Control of Energy Metabolism Through the Forkhead Transcription Factor Foxo1

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Abbreviations

kb	kilobase
Foxo1	Forkhead box O1
aP2	adipocyte fatty acid-binding protein
UCP1	uncoupling protein 1
IGF1	insulin like growth factor 1
CREB	cyclic AMP responsive element binding protein
CBP	CREB binding protein
PI3K	phosphatidylinositol 3'-kinase
PPARγ	peroxisome proliferator-activated receptor-γ
PGC1	PPARγ coactivator 1
C/EBP	CAAT/enhancer-binding protein
G6Pase	glucose-6-phosphatase
PEPCK	phosphoenolpyruvate carboxykinase
FFA	free fatty acid
GLUT4	glucose transporter 4
SREBP1	sterol regulatory element binding protein 1
TNFα	tumor necrosis factor α
IL	interleukin
LPL	lipoprotein lipase
WAT	white adipose tissue
BAT	brown adipose tissue
SDS	sodium dodecyl sulfate

PAGE	polyacrylamide gel electrophoresis
HEPES	N-2-hydroxyethylpiperazine-N'-2-ehanesulfonic acid
EGTA	ethyleneglycol-bis (beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid
EDTA	ethylenediaminetetraacetic acid
PBS	phosphate buffer salin
DMSO	dimethyl sulfoxide
DAB	3,3'-diamino benzidine tetrahydrochloride
PMSF	phenylmethylsulfonyl fluoride
ELISA	enzyme-linked immunosorbent assay
HOMA-R	homeostasis model assessment on insulin resistance
GTT	glucose tolerance test
ITT	insulin tolerance test
NTG	non transgenic
TG	transgenic
WT	wild type
RT-PCR	reverse transcription-polymerase chain reaction
min	minute

Contents

Chapter I:

Preface

A continuous supply of glucose is necessary to ensure proper function and survival of all organs. While hypoglycemia produces cellular death, chronic hyperglycemia also can result in organ damage. Therefore, the plasma glucose level is maintained in a narrow range around 5 mM, despite periods of feeding and fasting status (1). This tight control is governed by the balance between food intake, glucose production by the liver and uptake and metabolism by peripheral tissues such as skeletal muscle and adipose tissue. The glucose metabolism in feeding and fasting statuses is regulated by the organ cross-talk between tissues as indicated in Fig. 1 and Fig. 2. Insulin is one of the key hormones, involving whole-body glucose and lipid homeostasises. Liver and adipose tissue are key organs targeted by insulin, and they have important roles in overall glucose metabolism (1).

The liver keeps the blood glucose level nearly constant through breakdown of glycogen (glycogenolysis) and *de novo* synthesis of glucose from noncarbohydrate precursors such as lactate, glycerol, and alanine (gluconeogenesis) in fasted status, because hypoglycemia causes serious damage to brain and the neural systems (Fig. 1 and Fig. 2). Hepatic gluconeogenesis is finely mediated by hormonal signals, insulin and glucagon. In the fasting state, insulin levels drop while glucagon secretion goes up, resulting in increased gluconeogenesis. On the other hand, in the feeding state, insulin increases, promoting the glucose uptake in peripheral tissues, while suppressing glucose production in the liver. Accordingly, the expression of genes such as glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxylase (PEPCK),

which are key enzymes of gluconeogenesis, are repressed by insulin (2,3) (Fig. 3). Conversely, liver-specific disruption of insulin receptor knock out mice (LIRKO mice) exhibited a failure of insulin to suppress hepatic glucose production, partially because the expression level of G6Pase was abnormally increased in their liver (4). Thus, insulin and insulin receptor signaling in the liver have a potent role in the regulation of hepatic gluconeogenesis.

Adipose tissue is found in mammals in two different forms: white adipose tissue (WAT) and brown adipose tissue (BAT). WAT plays important metabolic roles by storing fat in the form of triglycerides in periods of energy excess such as postprandial status (Fig. 1), and releasing free fatty acids (FFA) and glycerol during energy deprivation (Fig. 2). Fat metabolism, including glyceroneogenesis, fatty acids re-esterification and lipolysis in WAT is important for overall energy flow in fasted status (Fig. 4). Most of all, fatty acids re-esterification and lipolysis of triglyceride are important mechanisms for the control of concentration of whole-body FFA, which is reconsidered as an adipocytokine (see below) as well as energy source in muscle and ketogenesis in liver for long term starve (5). Fatty acids re-esterification requires glycerol 3-phosphate, which can be synthesized from noncarbohydrate precursors like pyruvate, lactate or amino acids in the fasted status (glyceroneogenesis) (5,6). Therefore, even though the adipose tissue could account for less than 10% of whole-body glucose uptake, glucose metabolism in this tissue plays a potent role in fatty acid re-esterification. On the other hand, WAT-derived secreting molecules such as FFA, leptin, adiponectin, tumor necrosis factor α (TNFα), interleukin 6 (IL6), resistin, and recently identified visfatin, have been characterized (7-13). Some of these molecules associate with obesity and insulin resistance. BAT is multilocular lipid storage

comprising a large number of mitochondria and plays a crucial role in non-shivering thermogenesis, which could physiologically defense against obesity and type-2 diabetes through energy expenditure (14). Regulation of whole-body glucose homeostasis is strongly associated with insulin response in WAT and BAT. Insulin promotes differentiation of preadipocyte, revealed using a preadipocyte culture system (15). Fat specific disruption of insulin receptor knock out mice (FIRKO mice) displayed impaired adipocyte differentiation and marked adipose tissue hypotrophy. In addition, they exhibited promoting glucose tolerance and insulin sensitivity (16,17). On the other hand, BAT specific insulin receptor knock out mice (BATIRKO mice) exhibited diabetic phenotype without insulin resistance (18). Thus, the insulin has been realized to be critical roles in adipose metabolism and overall insulin sensitivity, but its signal cascade resulting in the affect of transcriptional regulation largely remains elusive.

