Study on genes for the onset of type 2 diabetes associated with obesity using new animal models

Daiki Sasaki

Department of Biotechnology
Division of Engineering
Kyoto Sangyo University

Contents

General Introduction **1**

Abbreviation **4**

Chapter1

A new animal model possessing one diabetogenic QTL （quantitative trait loci） and an obese locus reveals to confound hyperglycemia and insulin resistance

1-1. Introduction **6**

1-2. Materials and Methods **8**

1-2-1. Animals 8

1-2-2. Strain selection by PCR 8

1-2-3. Histologic examination 9

1-2-4. Oral glucose tolerance test (OGTT) 9

1-2-5. Insulin tolerance test (ITT) 9

1-2-6. Metabolic assays 10

1-2-7. Isolation of islets from rat pancreas 10

1-2-8. Statistical Analysis 10

1-3. Results **12**

1-3-1. New development of the rat strains 12

1-3-2. Characteristics of the rat strains 13
1-3-3. Metabolic assays 14
1-3-4. Glucose intolerance in F344-*fa-nidd2* strain 15
1-3-5. Plasma insulin level and insulin tolerance test (ITT) 16
1-3-6. Islet study 16
1-4. Discussion 18

Chapter 2

New animal models reveal that coenzyme Q2 (*Coq2*) and placenta-specific 8 (*Plac8*) are candidate genes for the onset of type 2 diabetes associated with obesity in rats
2-1. Introduction 21
2-2. Materials and Methods 23
2-2-1. Sample collection 23
2-2-2. Quantitative real-time PCR (RT-qPCR) 23
2-2-3. Western blot 23
2-2-4. DNA sequence analysis 24
2-2-5. Statistical Analysis 24
2-3. Results 26
2-3-1. Screening to identify diabetogenic genes associated with obesity in the *Nidd2*/*of* QTL region. 26
2-3-2. Obesity-specific gene expresson 27
2-3-3. Western blotting of COQ2 and PLAC8 in the livers of obese strains 27

2-3-4. DNA sequence analysis of Coq2 and Plac8 in F344-fa and F344-fa-nidd2 rat strains 27

2-4. Discussion **29**

Tables **35**

Figures and Legends **39**

References **52**

General Introduction

Type 2 diabetes mellitus, which is a major public health problem in many countries with about 380 million people affected in the world, is a phenotypically and genetically heterogeneous disease. It is a multifactorial and polygenic disorder except early-onset monogenic forms of type 2 diabetes including maturity-onset diabetes of the young (1). Genetic predisposition is a major risk factor for type 2 diabetes as indicated by numerous studies showing higher concordance rates in monozygotic than dizygotic twins (2, 3), and correlation between risk of diabetes and ethnic ancestry in admixed populations in a common environment (4, 5). Moreover, environmental factors superimposed on genetic susceptibility are involved in the development of type 2 diabetes. In particular, obesity has been known as a major risk factor for type 2 diabetes in human, and explains the increased prevalence of the disease observed during this century (6, 7). It is associated with 70% of all patients suffering from type 2 diabetes (8) and in many patients, type 2 diabetes can be largely controlled by weight reduction (9), indicating the integral relationship between obesity and type 2 diabetes.

Genetic predisposition and obesity are likely to interact in some etiological steps for the development of type 2 diabetes (10). However, the mechanisms of this interaction remain unknown. It is plausible that understanding the relationship between an individual locus responsible for type 2 diabetes and obesity is important as a first step for elucidation of the mechanisms of the complex interaction. The use of inbred animal models of obese type of type 2 diabetes is an essential component of investigation

for this relationship using the strategy of molecular and statistical genetics. The Goto-Kakizaki (GK) rat revealed the genetic basis of a non-obese model of type 2 diabetes (11, 12), but this rat model cannot be used to address the interaction between genetic susceptibility to type 2 diabetes and obesity.

The Otsuka Long-Evans Tokushima Fatty (OLETF) rat has been established by selective breeding based on impaired glucose tolerance (13). The OLETF rat develops late onset hyperglycemia, a chronic course of disease, mild obesity, hyperplastic foci of pancreatic islets and renal complication, and has been considered as one of the best models for human type 2 diabetes with mild obesity. Prevention of obesity by exercise training (14) or a calorie-restricted diet (15) can prevent type 2 diabetes almost completely in the OLETF rat as in many humans with type 2 diabetes, indicating that obesity is an important risk factor for type 2 diabetes development in this rat. Thus the OLETF rat is useful for examining the interaction between genetic susceptibility to type 2 diabetes and obesity.

Traditional genetic analysis in the OLETF have been based on mapping QTL (Quantitative Trait Loci) using microsatellite markers, followed by genetic isolation of QTL (Quantitative Trait Loci) in congenic strains (16, 17). Although the molecular characterization of the loci are yet to come, the careful characterization of the congenic rats have provided some useful insight into the potential mechanisms in which the causative mutations operate (18). A double congenic rat strain having one diabetogenic QTL (Quantitative Trait Loci) and a obese locus designated F344-*fa-nidd2,* which was generated by crossing F344-*nidd2* and F344-*fa* congenic rats, showed that the single *Nidd2/of* QTL (Quantitative Trait

Loci) was sufficient to induce severe hyperglycaemia after glucose loading in under condition of obesity (19). These findings support the conclusion that the *Nidd2/of* QTL (Quantitative Trait Loci) region includes a strong diabetogenic gene associated with obesity. In the present study, I compared the expression of genes for *Nidd2/of* QTL (Quantitative Trait Loci) region between the F344-*fa* and F344-*fa-nidd2* strains to identify candidate genes related to diabetes associated with obesity and found two candidate genes.

Abbreviation

ANOVA : analysis of variance

AUC : area under the curve

Abcg3l3 : ATP-binding cassette, subfamily G, member 3-like 3

Coq10 : coenzyme Q-binding protein COQ10

Coq2 : coenzyme Q2 (4-hydroxybenzoate polyprenyltransferase)

F344 rat : Fischer344 rat

GWAS : genome-wide association studies

Gapdh : Glyceraldehyde-3-phosphate dehydrogenase

Hpse : heparanase

ITT : insulin tolerance test

Lepr$^{-/-}$ locus : the ZuckerFatty-derived leptin rreceptor mutation

Mapk10 (JNK3) : mitogen-activated protein kinase 10

NEFA : non-esterified fatty acids

OGTT : oral glucose tolerance test

OLETF rat : Otsuka Long-Evans Tokushima Fatty rat

Plac8 (Onzin) : placenta-specific 8

QTLs : quantitative trait loci

T2DM : type 2 diabetes mellitus

TCHO : total cholesterol

TG : triglycerides

Chapter 1

A new animal model possessing one diabetogenic QTL (Quantitative Trait Loci) and an obese locus reveals to confound hyperglycemia and insulin resistance

1-1. Introduction

Type 2 diabetes mellitus (T2DM) has become a major public health problem all over the world. Environmental and behavioral factors such as lifestyle, rich nutrition and obesity have been heavily involved for onset of type 2 diabetes (20). Obesity is particularly associated with development of type 2 diabetes (21, 22). Furthermore, in part, type 2 diabetes is inherited. Family studies revealed that people with positive family history of type 2 diabetes are likely to develop the type 2 diabetes than people without it. Obesity and genetic susceptibility are major risk factor associated with type 2 diabetes (23). However, little is known about how these factors relate for onset of type 2 diabetes. Many genes responsible for type 2 diabetes or obesity have been identified in recent genome–wide association studies (24, 25, 26, 27, 28), but only a few loci are common between obesity and type 2 diabetes (28). Because it is difficult to directly search for diabetogenic genes associated with obesity and to investigate the molecular mechanisms of the induction of type 2 diabetes in obese human, use of inbred animal models is an essential component of genetic investigations (29).

The Otsuka Long-Evans Tokushima Fatty (OLETF) rat serves as a genetic animal model of late-onset hyperglycaemia, insulin resistance and renal complications associated with mild obesity that are very similar to

those observed in human with type 2 diabetes (13). The strategic development of new animal models using the OLETF rat has served as a crucial component of investigations of the influence of obesity on the onset of type 2 diabetes. For example, 14 hyperglycaemic quantitative trait loci (QTLs) were identified by intercrossing OLETF and F344 rats (16, 30, 31). Subsequently, congenic strains for each hyperglycaemic QTL (Quantitative Trait Loci) were constructed by introgressing the OLETF-derived QTL (Quantitative Trait Loci)s into the F344 background (17). OLETF rats develop type 2 diabetes with weight gain ; thus, I further generated obese congenic rats by introgressing the ZuckerFatty-derived leptin receptor mutation (*Lepr*$^{-/-}$ locus) into F344 rats (designated F344-*fa*) (19, 32). These congenic strains make it possible to search for QTL (Quantitative Trait Loci)s that exhibit strong hyperglycaemia under conditions of obesity using homozygotes of double congenic rats generated by crossing each hyperglycaemic QTL (Quantitative Trait Loci) congenic with the obese congenic line.

In this study, I looked at *Nidd2/of* QTL (Quantitative Trait Loci) locus. *Nidd2/of* QTL (Quantitative Trait Loci) locus on rat chromosome 14 is located on the p-arm telomere site in the interval bounded by the markers *D14Rat23* and *D14Mit1* (approximately 12 Mbp) (16). In this chapter, I described that I generated double congenic obese rats strain introgrossed *Nidd2/of* QTL (Quantitative Trait Loci) locus and *Lepr*$^{-/-}$ locus into F344 rats and investigated effects of *Nidd2/of* QTL (Quantitative Trait Loci) locus to onset of type 2 diabetes under obese condition.

1-2. Materials and Methods

1-2-1. Animals

All rats were maintained under specific pathogen-free conditions in the animal facility of Kyoto Sangyo University. The rats were housed in plastic cages containing sterilized woodchips for bedding in air-conditioned rooms at 23℃ ± 2℃ and 55% ± 10% humidity under a 12-h light/dark cycle (7:00-19:00 h). The rats had free access to tap water and standard laboratory chow (MF ; Oriental Yeast Co., Tokyo, Japan). The Institutional Animal Care and Use Committee of Kyoto Sangyo University approved the Protocols for animal care and experimentation.

Male inbred Fisher-344/Slc (F344) rats purchased from SLC Inc. (Shizuoka, Japan) were used in this experiment. The F344-*fa* congenic strain with the *fa*/*fa* locus ($Lepr^{-/-}$) (original name : F.ZF-*Lepr*) was used as the obese control (19). Because *fa*/*fa* rats are sterile, the heterozygotes were maintained by selecting them from each generation in our animal facility. The F344-*nidd2* congenic strain with the *Nidd2*/*of* QTL (Quantitative Trait Loci) (original name : F.O-*Nidd2*/*of*) was also bred in our animal facility (17). The F344-*fa*-*nidd2* double congenic strain with the fa/fa and *Nidd2*/*of* loci (original name : F.ZF-*Lepr&Nidd2of*) was generated by crossing the F344-*fa* and F344-*nidd2* starins and then selecting *fa*/*fa*-*Nidd2*/*of* homozygotes. The F344 and F344-*nidd2* rats are lean strains, whereas the F344-*fa* and F344-*fa*-*nidd2* rats are obese strains.

1-2-2. Strain selection by PCR

Since the F344-*fa* rats were sterile, F344-*fa* rats were maintained by heterozygote of *Lepr* gene. In order to determine their genotypes, Lepr

gene was amplified by PCR using a specific primer for Lepr, and then the products were treated by *MspI* restriction enzyme, Moreover, specific primers for *Nidd2/of* QTL (Quantitative Trait Loci) region were used to avoid contamination of other segment by crossover in the region (Table 1).

1-2-3. Histologic examination

The tissues were fixed in 10% phosphate-buffered formalin, embedded in paraffin blocks, sectioned in 3-μm thickness and stained with hematoxylin and eosin.

1-2-4. Oral glucose tolerance test (OGTT)

At 20 weeks of age, OGTT was performed by injecting glucose (2g/kg in a 2.8M solution) in overnight-fasted rats. Blood samples were obtained from the tail veins at 0 (fasting), 30, 60, 90 and 120 min. Blood glucose levels were determined directly using the glucose oxidase method with Glutest Neo test strips (Sanwa Chemical Co. Nagoya, Japan). The area under the curve (AUC) was calculated according to the trapezoid rule from the glucose measurements at each time and is expressed as mg/dl x min. The blood samples were collected from tail veins using heparinized capillary tubes and then centrifuged to obtain plasma. Plasma insulin levels were determined using an ELISA kit that detects rat insulin (Shibayagi Co., Ltd, Shibukawa, Japan).

1-2-5. Insulin tolerance test (ITT)

The insulin tolerance test (ITT) was performed by injecting human insulin (1 U/kg, Humulin R, Eli Lilly, Indianapolis, Ind., USA) intraperitoneally into rats (16 weeks of age) after overnight fasting (33).

Blood glucose levels at 0 (fasting), 30, 60, 90, 120 min were measured directly as described above.

1-2-6. Metabolic assays

Body weights and abdominal fat weights were determined. The serum levels of total cholesterol (TCHO), triglycerides (TG), non-esterified fatty acids (NEFA) were determined using T-Cho-E test kit, TG-E test kits and NEFA-C test kits respectively from Wako Chem. Ltd. Osaka, Japan.

1-2-7. Isolation of islets from rat pancreas

Animals were anesthetized with a mixture of 3% isoflurane in oxygen using a vaporizer. Then islet isolation was carried out as described by Li et al. (34) with slight modification. The bile duct was cannulated by 23G needle with 10 ml of cold Hnak's balanced solution (HBSS, Sigma-Aldrich, Saint Louis, Missouri, USA) containing 0.1 mg/ml of collagenase XI (Sigma-Aldrich, Saint Louis, Missouri, USA). After removing the pancreas and placing it in a 50 ml tube, the tube was placed in a water bath at 37 °C for 23 min. Digestion was stopped by addition of supplemented RPMI 1640 at 11 mM glucose with 1% BSA. Islet purification was performed by using micro-capillary selecting and sucking method to isolate the islets under a microsope, same as an embryo transfer protocol.

1-2-8. Statistical Analysis

Data are presented as the mean ± SEM. The statistical significance of differences was evaluated using the Student unpaired *t* test for comparing two groups and one-way ANOVA with a posthoc test (Scheffe's test) for

comparing three or more groups (StatView, SAS Institute, Inc.). A value of $p<0.05$ was defined as statistically significant.

