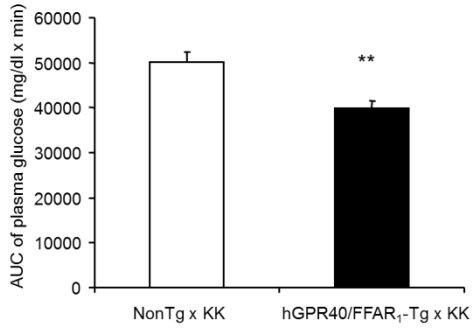
B


NonTg x KK
Tg x KK
Plasma glucose (mg/dl)
0
200
400
600
800
0
30
60
90
120
Time (min)

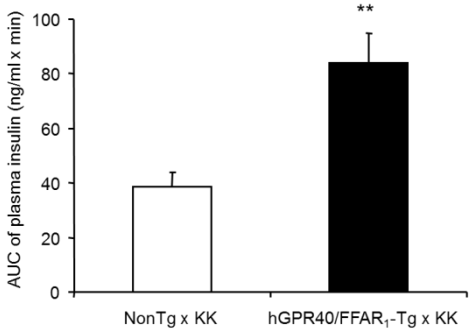
C


NonTg x KK
Tg x KK
Plasma insulin (ng/ml)
0
1.0
2.0
3.0
4.0
5.0
0
30
60
90
120
Time (min)

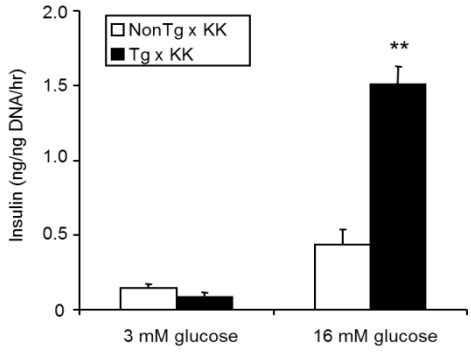
D


AUC of plasma glucose (mg/dl x min)
0
10000
20000
30000
40000
50000
60000
**
NonTg x KK
hGPR40/FFAR1-Tg x KK

E


AUC of plasma insulin (ng/ml x min)
0
20
40
60
80
100
**
NonTg x KK
hGPR40/FFAR1-Tg x KK

F


NonTg x KK
Tg x KK
Insulin (ng/ng DNA/hr)
0
0.5
1.0
1.5
2.0
**
3 mM glucose
16 mM glucose

G

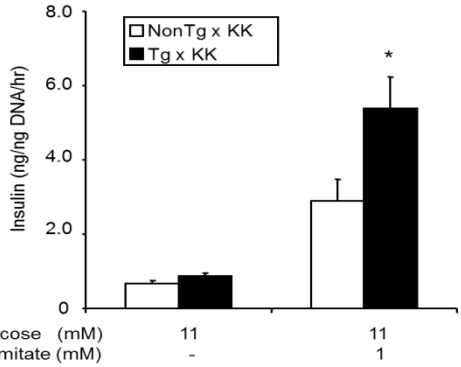
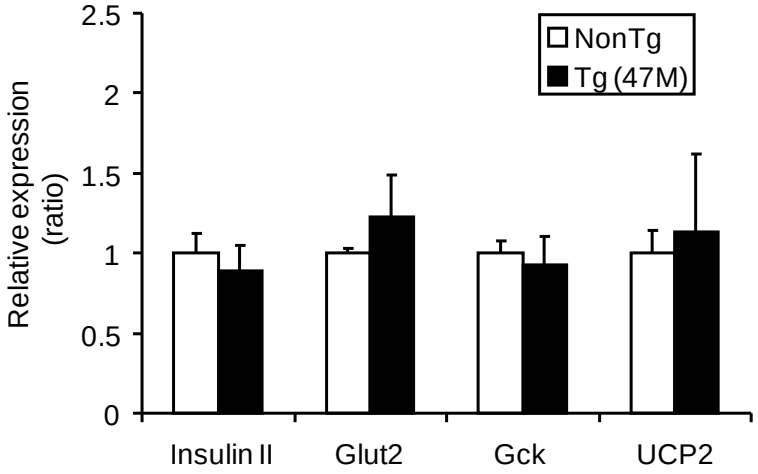

NonTg x KK
Tg x KK
Insulin (ng/ng DNA/hr)
0
2.0
4.0
6.0
8.0
*
Glucose (mM)
Palmitate (mM)
11
-
11
1

Figure 15. Gene expression profiles in islets from hGPR40/FFAR$_1$-Tg and NonTg mice.

