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Transgenic expression of a mutated cyclin-dependent kinase 4 (CDK4/R24C) in pancreatic β-cells prevents progression of diabetes in *db/db* mice

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ABSTRACT

In an attempt to rectify the hyperglycemic state in obese insulin resistant *db/db* mice, a transgenic line was generated (*db/db-CDK4*^{R24C}) that expresses a constitutively active form of cyclin-dependent kinase 4 (CDK4/R24C) under the control of the insulin promoter. Compared with non-transgenic *db/db* littermates, adult *db/db-CDK4*^{R24C} mice show near-complete glycemic normalization and improved plasma lipid concentrations, but are also more susceptible to weight gain and have significantly lower plasma adiponection levels. They have striking islet hypertrophy and β-cell hyperplasia, and retain an insulin secretory response during the glucose tolerance test. We examined the expression of several key regulatory transcription factor genes involved in lipid and glucose metabolism in insulin target tissues of *db/db-CDK4*^{R24C} as well as *db/db* mice, and found that the expression levels of members of the *peroxisome proliferator-activated receptor* (*PPAR*) family are highly associated with metabolic alterations in a gene- and tissue-specific manner. We show for the first time that the *Ppar-δ* in skeletal muscle and white adipose tissues is transcriptionally down-regulated in *db/db* mice. The *db/db-CDK4*^{R24C} mice present a novel model of leptin-resistant obesity with compensatory hyperinsulinemia and normalized blood glucose levels, and thus may be useful for future studies that aim to dissect relationships between insulin and leptin signaling.

1. Introduction

C57BL/KsJ (BKS) mice carrying a homozygous *db/db* mutation (*db/db* mice) are characterized by the absence of the long form of the leptin receptor (Ob-Rb), which leads to extreme obesity and the early onset of hyperglycemia [1,2]. In these mice, the increase in body weight is thought to be a result of not only hyperphagia, but also of other metabolic factors, such as an increase in the efficiency of food utilization and reduced physical activity [3]. Phenotypically, *db/db* mice are identical to leptin deficient C57BL/6J *ob/ob* mice (*ob/ob* mice) [1,2]. However, the clinical symptoms and course of the diabetes are known to

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be far more severe in *db/db* mice [4,5]. During the early phase (4–10 weeks), *db/db* mice exhibit hyperinsulinemia as a compensatory reaction to hyperphagia and insulin resistance in the peripheral tissues. However, highly elevated blood glucose levels, an indication of insufficient insulin production for the body's needs, are also usually observed even at this phase. During the late phase (>12 weeks), the decrease in the plasma insulin level is correlated with a degeneration of β-cells and atrophy of the pancreatic islets. The *db/db* mice then exhibit marked hyperglycemia and a number of diabetes-related metabolic phenotypes, resulting in a shortened lifespan.

Since disturbances in glucose and lipid metabolism in *db/db* mice resemble those fond in human type 2 diabetes (T2D) patients, *db/db* mice have been extensively used for the investigation of T2D and related metabolic traits [6]. However, multiple factors including leptin resistance, obesity, insulin resistance, β-cell dysfunction and resulting hyperglycemia, which are physiologically interconnected at multiple levels, are likely contributors to the metabolic abnormalities in *db/db* mice. Thus, it is extremely difficult to accurately determine the relative contribution of each of these factors. Hyperglycemia may be treated by the administration of hypoglycemic agents, external insulin injections, or transplantation of the pancreas or islets; however, such treatment strategies in *db/db* mice are usually insufficient to improve glycemic control, and are often hampered by several problems, such as recurrent episodes of hypoglycemia. For a few examples, *db/db* mice with a targeted gene deletion of cyclin-dependent kinase (Cdk) inhibitor $p27^{Kip1}$ (*Cdkn1b*) have been described as showing amelioration of hyperglycemia via increased pancreatic islet mass and the maintenance of compensatory hyperinsulinemia [7]. In addition, an early study showed that, as a cell-based gene therapy model, subcutaneous transplantation of MIN6 (a murine insulinoma cell-line) cells into *db/db* mice normalized glucose tolerance and prevented fatty liver development [8].

In our previous study, we have generated transgenic mice (*CDK4*^{R24C}*-Tg* mice) expressing a constitutively active form of cyclin-dependent kinase 4 (CDK4/R24C; an arginine-to-cysteine exchange at residue 24) under the control of the insulin promoter, and showed that these mice display pancreatic islet hyperplasia due to the extreme proliferation of β-cells [9]. These mice were superior to their littermates in terms of glucose tolerance and insulin secretion in response to intraperitoneal injection of glucose (ipGTT). Hypoglycemia was not observed even during fasting, and the transgenic mice did not develop tumors, such as insulinoma, over a 1-year observation period. In this study, in an effort to prevent or reverse β-cell loss and islet atrophy and to ameliorate the hyperglycemic state in *db/db* mice, a *db/db* line expressing the *CDK4*^{R24C} transgene (*db/db-CDK4*^{R24C} mice) was produced through cross-mating experiments. These mice exhibit near-complete glycemic normalization, with excessive insulin hypersecretion during ipGTT. As a first step in defining a metabolic phenotype in these mice, the expression levels of peroxisome proliferator-activated receptor genes (*PPARs*), sterol regulatory element binding protein genes (*SREBPs*) and carbohydrate-response element-binding protein gene (*ChREBP*), the main regulatory transcription factor genes involved in lipid and glucose metabolism [10,11], were measured in the major insulin target tissues (liver, skeletal muscle and white adipose tissue).