1-3. Results

1-3-1. New development of the rat strains

Nidd2/of QTL (Quantitative Trait Loci) locus was identified in OLETF rats and located on chromosome 14. In our previous study, F344-*nidd2* single congenic rat introgressed *Nidd2/of* QTL (Quantitative Trait Loci) locus into F344 rat showed bit higher blood glucose levels than F344 rats, but its levels were not sufficiently higher for a study of diabetes. The original diabetic animal model, OLETF rats showed diabetic features only under condition of obesity. Because F344-*nidd2* single congenic rats were lean animals, these animals did not showed hyperglycemia. In order to investigate the effect of *Nidd2/of* QTL (Quantitative Trait Loci) locus under obese condition, I generated the single obese congenic strain (F344-*fa* rat) introgressed leptin receptor-deficient locus ($Lepr^{-/-}$) into F344 rats. Furthermore, to generate the double congenic strain having both the *Nidd2/of* QTL (Quantitative Trait Loci) locus and $Lepr^{-/-}$ locus (F344-*fa-nidd2* rats), I crossed F344-*fa* and F344-*nidd2* single congenic rats. These offspring were selected by PCR with specific primers (see, Material and Methods). All strains used in this study were shown in Fig. 1. F344 and F344-*nidd2* strains were lean, while F344-*fa* and F344-*fa-nidd2* strains were obese, though the body length of F344-*nidd2* and F344-*fa-nidd2* strains showed a bit shorter than their control strains, F344 and F344-*fa*, respectively.

However, F344-*fa* and F344-*fa-nidd2* obese congenic strains were sterile. I maintained both strains with heterozygotes of leptin receptor deficient locus ($Lepr^{+/-}$) for generating the $Lepr^{-/-}$ homozygotes. When I crossed heterozygotes to generate homozygotes, I could get three types of

genotypes; +/+, +/-, and -/-. I needed to identify the genotype of *Lepr* gene. Since the *Lepr* gene deficiency is missence mutation, which mutate 806A (CAG) to C (CCG) in leptin receptor gene (32). I used the specific primer containing this region (Table 1) for PCR primers. After PCR amplification, *MSP1* restriction enzyme was used to recognize the CCGG sequence of *Lepr* mutation and to cleave this sequence. As shown in Fig. 2A, the wild type homozygotes ($Lepr^{+/+}$) have heavier molecular band compared with $Lepr^{-/-}$ derived from F344-*fa* rats. In the heterozygotes ($Lepr^{+/-}$), two bands were recognized. Homozygote mutation ($Lepr^{-/-}$) and heterozygotes mutation ($Lepr^{+/-}$) were selected by this methods, and then maintained these strains. Genotype of *Nidd2/of* region in F344-fa-nidd2 rats was also screened by using some microsatellite markers specific for the *Nidd2/of* QTL (Quantitative Trait Loci) region to avoid an insertion of another DNA segment by crossing over: *D14Wox1, D14Wox14, D14Rat8, D14Rat12* and *D14Rat23* (Fig. 2B).

1-3-2. Characteristics of the rat strains

The body weight of F344-*nidd2* single congenic rats were significantly lower than that of the F344 rats after 12 weeks of birth ($p > 0.001$) (Fig. 3A). Furthermore, the body weight of F344-*fa-nidd2* double congenic rats were significantly lower than that of F344-*fa* after 10 weeks of birth ($p > 0.05$) (Fig. 3A). However no significant difference in food intake was observed between the F344 and F344-*nidd2* lean rats (95.25 g/week ± 11.02 vs 109.77 g/week ± 13.61: $p = 0.59$) or between the F344-*fa* and F344-*fa-nidd2* obese rats (169.31 g/week ± 5.21 vs 164.3 g/week ± 2.82: $p = 0.45$) at 14 weeks of age (Fig. 3B). No significant difference in body mass index (BMI) was also observed between the F344

and F344-*nidd2* lean rats (0.48 ± 0.0076 vs 0.5 ± 0.0089: $p = 0.26$) or between the F344-*fa* and F344-*fa-nidd2* obese rats (0.73 ± 0.0087 vs 0.72 ± 0.011: $p = 0.38$) at 13 weeks of age (Fig. 4). It should be noted that the BMI was identical between F344-*fa* and F344-*fa-nidd2* rats, in spite of the large difference of their body weights between F344-*fa* and F344-*fa-nidd2* rat. The main reason may be due to a shorter body length in F344-*fa-nidd2* rat than in F344-*fa* rat,

1-3-3. Metabolic assays

The adiposity index of mesenteric fat pad was not significant difference between F344 and F344-*nidd2* single congenic lean rats ($p = 0.2$) (Table 2). However, F344-*fa-nidd2* double congenic rats were significantly lower levels compared with F344-*fa* obese control rats ($p < 0.05$). The adiposity index of retroperitoneal fat pad was not significant difference between F344 and F344-*nidd2* single congenic lean rats ($p = 0.77$). However, F344-*fa-nidd2* double congenic rats were significantly lower levels compared with F344-*fa* obese control rats ($p < 0.01$). The adiposity index of epididymal fat pad was significantly lower in F344-*nidd2* single congenic lean rats compared with F344 control rats ($p < 0.05$). No significantly difference was observed between F344-*fa* and F344-*fa-nidd2* double congenic obese rats ($p = 0.13$). Non-esterified free fatty acid (NEFA), triglyceride (TG) and total cholesterol (TCHO) were measured, because abdominal fat pad was lower in F344-*nidd2* and F344-*nidd2-fa* strains compared with F344 lean and F344-*fa* obese control strains, respectively. NEFA ($p = 0.27$) and TCHO ($p = 0.65$) were no significant difference between F344 and F344-*nidd2* single congenic strains. However, TG level was significantly lower in F344-*nidd2* single

congenic strain than in F344 control stains ($p < 0.05$). In obese strains rat, TG ($p < 0.01$) and TCHO ($p<0.01$) were significantly lower in F344-*fa-nidd2* double congenic obese strains rat compared with F344-*fa* obese control rat. However, NEFA was no significant difference between F344-*fa* and F344-*fa-nidd2* double congenic obese strain ($p = 0.11$). Although several metabolic parameters showed the significant differences between F344-*fa* and F344-*fa-nidd2* strains, both of strains showed hepatic steatosis with almost same grade (Fig.5). From the results of BMI, NEFA value and histological point of view, in spite of the difference of body weight between two strains, F344-*fa* rat is considered as the control rat of F344-*fa-nidd2* rat.

1-3-4. Glucose intolerance in F344-*fa-nidd2* strain

The blood glucose levels of lean F344 and F344-*nidd2* single congenic rats were normal, with no significant difference between them (Fig. 6a). In contrast, the glucose levels of the obese F344-*fa* control and F344-*fa-nidd2* double congenic rats were significantly higher than those of the lean F344 and F344-*nidd2* rats, respectively, at all time points after glucose loading. Moreover, blood glucose levels were significantly elevated in F344-*fa-nidd2* double congenic rats compared with F344-*fa* rats at 60, 90 and 120 min after glucose loading ($p < 0.0001$ vs F344-*obese*).

From the AUC values, there was no significant difference between the glucose levels of lean F344 and F344-*nidd2* rats. In contrast, there was a significant difference ($p < 0.0001$) in the glucose AUC between obese F344-*fa* and F344-*fa-nidd2* rats (Fig. 6B). These results indicate that the F344-*fa-nidd2* double congenic rats developed severe glucose intolerance.

1-3-5. Plasma insulin level and insulin tolerance test (ITT)

The serum insulin levels of F344 and F344-*nidd2* single congenic strain were no significant difference (Fig. 7A). While the plasma insulin levels of F344-*fa-nidd2* double congenic rats were significantly higher than those of F344-*fa* control rats after fasting (14.68 ± 1.85 ng/ml vs 29.05 ± 1.59 ng/ml: $p < 0.001$), and 90min (23.99 ± 3.02 ng/ml vs 37.49 ± 2.64: $p < 0.01$), after glucose loading (Fig. 7A). These results suggest that the F344-*fa-nidd2* rats were insulin resistant.

I performed insulin tolerance test (ITT) to determine whether the f344-*fa-nidd2* rats developed insulin resistance. As shown in Fig. 7B, the glucose-lowering effect by intraperitoneal administration of insulin was almost same levels between F344 and F344-*nidd2* lean rats, while the obese strain, F344-*fa* rats showed lower level for glucose-lowering effect than the lean F344 rats, and there was the significant difference between F344 and F344-*fa* strains at 0, 90 and 120 min, suggesting that obesity is necessary to induce insulin resistance. However, despite the lower body weight gain, the hypoglycaemic response to insulin at 30, 60 and 120 min after insulin injection was significantly lower in F344-*fa-nidd2* rats than in F344-*fa* rats. These results indicate severe insulin resistance in F344-*fa-nidd2* rats and show that insulin resistance requires a genetic component as well as obesity. Furthermore, the results indicate that the *Nidd2/of* QTL (Quantitative Trait Loci) region in F344-*fa-nidd2* rats includes a diabetogenic gene responsible for obesity.

1-3-6. Islet study

As shown in Fig 8A, islets were isolated from rat pancreas by bile

duct perfusion and collagenase digestion as described by Methods. Islets were almost intact. Islet size was significantly larger in F344-*nidd2* and F344-*fa-nidd2* than their control lines, F344 and F344-*fa*, respectively (Fig. 8B).

After isolation, they were cultured floating in RPMI at 11 mM glucose concentration and studied within 2 days. The islets were then pretreated in RPMI at 3 mM glucose for 1 hour and then the same medium was added and incubated at 37°C for another 1 hour after removing the old medium. The culture medium was harvested and stored and then added RPMI at 20 mM glucose medium and cultured for 1 hour. The medium was harvested after 1 hour incubation. Insulin concentrations of both medium (3 mM glucose and 20 mM glucose) were measured by insulin ELISA kit. As shown in Fig. 9, F344-*fa-nidd2* double congenic strain was significantly higher in insulin production than F344-*fa* single obese congenic strain at both 3 mM (8.9 ± 1.63 vs 20.26 ± 2.12: $p < 0.01$) and 20 mM (97.08 ± 4.9 vs 171.45 ± 23.74: $p < 0.05$) glucose concentration. These data suggest that Nidd2/of QTL (Quantitative Trait Loci) region includes genes which induce hyperglycemia and hyperinsulinemia.

1-4. Discussion

Until now, several obese and type 2 diabetes animals were reported as human disease models. KK-Ay obese mice having agouti-yellow gene (35) and animal models of obese type 2 diabetes introgressed leptin or leptin receptor deficient gene (*Lepr*$^{-/-}$) (36, 37, 38, 39) were proposed. However, it is difficult to search directly for diabatogenic genes associated with obesity using these animal models, because these animals include many diabetogenic genes themselves. To resolve such problems, separation of multigenes QTLs (Quantitative Trait Loci) into single QTL (Quantitative Trait Loci) was essential. I have planed a new strategy to make it possible to investigate a diabetogenic gene associated with obesity. Namely, I made several congenic lines which have one hyperglycemic QTL (Quantitative Trait Loci) (*Nidd/of*) in F344 strain, and an obese congenic line introgressed *Lepr*$^{-/-}$ gene into F344. I crossed the *Nidd/of* congenic with the obese congenic to make a strain which has both hyperglycemic gene and obese gene. In that process, I used the OLETF rat as the resorce of hyperglycemic QTLs (Quantitative Trait Loci), because OLETF rat is known as an excellent animal model of human type 2 diabetes associated with obesity (13). 14 loci were identified in OLETF rat (17, 30, 40). 14 congenic lines intorogressed each diabetogenic locus into lean F344 rat was already generated (17). Furthermore, obese congenic rat intorogressed leptin receptor deficient locus into lean F344 rat was also generated (19). Obese double congenic rat having each diabetogenic locus and leptin deficient locus was generated. It was possible to search effects of each diabetogenic locus under condition of obesity. In the present study, I propose the new strategic animal models that can analyze the diabetogenic

QTL (Quantitative Trait Loci) (*Nidd2/of*) associated with obesity.

F344-*fa-nidd2* double congenic strain was significantly lower body weight compared with F344-*fa* single obese congenic strain. This was due to a gene effect originated from *Nidd2/of* QTL (Quantitative Trait Loci) itself, because F344-*nidd2* was already smaller than F344 in the body weight. However, BMI values showed insignificant difference between them. Therefor, I regarded that F344-*fa* single obese congenic strain was an excellent obese control for F344-*fa-nidd2* double congenic strain.

F344-*fa* single obese strain introgressed leptin receptor deficient locus into F344 showed the hyperglyceamia compared with F344 lean control strain rats. I should remark that the blood glucose levels of F344-*fa* single obese strain rats showed over 140mg/dl after 120min glucose loading. This data indicates that obese control strain becomes prediabetic condition but not type 2 diabetes. The blood glucose levels of F344-*fa-nidd2* double congenic strain rats exhibited a remarkable hyperglyceamia with approximately 240 mg/dl 120min after glucose loading, and this levels could diagnose with type 2 diabetes. These data strongly indicates that *Nidd2/of* QTL (Quantitative Trait Loci) locus can develop type 2 diabetes under condition of obesity. Furthermore, I are searching similarly *Nidd1/of* and *Nidd4/of* QTL (Quantitative Trait Loci) locus under condition of obesity, but even if these congenic strain rats become obese, the blood glucose levels did not increased as I had expected (unpublished data).

In summary, I generated the new strategic animal model for onset of type 2 diabetes associated with obesity. Single lean congenic strain intorogressed *Nidd2/of* QTL (Quantitative Trait Loci) locus located on rat chromosome 14 were insignificant difference compared with F344 lean

control. However, we first showed that F344-*fa-nidd2* double congenic strain having both leptin deficient locus and *Nidd2*/*of* QTL (Quantitative Trait Loci) locus showed remarkable hyperglyceamia, hyperinsulineamia and higher insulin resistance compared with F344-*fa* obese control rats. These data clearly indicated that diabetogenic genes associated with obesity were existed in *Nidd2*/*of* QTL (Quantitative Trait Loci) locus.