There are fundamental biologic questions in glucose metabolism, how insulin signaling coordinates the physiology of animals and what is a responsible factor for the effect of the signal. The breakthrough of molecular details in target of insulin in the cell have recently been revealed by genetic analysis using *Caenorhabditis elegans* (*C. elegans*). The *C. elegans daf-2* pathway controls longevity and metabolism, and is orthologous to the mammalian insulin and IGF1 signaling cascade (19). Decreased *daf-2* signaling causes life-span extension, increased fat storage, and constitutive arrest at the dauer diapause stage. These phenotypes were suppressed by a mutation in the allele of *daf-16*, indicating that *daf-16* is negatively regulated by *daf-2* signaling and is the major downstream effector (20-22). The *daf-16* is the ortholog of mammalian FOXO family of forkhead transcription factors (23), and is regulated by nutritional conditions (24). Notably, Libina N *et al.* (25) have currently shown that *daf-16* activity in the intestine of

C. elegans, which corresponds to the animal's adipose tissue, was sufficient to extend lifespan. Furthermore, overexpressed *Drosophila melanogaster* dFOXO in adult fat body corresponding to the animal's liver and adipose tissue exhibited longevity, accompanied with regulation of fecundity, overall insulin sensitivity and lipid metabolism (26,27). These observations provide the common theme that FOXO proteins in mammalian liver and adipose tissue may regulate metabolism and life span under the various nutrient conditions. Therefore, I speculated that animal's FOXO proteins possess a critical role in energy homeostasis.

A FOXO family of forkhead transcription factor Foxo1 (Forkhead box O1) is expressed in mammalian insulin-responsible tissues, such as liver, skeletal muscle, WAT, BAT, hypothalamus and β cells of the islets of Langerhance in the pancreas (28-30). Recent biochemical studies have revealed that Foxo1 is an important target for mediating the effects of insulin or several growth factors such as insulin like growth factor 1 (IGF1) on the expression of genes involving metabolism, stress response, apoptosis and cell cycle downstream from phosphatidylinositol 3-kinase (PI3K) and Akt (also called as protein kinase B; PKB) (31). In response to insulin/IGF1, Foxo1 is phophorylated at Thr-24, Ser-253 and Ser-316 by Akt, leading to nuclear exclusion (32,33) and subsequent degradation through SCF^{Skp2}-mediated ubiquitination (34-36). In addition to phosphorylation, Daitoku *et al.* (36) and others (37-41) have shown that reversible acetylation of Foxo1 mediated by CBP/p300 and Sir2/SIRT1 is involved in modulating its transactivation function. Furthermore, a recent report has found that acetylation of Foxo1 weakens its DNA binding and facilitates PKB-dependent phosphorylation at Ser-253 (42). Together, I summarized the molecular mechanism of posttranslational modification of Foxo1 in Fig. 5.

From a physiological point of view, the genetic analysis with gain- and loss-of-function alleles has identified Foxo1 as a key modulator of hepatic gluconeogenesis under the insulin-Akt signaling pathway (43,44). Supporting these findings, hepatic production of Foxo1-Δ256, which mutant interferes with Foxo1 function, rescued a diabetic phenotype in *db/db* mice by reducing the G6Pase gene expression (45). On the other hand, Foxo1 haploinsufficiency protected against insulin resistance caused by genetically defective insulin signaling or diet induced obese, associated with increasing an adipogenic gene, peroxisome proliferator-activated receptor-γ (PPARγ) in WAT (46).

The outstanding questions in the regulation of energy metabolism of Foxo1 are how the nutrient responses due to fasting or feeding control Foxo1 *in vivo* and how Foxo1 have physiological function in adipose tissue. First of all, I demonstrated the mechanism of nutrient control of gluconeogenic gene expression through phosphorylation and translocation of Foxo1 *in vivo* (Chapter II). Furthermore, I revealed the physiological functions of Foxo1 targeted in the adipose tissue in transgenic mice, including the control of mass in WAT and BAT, regulation of fatty acids re-esterification, insulin sensitivity and energy metabolism (Chapter III). Finally, I will discuss an integrative physiological function of Foxo1 in glucose and lipid metabolisms in combination with Chapters II and III (Chapter IV), in which Foxo1 strongly participates in overall energy metabolism.