The hGPR40/FFAR$_1$-Tg (47M) and NonTg mice were fed on 60 kcal% fat diet for 9 weeks. At each group, the islets from more than three mice were collected, and mRNA levels of indicated genes were estimated by quantitative real-time PCR analyses at duplicate. mRNA levels of Actin were used as an internal control. Ratios in hGPR40/FFAR$_1$-Tg mice were with respect to values in NonTg mice. All values are means ± S.E. (n = 3-4). Gck; glucokinase.


2.5
2
1.5
1
0.5
0
Relative expression (ratio)
NonTg
Tg (47M)
Insulin II
Glut2
Gck
UCP2

General Discussion

To clarify whether loss of function of GPR40/$FFAR_1$ exacerbates diabetes or not, I have generated GPR40/$FFAR_1$ knockout mice and examined their phenotypes *in vitro* and *in vivo* under high-fat-diet feeding in chapter 1. Moreover, to clarify the function of GPR40/$FFAR_1$ in pancreatic β-cells more extensively, I generated transgenic mice overexpressing the human GPR40/$FFAR_1$ gene under control of the insulin II promoter (hGPR40/$FFAR_1$-Tg) and examined the role of GPR40/$FFAR_1$ in the regulation of insulin secretion and glucose homeostasis in chapter 2.

Type 2 diabetes mellitus is characterized by elevated plasma glucose levels arising from increased peripheral insulin resistance and impaired insulin secretion. GPR40/$FFAR_1$ was identified as a receptor for medium- and long-chain FFAs and was preferentially expressed at high levels in pancreatic β-cells. GPR40/$FFAR_1$ lead to glucose dependent augmentation of insulin secretion *in vitro*, and GPR40/$FFAR_1$ agonist may be the candidate of new drug for the treatment of type 2 diabetes and the mechanism of insulin secretion via GPR40/$FFAR_1$ was clarified *in vitro*, whearas the function of GPR40/$FFAR_1$ *in vivo* was not clarified yet.

In the first chapter, GPR40/$FFAR_1$ knockout mice (KO) mice were generated and evaluated their phenotypes. GPR40/$FFAR_1$ KO mice developed glucose intolerance to a similar degree as GPR40/$FFAR_1$ wild-type (WT) mice on a high-fat diet condition and diabetogenic KK gene background, so the lack of GPR40/$FFAR_1$ does not exacerbate glucose intolerance and insulin resistance. Interestingly, glucose-induced insulin secretion under high palmitate concentration was significantly lower in KO than in WT islets, so GPR40/$FFAR_1$ has a major role in regulating fatty acid-augmented insulin secretion. These findings indicate that loss of GPR40/$FFAR_1$ function does not

contribute to induce or exacerbate diabetes, and especially GPR40/$FFAR_1$ is selectively involved in fatty-acid-augmented insulin secretion.

Some of the activities attributed to fatty acids are mediated through their interaction with a number of G-protein-coupled receptors. GPR40/$FFFAR_1$, GPR84, and GPR120 are activated by medium- to long-chain fatty acids (32), so it was speculated that these receptors might have compensatory activities in lack of GPR40/$FFFAR_1$ condition *in vivo*. I reasoned that because the high-fat diet used in this *in vivo* experiment contained not only saturated fatty acids but also unsaturated fatty acids, it might not be selective condition for GPR40/$FFFAR_1$. Recently, it has been reported that GPR40/$FFFAR_1$ deficiency protected from CLA (conjugated linoleic acid)-induced insulin resistance and hepatic steatosis, which suggested that GPR40/$FFAR_1$ deficiency might link with the lack of CLA-induced augmentation of insulin secretion and GPR40/$FFAR_1$ selectively resposense to the selective ligand of GPR40/$FFAR_1$, linoleic acid, *in vivo* (72). Indeed, CLAs directly stimulate insulin secretion through GPR40/$FFAR_1$ (73).