Chapter 2

New animal models reveal that coenzyme Q2 (*Coq2*) and placenta-specific 8 (*Plac8*) are candidate genes for the onset of type 2 diabetes associated with obesity in rats

2-1. Introduction

Obesity is associated with the development of type 2 diabetes mellitus (21), and genetic predisposition and obesity are the major risk factors for this disease. Interactions between these two factors possibly contribute to the onset of type 2 diabetes; however, the gene(s) or mechanisms involved are poorly understood. In fact, it had been impossible to map a locus about multiple genes on a chromosomal region before the discovery of microsatellites DNA markers (41) and the development of the microsatellite DNA markers in rodents (42) and human (43). The microsatellites made it possible to map many quantitative trait loci (QTLs), such as asthma (44, 45, 46), obesity (47, 48, 49), hypertension (50, 51, 52) and diabetes (16, 53, 54, 55, 56, 57), on chromosome region with several computer softwares for mapping such as Mapmaker/QTL (Quantitative Trait Loci) (58) and Map Manager QTX (59). QTL (Quantitative Trait Loci) analysis could map many quantitative trait loci on their chromosomes, however, it only mapped the locus as the region. It could not determine the locus as a single gene. Namely, one QTL (Quantitative Trait Loci) generally included more than 50 to 100 genes in the QTL (Quantitative Trait Loci) region. It was very hard to decide a causative gene from the

QTL (Quantitative Trait Loci) region. So, many genes responsible for type 2 diabetes or obesity have been identified in recent genome-wide association studies (GWAS) (24, 25, 26, 27, 28). However, it has been also hard to determine a diabetogenic gene associated with obesity in human by the GWAS. As it was also hard to determine a causative gene from the QTL (Quantitative Trait Loci) region in animal models of human disease such as diabetes. This was the reason why I have developed new animal models with a double congenic strategy having obesity and the individual *Nidd/of* locus from the OLETF rat, because OLETF rat only showed diabetes when the strain reached the body weight of more than 600 g. Then, I could define a locus (loci) raising blood glucose level abnormally under condition of obesity after glucose loading, as comparing the mRNA expression of all genes in *Nidd2/of* QTL (Quantitative Trait Loci) region between double congenic and single obese control.

As described in Chapter 1, the F344-*fa-nidd2* double congenic rat showed extremely higher plasma glucose levels compared with the F344-*fa* obese control rat, clearly indicating that *Nidd2/of* QTL (Q Quantitative Trait Loci locus include a diabetogenic gene (genes). The *Nidd2/of* locus on chromosome 14 is located on the p-arm telomere site in the interval bounded by the markers *D14Rat23* and *D14Mit1* (approximately 12 Mbp) (16). Approximately 60 genes reside in the *Nidd2/of* QTL (Quantitative Trait Loci) region. In the present study, I compared the expression of the genes between the F344-*fa* and F344-*fa-nidd2* strains to identify candidate genes related to diabetes associated with obesity and found two candidate genes.

2-2. Materials and Methods

2-2-1. Sample collection

At 25 weeks, rats were decapitated after overnight fasting or at 60 min after administering glucose. The liver and skeletal muscle were dissected and stored at -80°C. The retroperitineal, mesenteric and epididymal fat pads were removed and weighted. The adiposity index was calculated from each fat pad and body weight (percentage of fat pad weight/body weight).

2-2-2. Quantitative real-time PCR (RT-qPCR)

Liver, fat and muscle were homogenized using ISOGEN II regent (Wako Pure Chemical industries Ltd. Osaka, Japan), and RNA was obtained from each tissue using ethanol precipitation methods. RT-qPCR reactions were performed using Fast SYBR Green Master Mix (Applied Biosystems, Tokyo, Japan), and a calibration curve method was used to analyze the data. The cDNA sequences were acquired from the genome database of the United States National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Primers were designed using Primer3Plus (http://primer3plus.com) and Amplify-3 (http://engels.genetics.wisc.edu/amplify/) computer software. The relative expression levels were compared by normalization to the expression levels of Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) (Takara Bio Inc. Ohtu, Japan).

2-2-3. Western blot

Western blot analysis was performed as described by Towbin et al.

(60) with slight modifications. Tissue homogenates were centrifuged and the supernatants were subjected to electrophoresis in 15% SDS-PAGE. The proteins separated in the gel were transferred electrophoretically to a polyvinyl difluoride (PVDF) membrane sheet (Immobilon-P, Millipore Co., Billerica, USA), which was blocked with 5% nonfat dry milk/0.1% Tween 20 in TBS. After washing, the membrane was incubated with antibodies against COQ2 (ARP47180-P050, Aviva Systems Biology Co., San Diego, USA), PLAC8 (12284-1-AP, Protein Tech Inc., Chicago, USA), or β-actin (GTX 629630, Gene Tex Inc., Los Angeles, USA) in 5%nonfat dry milk/0.1% Tween 20 in TBS. Antigen-antibody complexes were detected using peroxidase conjugated secondary antibodies (SC-3837, Santa Cruz Biotechnology, Inc., Texas, USA and 474-1806, Kirkegaard & Perry Laboratories, Inc., Washington, DC, USA). Band intensities were analyzed using a Molecular Imager ChemiDoc XRS + (Bio-Rad Laboratories, Inc., Berkeley, USA).

2-2-4. DNA sequence analysis

Genomic DNA was extracted from the tail tissue, and RNA was extracted from liver, femoral skeltal muscle, and epididymal fat of the rat strains. Specific Primers were designed using Primer3Plus. PCR amplification was performed with KOD-Plus-Ver.2 (Toyobo Co., Ltd., Japan). Cycle sequence PCR was performed using a Big Dye Terminator v3.1 Cycle Sequence Kit (Applied Biosystems by Life Technologies, Tokyo, Japan). DNA sequencing was performed using a 3500 Genetic Analyzer (Applied Biosystems by Life Technologies, Tokyo, Japan).

2-2-5. Statistical Analysis

Data are presented as the mean ± SEM. The statistical significance of differences was evaluated using the Student unpaired *t* test for comparing two groups and one-way ANOVA for comparing three or more groups (StatView, SAS Institute, Inc.). A value of $p < 0.05$ was defined as statistically significant.

2-3. Results

2-3-1. Screening to identify diabetogenic genes associated with obesity in the *Nidd2/of* QTL (Quantitative Trait Loci) region.

The *Nidd2/of* QTL (Quantitative Trait Loci) on chromosome 14 peaks near *D14Rat3* (10.67Mbp) and linked to high levels at 60 min after glucose challenge in OLETF rats (16, 31). Because the 1.5-LOD support intervals were within the 95% confidence intervals (61), I used RT-qPCR to analyze the expression levels of genes between the *D14Rat23* and *D14Mit1* markers in the F344-*fa* and F344-*fa-nidd2* strains. I found that approximately 60 genes were expressed in liver. In Table 3, we generated the specific primers for approximately 60 genes expressed in liver using Prier3Plus and Amplify3 computer software. Given that high glucose levels were induced in overnight-fasted F344-*fa-nidd2* rats after glucose loading, I determined whether there were any genes within the region bounded by the markers *D14Rat23* and *D14 Mit1* that were differentially expressed in the livers of obese control F344-*fa* and F344-fa-*nidd2* rats. As a result, I detected significant differences in the mRNAs levels of five genes: *Abcg3l3* (6.04-6.08 Mbp, p = 0.0062), *Mapk10* (8.05-8.34 Mbp, p = 0.00088), *Hpse* (10.48-10.52 Mbp, p = 00119), *Coq2* (10.52-10.52 Mbp, p = 0.00122), and *Plac8* (10.64-10.66 Mbp, p = 0.00387) (Fig. 10).

2-3-2. Obesity-specific gene expresson

To examine whether the differential expression of five genes was specific for the obesity phenotype, I compared the mRNA levels between lean F344 and F344-*nidd2* rat strains under fasting and postprandial conditions. As shown in Fig. 11A-C, the *Abcg3l3*, *Hpse*, and *Mapk10* genes

were significantly differentially expressed in the liver of lean F344 vs F344-*nidd2* rat strains, respectively, as well as in obese F344-*fa* vs F344-*fa-nidd2* rat strains, respectively. This result clearly indicated that the expression of these genes was not specific for obesity; therefore, I ruled them out as candidate genes. In contrast, the levels of *Coq2* and *Plac8* mRNAs were significantly different in the livers of obese F344-*fa* vs F344-*fa-nidd2* rat strains, but were not significantly different ($p = 0.79$ and $p = 0.45$, respectively) between lean F344 and F344-*nidd2* rat strains (Fig. 12A, B), indicating that the differential expression of these responds specifically to obesity. Therefore, I focused on *Coq2* and *Plac8* as candidate mediators of the onset of type 2 diabetes in obese rats.

2-3-3. Western blotting of COQ2 and PLAC8 in the livers of obese strains

COQ2 protein levels were significantly lower in F344-*fa-nidd2* rat strains than F344-*fa* control rat strains under both fasting ($p = 0.023$) and postprandial conditions ($p = 0.000012$) (Fig. 13A, B), reflecting the relative mRNA expression levels of *Coq2*. As expected from the mRNA expression levels of *Plac8*, there was no significant difference in PLAC8 contents under fasting conditions between F344-*fa* and F344-*fa-nidd2* rat strains. Whereas the levels of PLAC8 were significantly reduced under postprandial conditions in F344-*fa-nidd2* rat strains compared with that in F344-*fa* rat strains ($p = 0.019$) (Fig. 13C, D).

2-3-4. DNA sequence analysis of Coq2 and Plac8 in F344-fa and F344-fa-nidd2 rat strains

I postulated that the sequence of the *Coq2* and *Plac8* OLETF alleles

was altered, resulting in decreased mRNA or protein expression under the condition of obesity. The sequences of the seven *Coq2* exons of the OLETF and F344 alleles were identical. I further sequenced the genomic locus from the 5' end to approximately 600 bp upstream. I observed no difference in the exon and promoter region between the OLETF and F344 alleles (data not shown). The sequences of the five *Plac8* exons of OLETF and F344 alleles were identical. I further sequenced the genome locus from 5' end to approximately 600 bp upstream. However, the sequence of Plac8 allele at positions -419 (G to A) and -577 (C to T) in OLETF rats differed from that in F344 and five other inbred rat strains (Table 4).

2-4. Discussion

Obesity is important but not the only determinant for the onset of type 2 diabetes in OLETF rats (62), indicating that genetic factors must also contribute to this disease in these rats. I hypothesized that some of the 14 hyperglycaemic QTLs (Quantitative Trait Loci) previously identified in OLETF rats (16, 31) contribute to the onset of type 2 diabetes under the condition of obesity. In our previous study, the single QTL (Quantitative Trait Loci) *Nidd2/of* was sufficient to induce severe hyperglycaemia after glucose loading in obese rats using the double congenic strain F344-*fa-nidd2* (*Lepr*$^{-/-}$ and *Nidd2/of*) (19). Wally et al. reported that approximately one-third of the transcripts were differentially expressed in lean and obese siblings (63), suggesting that obesity control is essential to eliminate genes that are related to obesity. In the present study, I analyzed the expression of genes located in the *Nidd2/of* QTL (Quantitative Trait Loci) region of F344-*fa* (*Lepr*$^{-/-}$) control rat strains and F344-*fa-nidd2* rat strains. The major finding of this study is that *Coq2* (also known as polyprenyl transferase) and *Plac8* (also known as Onzin) are strong candidates for the onset of type 2 diabetes associated with obesity, although the detailed mechanisms of its function require further study.

The COQ2 enzyme mediates the conjugation of the benzoquinone ring with the completed side chain, and thus plays a central role in the biosynthesis of COQ (64, 65). COQ10 protein deficiency due to a mutation in the COQ2 protein has been associated with familial and sporadic multiple-system atrophy (66, 67). Although I observed that the expression level of the COQ2 protein was significantly lower in F344-*fa-nidd2* rat strains than in F344-*fa* control rat strains, the exon and promoter region of

the *Coq2* gene did not include mutation in F344-*fa-nidd2* rat strains. Because enhancer affects the expression on mRNA, the cause of the reduction in the expression levels of the COQ2 protein in F344-*fa-nidd2* rat strains may be due to changes in the enhancer region , although the reasons for the reduction require further study.

Beneficial effects of ubiquinone (COQ10) supplementation have been reported for most disorders associated with metabolic syndrome, e.g., hypertension, diabetes, insulin resistance and obesity. In a previous study, in hypertensive patients with coronary artery disease, a significant decline in fasting and postprandial plasma insulin and glucose levels was found in the COQ10 supplementation group compared with the control group (68). In hypertensive obese rat (SHR/cp), COQ10 supplementation prevented an elevation in plasma insulin levels but did not affect the elevation in glucose levels (69). Amin et al. also reported that COQ10 supplementation improved insulin sensitivity and hyperglycaemia in high-fat, high-ffructose diet fed Wister rats injected with streptozotocin (70).

As mentioned above, ubiquinone is a well-known antioxidant; thus, a reduction in COQ2 levels may lead to a decrease in so-called ubiquinone levels, which may then induce deterioration of insulin sensitivity in F344-*fa-nidd2* double congenic rat strains. COQ2 was reduced in phenotypes with increased insulin levels or insulin resistance under both fasting and postprandial conditions in F344-*fa-nidd2* rats, which supports the idea that the reduction in COQ2 levels may be involved in the phenotype of insulin resistance.

I also speculate that the *Plac8* gene may be related to blood glucose levels, because fasting glucose levels were not significantly different between F344-*fa-nidd2* rat strains and F344-*fa* control rat strains, whereas

postprandial glucose levels were significantly different (Fig. 6A). In addition, the differential expression of the *Plac8* gene was observed only under postprandial conditions in the obese strains (Fig. 12B).

Plac8 was discovered as an mRNA enriched in mouse placenta, and its sequence is highly conserved in humans. The predicted mouse and human proteins (112 and 108 amino acid residues) contain a putative signal peptide (71). *Plac8* can considerably affect growth, cell cycle progression and apoptosis in several different cell types, and inhibition of endogenous PLAC8 expression results in a reduced growth rate (72). This may account for the smaller body weight of the F344-*nidd2* and F344-*fa-nidd2* rat strains in comparison with the F344 and F344-*fa* rat strains, respectively. It should be noted that despite body weight and abdominal fat pad weight being significantly lighter and TCHO and TG levels being significantly lower in F344-*fa-nidd2* obese rat strains than in F344-*fa* obese control rat strains (Table 2), F344-*fa-nidd2* double congenic rat strains exhibited significantly higher plasma glucose and insulin levels than F344-*fa* obese control rat strains. These results indicate that genetic factor(s) must be crucial for the onset of type 2 diabetes in obese rat strains. Thus, this new animal model generated using a classical strategy provides a powerful tool for examining gene-environment interactions in obesity and type 2 diabetes.