2. Materials and methods

2.1. Animals

All animal experiments and procedures used in this study were approved by the Institutional Animal Care and Use Committee of the University of Tokushima. All mice were maintained in 12:12 light-dark cycles, with free access to water and standard chow (Oriental MF; Oriental Yeast Company, Tokyo, Japan). *CDK4*^{R24C}*-Tg* mice expressing a constitutively active form of human *CDK4* (R24C) in pancreatic β-cells were generated and maintained on the C57BL/6 background as previously described [9]. To generate *db/db-CDK4*^{R24C} mice, male *CDK4*^{R24C}*-Tg* and female C57BL/KsJ *db/m+* (*m*; misty) mice (BKS.Cg-*m+/+Lepr*^{db}/Jcl; purchased from CLEA, Tokyo, Japan) were initially mated to obtain male heterozygous transgenic mice (*db/m+-CDK4*^{R24C}), and then crossed with female db/m+ mice. Since the C57BL/KsJ strain is reportedly a mixture of 84% C57BL/6J-like genes and 16% DBA/2J-like genes [12,13], approximately 4% of the chromosomal regions of *db/db-CDK4*^{R24C} mice and their littermates are expected to be from the DBA/2J strain.

At 4 weeks of age, all experimental animals were typed for the presence/absence of the *CDK4*^{R24C} transgene or *db* mutation, using genomic DNA purified from a tail biopsy or blood sample on FTA cards (Whatman, Tokyo, Japan). A detailed description of these PCR-based genotyping protocols has been described elsewhere [9,14].

2.2. Phenotypic characterization

Body weight was measured longitudinally. Blood for biochemical assays was collected from the retro-orbital sinus with heparin-coated micropipettes. Blood glucose concentrations were measured with a blood glucose analyzer (Antsense III; Horiba Industry, Kyoto, Japan). Plasma non-esterified free fatty acid (FFA), triglyceride (TG) and total cholesterol (TC) concentrations were determined with the NEFA C-test (Wako, Osaka, Japan), Lipidos Liquid (Toyobo, Osaka, Japan) and Cholesterol E-test (Wako, Osaka, Japan), respectively. Plasma adiponectin levels were determined using the Mouse Adiponectin ELISA Kit (Otsuka, Pharmaceuticals, Tokushima, Japan).

Intraperitoneal glucose tolerance tests (ipGTTs) were performed as described previously [9]. Briefly, male mice at 12 weeks of age were fasted overnight (approximately 16 h) with free access to water. Next morning, after body weight and a baseline blood sample were taken, mice were given an intraperitoneal injection of 1 mg glucose per gram of body weight. Subsequently, blood samples were taken from the retro-orbital sinus at 15, 30, 60, and 120 min. Plasma samples were obtained by centrifugation, and stored at −80 °C until used. Plasma insulin concentrations were measured using the Mouse Insulin ELISA Kit U-type or T-type (Shibayagi, Gunma, Japan). The homeostasis model assessment-insulin resistance (HOMA-IR) index was not calculated in this study, because the use of HOMA-IR values in non-human animal models has not been adequately validated, and is controversial [15,16]. Hepatic TG content was measured as previously described [17]. Briefly, hepatic TG was extracted from frozen liver tissue using chloroform/methanol (2:1), dried at −80 °C and resolved

with isopropanol. Aliquots were then used in the Lipidos Liquid (Toyobo) assay, with minor modifications.

2.3. Histological analysis

Male mice were sacrificed at 12 weeks of age and the liver and pancreas were immediately removed and fixed in 10% formalin neutral buffer solution (Mildform 10N; Wako) overnight at 4 °C. The tissues were subsequently placed in 20% sucrose in phosphate buffered saline, embedded in Tissue-Tek OCT compound (Sakura, Tokyo, Japan) and immediately frozen in a dry-ice/isopentane bath. For histological examination, 5-μm cryostat sections were prepared and stained with hematoxylin and eosin (H&E).

Immunostaining for insulin was carried out with HRP-conjugated Anti-Insulin Affibody (Abcam, Cambridge, MA, USA) according to the manufacturer's protocol. The staining was developed with DAB substrate and the tissue sections were counter-stained with hematoxylin. Morphometric evaluation of the β-cell area was performed on insulin-stained sections using WinROOF image software (Ver. 5.0; Mitani Corp., Fukui, Japan). The percentage of β-cell area in the pancreas was calculated by dividing the area of all insulin-positive cells in one section by the total area of this section and multiplying this ratio by 100.

2.4. RNA isolation and quantitative real-time RT-PCR

Following sacrifice, tissues (liver, skeletal muscle and epididymal white fat) were removed, quick-frozen in liquid nitrogen, and stored at −80 °C prior to extraction of total RNA. Total RNA was isolated from liver and skeletal muscle using Trizol reagent (Invitrogen, Tokyo, Japan) according to the manufacturer's protocol. The RNeasy Lipid Tissue Mini Kit (QIAGEN, Tokyo, Japan) was used to isolate RNA from epididymal white fat. To eliminate residual genomic DNA, the purified total RNA was treated with RNase-free DNase I and re-purified following the QIAGEN RNeasy Mini "RNA Cleanup" protocol (QIAGEN).