In the second chapter, the function of GPR40/$FFAR_1$ transgenic (Tg) mice which human GPR40/$FFAR_1$ gene was overexpressed by using insulin II promoter was analysed. In comparison with nontransgenic (NonTg) littermates, GPR40/$FFAR_1$ Tg mice showed the improvement of glucose tolerance with augmented insulin secretion on a high-fat diet condition and diabetogenic KK gene background. Isolated islets from hGPR40/$FFAR_1$-Tg mice enhanced robust insulin secretion in response to high glucose without change of that in low glucose stimulation. In addition, hGPR40/$FFAR_1$-Tg islets significantly increased insulin secretion against a naturally occurring agonist palmitate in the presence of glucose. These results indicate that GPR40/$FFAR_1$ have a role in

regulating glucose-stimulated insulin secretion *in vitro* and plasma glucose levels *in vivo*, but its detailed mechanism has not been clarified yet. GPR40/$FFAR_1$ activation by fatty acids stimulated the $G\alpha_q$-PLC signaling pathway, resulting in the activation of PLC, production of IP_3, and activation of DAG which leads to release of calcium from the endoplasmic reticulum (29, 32-34). Glucose and fatty acids could also augment insulin secretion through pathways involving PKC (63, 64). Recently, it has been reported that GPR40/$FFAR_1$ signaling activated enhances GSIS pathway via dual potentiating mechanisms in which IP3 amplifies glucose-induced Ca^{2+} oscillations and DAG/PKC augments downstream secretory mechanisms independent of Ca^{2+} oscillations(74). Fasiglifam (TAK‐875), an oral, highly potent and selective GPR40/$FFAR_1$ agonist was developed as a first in class agent (75). Fasiglifam was effective in lowering plasma glucose levels in a 12 week phase 2 randomized, double‐blind, placebo control and active comparator (glimepiride) trial (76). Whereas concerns about liver safety arose and Fasiglifam clinical development program was terminated in phase 3 (77), my research could contribute to the drug discovery of Fasiglifam from rodents to human.

In conclusion, GPR40/$FFAR_1$ is concerned with overt increase in insulin secretion in the presence of elevated glucose such as during the postprandial period, and I clarified the physiological role of GPR40/$FFAR_1$ *in vivo* with genetically modified anumals. These data supported the concept that GPR40/$FFAR_1$ agonists might be effective insulin secretagogues for the treatment of type 2 diabetes with low risk of hypoglycemia.

Acknowledgements

I am deeply grateful to Professors Osamu Numata, Tomoki Chiba, and Kazuto Nakata, and Associate Professor Kazuichi Sakamoto, University of Tsukuba, for guiding my work and valuable discussions through my doctoral program.

I am very thankful to Drs. Yoshihiko Kaisho, Shigehisa Taketomi, Hiroyuki Odaka, Seigo Izumo, Yukio Yamada, Masakuni Noda, and Koji Takeuchi, Nobuhiro Nishigaki, Takeda Pharmaceutical Company Limited, for guiding my work and valuable discussions through my work.

I appreciate Ritsuko Takami, Kimihiko Iwachidow, Yoshitaka Yasuhara, Dr. Kazuhiro Ogi, Hideyuki Tanaka, Mitsugu Nakata, Takashi Yano, Kozo Shimakawa Dr. Shuji Hinuma, Dr. Nobuhiro Suzuki, Hisao Kasuga, and Dr. Yasuaki Itoh, Takeda Pharmaceutical Company Limited, for their contributions and helpful supports.

I also thank Drs. Yoshiyuki Tsujihata, Ryo Ito, and Masaki Hosoya, Takeda Pharmaceutical Company Limited, for valuable suggestions and comments.

I also thank Kazuhiro Hosono, Kunio Matsuoka, Takeda Pharmaceutical Company Limited, for their technical support.

Finally, I would like to appreciate my family for supporting my life in University of Tsukuba.

References

1. Federation. ID. International Diabetes Federation. IDF Diabetes Atlas, 6th ed 2013.

2.Muoio DM, Newgard CB. Mechanisms of disease: molecular and metabolic mechanisms of insulin resistance and beta-cell failure in type 2 diabetes. Nat Rev Mol Cell Biol. 2008 Mar;9(3):193-205.

3.World health Organization. Prevention of diabetes mellitus. Report of a WHO study group. 1994;844.

4.Giorgino F, Laviola L, Leonardini A. Pathophysiology of type 2 diabetes: rationale for different oral antidiabetic treatment strategies. Diabetes Res Clin Pract. 2005 Jun;68 Suppl1:S22-9.