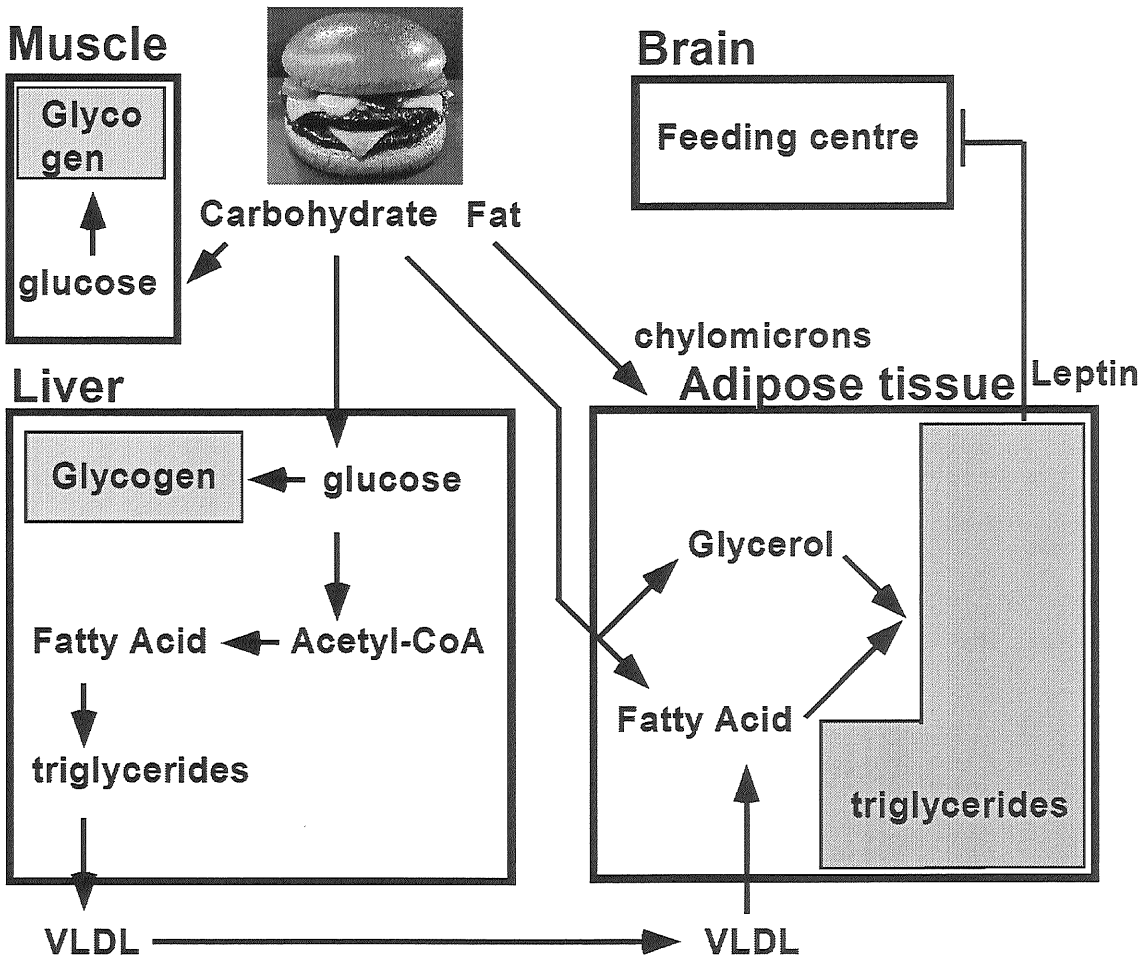

Fig. 1 Overview of the metabolic roles between tissues during feeding status. In the fed status, carbohydrates and fat enter the circulation in the form of glucose and chylomicrons, respectively. Most glucose is taken up by the muscle and liver and, if glycogen stores are already filled, used for lipogenesis in the liver. Fatty acids are converted to triglycerides and packed into very low density lipoproteins (VLDL) following a transport into adipose tissue. Uptakes of glucose and fatty acids in the adipose tissue are increased, and their subsequent conversion to triglycerides. Triglyceride storage causes increased production of hormone leptin in the adipose tissue. Leptin serves as an important signal from adipose tissue to brain to limit further food intake.

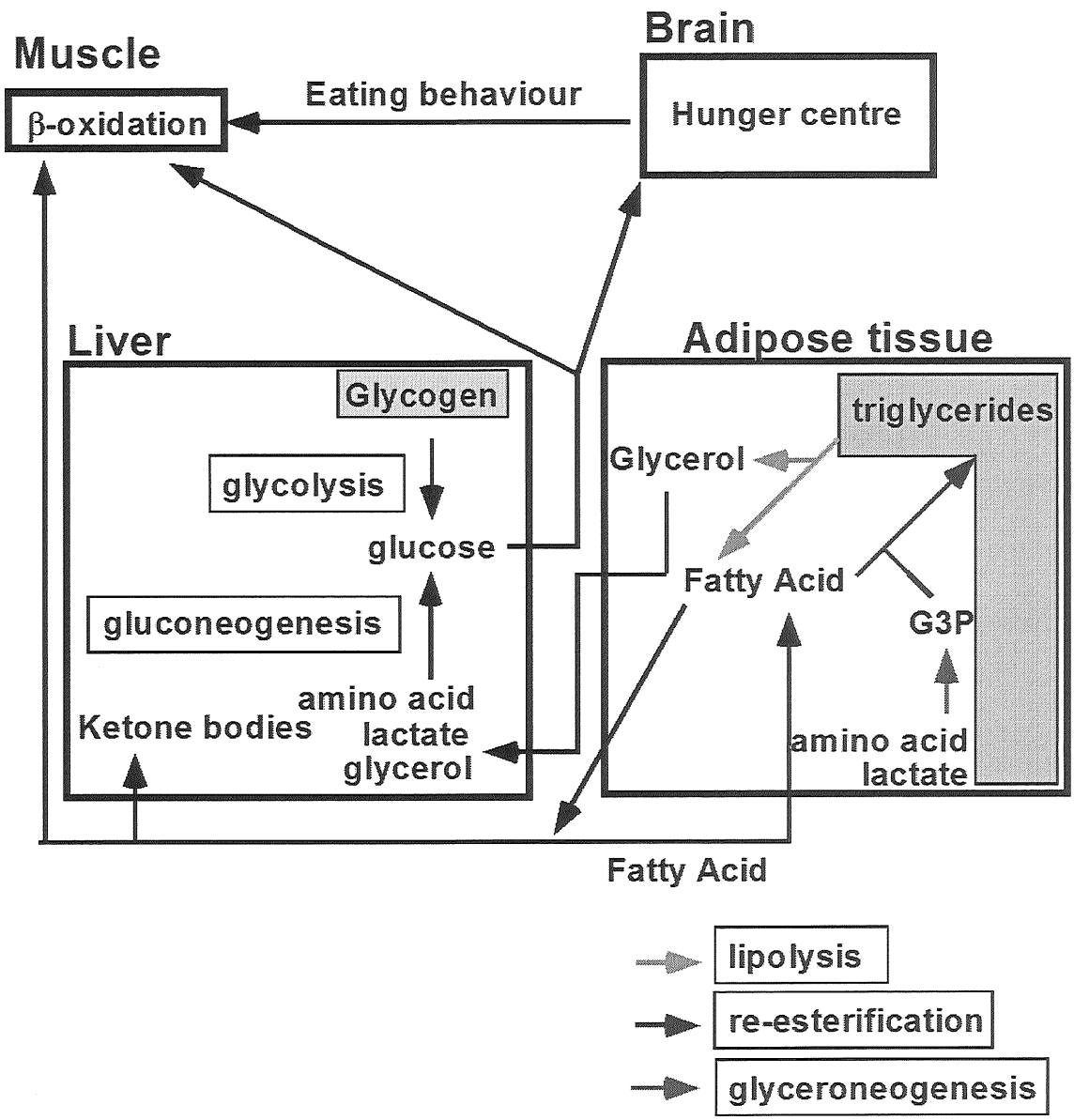

Fig. 2 Overview of the metabolic roles between tissues during fasting status. In the early fasted period, liver can produce glucose by breaking down glycogen (glycogenolysis). Under prolonged fasting, trigycerides are hydrolyzed to fatty acids and glycerol, but some of the released fatty acids are re-esterified to triglycerides that requires synthesis of glycerol (glyceroneogenesis). Fatty acids are used in muscle for β-oxidation. On the other hands, the liver can produce glucose by de novo synthesis of glucose from glycerol, lactate and amino acid (gluconeogenesis). In more prolonged fasted status, fatty acids are oxidized to ketone bodies in the liver, when brain uses them as an energy source instead of glucose. G3P, glycerol 3-phophate