First-strand cDNA was synthesized from 1 μg of total RNA using the Superscript III First-Strand cDNA Synthesis System (Invitrogen) and random hexamers as primers. Quantitative RT-PCR was performed on an ABI 7900HT system (Applied Biosystems, Tokyo, Japan), using pre-designed TaqMan gene expression assays (assay IDs: *Ppar-α*, Mm00440939_m1; *Ppar-γ*, Mm00440945_m1; *Ppar-δ*, Mm00803186_g1; *Srebp-1*, Mm01138344_m1; *Srebp-2*, Mm01306293_m1; *Chrebp*, Mm00498811_m1; *18S* rRNA, Mm00507222_s1). RT-PCR reactions were performed in triplicate for each gene in a mixture containing 1–3 μl of diluted cDNA, 250 nM probes, 900 nM primers, and 5 μl of 2× Taqman Universal PCR Master Mix (Applied Biosystems), with the following amplification parameters: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. The quantity of the target in each sample was normalized to that of the reference control (*18S* rRNA) using the comparative ($2^{-\Delta\Delta Ct}$) method following the manufacturer's instructions.

2.5. Statistical analysis

Data were expressed as the mean ± standard deviations (S.D.). Statistical analyses were performed using the two-tailed Student's *t*-test.

3. Results

3.1. Changes in body weight and blood glucose concentration

The *db/db-CDK4*^{R24C} mice, both male and female, were born healthy and in normal Mendelian ratios (data not

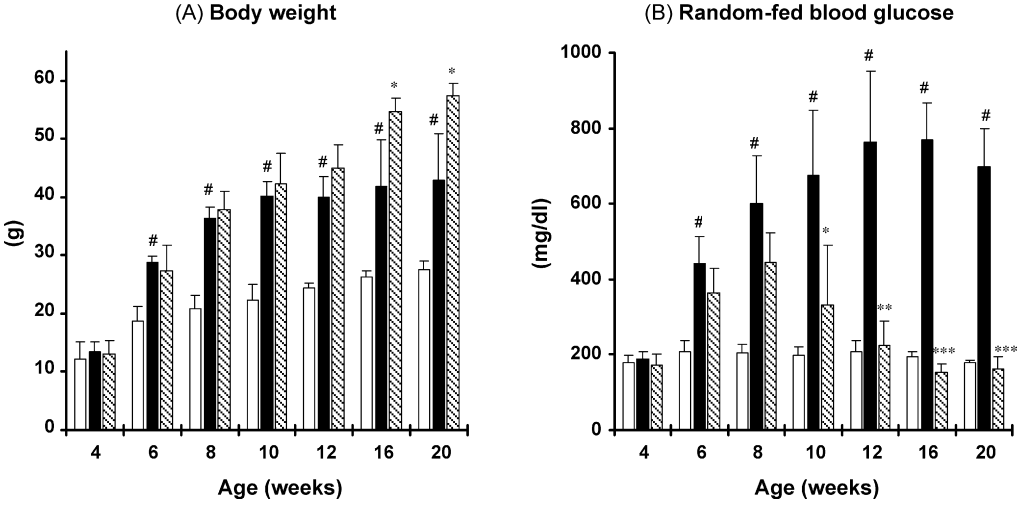

Fig. 1 – Changes in body weight (A) and random-fed blood glucose level (B) in wild-type, *db/db* and *db/db-CDK4*^{R24C} male mice. (A) The body weights of wild-type (open bars, *n* = 7), *db/db* (closed bars, *n* = 5) and *db/db-CDK4*^{R24C} (hatched bars, *n* = 8) mice were measured every 2 weeks for 12 weeks and every 4 weeks, thereafter. All of the mice were male and maintained on normal rodent chow and water ad lib. Data are expressed as the mean ± S.D. $^{\#}P < 0.001$ versus wild-type animals; $^{*}P < 0.05$ versus *db/db* mice. (B) Random blood glucose levels were measured once every 2–4 weeks in non-fasted wild-type (open bars, *n* = 7), *db/db* (closed bars, *n* = 5) and *db/db-CDK4*^{R24C} (hatched bars, *n* = 8) male mice fed on normal chow. Data are expressed as the mean ± S.D. $^{\#}P < 0.001$ versus wild-type animals; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus *db/db* mice.

Table 1 – Metabolic phenotype in wild-type, *db/db* and *db/db-CDK4*^{R24C} male mice (12 weeks of age)

	Wild-type	*db/db*	*db/db-CDK4*^{R24C}
Plasma lipid (in fasting state, $n = 6$ per group)			
TC (mg/dl)	64.7 ± 13.8	128.5 ± 13.4$^{###}$	143.6 ± 16.0
TG (mg/dl)	14.7 ± 4.2	111.6 ± 35.4$^{###}$	46.6 ± 8.6**
FFA (mequiv./l)	0.70 ± 0.21	1.40 ± 0.34$^{##}$	0.74 ± 0.12**
ipGTT ($n = 3$–5 per group)			
Glucose (mg/dl)			
0-min	122.3 ± 21.8	413.3 ± 124.8$^{#}$	106.8 ± 10.1**
15-min	304.0 ± 24.6	792.7 ± 119.0$^{##}$	287.8 ± 61.2***
30-min	299.0 ± 27.7	957.4 ± 13.6$^{###}$	290.6 ± 88.1***
60-min	255.3 ± 29.5	989.5 ± 129.4$^{###}$	267.2 ± 114.4***
120-min	222.3 ± 41.1	850.6 ± 111.1$^{###}$	206.7 ± 69.7**
Insulin (ng/ml)			
0-min	BAS[a]	3.17 ± 1.62$^{#}$	22.0 ± 9.76*
15-min	0.29 ± 0.34	3.87 ± 2.59	54.8 ± 20.6**
30-min	0.35 ± 0.33	2.19 ± 1.96	34.7 ± 17.4*
60-min	0.31 ± 0.10	2.30 ± 1.83	23.0 ± 6.05**
120-min	0.48 ± 0.19	2.63 ± 1.19$^{#}$	9.90 ± 3.99*
Adiponection ($n = 6$; μg/ml)	18.4 ± 1.6	12.8 ± 1.4$^{###}$	8.7 ± 0.9***