I hypothesized that the sequence of *Plac8* mRNAs differ between F344-*fa* and F344-*fa-nidd2* rat; however, our data show that they are in fact identical. This is not surprising, because many genes associated with type 2 diabetes harbour single-nucleotide polymorphisms (27). I therefore examined if there were sequence differences in the regulatory regions of Plac8 and found unique substitutions at positions -419 (G to A) and -577

(C to T) in the OLETF allele.

In particular, the G to A transversion is intriguing, because GGAGGCAGAGGC is a highly conserved sequence assigned as the consensus of class 3 sequences among humans and rodents, and its G-rich GGCAGA motif is a CTCF zinc finger protein-binding site (73). The formation of different CTCF-DNA complexes, some of which are methylation sensitive, mediates functions such as gene activation, repression and silencing, as well as chromatin insulation (74). The G-rich motif is not present in the OLETF allele, which may account for the difference in *Plac8* expression between F344-*fa* and F344-*fa-nidd2* rat strains under glucose loading.

Plac8-knockout mice are intolerant to cold temperatures and late-onset obesity, and their brown adipocytes exhibit abnormal morphology and impaired function (75). PLAC8 is expressed at very high levels in phagocytes such as macrophages and neutrophils and is essential for the optimal elimination of bacteria by these cells using *Plac8*-knockout mice (76). These results suggest that inactivity or decrease of Plac8 may result in the loss of host defence functions. Furthermore, glucose levels are indistinguishable between wild-type and Plac8-knockout mice (77), suggesting that *Plac8* function is independent of the regulation of glucose metabolism. However, I show here that differences in *Plac8* expression between F344-*fa* and F344-*fa-nidd2* rat strains are associated with obesity and glucose loading. Therefore, it will be interesting to generate obese *Plac8*-knockout mice, and for example, introgress the $Lepr^{-/-}$ locus.

In summary, our study shows that the strategic development of new animal models such as double congenic rats combining the *Nidd2/of* QTL (Quantitative Trait Loci) with obesity is useful for identifying genes that

contribute to the onset of type 2 diabetes associated with obesity. Here, I demonstrate that *Coq2* and *Plac8* are candidates for such genes, and I are currently performing further research to confirm this.

Acknowledgments

I would like to express my sincere gratitude to my supervisor, Professor Kozo Matsumoto, Laboratory of Genetics in Experimental Medicine, Faculty of Life Science, Kyoto Sangyo University, for a great helpful on the entire this work, critical reading of the manuscript, and everything that he done for me. I also very grateful to Dr. Jyunichi Takahashi and Takakazu Kaneko, Faculty of Life Science, Kyoto Sangyo University, for kind and valuable advise on my study. I would like to thank my entire colleague in Matsumoto's laboratory, including Mr. Jun Koto, Ms. Risa Watadani, Mr. Yoshiyasu Okano, Mr. Keiya Tanimura, Ms. Kaori Hayashi, Ms. Mei Yoshida for their kind and help to perform my research.

Tables

Table1 : Specific primers for Lepr and Nidd2/of QTL region.

		Location	Sence	Anti-sense
Lep r	*r.LeprZF*	5: 120,503,475-120,682,283	TTGCGTATGGAAGTCACAGATGAT	AGGCACACACCTTTAATCCCAGTA
Nidd 2	*D14Rat23*	*14: 2,227,825-2,227,992*	TCGGAGGGAGTGACACATAA	TGTCCCTCAGTTCAGAATACCC
Nidd 2	*D14Wox1*	*14: 10,443,917-10,444,095*	CTGACACAATGCCTCCCTC	AGCACAGCATAATTACACAGG
Nidd 2	*D14Wox14*	*14: 22,009,849-22,009,966*	ACTTGATTACACACACAAACACAGA	CTTTGCTTTCTTTTAGCCATTT
Nidd 2	*D14Rat8*	*14: 32,593,926-32,594,049*	TCCAAATTTTCTTTTCCTGTCTTT	CCTGTAACCATGAAGCAGCA
Nidd 2	*D14Rat12*	*14: 61,783,047-61,783,215*	GGCTTGCTTTATCCACCAAA	GGACCATGGAGTTGATGAGAA

Table 2. Comparison of body weights, fat weights and metabolic parameters in F344, F344-*nidd2*, F344-*fa*, and F344-*fa-nidd2* rats at 25 weeks of ages

	F344	F344-*nidd2*	F344-*fa*	F344-*fa-nidd2*
Body weight (g)	380.20 ± 12.73 (n=20)	301.64 ± 7.17 (n=22)†††	590.04 ± 7.75 (n=16)	461.50 ± 13.65 (n=5)***
Fat weight (g)				
Retroperitoneal fat	9.35 ± 0.77 (n=5)	7.81 ± 0.41 (n=12)	33.57 ± 0.83 (n=5)	22.60 ± 0.62 (n=9)***
Mesenteric fat	7.67 ± 0.30 (n=10)	5.23 ± 0.57 (n=5)††	13.89 ± 0.44 (n=10)	11.45 ± 0.64 (n=7)**
Epididymal fat	9.79 ± 1.12 (n=5)	6.45 ± 0.27 (n=12)†††	14.16 ± 0.42 (n=5)	10.06 ± 0.45 (n=9)***
Adiposity index (%)				
Retroperitoneal fat	2.4 ± 0.18	2.5 ± 0.18	5.8 ± 0.20	4.9 ± 0.06**
Mesenteric fat	1.9 ± 0.08	1.7 ± 0.13	2.4 ± 0.09	2.0 ± 0.08*
Epididymal fat	2.7 ± 0.22	2.1 ± 0.10†	2.5 ± 0.05	2.3 ± 0.80
TCHO (mg/mL)	45.77 ± 4.03 (n=7)	42.69 ± 5.25 (n=7)	426.24±30.04 (n=10)	277.94 ± 40.79 (n=10)**
TG (mg/dL)	130.17 ± 15.88 (n=7)	80.54 ± 8.04 (n=7)†	2342.86±269.19 (n=10)	1240.6±209.56 (n=10)**
NEFA ((mEq/L)	0.44 ± 0.03 (n=7)	0.39 ± 0.03 (n=7)	1.62±0.24 (n=10)	1.23±0.24 (n=10)

†††p<0.0001 : F344 vs F344-*nidd2* **p<0.01, ***p<0.001 : F344-*fa* vs F344-*fa-nidd2*

Table 3 : gene specific primers

Gene Name	Location(Mb)	Forward Primer (5'–3')	Reverse Primer (3' –5')
Cplx1	1.72	TCTGCCCAAAGAGATGACG	AGGAACAGGATGCAGGATTG
Pcgf3	1.84	ACGATTACCACAGGAGTGACG	ATCTGATCCACTTCCGCTTG
Atp5i	1.86	TTCAGGTCTCTCCGCTCATC	TTCCTCCGCTGCTATTCTTC
Pde6b	1.92	AGAGATCCTGCCCATGTTTG	TCTTCTTCTCTTCCTCCAGTGC
Pigg	1.98	GGCATCTCAAATGGTTCAGC	GCTTTGGGGAAAACACACTC
Dr1	2.02	TATCTTCCGAAGCCAACGAG	TGCTTGGATGACATGCTCTG
Ccdc18	2.17	CAGAATCGGAAAGGAGGATG	GTGACAGGATTTTCCGCTTG
Tmed5	2.17	TGATTTCCACCTTGCCTCTC	CATGTAGTCGCCATCTTCAGTC
Fam69a	2.32	AGTTCTTGCTCCTTGTGGTTG	TGCCTATCAGGTTCATGTGG
Rpl5	2.41	TGGCCTGACAAACTATGCTG	CTGACCGTCTATGCTTTCCAC
Ube2d4	2.47	GGGAATGGACTCAGAAGTATGC	TGGGTTCACAGCAAACAGTC
Gfi1	2.6	CAATCAGAGTGGGTGAAACG	ATAACCTGTGGCCAACAACC
Rpap2	2.76	TATGGGTTCGAGAGGAAGAGAG	GCATCAAAGGCACCATAAGC
Glmn	2.8	TTACATTCCGCCCTCTTCAC	TTCATCCCAGCGTTTTCC
Lpcat2b	2.9	CCCTGCTTATCAAGCTTTGG	TTGGTCCCCAAGTTCACATC
Ephx4	2.93	GTCGTTGCACTGGATTTGAG	CCCAAGGAGTCCAAAACATC
Brdt	3.01	CAAAAGACAGCCGTTTCCTC	GCCTCTTGTGCCATGTTTAAG
Tgfbr3	3.02	CACTTACCACCGCAACATTC	AGGCACTTTTGGAGTTGGTG
Cdc7	3.44	AGCGATTGACATGTGGTCTG	CATCGCTGGCCTTGTAAAAC
Znf644	3.63	CGCTAAAACCCACGAGAAAG	TCGATGACATTCCAGCTCAC
Barhl2	3.89	TACAGCACCAGCGTTTCTTC	AGCTTGGTTTTGCTGTCCTC
Lrrc8d	5.12	CTGCTGGAAATTGAGCTTGG	TAACAACCGCCAGGTAATCC
Lrrc8c	5.41	TGCTCTGGTTTCCAAAGTCC	CAAAGCACAGGTAGCAAAAGG
Lrrc8b	5.45	TGTCCAAGCATTTGGGTACA	ACAAAGGGCCAAACACAAAG
Abcg3l2	5.62	CTACATGAGCACACCAGTGG	AGAGCTCATGCTCATTCTTC
Abcg3	5.88	CTACATGAGCACACCAGTGG	CTATGAAGTACATGAGCTCT
Abcg3l3	5.94	CTACATGAGCACACCAGTGG	CCCTTGACACCTGTTTTCAT
Pkd2	6.4	CCAGCAGAAAGCAGAAATGG	AATGGCGTCTACGGTGTTTC
Sparcl1	6.76	AGGGGACACACTTGCAAAAC	CTTGGTAGCAAACAGGTGACAG
Nudt9	6.85	ATGAGACAGGGGAGACGATG	TCTCTGCCACGAGTTTGATG
Hsd17b11	6.85	TTCGAAGTCAATGTGCTTGC	GCAGCCAGTTCATCAGTCAA
Hsd17b13	6.9	CATCCTGGAGCTTCTCTTGC	CGGCCACAGACTTTCTTCTC
Klhl8	6.95	TCAACATCGACAGCGAGAAG	TCCAGCCATTTAGGGTGATG
Aff1	7.13	GAGAAGGCTCCACTGTTTGC	TGGGTAGGCTGGTGGTAGAC
Slc10a6	7.27	GGTCTGAAACCATTCCAAGC	GAGGTCCATATCTCCATCAACC
Ptpn13	7.45	CTTTGGACCGAATTCGAGAG	TGGAAGTGCTGAACAGATCG
Ndufb4l1	7.55	AGTACAACGACCCCAAATGC	GTTCTTGGGAGTGGGTCTGA
Mapk10	7.68	CATTGCAGCATCCGTACATC	ATGGTGTGCTCCCTTTCATC
Arhgap24	8.08	CCAGCACAACAGACTCTCCA	TTTCACAGGAGGAGGTGGAC
Wdfy3	8.9	TCGCGTACGAAGACATCAAG	AGACCCTTGTTCCACACTGG
Cds1	9.14	TGGTTCCTTGTCCCCATATC	CAATGAATCCTTCCCAGGTC
Agpat9	10.06	TCCTGGTGAAAACCTTGGAG	AGCTGGGCTTTTGAGAACTG
Fam175a	10.08	AAAAGACTGTGGTGGGTTGG	AGCTCTGGGCTTGAAAGATG
Mrps18c	10.1	TCTGTGCCATGGAGAAGATG	GGGTTTTCCATTGGGATTG
Helq	10.14	CCTCACCAATCATGTGCAAC	TGTTGACAGCGTTCTTGTCC
Hpse	10.19	CCTTTGCAGCTGGCTTTATG	CGAAAAACACCTGCCTCATC
Coq2	10.21	TATTCTCATGCGTGGAGCAG	AGGACTGGAAGGCTGAAATG
Plac8	10.3	TCTGTGGGACCTTTTGCTTC	CACCGTTGTTCCACACAAAC
Cops4	10.36	TCAAGCGGCTCTCACAAAG	TGACGTTCTCGTTCACCATC
Lin54	10.39	TATCCGTCCCTTTCCTTCTG	GAACCCTCCCCAAATAATCG
Sec31a	10.46	CTGACCGGCAATTTTGAGAG	CACCTGCGATGGCTAATATG
Tmem150c	10.81	ATCCTCTTCGCCTTTCCAAC	ATTGGTACCAGATCCACCACTC
Enoph1	10.86	GAAGATTGCAGACAGCATCG	TTTTCTCGTCGTCGGTTAGC
Hnrnpdl	10.86	TCGGTTTGGGGAAGTTGTAG	TTGCCATCCAGTTTGTGTTC
Hnrpd	10.91	GATCCTAAAAGGGCCAAAGC	CCTCACCAAAACCACCAAAG
Bmp3	12.1	CAAGAGGATGCTGCTTTTCC	CAGTTCCTGCTGTGAAATCG

Table 4. Comparison of nucleotide sequence of part of up stream segment from 5' end of exon 1 of *Plac8* among rat strains

Strain	Sequences
	-577 ..... -419
OLETF	5'-ATCGGGGTT**T**GGAATAAT……GCTCCCAGGA**A**GCAGAGGCAGATGGGTC-3'
F344	5'-ATCGGGGTT**C**GGAATAAT……GCTCCCAGGA**G**GCAGAGGCAGATGGGTC-3'
ACI	5'-ATCGGGGTT**C**GGAATAAT……GCTCCCAGGA**G**GCAGAGGCAGATGGGTC-3'
BN	5'-ATCGGGGTT**C**GGAATAAT……GCTCCCAGGA**G**GCAGAGGCAGATGGGTC-3'
DA	5'-ATCGGGGTT**C**GGAATAAT……GCTCCCAGGA**G**GCAGAGGCAGATGGGTC-3'
Gunn	5'-ATCGGGGTT**C**GGAATAAT……GCTCCCAGGA**G**GCAGAGGCAGATGGGTC-3'
NAR	5'-ATCGGGGTT**C**GGAATAAT……GCTCCCAGGA**G**GCAGAGGCAGATGGGTC-3'

Figures and Legends

F344 **F344-*nidd2*** **F344-*fa*** **F344-*fa*-*nidd2***

Fig. 1 : Rat strains used in this study; F344, F344-*nidd2*, F344-*fa* and F344-*fa*-*nidd2*. F344 and F344-*nidd2* strains were lean, while F344-*fa* and F344-*fa*-*nidd2* strains were obese.