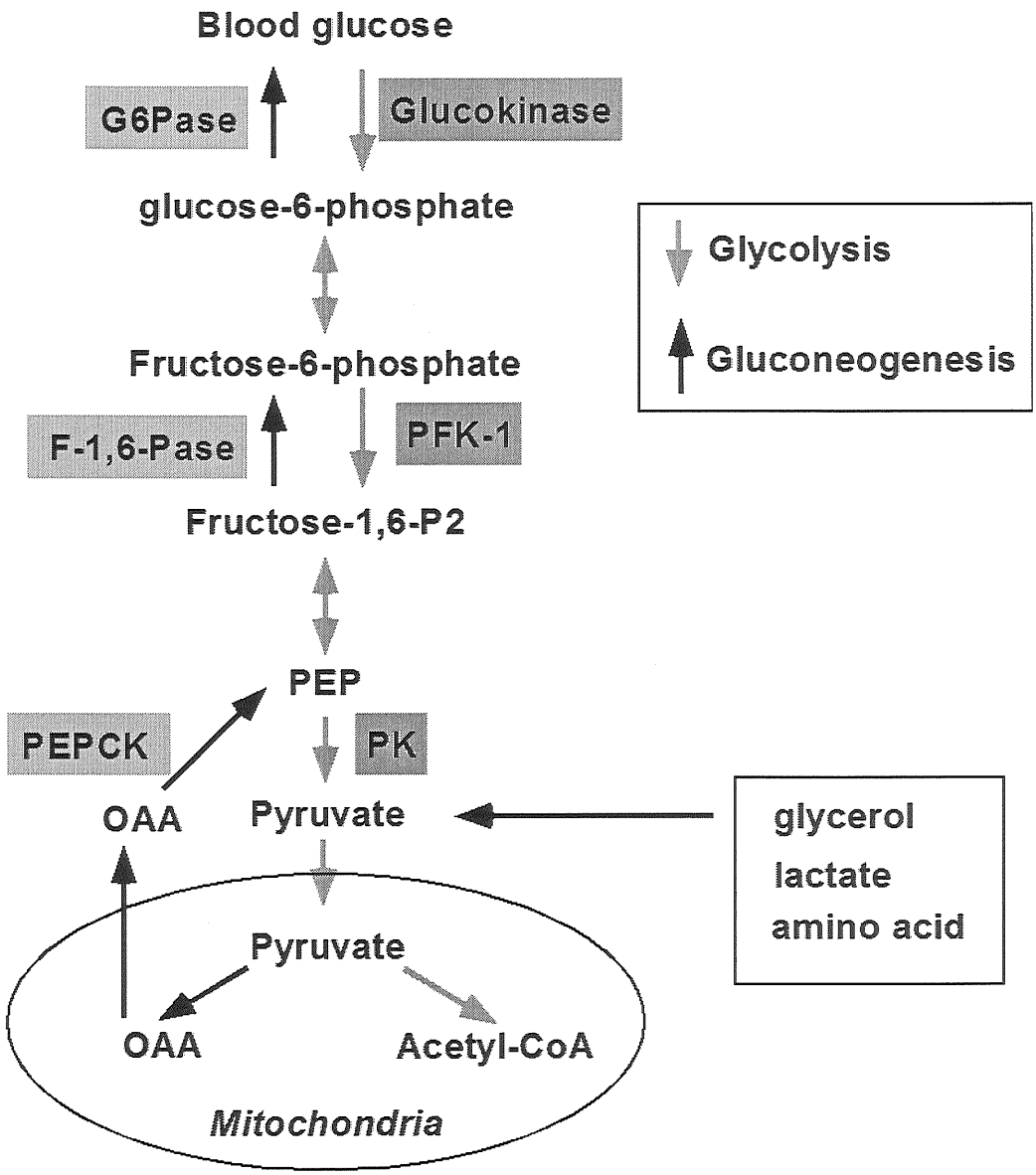

Fig. 3 Metabolic pathway of gluconeogenesis. Gluconeogenesis is controlled principally by the activity of rate-limiting enzymes, such as G6Pase, F-1,6,-Pase and PEPCK. G6Pase, glucose-6-phosphatase; F-1,6,-Pase, Fructose-1,6-bisphosphatase; PEPCK, phophoenolpyruvate carboxykinase; PFK-1, phosphofructokinase; PEP, phosphoenolpyruvate; OAA, oxaloacetate; PK, pyruvate kinase.