Data are means ± S.D. Significance was determined using two-tailed unpaired Student's *t*-test. $^{#}P < 0.05$, $^{##}P < 0.01$ and $^{###}P < 0.001$ compared with wild-type mice. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with *db/db* mice.

[a] The fasting plasma insulin levels of wild-type mice were below the assay sensitivity limit of 0.039 ng/ml. BAS, below assay sensitivity; TC, total cholesterol; TG, triglyceride; FFA, free fatty acid.

shown). Fig. 1A shows body weight changes in male *db/db-CDK4*^{R24C} mice fed a regular chow diet. Compared to littermate controls, *db/db* and *db/db-CDK4*^{R24C} mice become obese at around 6 weeks of age, and, until 12 weeks of age, there was no significant difference between mean body weights for the two *db/db* groups. However, *db/db-CDK4*^{R24C} mice displayed accelerated weight gain at 16 weeks or later, a significant increase compared to *db/db* littermates ($P < 0.05$).

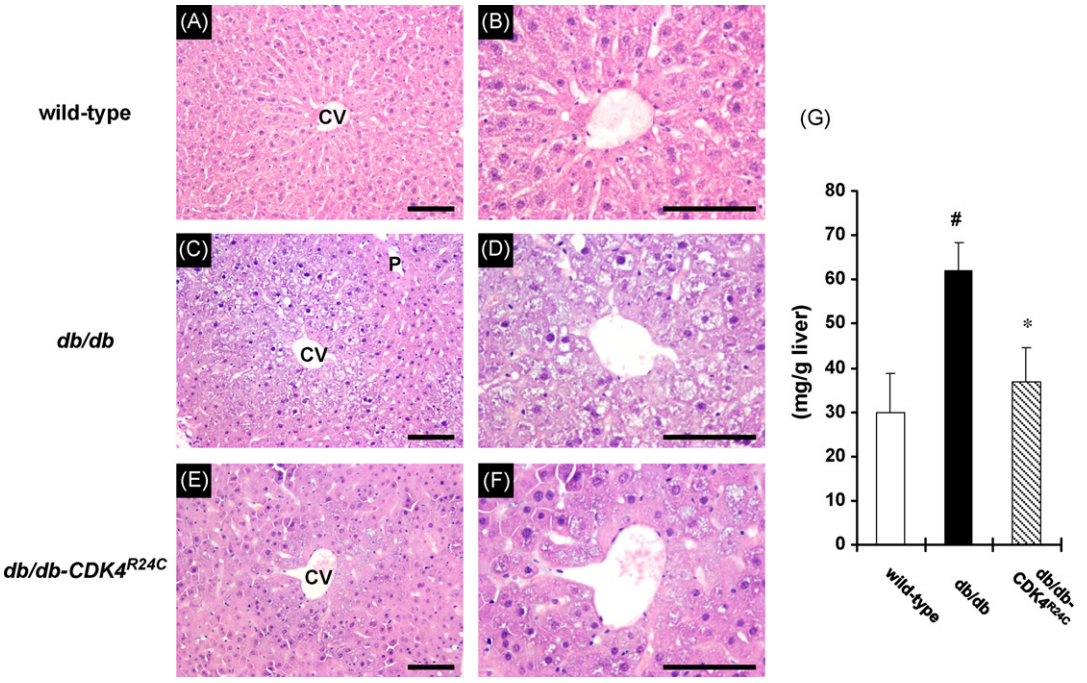

Fig. 2 – Hematoxylin-eosin (H&E) staining of liver slices, hepatic triglyceride (TG) contents in wild-type, *db/db* and *db/db-CDK4*^{R24C} mice. Liver sections from 12-week-old male wild-type (A and B), *db/db* (C and D) and *db/db-CDK4*^{R24C} (E and F) mice were stained with H&E and visualized either at low (A, C and E) or high magnifications (B, D and F). Scale bar = 100 μm. CV, central vein; P, portal vein. (G) Hepatic TG content was determined in 12-week-old male wild-type (open bars, $n = 3$), *db/db* (closed bars, $n = 4$) and *db/db-CDK4*^{R24C} (hatched bars, $n = 3$) mice as described under Section 2. Data are expressed as the mean ± S.D. $^{#}P < 0.01$ versus wild-type animals; $^{*}P < 0.01$ versus *db/db* mice.