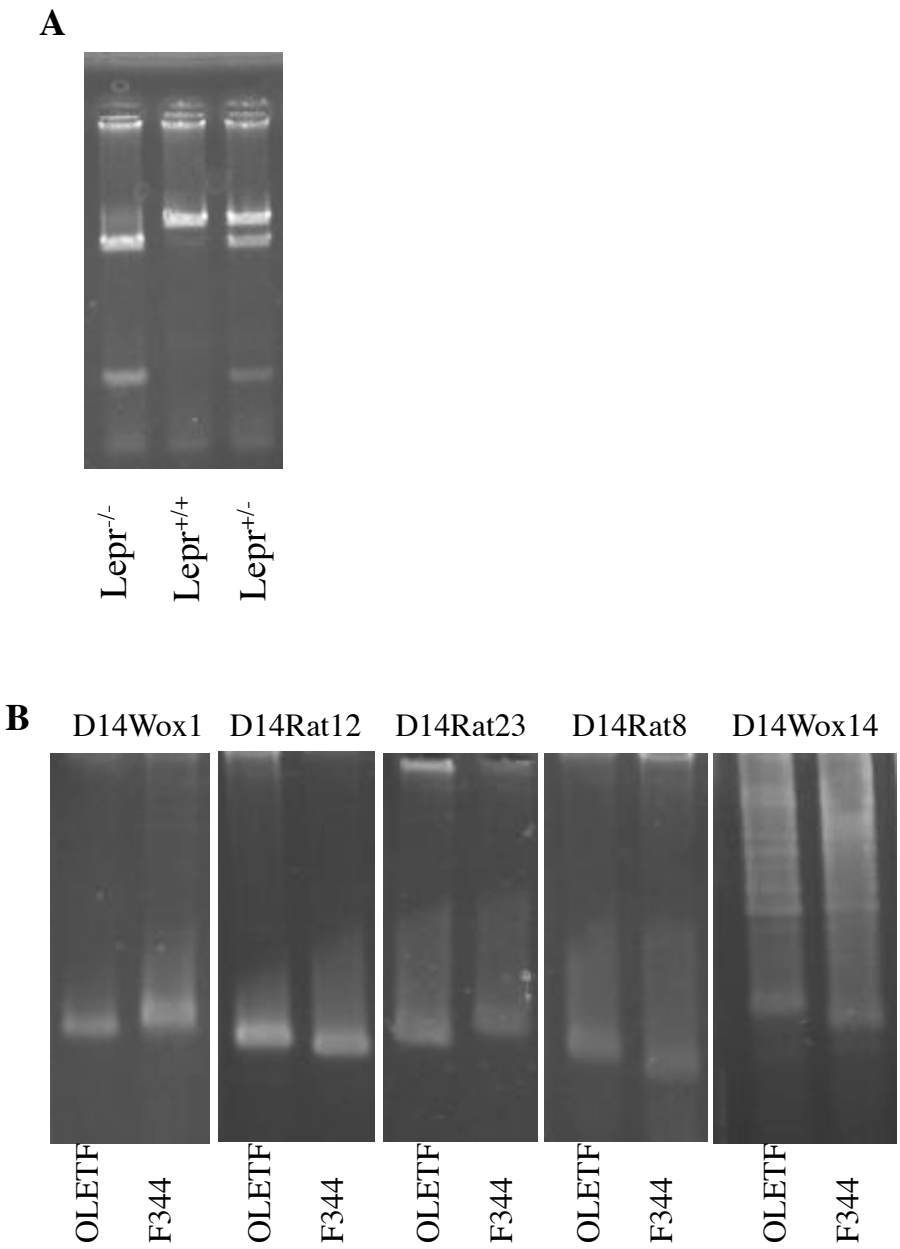


Fig. 2 : Genotyping of leptin receptor defficient locus (Lepr) and *Nidd2/of* QTL locus.
A) *Lepr*$^{-/-}$ was cut and showed faster band. But *Lepr*$^{+/+}$ was not cleaved and showed slower band. *Lepr*$^{+/-}$ was showed both faster and heavier bands.
B*) Nidd2/of* QTL locus were checked using specific primers ; *D14Wox1*, *D14Rat12*, *D14Rat23*, *D14Rat8* and *D14Wox14*.

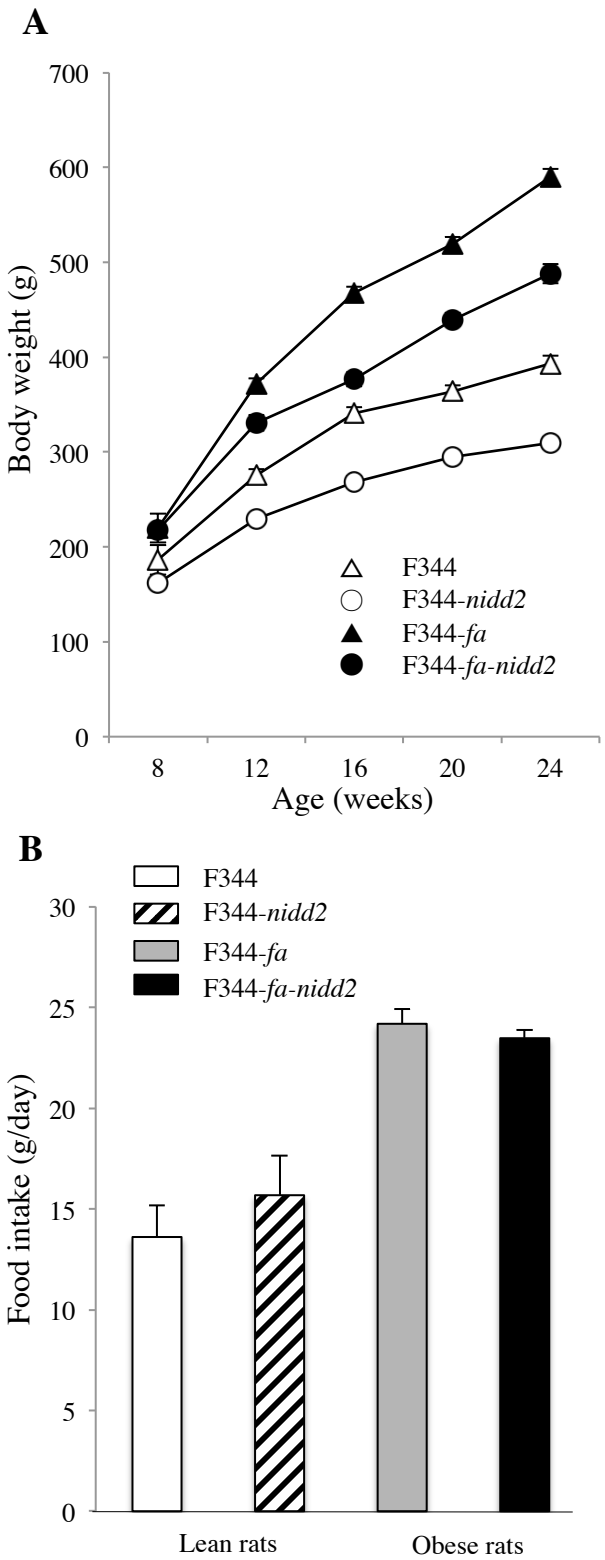


Fig. 3 : Body weight and food intake.
A) Body weight was measured for F344, F344-*nidd2*, F344-*fa* and F344-*fa-nidd2* rats from 8 to 24 weeks (n=5-22).
B) Food intake by the rats was measured at 14 weeks (n=5-8).

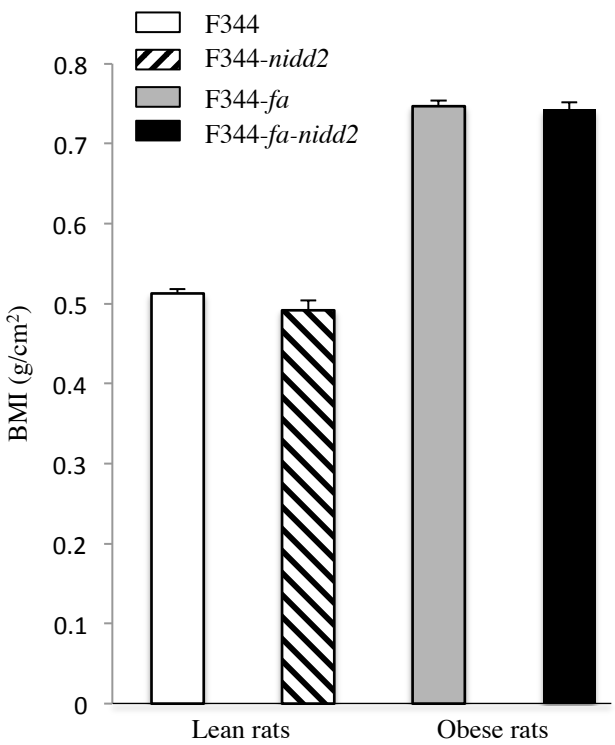


Fig. 4 : Body mass index (BMI) was measured at 13 weeks (n=5-8).

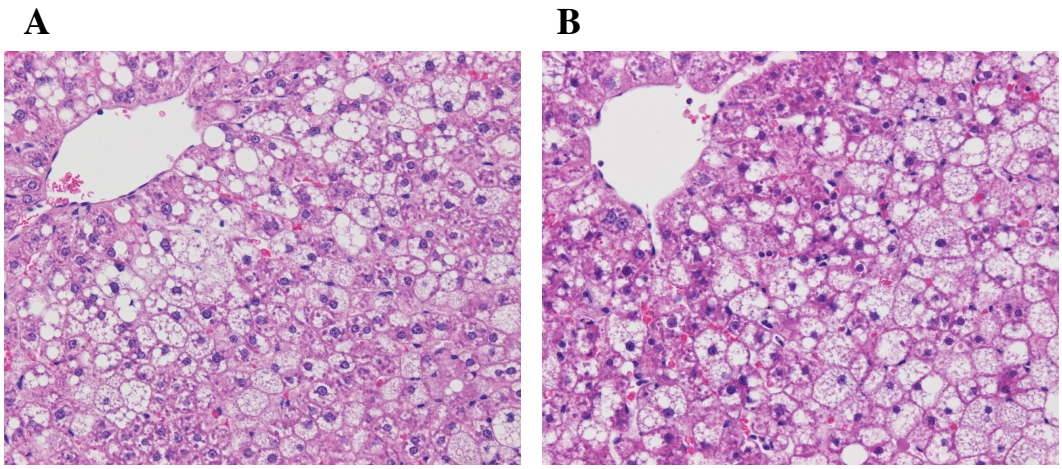


Fig. 5: Sections of rat livers were stained with hematokylin and eosin.
A) F344-fa rat as obese control
B) F344-fa-nidd2 double congenic rat

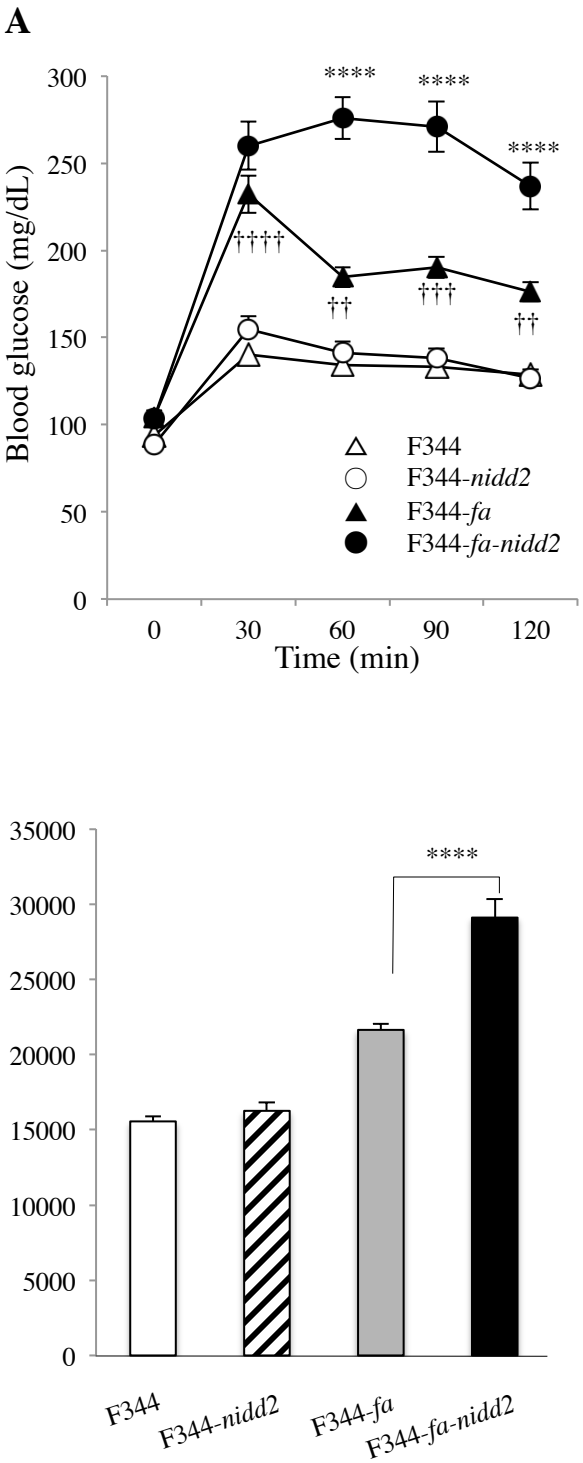


Fig. 6 : Analysis of the phenotypes of F344, F344-*nidd2*, F344-*fa* and F344-*fa-nidd2* rats.

A) Glucose levels at 20 weeks of age were measured during the OGTT for overnight- fasted F344 (n=21, white triangle), F344-nidd2 (n=22, white circle), F344-fa (n=32, black triangle) and F344-fa-nidd2 (n=16, black circle) rats.

B) The AUC of blood glucose levels were calculated from the results of the OGTT at all sampling times.

***p*<0.01, ****p*<0.001 and *****p*<0.0001vs F344-*fa* as the obese control rats ; ††*p*<0.01, †††*p*<0.001 and ††††*p*<0.0001 vs F344 rats.