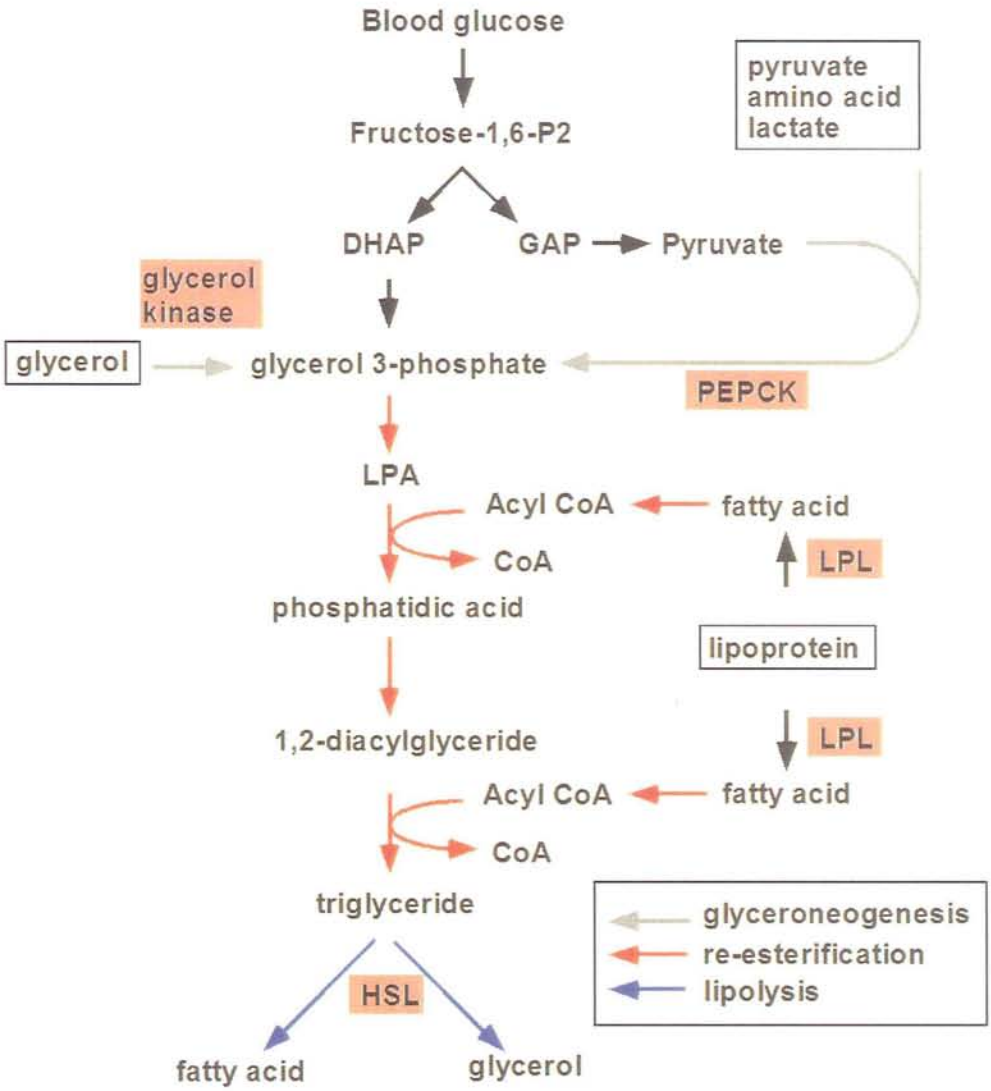

Fig. 4 Metabolic pathway of glyceroneogenesis, fatty acid re-esterification and lipolysis. The pathway of glyceroneogenesis is involved in the generation of glycerol 3-phosphate from precursors including glycerol, pyruvate, amino acid, lactate and glucose. Glyceroneogenesis is controlled principally by the activity of PEPCK. The pathway of re-esterification is involved in the generation of triglyceride from fatty acid and glycerol 3-phosphate. Conversely, the pathway of lipolysis is involved in the generation of fatty acid and glycerol from triglyceride. PEPCK, phophoenolpyruvate carboxykinase; GAP, glyceraldehydes-3-phosphate; DHAP, dihydroxyacetone phosphate; LPA, lisophosphatidic acid; HSL, hormone sensitive lipase; LPL, lipoprotein lipase