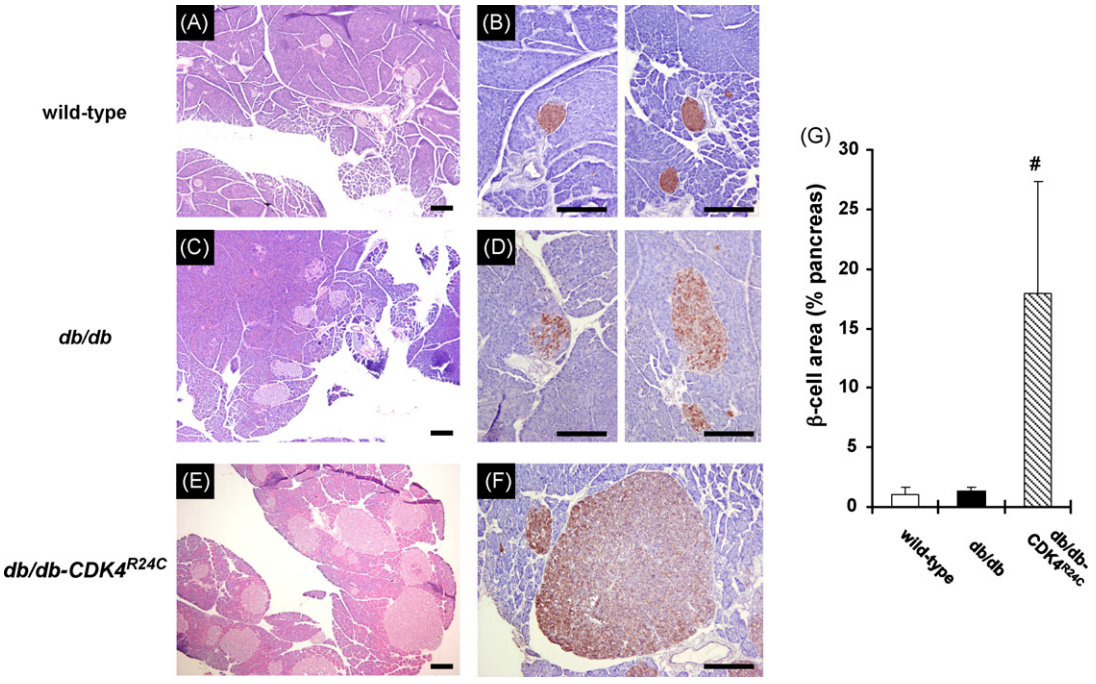

Fig. 3 – Pancreatic islet sections from wild-type, *db/db* and *db/db-CDK4*^{R24C} mice. Pancreatic sections from 12-week-old male wild-type (A), *db/db* (C) and *db/db-CDK4*^{R24C} (E) mice were stained with H&E. Serial sections were stained immunohistochemically for insulin, counter-stained with hematoxylin and visualized at higher magnification; wild-type (B), *db/db* (D) and *db/db-CDK4*^{R24C} (F) mice. Scale bar = 200 μm. (G) The percentage β-cell fraction in the pancreas was calculated from the ratio of the insulin-positive area to the total area of the tissue section (n = 4–5 for each group). $^{\#}P < 0.01$ versus wild-type or *db/db* mice.

Changes in blood glucose level are shown in Fig. 1B. In *db/db* mice, random-fed blood glucose levels were normal just after weaning (4 weeks of age) but markedly increased over time, and severe hyperglycemia was sustained at least until 20 weeks of age. The *db/db-CDK4*^{R24C} mice showed a similar increase in blood glucose levels until 8 weeks of age, though mean blood glucose levels were slightly lower than those of *db/db* mice, and then exhibited a significant decrease thereafter when compared to *db/db* mice. At 12 weeks of age, *db/db-CDK4*^{R24C} mice displayed normal non-fasting blood glucose levels (224.3 ± 65.1 mg/dl).

3.2. *Plasma lipid profile, liver histology and hepatic TG content of db/db-CDK4*^{R24C} *mice*

At 12 weeks of age, *db/db* mice had markedly elevated fasting plasma levels of TC, TG and FFA (Table 1). These mice also displayed hepatic steatosis in a pericentral distribution expanding toward periportal zones (Fig. 2C and D), which is consistent with higher liver TG content in these mice as compared to wild-type mice (Fig. 2G; 62.1 ± 6.2 versus 29.9 ± 8.9 mg/g tissue, $P < 0.01$). Conversely, *db/db-CDK4*^{R24C} mice had significantly lower plasma TG and FFA levels than their *db/db* littermates ($P < 0.01$ for both; Table 1), but exhibited a trend toward increased TC levels. The degree of histological steatosis (Fig. 2E and F), as well as liver TG content (Fig. 2G; 36.8 ± 7.6 mg/g tissue, $P < 0.01$ versus *db/db* liver), were significantly less pronounced in *db/db-CDK4*^{R24C} than in *db/db* mice.

3.3. *Results of an ipGTT and pancreatic histology of db/db-CDK4*^{R24C} *mice*

Glucose and insulin levels were measured during an ipGTT after 16-h fasting in wild-type, *db/db* and *db/db-CDK4*^{R24C} male mice at 12 weeks of age (Table 1). A marked elevation in both basal (fasting) and post-challenge blood glucose levels was detected in *db/db* mice. They had high baseline fasting insulin levels (3.17 ± 1.62 ng/ml), but without significant potentiation of glucose-stimulated insulin secretion. In contrast, the *db/db-CDK4*^{R24C} mice showed normal glucose tolerance accompanied by increased fasting insulin level (22.0 ± 9.76 ng/ml) and marked enhancement of glucose-stimulated insulin secretion (Table 1).