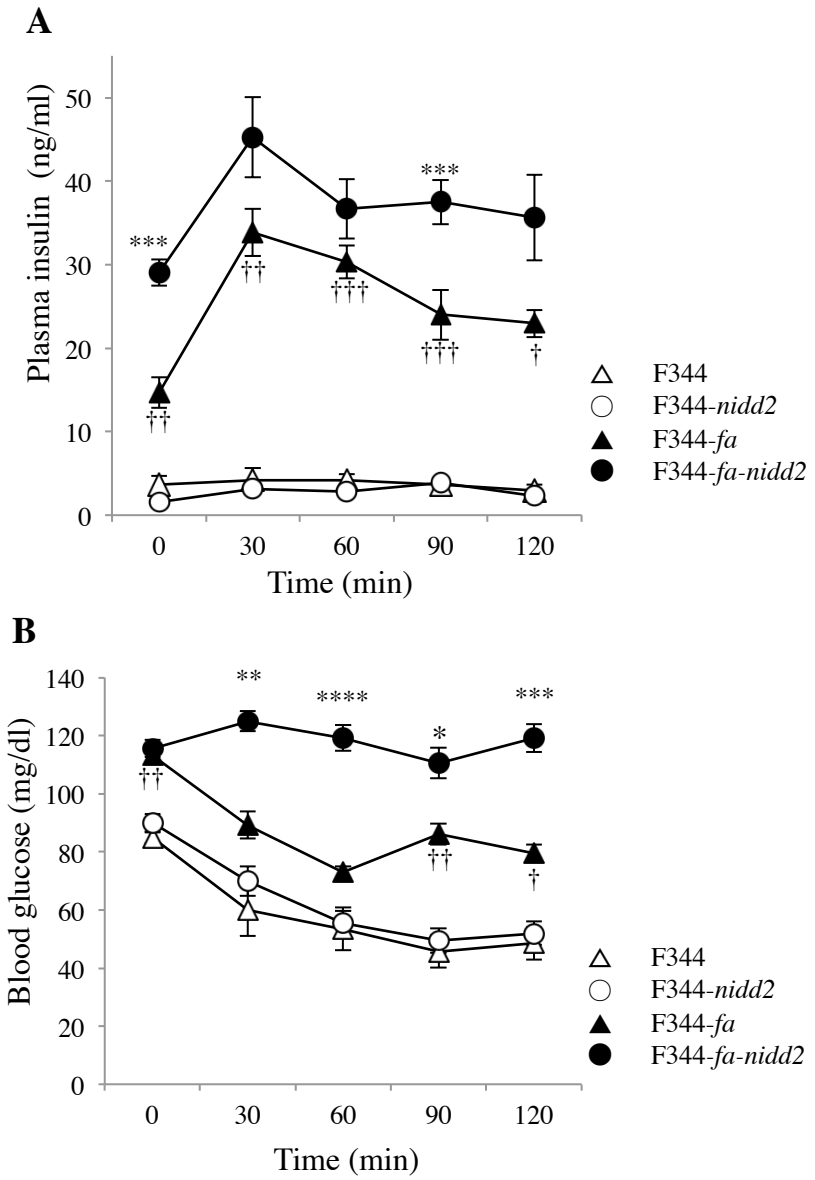
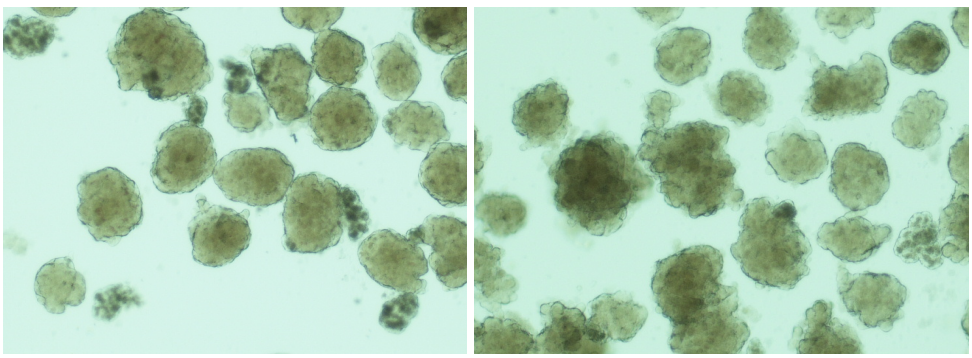


Fig. 7 : Analysis of the phenotypes of F344, F344-*nidd2*, F344-*fa* and F344-*fa-nidd2* rats.

A) Insulin levels during the OGTT were determined in the rats (n=6-9) at 20 weeks of age using ELISA.

B) The insulin tolerance test (1U/kg body weight) was performed in overnight-fasted rats (n=6-12) at 16 weeks of age. Data are presented as the mean ± SEM. **p*<0.05, ***p*<0.01, ****p*<0.001 and *****p*<0.0001vs F344-*fa* as the obese control rats ; ††*p*<0.01, †††*p*<0.001 and ††††*p*<0.0001 vs F344 rats.

A



F344-*fa* F344-*fa-nidd2*

B

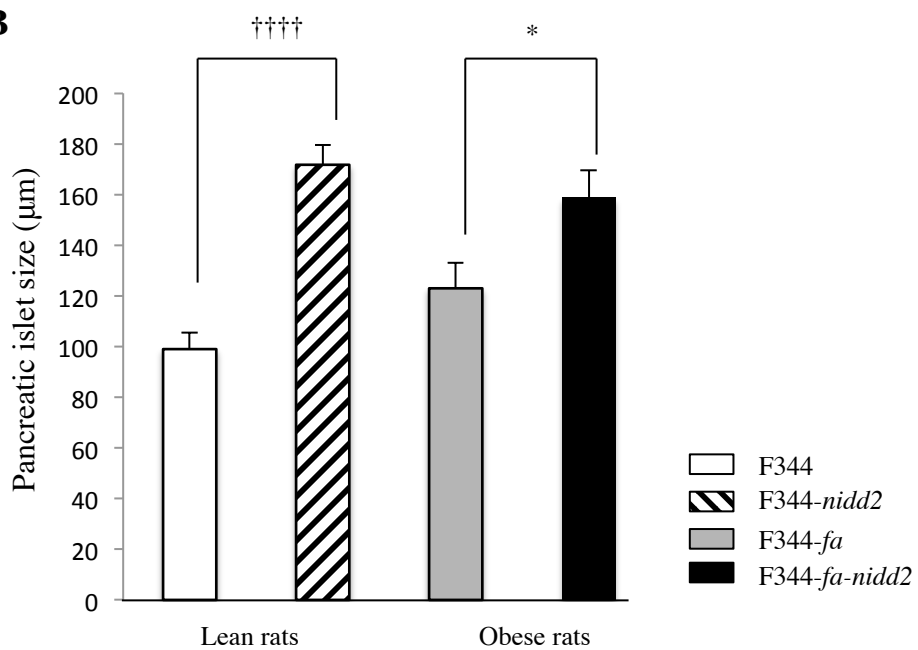


Fig. 8 Islets isolation and their size in F344, F344-*nidd2*, F344-*fa* and F344-*fa-nidd2* rats.
A) Islet isolation was carried out by bile duct perfusion and collagenase digestion.
B) Thirty islet cells of each strain were measured their diameters.

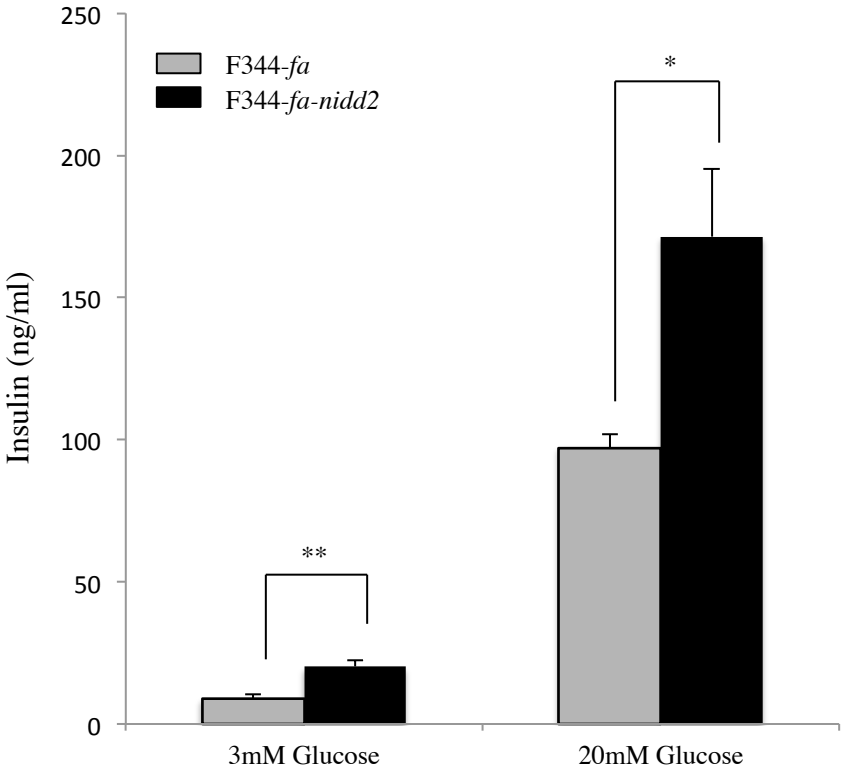


Fig. 9 Glucose challenge to pancreatic islets from F344-*fa* and F344-*fa-nidd2* rats in RPMI culture medium at 3 mM and 20 mM glucose, respectively. Islets from each strain were divided into six wells including 10 islets each well. The insulin content in each well was measured duplicately.

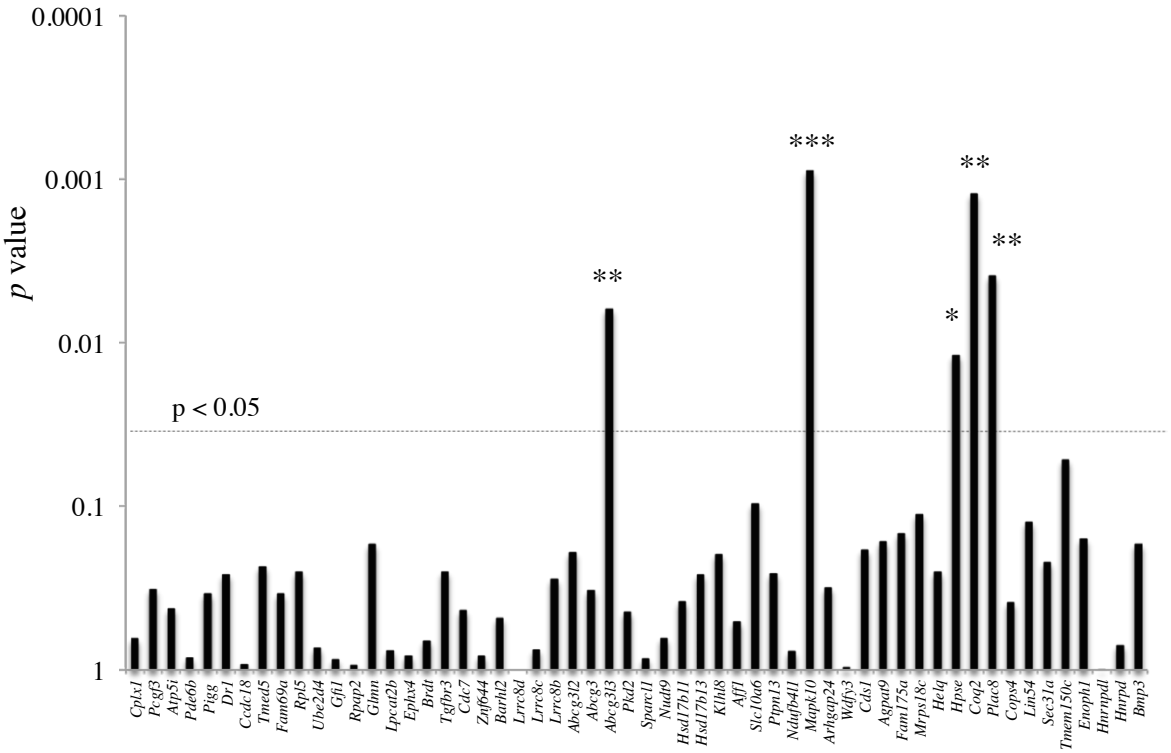


Fig. 10 : RT-qPCR analysis of genes within the *Nidd2/of* QTL region.
Differences in gene expression levels were measured between F344-*fa* (n=7) and F344-*fa-nidd2* (n=8) rats at 25 weeks of age.
The mRNA levels were quantified using RT-qPCR and were normalized to *Gapdh* mRNA levels.
The rats were sacrificed at 60 min after glucose loading , and liver RNAs were prepared immediately.
Data are presented as the mean ± SEM.
**p*<0.05, ***p*<0.01 and ****p*<0.001