5.Khaw KT, Wareham N, Luben R, Bingham S, Oakes S, Welch A, Day N. Glycated haemoglobin, diabetes, and mortality in men in Norfolk cohort of european prospective investigation of cancer and nutrition (EPIC-Norfolk). BMJ. 2001 Jan 6;322(7277):15-8.

6.DeFronzo RA, Barzilai N, Simonson DC. Mechanism of metformin action in obese and lean noninsulin-dependent diabetic subjects. J Clin Endocrinol Metab. 1991 Dec;73(6):1294-301.

7.Waugh J, Keating GM, Plosker GL, Easthope S, Robinson DM. Pioglitazone: a review of its use in type 2 diabetes mellitus. Drugs. 2006;66(1):85-109.

8.Krentz AJ, Bailey CJ. Oral antidiabetic agents: current role in type 2 diabetes mellitus. Drugs. 2005;65(3):385-411.

9.Chen X, Zheng Y, Shen Y. Voglibose (Basen, AO-128), one of the most important alpha-glucosidase inhibitors. Curr Med Chem. 2006;13(1):109-16.

10.Van de Laar FA, Lucassen PL, Akkermans RP, Van de Lisdonk EH, Rutten GE, Van Weel C. Alpha-glucosidase inhibitors for type 2 diabetes mellitus. Cochrane Database Syst Rev. 2005(2):CD003639.

11.Matsuo T, Odaka H, Ikeda H. Effect of an intestinal disaccharidase inhibitor (AO-128) on obesity and diabetes. Am J Clin Nutr. 1992 Jan;55(1 Suppl):314S-7S.

12.Moritoh Y, Takeuchi K, Asakawa T, Kataoka O, Odaka H. Chronic administration of alogliptin, a novel, potent, and highly selective dipeptidyl peptidase-4 inhibitor, improves glycemic control and beta-cell function in obese diabetic ob/ob mice. Eur J Pharmacol. 2008 Jul 7;588(2-3):325-32.

13.Christopher R, Covington P, Davenport M, Fleck P, Mekki QA, Wann ER, Karim A.

Pharmacokinetics, pharmacodynamics, and tolerability of single increasing doses of the dipeptidyl peptidase-4 inhibitor alogliptin in healthy male subjects. Clin Ther. 2008 Mar;30(3):513-27.

14.Feng J, Zhang Z, Wallace MB, Stafford JA, Kaldor SW, Kassel DB, Navre M, Shi L, Skene RJ, Asakawa T, Takeuchi K, Xu R, Webb DR, Gwaltney SL, 2nd. Discovery of alogliptin: a potent, selective, bioavailable, and efficacious inhibitor of dipeptidyl peptidase IV. J Med Chem. 2007 May 17;50(10):2297-300.

15.DeFronzo RA, Fleck PR, Wilson CA, Mekki Q. Efficacy and safety of the dipeptidyl peptidase-4 inhibitor alogliptin in patients with type 2 diabetes and inadequate glycemic control: a randomized, double-blind, placebo-controlled study. Diabetes Care. 2008 Dec;31(12):2315-7.

16.Covington P, Christopher R, Davenport M, Fleck P, Mekki QA, Wann ER, Karim A. Pharmacokinetic, pharmacodynamic, and tolerability profiles of the dipeptidyl peptidase-4 inhibitor alogliptin: a randomized, double-blind, placebo-controlled, multiple-dose study in adult patients with type 2 diabetes. Clin Ther. 2008 Mar;30(3):499-512.

17.Lambeir AM, Durinx C, Scharpe S, De Meester I. Dipeptidyl-peptidase IV from bench to bedside: an update on structural properties, functions, and clinical aspects of the enzyme DPP IV. Crit Rev Clin Lab Sci. 2003 Jun;40(3):209-94.

18.Drucker DJ, Nauck MA. The incretin system: glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes. Lancet. 2006 Nov 11;368(9548):1696-705.

19.Ferrannini E, Solini A. SGLT2 inhibition in diabetes mellitus: rationale and clinical prospects. Nat Rev Endocrinol. 2012 Aug;8(8):495-502.