Fig. 3 shows typical H&E staining and insulin immunostaining of pancreatic tissues in wild-type, *db/db*, and *db/db-CDK4*^{R24C} mice. Morphologically, islets in the *db/db* pancreas generally showed hypertrophy, having various sizes and shapes, and most of the β-cells were extensively degranulated (Fig. 3C and D) as compared to islets in normal littermates (Fig. 3A and B). Both the size and the number of islets, as well as insulin immunopositivity, were markedly increased in *db/db-CDK4*^{R24C} mice (Fig. 3E and F). The β-cell (insulin-positive) area from wild-type, *db/db*, and *db/db-CDK4*^{R24C} mice comprised $0.99 \pm 0.57\%$, $1.29 \pm 0.37\%$ and $18.0 \pm 9.39\%$ of the total

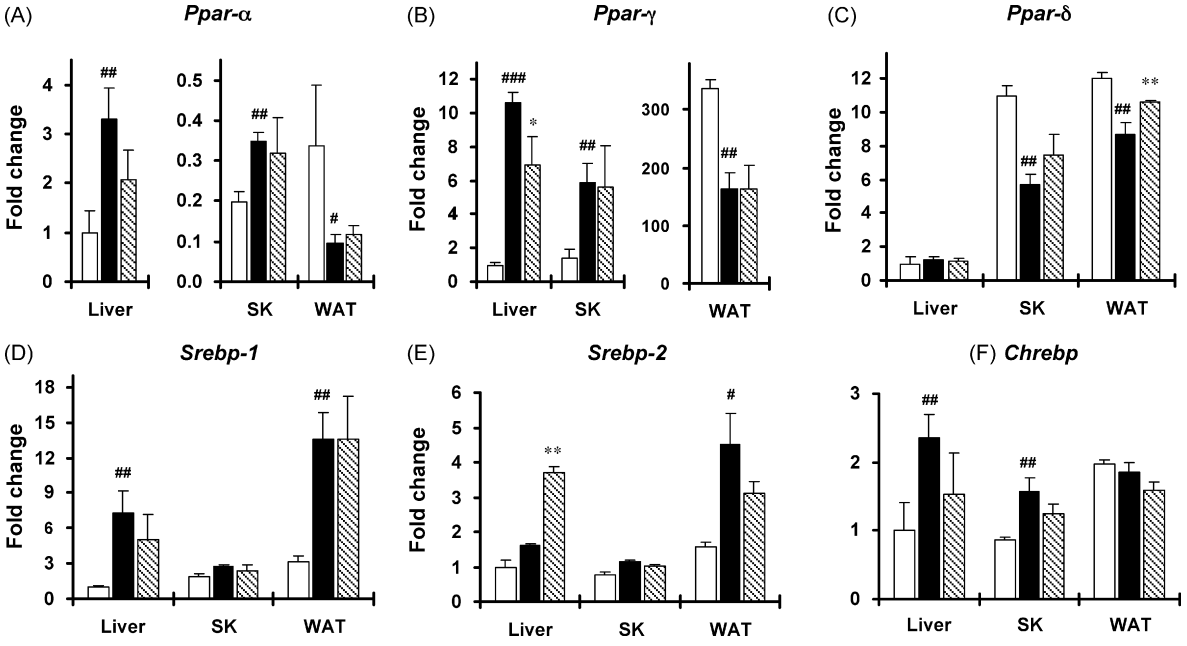

Fig. 4 – Expression of PPAR, SREBP and ChREBP transcription factors in insulin target tissues. The quantitative expression levels of *Ppar-α* (A), *-γ* (B), *-δ* (C), *Srebp-1* (D), *-2* (E) and *Chrebp* (F) were measured in tissues obtained from 12-week-old male wild-type (open bars, *n* = 3), *db/db* (closed bars, *n* = 4) and *db/db-CDK4*^{R24C} (hatched bars, *n* = 3) mice as described under Section 2. After normalization to a reference control (*18S* rRNA), the mRNA level for each gene in liver tissue was arbitrarily set at 1.0. Data are expressed as the mean ± S.D. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ versus wild-type animals; $^{*}P < 0.05$ and $^{**}P < 0.01$ versus *db/db* mice. SK, skeletal (soleus) muscle; WAT, white adipose tissue (epididymal fat).

pancreatic area, respectively ($P < 0.01$ versus wild-type or *db/db* mice; Fig. 3G).

3.4. *Plasma adiponectin concentrations*

It has been reported that plasma adiponectin concentrations correlate inversely with body mass status and positively with insulin sensitivity [18,19]. At 12 weeks of age, the plasma adiponectin level in each group of mice was inversely related to their fasting insulin levels, showing a significantly lower value in obese diabetic *db/db* mice (12.8 ± 1.4 μg/ml, $P < 0.001$ versus wild-type mice; Table 1) and a further decline in *db/db-CDK4*^{R24C} mice (8.7 ± 0.9 μg/ml, $P < 0.001$ versus *db/db* mice).