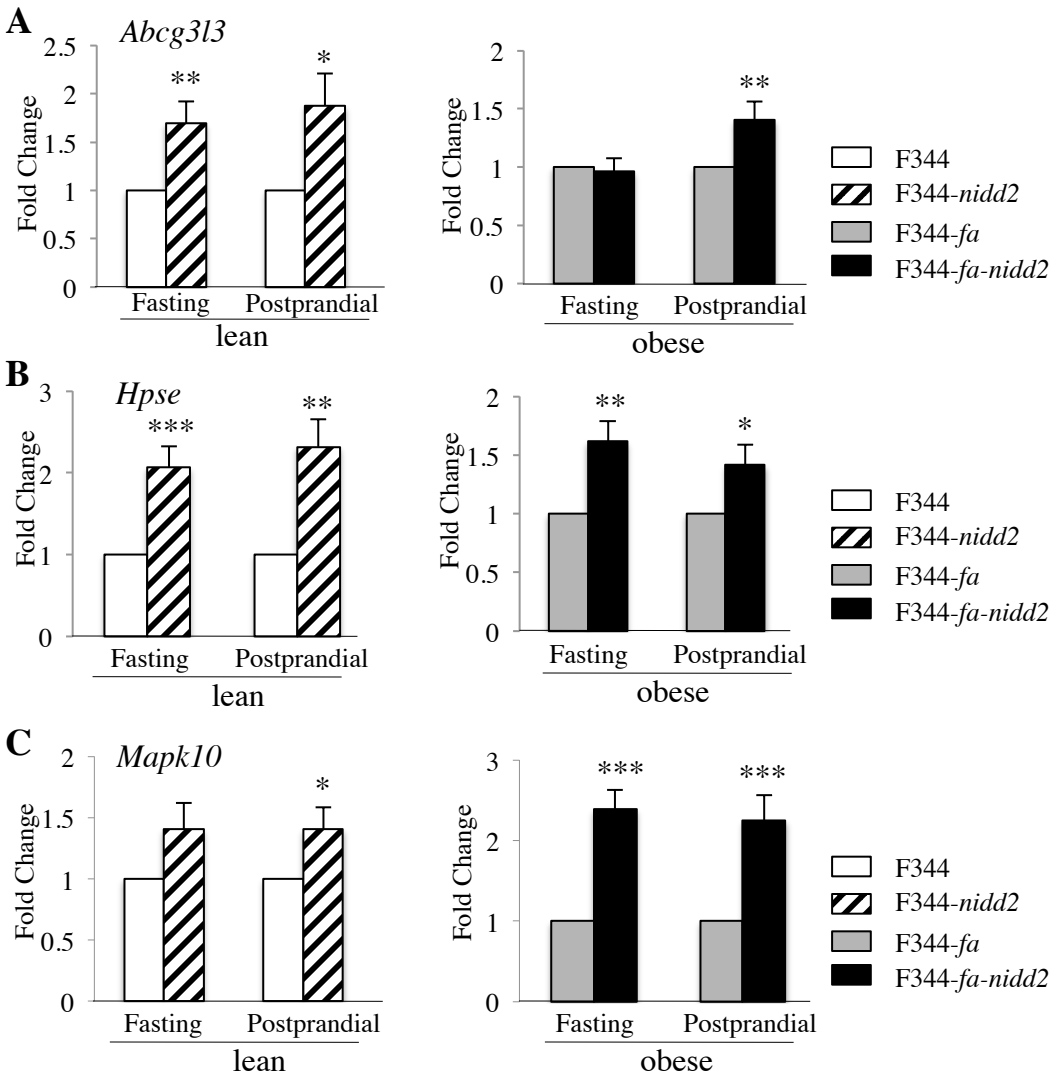


Fig. 11 : Relative expression levels of *Abcg3l3*, *Hpse*, *Mapk10*, *Coq2* and *Plac8* mRNA in overnight-fasting lean and obese rats (25 weeks of age) at 0 and 60 min after glucose loading.

A) Relative expression levels of *Abcg3l3* mRNA in F344 (n=8, *white column*), F344-*nidd2* (n=5, *hatched column*), F344-*fa* (n=8, *grey column*) and F344-*fa-nidd2* (n=8, *black column*) rats.

The mRNA levels were quantified using RT-qPCR and normalized to the levels of *Gapdh* mRNA.

B) Relative expression levels of *Hpse* mRNA.

C) Relative expression levels of *Mapk10* mRNA.

Data are presented as the mean ± SEM.

**p*<0.05, ***p*<0.01 and ****p*<0.001 vs F344 in lean rats and versus F344-*fa* in obese rats.

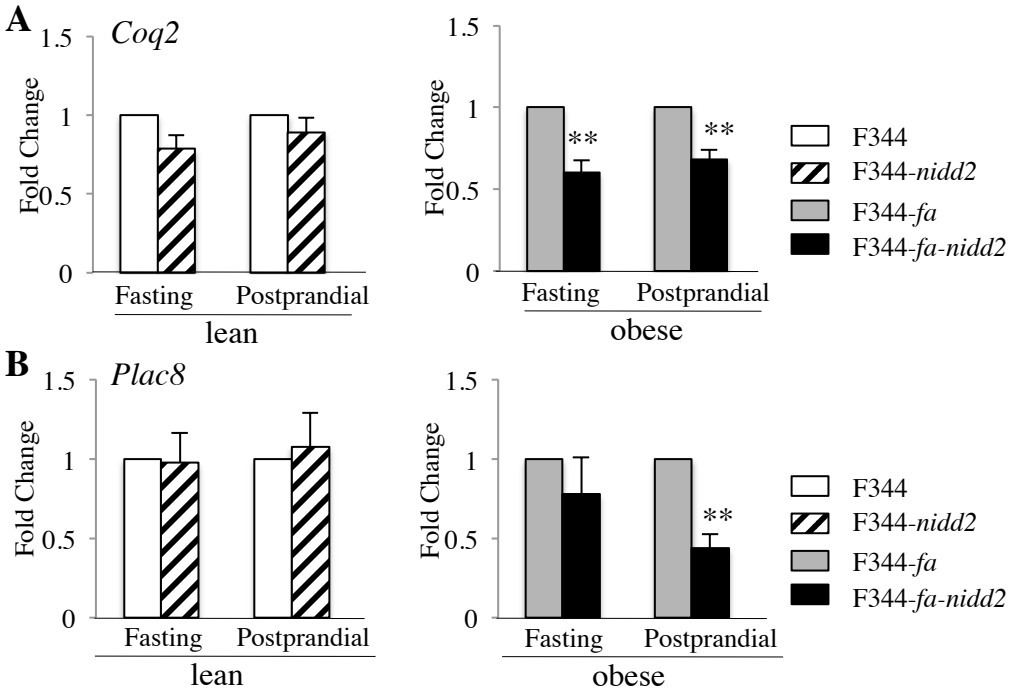


Fig. 12 : Relative expression levels of *Abcg3l3*, *Hpse*, *Mapk10*, *Coq2* and *Plac8* mRNA in overnight-fasting lean and obese rats (25 weeks of age) at 0 and 60 min after glucose loading.
A) Relative expression levels of *Coq2* mRNA in F344 (n=8, *white column*), F344-*nidd2* (n=5, *hatched column*), F344-*fa* (n=8, *grey column*) and F344-*fa-nidd2* (n=8, *black column*) rats.
The mRNA levels were quantified using RT-qPCR and normalized to the levels of *Gapdh* mRNA.
B) Relative expression levels of *Plac8* mRNA.
Data are presented as the mean ± SEM.
**p*<0.05, ***p*<0.01 and ****p*<0.001 vs F344 in lean rats and versus F344-*fa* in obese rats.

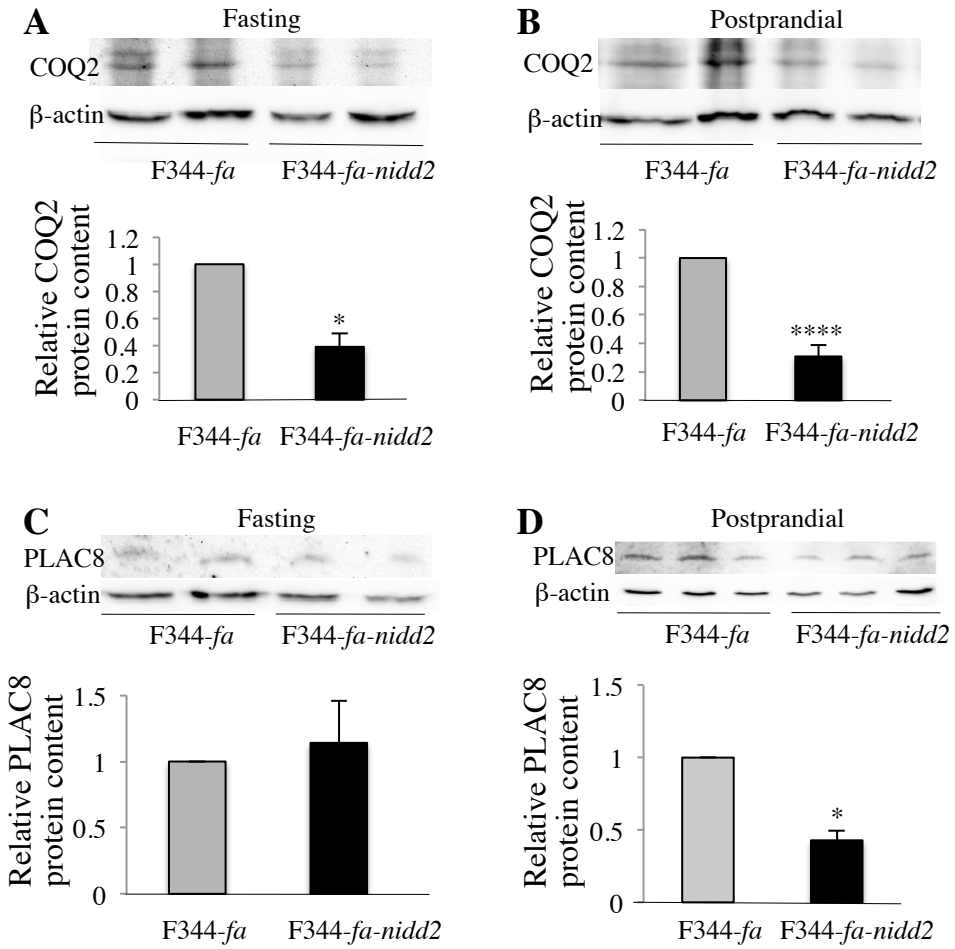


Fig. 13 : Western blot analysis of COQ2 and PLAC8 expression in crude liver extracts of obese F344-fa (n=7) and F344-fa-nidd2 (n=8) under both fasting and postprandial (60 min after glucose loading) conditions.
a) and **b**) Relative COQ2 protein contents under fasting postprandial conditions.
c) and **d**) Relative PLAC8 protein contents under fasting postprandial conditions, respectively.
The protein levels were normalized to those of β-actin.
Data are presented as the mean ± SEM.
**p*<0.05 and *****p*<0.001 vs F344-*fa* rats.

Reference

1. Froguel P, Zouali H, Vionnet N, Velho G, Vaxillaire M, Sun F, *et al*. Familial hyperglycemia due to mutations in glucokinase. Definition of a subtype of diabetes mellitus. *The New England journal of medicine* 1993;**328:** 697-702.

2. Barnett AH, Eff C, Leslie RD, Pyke DA. Diabetes in identical twins. A study of 200 pairs. *Diabetologia* 1981;**20:** 87-93.

3. Newman B, Selby JV, King MC, Slemenda C, Fabsitz R, Friedman GD. Concordance for type 2 (non-insulin-dependent) diabetes mellitus in male twins. *Diabetologia* 1987;**30:** 763-768.

4. Zimmet P, Canteloube D, Genelle B, LeGonidec G, Couzigou P, Peghini M, *et al*. The prevalence of diabetes mellitus and impaired glucose tolerance in Melanesians and part-Polynesians in rural New Caledonia and Ouvea (Loyalty Islands). *Diabetologia* 1982;**23:** 393-398.

5. Chakraborty R, Ferrell RE, Stern MP, Haffner SM, Hazuda HP, Rosenthal M. Relationship of prevalence of non-insulin-dependent diabetes mellitus to Amerindian admixture in the Mexican Americans of San Antonio, Texas. *Genetic epidemiology* 1986;**3:** 435-454.

6. Friedman JM, Leibel RL. Tackling a weighty problem. *Cell* 1992;**69:** 217-220.

7. Ludvik B, Nolan JJ, Baloga J, Sacks D, Olefsky J. Effect of obesity on insulin resistance in normal subjects and patients with NIDDM. *Diabetes* 1995;**44:** 1121-1125.

8. Campbell PJ, Carlson MG. Impact of obesity on insulin action in NIDDM. *Diabetes* 1993;**42:** 405-410.

9. Henry RR, Wallace P, Olefsky JM. Effects of weight loss on mechanisms of hyperglycemia in obese non-insulin-dependent diabetes mellitus. *Diabetes* 1986;**35:** 990-998.

10. Knowler WC, Saad MF, Pettitt DJ, Nelson RG, Bennett PH. Determinants of diabetes mellitus in the Pima Indians. *Diabetes care* 1993;**16:** 216-227.

11. Galli J, Li LS, Glaser A, Ostenson CG, Jiao H, Fakhrai-Rad H, *et al*. Genetic analysis of non-insulin dependent diabetes mellitus in the GK rat. *Nature genetics* 1996;**12:** 31-37.

12. Gauguier D, Froguel P, Parent V, Bernard C, Bihoreau MT, Portha B, *et al*. Chromosomal mapping of genetic loci associated with non-insulin dependent diabetes in the GK rat. *Nature genetics* 1996;**12:** 38-43.

13. Kawano K, Hirashima T, Mori S, Saitoh Y, Kurosumi M, Natori T. Spontaneous long-term hyperglycemic rat with diabetic complications. Otsuka Long-Evans Tokushima Fatty (OLETF) strain. *Diabetes* 1992;**41:** 1422-1428.

14. Shima K, Shi K, Sano T, Iwami T, Mizuno A, Noma Y. Is exercise training effective in preventing diabetes mellitus in the Otsuka-Long-Evans-Tokushima fatty rat, a model of spontaneous non-insulin-dependent diabetes mellitus? *Metabolism: clinical and experimental* 1993;**42:** 971-977.

15. Okauchi N, Mizuno A, Zhu M, Ishida K, Sano T, Noma Y, *et al*. Effects of obesity and inheritance on the development of non-insulin-dependent diabetes mellitus in Otsuka-Long-Evans-Tokushima fatty rats. *Diabetes research and clinical practice* 1995;**29:** 1-10.

16. Moralejo DH, Wei S, Wei K, Weksler-Zangen S, Koike G, Jacob HJ, *et al*. Identification of quantitative trait loci for non-insulin-dependent diabetes mellitus that interact with body weight in the Otsuka Long-Evans Tokushima Fatty rat. *Proceedings of the Association of American Physicians* 1998;**110:** 545-558.

17. Kose H, Moralejo DH, Ogino T, Mizuno A, Yamada T, Matsumoto K. Examination of OLETF-derived non-insulin-dependent diabetes mellitus QTL by construction of a series of congenic rats. *Mammalian genome : official journal of the International Mammalian Genome Society* 2002;**13:** 558-562.

18. Kose H, Bando Y, Izumi K, Yamada T, Matsumoto K. Epistasis between hyperglycemic QTLs revealed in a double congenic of the OLETF rat. *Mammalian genome : official journal of the International Mammalian Genome Society* 2007;**18:** 609-615.

19. Kose H, Yamada T, Matsumoto K. Single diabetic QTL derived from OLETF rat is a sufficient agent for severe diabetic phenotype in combination with leptin-signaling deficiency. *Experimental diabetes research* 2012;**2012:** 858121.

20. Zimmet P, Alberti KG, Shaw J. Global and societal implications of the diabetes epidemic. *Nature* 2001;**414:** 782-787.