20.Hollander PA, Schwartz SL, Gatlin MR, Haas SJ, Zheng H, Foley JE, Dunning BE. Importance of early insulin secretion: comparison of nateglinide and glyburide in previously diet-treated patients with type 2 diabetes. Diabetes Care. 2001 Jun;24(6):983-8.

21.Doyle ME, Egan JM. Pharmacological agents that directly modulate insulin secretion. Pharmacol Rev. 2003 Mar;55(1):105-31.

22.Prentki M, Vischer S, Glennon MC, Regazzi R, Deeney JT, Corkey BE. Malonyl-CoA and long chain acyl-CoA esters as metabolic coupling factors in

nutrient-induced insulin secretion. J Biol Chem. 1992 Mar 25;267(9):5802-10.
23.Yaney GC, Corkey BE. Fatty acid metabolism and insulin secretion in pancreatic beta cells. Diabetologia. 2003 Oct;46(10):1297-312.
24.Stein DT, Esser V, Stevenson BE, Lane KE, Whiteside JH, Daniels MB, Chen S, McGarry JD. Essentiality of circulating fatty acids for glucose-stimulated insulin secretion in the fasted rat. J Clin Invest. 1996 Jun 15;97(12):2728-35.
25.Dobbins RL, Chester MW, Daniels MB, McGarry JD, Stein DT. Circulating fatty acids are essential for efficient glucose-stimulated insulin secretion after prolonged fasting in humans. Diabetes. 1998 Oct;47(10):1613-8.
26.Itoh Y, Kawamata Y, Harada M, Kobayashi M, Fujii R, Fukusumi S, Ogi K, Hosoya M, Tanaka Y, Uejima H, Tanaka H, Maruyama M, Satoh R, Okubo S, Kizawa H, Komatsu H, Matsumura F, Noguchi Y, Shinohara T, Hinuma S, Fujisawa Y, Fujino M. Free fatty acids regulate insulin secretion from pancreatic beta cells through GPR40. Nature. 2003 Mar 13;422(6928):173-6.
27.Briscoe CP, Tadayyon M, Andrews JL, Benson WG, Chambers JK, Eilert MM, Ellis C, Elshourbagy NA, Goetz AS, Minnick DT, Murdock PR, Sauls HR, Jr., Shabon U, Spinage LD, Strum JC, Szekeres PG, Tan KB, Way JM, Ignar DM, Wilson S, Muir AI. The orphan G protein-coupled receptor GPR40 is activated by medium and long chain fatty acids. J Biol Chem. 2003 Mar 28;278(13):11303-11.
28.Kotarsky K, Nilsson NE, Flodgren E, Owman C, Olde B. A human cell surface receptor activated by free fatty acids and thiazolidinedione drugs. Biochem Biophys Res Commun. 2003 Feb 7;301(2):406-10.
29.Fujiwara K, Maekawa F, Yada T. Oleic acid interacts with GPR40 to induce Ca2+ signaling in rat islet beta-cells: mediation by PLC and L-type Ca2+ channel and link to insulin release. Am J Physiol Endocrinol Metab. 2005 Oct;289(4):E670-7.
30.Tomita T, Masuzaki H, Noguchi M, Iwakura H, Fujikura J, Tanaka T, Ebihara K, Kawamura J, Komoto I, Kawaguchi Y, Fujimoto K, Doi R, Shimada Y, Hosoda K, Imamura M, Nakao K. GPR40 gene expression in human pancreas and insulinoma. Biochem Biophys Res Commun. 2005 Dec 30;338(4):1788-90.
31.Tomita T, Masuzaki H, Iwakura H, Fujikura J, Noguchi M, Tanaka T, Ebihara K, Kawamura J, Komoto I, Kawaguchi Y, Fujimoto K, Doi R, Shimada Y, Hosoda K, Imamura M, Nakao K. Expression of the gene for a membrane-bound fatty acid receptor

in the pancreas and islet cell tumours in humans: evidence for GPR40 expression in pancreatic beta cells and implications for insulin secretion. Diabetologia. 2006 May;49(5):962-8.

32.Brown AJ, Jupe S, Briscoe CP. A family of fatty acid binding receptors. DNA Cell Biol. 2005 Jan;24(1):54-61.

33.Shapiro H, Shachar S, Sekler I, Hershfinkel M, Walker MD. Role of GPR40 in fatty acid action on the beta cell line INS-1E. Biochem Biophys Res Commun. 2005 Sep 16;335(1):97-104.