3.5. *Differential gene expression pattern of the members of the PPAR, SREBP and ChREBP families*

Members of the *PPAR*, *SREBP* and *ChREBP* families are important transcription factors regulating glucose and lipid metabolism. Therefore, their expression profiles in the insulin target tissues of *db/db* or *db/db-CDK4*^{R24C} mice were of interest. As previously indicated [20], in normal mice, three *PPAR* members show unique and distinct tissue expression patterns (Fig. 4): i.e. *Ppar-α* is expressed highly in the liver, *Ppar-γ* predominantly in white adipose tissue, and *Ppar-δ* in skeletal muscle and white adipose tissues. Intriguingly, the expression changes and patterns of *PPARs* that occurred under fasting conditions in *db/db* mice also differed among tissues. The expression levels of *Ppar-α* and *-γ* were correlated and elevated in liver and skeletal muscle from *db/db* mice, whereas they were reciprocally reduced in white adipose tissue (Fig. 4A and B). *Ppar-δ* was down-regulated in *db/db* skeletal muscle and white adipose tissues, but not in the liver (Fig. 4C). In *db/db-CDK4*^{R24C} mice, expression changes toward wild-type levels were observed with *Ppar-α* and *-γ* (in the liver), and *Ppar-δ* (in skeletal muscle and white adipose tissues).

In fasted wild-type control mice, the expression levels of *Srebps* and *Chrebp* were relatively low, an observation consistent with previous reports [21,22], and there were no obvious differences among tissues (Fig. 4D–F). However, they were generally higher in *db/db* mice, and were increased at least twofold over wild-type for hepatic and adipose *Srebp-1* (7.31- and 4.27-fold increase, respectively), adipose *Srebp-2* (2.83-fold), and hepatic *Chrebp* (2.36-fold). In *db/db-CDK4*^{R24C} mice, though not statistically significant, a decreasing trend in expression was observed for hepatic *Srebp-1*, adipose *Srebp-2* and hepatic *Chrebp*. Conversely, *Srebp-2* expression was significantly increased in *db/db-CDK4*^{R24C} liver tissues (3.69- and 2.27-fold versus wild-type and *db/db*, respectively, $P < 0.01$; Fig. 4E).

4. Discussion

In T2D, insulin resistance is thought to be an important initiating pathogenic mechanism, and hyperglycemia becomes overt when pancreatic β-cells fail to compensate for the insulin resistance through an increase in β-cell mass, an increase in secretory capacity, or both [23,24]. Thus, the capacity of the β-cell mass to increase in the face of insulin

resistance is very important in the pathophysiology of hyperglycemia in T2D. Intriguingly, a recent study revealed that mice with a pancreas-specific knockout of the leptin receptor (pancreas-specific Ob-R KO mice), when fed normal chow, manifested improved glucose tolerance, enhanced first-phase insulin response to glucose, and increased β-cell size and islet hyperplasia, whereas on a high-fat diet they exhibited significantly greater glucose intolerance as compared to controls because of their inability to increase β-cell mass [25]. These findings suggest that leptin signaling or leptin resistance within β-cells is an important contributing factor to hyperinsulinemia, β-cell failure, and consequent hyperglycemia in the obese state. In this study, 12-week-old male *db/db-CDK4*^{R24C} mice achieved near-normal glycemia with escalating hyperinsulinemia. They had striking islet hypertrophy and β-cell hyperplasia, but their insulin-producing cells behave like differentiated β-cells with regard to insulin secretion in response to glucose during ipGTT. These results suggest that *CDK4*^{R24C} overexpression was sufficient to overcome the genetic leptin-resistance in *db/db* β-cells.

Furthermore, it was recently shown that secreted insulin itself can serve as an autocrine feedback signal for β-cell function and growth, and that insulin resistance at the β-cell level might affect this modulation [23]. Supporting this notion, mice with a β-cell specific knockout of the insulin receptor (IR; βIRKO) [26], as well as insulin receptor substrate 2 (*Irs2*) knockout mice [27,28], exhibit an increased susceptibility to developing T2D, due to either decreased islet growth in adults or lack of compensatory β-cell hyperplasia. Based on these findings, we speculate that, in *db/db-CDK4*^{R24C} mice, their sustained high insulin levels may also account, at least in part, for the acceleration of β-cell mass growth.

Our *db/db-CDK4*^{R24C} mice present a unique model of leptin-resistant obesity that is associated with severe insulin resistance but compensatory hyperinsulinemia and normalized blood glucose levels. These phenotypes might resemble those of other animal models such as the obese *ob/ob* mouse [1] and the fatty *fa/fa* Zucker rat [29], although, reportedly, they usually develop moderate hyperglycemia. The *db/db-CDK4*^{R24C} mice may also be a good representation of human obese prediabetic subjects or inadequately insulin-treated obese T2D patients, resulting in iatrogenic hyperinsulinemia and further weight gain. These mice also had a lipid profile that differed from that of diabetic *db/db* mice: i.e., significantly low plasma TG and FFA levels associated with reduced hepatic TG content. These changes are probably attributable to complex mechanisms involving decreased peripheral lipolysis, reduced hepatic *de novo* lipogenesis, and possibly elevated very-low-density lipoprotein (VLDL) formation and fatty acid uptake.