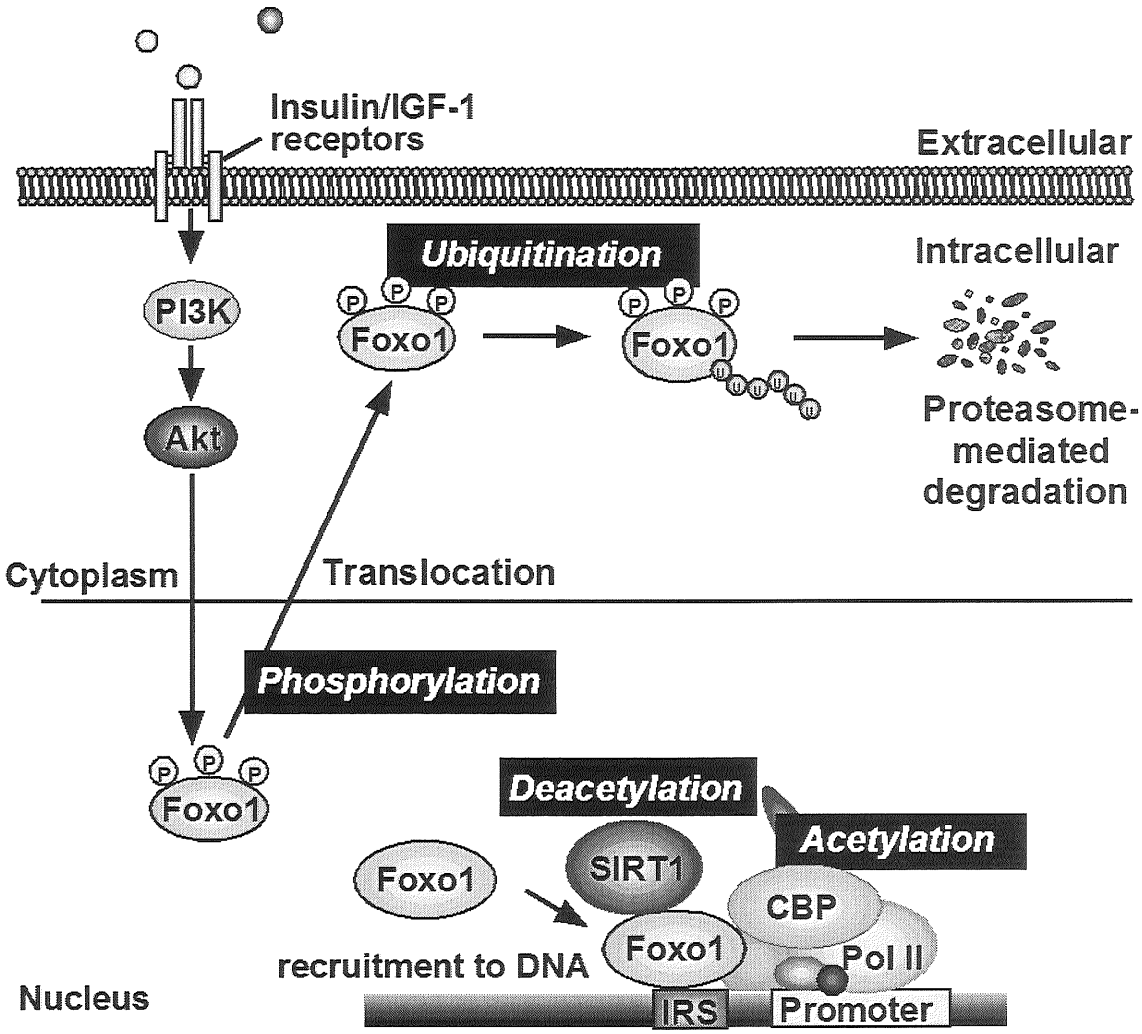

Fig. 5 A schematic model for regulation of Foxo1 mediated by posttranslational modification such as phosphorylation, acetylation, deacetylation and ubiquitination. Foxo1 is an important target for mediating effect of insulin/IGF1 on gene expression downstream from PI3K and Akt. In the absence of phosphorylation, Foxo1 localizes to the nucleus and interacts with target gene promoters (IRS, insulin response sequence). In this state, Foxo1 can be associated with reversible acetylation by CBP and SIRT1, involving in modulation its transactivation function. In contrast, in response to insulin/IGF1, Foxo1 is phosphorylated by Akt, thus leading to nuclear exclusion and subsequent degradation ubiqutination.

Chapter II

Nutrient control of gluconeogenic gene expression through phosphorylation and translocation of Foxo1

Summary

The nutrient response mediated by feeding or fasting plays an important role in controlling gluconeogenic gene expression such as G6Pase and PEPCK. Foxo1 is a key regulator that stimulates the expression of gluconeogenic genes in the nucleus but is phosphorylated by Akt and translocated to the cytoplasm in response to insulin. Although it has been widely accepted that the cellular signaling of insulin represses Foxo1 function through the Akt-dependent phosphorylation, the molecular mechanism how the nutrient response including feeding or fasting modulates Foxo1 function remains unknown *in vivo*. Here, I investigated the consequences of the nutritional changes in the Akt-mediated Foxo1 phosphorylation and translocation in the liver with control C57BL/6 and diabetic *db/db* mice. I found that feeding promotes the phosphorylation and nuclear exclusion of Foxo1, whereas fasting counteracted them in C57BL/6 mice. Notably, *db/db* mice exhibited constitutive phosphorylation but dominant nuclear accumulation of Foxo1, even though CREB phosphorylation normally occurred in the fasted status. Furthermore, in contrast to C57BL/6 mice, downregulation of G6Pase and PEPCK expression during feeding is not observed in *db/db* mice. Thus, I suggest that the accurate regulation of Foxo1 via the Akt-dependent phosphorylation is required for the physiological adaptation to the different nutritional statuses.

Introduction

Hepatic gluconeogenesis is strictly controlled by the activities of two rate-limiting enzymes in the liver, G6Pase and PEPCK. The genes encoding these proteins are regulated at the transcriptional level by key hormones, which are precisely secreted in response to various nutritional statuses mediated by fasting or feeding (2). In the fasted status, glucagon is secreted and robustly stimulates gluconeogenesis to maintain blood glucose levels within a basal range, while in the fed status, insulin markedly increase and powerfully suppress gluconeogenesis for protecting the body against hyperglycemia. On the other hand, aberrant hormonal responses to the nutritional statuses are implicated in the pathogenesis of type-2 diabetes, producing excessive hepatic glucose, and resulting in the fasting hyperglycemia and the exaggerated postprandial hyperglycemia (47). It is well established that Foxo1 plays a critical role in mediating the effects of insulin on the gene expression of G6Pase and PEPCK (48-50). However, the nutrient responses due to fasting or feeding control the Foxo1 function remains unsolved *in vivo*.

In this study, I sought to address the two questions; the first is whether the endogenous Foxo1 protein is actually regulated by the nutrient response, the second is whether the nutritional regulation of Foxo1 is also observed in diabetic mice. To this end, I examined the phosphorylation levels and the intracellular localization of Foxo1 during *ad libitum* feeding, fasting or refeeding in control C57BL/6 and diabetic *db/db* mice. I demonstrated that refeeding induced the Akt-dependent phosphorylation and nuclear exclusion of Foxo1 in C57BL/6 mice. Conversely, constitutive phosphorylation but dominant nuclear accumulation of Foxo1 was observed throughout each nutritional status in *db/db* mice. Moreover, *db/db* mice did not exhibit the downregulation of the

G6Pase and PEPCK gene expression even in the fed status. These results support the notion that the fine modulation of Foxo1 function under different nutrient signaling, at least in part, contributes to glucose homeostasis.

Materials and Methods

Animals

C57BL/6J and *db/db* male mice at 6 weeks of age were purchased from CLEA (Osaka, Japan) and adapted to the environment for 2 weeks before initiation of the studies. Animals were fed standard rodent diet and water *ad libitum* in sterile cages (one mice per cage) maintained under a light / dark cycle of 12 h (lights on at 7:00 h). In the feeding experiment, mice were killed at the status fed *ad libitum* (18:00 h), starved for 16 h (10:00 h), or starved for 16 h followed by refeeding high -sucrose/fat-free diet for 4 h (14:00 h). Care of experimental animals was within institutional guideline approved by the Laboratory Animal Resource Center at the University of Tsukuba.