34.Gromada J. The free fatty acid receptor GPR40 generates excitement in pancreatic beta-cells. Endocrinology. 2006 Feb;147(2):672-3.

35.Kotarsky K, Nilsson NE, Olde B, Owman C. Progress in methodology. Improved reporter gene assays used to identify ligands acting on orphan seven-transmembrane receptors. Pharmacol Toxicol. 2003 Dec;93(6):249-58.

36.Schnell S, Schaefer M, Schofl C. Free fatty acids increase cytosolic free calcium and stimulate insulin secretion from beta-cells through activation of GPR40. Mol Cell Endocrinol. 2007 Jan 15;263(1-2):173-80.

37.Tan CP, Feng Y, Zhou YP, Eiermann GJ, Petrov A, Zhou C, Lin S, Salituro G, Meinke P, Mosley R, Akiyama TE, Einstein M, Kumar S, Berger JP, Mills SG, Thornberry NA, Yang L, Howard AD. Selective small-molecule agonists of G protein-coupled receptor 40 promote glucose-dependent insulin secretion and reduce blood glucose in mice. Diabetes. 2008 Aug;57(8):2211-9.

38.Briscoe CP, Peat AJ, McKeown SC, Corbett DF, Goetz AS, Littleton TR, McCoy DC, Kenakin TP, Andrews JL, Ammala C, Fornwald JA, Ignar DM, Jenkinson S. Pharmacological regulation of insulin secretion in MIN6 cells through the fatty acid receptor GPR40: identification of agonist and antagonist small molecules. Br J Pharmacol. 2006 Jul;148(5):619-28.

39.Song F, Lu S, Gunnet J, Xu JZ, Wines P, Proost J, Liang Y, Baumann C, Lenhard J, Murray WV, Demarest KT, Kuo GH. Synthesis and biological evaluation of 3-aryl-3-(4-phenoxy)-propionic acid as a novel series of G protein-coupled receptor 40 agonists. J Med Chem. 2007 Jun 14;50(12):2807-17.

40.Nagasumi K, Esaki R, Iwachidow K, Yasuhara Y, Ogi K, Tanaka H, Nakata M, Yano T, Shimakawa K, Taketomi S, Takeuchi K, Odaka H, Kaisho Y. Overexpression of

GPR40 in pancreatic beta-cells augments glucose-stimulated insulin secretion and improves glucose tolerance in normal and diabetic mice. Diabetes. 2009 May;58(5):1067-76.

41.Tsujihata Y, Ito R, Suzuki M, Harada A, Negoro N, Yasuma T, Momose Y, Takeuchi K. TAK-875, an orally available G protein-coupled receptor 40/free fatty acid receptor 1 agonist, enhances glucose-dependent insulin secretion and improves both postprandial and fasting hyperglycemia in type 2 diabetic rats. J Pharmacol Exp Ther. 2011 Oct;339(1):228-37.

42.Shimabukuro M, Zhou YT, Levi M, Unger RH. Fatty acid-induced beta cell apoptosis: a link between obesity and diabetes. Proc Natl Acad Sci U S A. 1998 Mar 3;95(5):2498-502.

43.Poitout V, Robertson RP. Minireview: Secondary beta-cell failure in type 2 diabetes--a convergence of glucotoxicity and lipotoxicity. Endocrinology. 2002 Feb;143(2):339-42.

44.Robertson RP, Zhang HJ, Pyzdrowski KL, Walseth TF. Preservation of insulin mRNA levels and insulin secretion in HIT cells by avoidance of chronic exposure to high glucose concentrations. J Clin Invest. 1992 Aug;90(2):320-5.

45.Steneberg P, Rubins N, Bartoov-Shifman R, Walker MD, Edlund H. The FFA receptor GPR40 links hyperinsulinemia, hepatic steatosis, and impaired glucose homeostasis in mouse. Cell Metab. 2005 Apr;1(4):245-58.

46.Lan H, Hoos LM, Liu L, Tetzloff G, Hu W, Abbondanzo SJ, Vassileva G, Gustafson EL, Hedrick JA, Davis HR. Lack of FFAR1/GPR40 does not protect mice from high-fat diet-induced metabolic disease. Diabetes. 2008 Nov;57(11):2999-3006.