Many previous studies, including those utilizing various tissue-specific IR knockout mice, have indicated that insulin resistance is not a uniform systemic alteration, but varies considerably among different tissues [30,31]. Consistent with this idea, organs in *db/db-CDK4*^{R24C} mice appeared to have different degrees of compensation to insulin resistance. Fasting hyperglycemia is a hallmark of T2D and this appears to be largely attributable to increased hepatic gluconeogenesis. At 12 weeks of age, *db/db-CDK4*^{R24C} mice showed normal fasting blood glucose levels, perhaps due to successful inhibition of hepatic glucose production. It is noteworthy that these mice displayed marked peripheral insulin concentration (mean fasting insulin 22.0 ± 9.76 ng/dl; Table 1), and it is anticipated that they have much higher insulin levels in portal vein blood or within the liver sinusoids. We thus assume that hepatic insulin resistance was almost fully compensated for by hyperinsulinemia. It has been suggested that overexpressions of *PPAR-γ*, *SREBP-1* and *ChREBP* could contribute to the pathogenesis of insulin resistance and steatosis in liver [17,32,33]. Consistently, our expression data showed that hepatic expressions of these transcription factors was increased in the *db/db* mouse liver, and that expression changes toward wild-type levels, though only partial, were observed in euglycemic *db/db-CDK4*^{R24C} mice.

While hepatic insulin resistance appeared to be improved, the lower plasma adiponection levels in *db/db-CDK4*^{R24C} mice as compared to those in *db/db* mice raise the possibility that whole-body insulin resistance or insulin resistance in non-hepatic peripheral tissues might persist and may even worsen in these mice. It has been shown that skeletal muscle contributes considerably to the maintenance of systemic substrates and energy balance [34,35]. The muscle-specific IR knockout (MIRKO) mice shows severe insulin resistance in skeletal muscle both *in vitro* and *in vivo*, a dramatic increase in total body fat mass, and elevated serum TG and FFA levels, but without developing hyperinsulinemia or overt T2D [36,37]. During euglycemic-hyperinsulinemic clamp tests, MIRKO mice showed increased glucose utilization in white adipose tissues, which ultimately leads to increased fat mass and weight gain. In addition, in genetically leptin-resistant obese diabetic ZDF rats, the degree of insulin sensitivity improvement in response to amelioration of hyperglycemia differs among tissues, and is high in the liver and very low in skeletal muscle [38]. These findings reaffirm that skeletal muscle is a primary site of insulin resistance, and that skeletal muscle-specific regulation of leptin or insulin signaling, their tightly coordinated cross-talk is important for the regulation of body fat mass and the development of obesity. It is tempting to speculate that a similar situation with MIRKO mice or ZDF rats may be mirrored in *db/db-CDK4*^{R24C} mice, which are characterized by sustained hyperinsulinemia-euglycemia and a significant body weight gain compared to *db/db* mice. However, for precise assessment of insulin sensitivity status in *db/db-CDK4*^{R24C} mice, and to localize the site of potential insulin resistance, further studies, such as insulin tolerance tests (ITT) or glucose clamp studies, are clearly warranted.

An examination of the expression levels of key transcription factor genes (*PPARs*, *SREBPs* and *ChREBP*) in several tissues of *db/db* and *db/db-CDK4*^{R24C} mice apparently supports their importance, particularly those of *PPAR* members, as potential determinants of insulin and leptin sensitivities in different tissues, as they were significantly altered in *db/db* mice. An interesting finding based on our work is that each individual *PPAR* member showed substantially diverse expression patterns in obese hyperglycemic *db/db* mice, which occurred in both a gene- and tissue-specific manner. For example, when compared to wild-type mice, *Ppar-α* and *-γ* were significantly up-regulated in liver and skeletal muscle tissues of *db/db* mice, whereas their expression levels in *db/db* white adipose tissues were reciprocally down-regulated. In contrast, *Ppar-δ* expression in *db/db* mice was not altered in the liver, but was down-

regulated in skeletal muscle and white adipose tissues. To the best of our knowledge, this is the first report of *db/db*-induced expression changes of *Ppar-γ* in white adipose and *Ppar-δ* in skeletal muscle and white adipose tissues. We assume that the observed expression changes may be either causal or reflect altered metabolic phenotypes; however, due to the preliminary nature of the study, it is difficult to draw definitive conclusions about cause-and-effect relationships.

Expression changes attributable to the $CDK4^{R24C}$ transgene were only seen in *Ppar-α* and *-γ* (in the liver) and *Ppar-δ* (in skeletal muscle and adipose tissues), although they were only marginal to small changes. These alterations in *Ppar-δ* expression are particularly interesting because previous studies demonstrated that its activation exerts many favorable effects on glucose tolerance and lipid metabolism, including a reduction of weight gain, an increase skeletal muscle metabolic rate and endurance and, consequently, an improvement in insulin sensitivity [10,39]. Further studies are warranted to broaden the experimental evidence for the role of *Ppar-δ*, especially in glucose and lipid metabolism in skeletal muscle.

In summary, *db/db-*$CDK4^{R24C}$ mice were created that had near-normal glycemia and compensatory hyperinsulinemia with marked β-cell hyperplasia. Future investigations, including clamp studies and *in vitro* experiments on a tissue-by-tissue basis, should address the physiologic explanation for the inter-tissue differences in insulin resistance, as well as differences in leptin action. This mouse model might also be utilized in studies of basic mechanisms of pancreatic β-cell growth and function. Our results also support the use of *CDK4* as a potential tool in engineering a source of β-cell mass for islet transplantation, or as a target of gene therapy for diabetes.

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Conflict of interest

There are no conflicts of interest.

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