Preparation of whole tissue extracts

Animals were killed and the removed livers were quickly frozen in liquid nitrogen. One hundred milligrams of the livers were homogenized using a polytron on ice in 2.5 ml of the buffer containing 50 mM HEPES-KOH (pH 7.9), 1% Nonidet P-40, 250 mM KCl, 5 mM EGTA, 1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF and protease inhibitors. Samples were rotated for 1 h at 4°C and centrifuged at 14,000 rpm for 20 min at 4°C. The supernatants were stored at -80°C until analysis. Total protein concentrations of the extracts were measured using a protein assay kit (BIO-RAD).

Fractionation of cytoplasmic and nuclear proteins

Cytoplasmic and nuclear protein extracts were fractionated using the NE-PER extraction reagents (Pierce). The livers from mice were cut into small pieces, and then

homogenized using tissue homogenizer for preparation of single cells. Thereafter, packed cell (40 mg) by centrifugation at 500 rpm for 3 min at 4°C was homogenized in 400 μl of the ice-cold CER-I solution (Pierce) supplied with protease inhibitors. Subsequent steps for fractionation were according to the manufacturer's instruction.

Antibodies and Western blotting

A rabbit polyclonal antibody specific for mouse Foxo1 was described previously (51). Antibodies for phospho-Foxo1 (Thr-24), phospho-Foxo1 (Ser-256), Akt, phospho-Akt (Ser-473), CREB and phospho-CREB (Ser-133) were purchased from Cell Signaling Technology (Beverly, MA).

Protein extracts were separated by 8% SDS-PAGE, followed by electrotransfer onto PVDF membrane and probed with indicated first antibodies. Chemiluminescent detection relied on horseradish peroxidase-conjugated secondary antibodies. The intensity of the protein bands was quantified by densitometry using Image quant software and expressed as the means ± S.E.

Immunohistochemistry

The livers were fixed in 4 % paraformaldehyde overnight at 4°C and then embedded in paraffin. The paraffin sections (4 μm) on slides were deparaffinized in xylene (3 min, three times), and rehydrated in a decreasing ethanol series diluted in distilled water (100%, 100%, 90%, 80%, 70%, 3 min each). We used a microwave-based antigen retrieval method to enhance the reactivity of an antibody as described previously (52). Endogenous peroxide activity was inactivated by 3% hydrogen peroxide in methanol for 15 min. The sections were blocked with 2.5%

normal goat serum in PBS containing 0.25% Triton X-100 for 30 min. The anti-Foxo1 (1:100 dilution) antibody was incubated with the sections at room temperature for 1 h in a humidity chamber. Peroxidase-based detection was performed using the DAKO's Envision system, followed by color reaction using the DAB substrate. Pictures were obtained using an optical microscope (Leica).

Total RNA extraction and Northern blot analysis

The livers were homogenized using a polytron and total RNA was isolated using the ISOGEN RNA isolation reagent (Nippon Gene). Fifteen micrograms of total RNA were denatured with glyoxal and DMSO, separated by electrophoresis and transferred to a nylon membrane (NEN Life Science Products). The membranes were hybridized with the ^{32}P-labeled probes under stringent conditions. Hybridization products were detected by an image analyzer (Tyhoon 8600, Amersham Biosciences) and standardized against human β-actin bands. A probe for Foxo1 was generated by PCR using primers as follows: forward, 5'-gaattcaattcgccacaatctgtccc-3', and reverse, 5'-ttagcctgacacccagctgtgtg-3'. Probes for G6Pase, PEPCK and β-actin were prepared as described previously (53). The intensity of the RNA bands was quantified by densitometry using Image quant software and expressed as the means ± S.E.

Results

Effects of nutritional statuses on Akt-mediated Foxo1 phosphorylation

To verify whether endogenous Foxo1 is actually regulated by nutritional statuses *in vivo*, I first examined the phosphorylation levels of Foxo1 at Thr-24 and Ser-253 residues, both of which are phosphorylated by Akt via the insulin-signaling pathway. I prepared the liver extracts from C57BL/6 and *db/db* mice subjected to the conditions of "*ad libitum* feeding", an overnight "fasting" or the following "refeeding" and analyzed the expression and phosphorylation levels of Foxo1 and Akt by Western blotting. Compared to *ad libitum* feeding, an overnight fasting attenuated the phosphorylation levels of Foxo1 at both Thr-24 and Ser-253, whereas refeeding restored these levels in C57BL/6 mice (Fig. 6A and C). It should be noted that the alteration of nutritional statuses showed no substantial effect on the amount of hepatic Foxo1 protein (Fig. 6A). To further assess whether the insulin-Akt-signaling pathway is also involved in the nutrient response, I examined the phosphorylation levels of Akt at Ser-473 under fasting or refeeding conditions in the liver. Consistent with the result of Foxo1, the phosphorylation level of Akt was attenuated during fasting, suggesting that the phosphorylation of Akt and Foxo1 is controlled under the different nutritional statuses, probably through the insulin-signaling pathway, in C57BL/6 mice.

Next, I employed *db/db* mice as a model of type-2 diabetes and investigated whether Foxo1 phosphorylation cascades could be normally transmitted by nutrient responses. Surprisingly, in contrast to the control C56BL/6 mice, the phosphorylation levels of Foxo1 were not decreased by fasting (Fig 6B and D). Moreover, the nutritional changes did not alter the phosphorylation levels of Akt in *db/db* mice. These findings

suggest that *db/db* mice are deficient in the nutrient-induced phosphorylation of both Foxo1 and Akt in the liver.

Effects of nutritional statuses on CREB phosphorylation

During a period of prolonged fasting, glucagon stimulates protein kinase A (PKA), which in turn phosphorylates the cyclic AMP (cAMP) response element binding (CREB) protein at Ser-133, thereby promoting the gluconeogenesis through the induction of the nuclear receptor coactivator PGC1 (54). To test whether the glucagon-signaling pathway actually responds to fasting, I monitored the phosphorylation levels of hepatic CREB protein in C57BL/6 and *db/db* mice. As expected, fasting increased CREB posphorylation in C57BL/6 mice, while refeeding slightly decreased it (Fig. 7A and C). Interestingly, in contrast to the constitutive posphorylation of Foxo1 and Akt throughout each nutritional status (Fig. 6B and D), *db/db* mice showed a marked increase in the level of CREB posphorylation by approximately 7-fold in the fasted status (Fig. 7B and D). Moreover, a drastic effect of refeeding on CREB posphorylation was also observed in *db/db* mice. These data indicate that the nutrient response is normally transmitted to CREB, probably via the glucagon-PKA signaling pathway, even in *db/db* mice.