47.Latour MG, Alquier T, Oseid E, Tremblay C, Jetton TL, Luo J, Lin DC, Poitout V. GPR40 is necessary but not sufficient for fatty acid stimulation of insulin secretion in vivo. Diabetes. 2007 Apr;56(4):1087-94.

48.Kebede M, Alquier T, Latour MG, Semache M, Tremblay C, Poitout V. The fatty acid receptor GPR40 plays a role in insulin secretion in vivo after high-fat feeding. Diabetes. 2008 Sep;57(9):2432-7.

49.Alquier T, Peyot ML, Latour MG, Kebede M, Sorensen CM, Gesta S, Ronald Kahn C, Smith RD, Jetton TL, Metz TO, Prentki M, Poitout V. Deletion of GPR40 impairs glucose-induced insulin secretion in vivo in mice without affecting intracellular fuel

metabolism in islets. Diabetes. 2009 Nov;58(11):2607-15.
50.Kondo K NT, Tomida T, Ezaki K 6: 107-112. Inbred strains resulting from Japanese mice. Bull Exp Animals. 1957(6):107-12.
51.Taketomi S, Ikeda H. KK and KKA^{y} mice. *In Animal Models of Diabetes. A Primer* Shima AAF, Shafrir E, Eds Amsterdam, the Netherlands: Harwood Academic Publishers. 2001:129-42.
52.Hayase M, Ogawa Y, Katsuura G, Shintaku H, Hosoda K, Nakao K. Regulation of obese gene expression in KK mice and congenic lethal yellow obese KKAy mice. Am J Physiol. 1996 Aug;271(2 Pt 1):E333-9.
53.Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia. 1985 Jul;28(7):412-9.
54.Sutton R, Peters M, McShane P, Gray DW, Morris PJ. Isolation of rat pancreatic islets by ductal injection of collagenase. Transplantation. 1986 Dec;42(6):689-91.
55.Magnan C, Collins S, Berthault MF, Kassis N, Vincent M, Gilbert M, Penicaud L, Ktorza A, Assimacopoulos-Jeannet F. Lipid infusion lowers sympathetic nervous activity and leads to increased beta-cell responsiveness to glucose. J Clin Invest. 1999 Feb;103(3):413-9.
56.Roduit R, Nolan C, Alarcon C, Moore P, Barbeau A, Delghingaro-Augusto V, Przybykowski E, Morin J, Masse F, Massie B, Ruderman N, Rhodes C, Poitout V, Prentki M. A role for the malonyl-CoA/long-chain acyl-CoA pathway of lipid signaling in the regulation of insulin secretion in response to both fuel and nonfuel stimuli. Diabetes. 2004 Apr;53(4):1007-19.
57.Hamid YH, Vissing H, Holst B, Urhammer SA, Pyke C, Hansen SK, Glumer C, Borch-Johnsen K, Jorgensen T, Schwartz TW, Pedersen O, Hansen T. Studies of relationships between variation of the human G protein-coupled receptor 40 Gene and Type 2 diabetes and insulin release. Diabet Med. 2005 Jan;22(1):74-80.
58.Ogawa T, Hirose H, Miyashita K, Saito I, Saruta T. GPR40 gene Arg211His polymorphism may contribute to the variation of insulin secretory capacity in Japanese men. Metabolism. 2005 Mar;54(3):296-9.
59.Vettor R, Granzotto M, De Stefani D, Trevellin E, Rossato M, Farina MG, Milan G, Pilon C, Nigro A, Federspil G, Vigneri R, Vitiello L, Rizzuto R, Baratta R, Frittitta L.