21. Kopelman PG. Obesity as a medical problem. *Nature* 2000;**404:**

635-643.

22. Kyrou I, Randeva HS, Weickert MO. Clinical Problems Caused by Obesity. In: De Groot LJ, Beck-Peccoz P, Chrousos G, Dungan K, Grossman A, Hershman JM, *et al*. (eds). *Endotext*. MDText.com, Inc.: South Dartmouth (MA), 2000.

23. Florez JC, Hirschhorn J, Altshuler D. The inherited basis of diabetes mellitus: implications for the genetic analysis of complex traits. *Annual review of genomics and human genetics* 2003;**4:** 257-291.

24. Kimber CH, Doney AS, Pearson ER, McCarthy MI, Hattersley AT, Leese GP, *et al*. TCF7L2 in the Go-DARTS study: evidence for a gene dose effect on both diabetes susceptibility and control of glucose levels. *Diabetologia* 2007;**50:** 1186-1191.

25. Saxena R, Voight BF, Lyssenko V, Burtt NP, de Bakker PI, Chen H, *et al*. Genome-wide association analysis identifies loci for type 2 diabetes and triglyceride levels. *Science (New York, NY)* 2007;**316:** 1331-1336.

26. Hertel JK, Johansson S, Raeder H, Midthjell K, Lyssenko V, Groop L, *et al*. Genetic analysis of recently identified type 2 diabetes loci in 1,638 unselected patients with type 2 diabetes and 1,858 control participants from a Norwegian population-based cohort (the HUNT study). *Diabetologia* 2008;**51:** 971-977.

27. Strawbridge RJ, Dupuis J, Prokopenko I, Barker A, Ahlqvist E, Rybin D, *et al*. Genome-wide association identifies nine common variants associated with fasting proinsulin levels and provides new insights into the pathophysiology of type 2 diabetes. *Diabetes* 2011;**60:** 2624-2634.

28. Grarup N, Sandholt CH, Hansen T, Pedersen O. Genetic susceptibility to type 2 diabetes and obesity: from genome-wide association studies to rare variants and beyond. *Diabetologia* 2014;**57:** 1528-1541.

29. Aitman TJ, Critser JK, Cuppen E, Dominiczak A, Fernandez-Suarez XM, Flint J, *et al*. Progress and prospects in rat genetics: a community view. *Nature genetics* 2008;**40:** 516-522.

30. Sugiura K, Miyake T, Taniguchi Y, Yamada T, Moralejo DH, Wei S, *et al*. Identification of novel non-insulin-dependent diabetes mellitus susceptibility loci in the Otsuka Long-Evans Tokushima fatty rat by MQM-mapping method. *Mammalian genome : official journal of the International Mammalian Genome Society* 1999;**10:** 1126-1131.

31. Wei S, Wei K, Moralejo DH, Ogino T, Koike G, Jacob HJ, *et al*. Mapping and characterization of quantitative trait loci for non-insulin-dependent diabetes mellitus with an improved genetic map in the Otsuka Long-Evans Tokushima fatty rat. *Mammalian genome : official journal of the International Mammalian Genome Society* 1999;**10:** 249-258.

32. Phillips MS, Liu Q, Hammond HA, Dugan V, Hey PJ, Caskey CJ, *et al*. Leptin receptor missense mutation in the fatty Zucker rat. *Nature genetics* 1996;**13:** 18-19.

33. Ueda H, Ikegami H, Kawaguchi Y, Fujisawa T, Nojima K, Babaya N, *et al*. Age-dependent changes in phenotypes and candidate gene analysis in a polygenic animal model of Type II diabetes mellitus; NSY mouse. *Diabetologia* 2000;**43:** 932-938.

34. Li DS, Yuan YH, Tu HJ, Liang QL, Dai LJ. A protocol for islet isolation from mouse pancreas. *Nature protocols* 2009;**4:** 1649-1652.

35. Okazaki M, Saito Y, Udaka Y, Maruyama M, Murakami H, Ota S, *et al*. Diabetic nephropathy in KK and KK-Ay mice. *Experimental animals / Japanese Association for Laboratory Animal Science* 2002;**51:** 191-196.

36. Masuyama T, Katsuda Y, Shinohara M. A novel model of obesity-related diabetes: introgression of the Lepr(fa) allele of the Zucker fatty rat into nonobese Spontaneously Diabetic Torii (SDT) rats. *Experimental animals / Japanese Association for Laboratory Animal Science* 2005;**54:** 13-20.

37. Gao Q, Jia Y, Yang G, Zhang X, Boddu PC, Petersen B, *et al*. PPARalpha-Deficient ob/ob Obese Mice Become More Obese and Manifest Severe Hepatic Steatosis Due to Decreased Fatty Acid Oxidation. *The American journal of pathology* 2015;**185:** 1396-1408.

38. Giesbertz P, Padberg I, Rein D, Ecker J, Hofle AS, Spanier B, *et al*. Metabolite profiling in plasma and tissues of ob/ob and db/db mice identifies novel markers of obesity and type 2 diabetes. *Diabetologia* 2015;**58:** 2133-2143.

39. Sasaki T, Kuroko M, Sekine S, Matsui S, Kikuchi O, Susanti VY, *et al*. Overexpression of insulin receptor partially improves obese and diabetic phenotypes in db/db mice. *Endocrine journal* 2015.

40. Ogino T, Moralejo DH, Zhu M, Toide K, Wei S, Wei K, *et al*. Identification of possible quantitative trait loci responsible for hyperglycaemia after 70% pancreatectomy using a spontaneously

diabetogenic rat. *Genetical research* 1999;**73:** 29-36.

41. Tautz D. Hypervariability of simple sequences as a general source for polymorphic DNA markers. *Nucleic acids research* 1989;**17:** 6463-6471.

42. Serikawa T, Kuramoto T, Hilbert P, Mori M, Yamada J, Dubay CJ, *et al*. Rat gene mapping using PCR-analyzed microsatellites. *Genetics* 1992;**131:** 701-721.

43. Hubert R, Weber JL, Schmitt K, Zhang L, Arnheim N. A new source of polymorphic DNA markers for sperm typing: analysis of microsatellite repeats in single cells. *American journal of human genetics* 1992;**51:** 985-991.

44. Kimura K, Noguchi E, Shibasaki M, Arinami T, Yokouchi Y, Takeda K, *et al*. Linkage and association of atopic asthma to markers on chromosome 13 in the Japanese population. *Human molecular genetics* 1999;**8:** 1487-1490.

45. De Sanctis GT, Singer JB, Jiao A, Yandava CN, Lee YH, Haynes TC, *et al*. Quantitative trait locus mapping of airway responsiveness to chromosomes 6 and 7 in inbred mice. *The American journal of physiology* 1999;**277:** L1118-1123.

46. Zhang Y, Leaves NI, Anderson GG, Ponting CP, Broxholme J, Holt R, *et al*. Positional cloning of a quantitative trait locus on chromosome 13q14 that influences immunoglobulin E levels and asthma. *Nature genetics* 2003;**34:** 181-186.

47. West DB, Goudey-Lefevre J, York B, Truett GE. Dietary obesity linked to genetic loci on chromosomes 9 and 15 in a polygenic

mouse model. *The Journal of clinical investigation* 1994;**94:** 1410-1416.

48. York B, Lei K, West DB. Sensitivity to dietary obesity linked to a locus on chromosome 15 in a CAST/Ei x C57BL/6J F2 intercross. *Mammalian genome : official journal of the International Mammalian Genome Society* 1996;**7:** 677-681.

49. Pomp D. Genetic dissection of obesity in polygenic animal models. *Behavior genetics* 1997;**27:** 285-306.

50. Clark JS, Jeffs B, Davidson AO, Lee WK, Anderson NH, Bihoreau MT, *et al*. Quantitative trait loci in genetically hypertensive rats. Possible sex specificity. *Hypertension* 1996;**28:** 898-906.

51. Samani NJ, Gauguier D, Vincent M, Kaiser MA, Bihoreau MT, Lodwick D, *et al*. Analysis of quantitative trait loci for blood pressure on rat chromosomes 2 and 13. Age-related differences in effect. *Hypertension* 1996;**28:** 1118-1122.

52. Baima J, Nicolaou M, Schwartz F, DeStefano AL, Manolis A, Gavras I, *et al*. Evidence for linkage between essential hypertension and a putative locus on human chromosome 17. *Hypertension* 1999;**34:** 4-7.

53. Galli J, Fakhrai-Rad H, Kamel A, Marcus C, Norgren S, Luthman H. Pathophysiological and genetic characterization of the major diabetes locus in GK rats. *Diabetes* 1999;**48:** 2463-2470.

54. Suto J, Matsuura S, Imamura K, Yamanaka H, Sekikawa K. Genetics of obesity in KK mouse and effects of A(y) allele on quantitative regulation. *Mammalian genome : official journal of the International*

Mammalian Genome Society 1998;**9:** 506-510.

55. Moralejo DH, Ogino T, Zhu M, Toide K, Wei S, Wei K, *et al*. A major quantitative trait locus co-localizing with cholecystokinin type A receptor gene influences poor pancreatic proliferation in a spontaneously diabetogenic rat. *Mammalian genome : official journal of the International Mammalian Genome Society* 1998;**9:** 794-798.

56. Mu JL, Naggert JK, Svenson KL, Collin GB, Kim JH, McFarland C, *et al*. Quantitative trait loci analysis for the differences in susceptibility to atherosclerosis and diabetes between inbred mouse strains C57BL/6J and C57BLKS/J. *Journal of lipid research* 1999;**40:** 1328-1335.

57. Yamada T, Miyake T, Sugiura K, Narita A, Wei K, Wei S, *et al*. Identification of epistatic interactions involved in non-insulin-dependent diabetes mellitus in the Otsuka Long-Evans Tokushima Fatty rat. *Experimental animals / Japanese Association for Laboratory Animal Science* 2001;**50:** 115-123.

58. Lander ES, Green P, Abrahamson J, Barlow A, Daly MJ, Lincoln SE, *et al*. MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. *Genomics* 1987;**1:** 174-181.

59. Manly KF, Cudmore RH, Jr., Meer JM. Map Manager QTX, cross-platform software for genetic mapping. *Mammalian genome : official journal of the International Mammalian Genome Society* 2001;**12:** 930-932.

60. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of

proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences of the United States of America* 1979;**76:** 4350-4354.

61. Dupuis J, Siegmund D. Statistical methods for mapping quantitative trait loci from a dense set of markers. *Genetics* 1999;**151:** 373-386.

62. Ishida K, Mizuno A, Murakami T, Shima K. Obesity is necessary but not sufficient for the development of diabetes mellitus. *Metabolism: clinical and experimental* 1996;**45:** 1288-1295.

63. Walley AJ, Jacobson P, Falchi M, Bottolo L, Andersson JC, Petretto E, *et al*. Differential coexpression analysis of obesity-associated networks in human subcutaneous adipose tissue. *International journal of obesity (2005)* 2012;**36:** 137-147.

64. Forsgren M, Attersand A, Lake S, Grunler J, Swiezewska E, Dallner G, *et al*. Isolation and functional expression of human COQ2, a gene encoding a polyprenyl transferase involved in the synthesis of CoQ. *The Biochemical journal* 2004;**382:** 519-526.

65. Lopez-Martin JM, Salviati L, Trevisson E, Montini G, DiMauro S, Quinzii C, *et al*. Missense mutation of the COQ2 gene causes defects of bioenergetics and de novo pyrimidine synthesis. *Human molecular genetics* 2007;**16:** 1091-1097.

66. Quinzii C, Naini A, Salviati L, Trevisson E, Navas P, Dimauro S, *et al*. A mutation in para-hydroxybenzoate-polyprenyl transferase (COQ2) causes primary coenzyme Q10 deficiency. *American journal of human genetics* 2006;**78:** 345-349.

67. Collaboration TM-SAR. Mutations in COQ2 in familial and sporadic

multiple-system atrophy. *The New England journal of medicine* 2013;**369:** 233-244.

68. Singh RB, Niaz MA, Rastogi SS, Shukla PK, Thakur AS. Effect of hydrosoluble coenzyme Q10 on blood pressures and insulin resistance in hypertensive patients with coronary artery disease. *Journal of human hypertension* 1999;**13:** 203-208.

69. Kunitomo M, Yamaguchi Y, Kagota S, Otsubo K. Beneficial effect of coenzyme Q10 on increased oxidative and nitrative stress and inflammation and individual metabolic components developing in a rat model of metabolic syndrome. *Journal of pharmacological sciences* 2008;**107:** 128-137.

70. Amin MM, Asaad GF, Abdel Salam RM, El-Abhar HS, Arbid MS. Novel CoQ10 antidiabetic mechanisms underlie its positive effect: modulation of insulin and adiponectine receptors, Tyrosine kinase, PI3K, glucose transporters, sRAGE and visfatin in insulin resistant/diabetic rats. *PloS one* 2014;**9:** e89169.

71. Galaviz-Hernandez C, Stagg C, de Ridder G, Tanaka TS, Ko MS, Schlessinger D, *et al*. Plac8 and Plac9, novel placental-enriched genes identified through microarray analysis. *Gene* 2003;**309:** 81-89.

72. Rogulski K, Li Y, Rothermund K, Pu L, Watkins S, Yi F, *et al*. Onzin, a c-Myc-repressed target, promotes survival and transformation by modulating the Akt-Mdm2-p53 pathway. *Oncogene* 2005;**24:** 7524-7541.

73. Kondrashov AS, Shabalina SA. Classification of common conserved sequences in mammalian intergenic regions. *Human molecular genetics* 2002;**11:** 669-674.

74. Ohlsson R, Renkawitz R, Lobanenkov V. CTCF is a uniquely versatile transcription regulator linked to epigenetics and disease. *Trends in genetics : TIG* 2001;**17:** 520-527.

75. Jimenez-Preitner M, Berney X, Uldry M, Vitali A, Cinti S, Ledford JG, *et al*. Plac8 is an inducer of C/EBPbeta required for brown fat differentiation, thermoregulation, and control of body weight. *Cell metabolism* 2011;**14:** 658-670.

76. Ledford JG, Kovarova M, Koller BH. Impaired host defense in mice lacking ONZIN. *Journal of immunology (Baltimore, Md : 1950)* 2007;**178:** 5132-5143.

77. Jimenez-Preitner M, Berney X, Thorens B. Plac8 is required for white adipocyte differentiation in vitro and cell number control in vivo. *PloS one* 2012;**7:** e48767.