Loss-of-function mutation of the GPR40 gene associates with abnormal stimulated insulin secretion by acting on intracellular calcium mobilization. J Clin Endocrinol Metab. 2008 Sep;93(9):3541-50.
60.Unger RH. The physiology of cellular liporegulation. Annu Rev Physiol. 2003;65:333-47.
61.McGarry JD, Dobbins RL. Fatty acids, lipotoxicity and insulin secretion. Diabetologia. 1999 Feb;42(2):128-38.
62.A N. Manipulating the Mouse Embryo: A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring harbor Laboratory Press. 2003.
63.Yaney GC, Korchak HM, Corkey BE. Long-chain acyl CoA regulation of protein kinase C and fatty acid potentiation of glucose-stimulated insulin secretion in clonal beta-cells. Endocrinology. 2000 Jun;141(6):1989-98.
64.Alcazar O, Qiu-yue Z, Gine E, Tamarit-Rodriguez J. Stimulation of islet protein kinase C translocation by palmitate requires metabolism of the fatty acid. Diabetes. 1997 Jul;46(7):1153-8.
65.Offield MF, Jetton TL, Labosky PA, Ray M, Stein RW, Magnuson MA, Hogan BL, Wright CV. PDX-1 is required for pancreatic outgrowth and differentiation of the rostral duodenum. Development. 1996 Mar;122(3):983-95.
66.Boden G. Role of fatty acids in the pathogenesis of insulin resistance and NIDDM. Diabetes. 1997 Jan;46(1):3-10.
67.Hotamisligil GS, Shargill NS, Spiegelman BM. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. Science. 1993 Jan 1;259(5091):87-91.
68.Cohen B, Novick D, Rubinstein M. Modulation of insulin activities by leptin. Science. 1996 Nov 15;274(5290):1185-8.
69.Duttaroy A, Zimliki CL, Gautam D, Cui Y, Mears D, Wess J. Muscarinic stimulation of pancreatic insulin and glucagon release is abolished in m3 muscarinic acetylcholine receptor-deficient mice. Diabetes. 2004 Jul;53(7):1714-20.
70.Gromada J, Hughes TE. Ringing the dinner bell for insulin: muscarinic M3 receptor activity in the control of pancreatic beta cell function. Cell Metab. 2006 Jun;3(6):390-2.
71.Gautam D, Han SJ, Hamdan FF, Jeon J, Li B, Li JH, Cui Y, Mears D, Lu H, Deng C, Heard T, Wess J. A critical role for beta cell M3 muscarinic acetylcholine receptors in

regulating insulin release and blood glucose homeostasis in vivo. Cell Metab. 2006 Jun;3(6):449-61.
72.Sartorius T, Drescher A, Panse M, Lastovicka P, Peter A, Weigert C, Kostenis E, Ullrich S, Haring HU. Mice Lacking Free Fatty Acid Receptor 1 (GPR40/FFAR1) are Protected Against Conjugated Linoleic Acid-Induced Fatty Liver but Develop Inflammation and Insulin Resistance in the Brain. Cell Physiol Biochem. 2015;35(6):2272-84.
73.Schmidt J, Liebscher K, Merten N, Grundmann M, Mielenz M, Sauerwein H, Christiansen E, Due-Hansen ME, Ulven T, Ullrich S, Gomeza J, Drewke C, Kostenis E. Conjugated linoleic acids mediate insulin release through islet G protein-coupled receptor FFA1/GPR40. J Biol Chem. 2011 Apr 8;286(14):11890-4.
74.Sakuma K, Yabuki C, Maruyama M, Abiru A, Komatsu H, Negoro N, Tsujihata Y, Takeuchi K, Habata Y, Mori M. Fasiglifam (TAK-875) has dual potentiating mechanisms via Galphaq-GPR40/FFAR1 signaling branches on glucose-dependent insulin secretion. Pharmacol Res Perspect. 2016 Jun;4(3):e00237.
75.Negoro N, Sasaki S, Mikami S, Ito M, Suzuki M, Tsujihata Y, Ito R, Harada A, Takeuchi K, Suzuki N, Miyazaki J, Santou T, Odani T, Kanzaki N, Funami M, Tanaka T, Kogame A, Matsunaga S, Yasuma T, Momose Y. Discovery of TAK-875: A Potent, Selective, and Orally Bioavailable GPR40 Agonist. ACS Med Chem Lett. 2010 Sep 09;1(6):290-4.
76.Burant CF, Viswanathan P, Marcinak J, Cao C, Vakilynejad M, Xie B, Leifke E. TAK-875 versus placebo or glimepiride in type 2 diabetes mellitus: a phase 2, randomised, double-blind, placebo-controlled trial. Lancet. 2012 Apr 14;379(9824):1403-11.
77.Kaku K, Enya K, Nakaya R, Ohira T, Matsuno R. Efficacy and safety of fasiglifam (TAK-875), a G protein-coupled receptor 40 agonist, in Japanese patients with type 2 diabetes inadequately controlled by diet and exercise: a randomized, double-blind, placebo-controlled, phase III trial. Diabetes Obes Metab. 2015 Jul;17(7):675-81.