Effects of nutritional statuses on Foxo1 localization

Since the insulin/Akt-mediated phosphorylation of Foxo1 has been shown to correlate with its intracellular localization in mammalian cultured cells (31-33), I investigated whether Foxo1 could be also regulated by fasting or feeding in the mouse liver using an immunohistochemical assay. As shown in Figure 8A, fasting caused an

extensive nuclear accumulation of Foxo1 in C57BL/6 mice. In contrast, Foxo1 predominantly localized in the nucleus in both fed *ad libitum* and fasted statuses on the sections from *db/db* mice (Fig. 8B).

To further support the above data, I next performed Western blot analysis using cytoplasmic and nuclear fractionations of the liver extracts. In C57BL/6 mice, the amount of cytoplasmic Foxo1 protein significantly decreased by fasting compared to the fed *ad libitum* status, while the nuclear Foxo1 was also reduced by the following refeeding (Fig. 9A and C), suggesting that the intracellular localization of Foxo1 is tightly controlled in response to nutrient responses. Remarkably, in agreement with the results from immnunohistochemisty, the nutritional changes did not affect the amounts of nuclear and cytoplasmic Foxo1 proteins in *db/db* mice (Fig. 9B and D). Taken together, these findings indicate that feeding stimulates the nuclear exit of Foxo1 in the liver, but this nutrient response to the Foxo1 translocation is impaired in *db/db* mice.

Effects of nutritional statuses on Foxo1-mediated transcription

To examine the effects of nutrient response on the transactivation function of Foxo1, I evaluated the expression levels of G6Pase and PEPCK genes, which are the targets of Foxo1. Consistent with the finding from Western blotting (Fig. 6A), no differences were observed in the mRNA levels of Foxo1 throughout each nutritional status in C57BL/6 mice (Fig. 10A); however, the levels of G6Pase and PEPCK were substantially induced in the fasted status (Fig. 10A and C). Moreover, refeeding entirely diminished the mRNA levels of two gluconeogenic genes. In contrast, neither fasting nor refeeding changed the expression levels of G6Pase and PEPCK in *db/db* mice (Fig. 10B and D), suggesting that it is also deficient in the nutrient responses to the

gluconeogenic gene expression as well as the deregulation of the Akt-mediated Foxo1 phosphorylation and translocation.

Discussion

My present study demonstrates a direct correlation between the nutritional statuses and the transactivation function of Foxo1 in the mouse liver. I have shown that fasting drastically decreases the phosphorylation level of Foxo1, whereas refeeding markedly increases it, thereby promoting the nuclear exclusion of Foxo1 (Fig. 6, 8, and 9). Accompanied by the phosphorylation and the intracellular translocation of Foxo1, refeeding reduces the expression levels of G6Pase and PEPCK, but not Foxo1 (Fig. 10). These results lead me to conclude that the metabolic information on the metabolic changes due to feeding or fasting is converted into the phosphorylation of Foxo1 via the Akt-dependent pathway, and an extent of modification represents the expression levels of gluconeogenic genes in the liver.

An alternative finding of this work is that diabetic *db/db* mice showed an aberrant nutrient control of Foxo1, namely its constitutive phosphorylation and nuclear accumulation throughout each nutritional status (Fig. 6, 8, and 9). Supporting these results from *db/db* mice, we also found an impaired nutrient control of Akt and Foxo1 in another diabetic model, New Zealand Obese mice (data not shown). Interestingly, Altomonte *et al.* (45) have reported that *db/db* mice exhibit a significant induction of hepatic Foxo1 protein as well as increased its nuclear localization, compared with their heterozygous littermates ($db/^{+}$). Considering this finding along with my present results, a plausible explanation for an excessive hepatic gluconeogenesis in *db/db* mice may be an abnormal regulation of Foxo1 in both transcriptional and posttranslational levels.

Despite the aberrant nutrient control of Akt and Foxo1 in *db/db* mice, I found that the nutrient response by feeding robustly correlates with the phosphorylation levels of

CREB (Fig. 7). This finding strongly suggests that *db/db* mice possess an adequate response to glucagon, which in turn phosphorylates hepatic CREB protein through the PKA-signaling pathway. Given the constitutive hyper-phosphorylation and abnormal nuclear accumulation of Foxo1 in the liver of *db/db* mice, a contributing factor to hyperglycemia in diabetes appears to be an impaired suppression of Foxo1 transactivation function in response to feeding through the insulin-signaling pathway.

One significant question of my results is the mechanism whereby the nutritional conditions do not alter the intracellular localization of Foxo1 despite its hyper-phosphorylation status in *db/db* mice. Notably, a recent study has revealed that c-Jun N-terminal kinase (JNK) directly phosphorylates FOXO4 and this leads to the nuclear translocation and the transcriptional activation (55). JNK has been shown as a mediator of an oxidative stress induced by tumor necrosis factor α and hyperglycemia and also revealed to be abnormally elevated in obesity and insulin resistance (56,57). In view of my present results, it is possible that an immoderate JNK activity might override the Akt-mediated nuclear exclusion of Foxo1, and subsequent increasing nuclear accumulation might result in sustained expression of the gluconeogenic genes in *db/db* mice.

In conclusion, this study presents a critical role for Foxo1 in response to a nutrient signaling. Impaired nutritional regulation of Akt-mediated Foxo1 phosphorylation and translocation could, at least in part, account for an uncontrolled expression of gluconeogenic genes. In addition to phosphorylation, Daitoku *et al.* (36) previously demonstrated that Foxo1 is acetylated by CBP, the modification of which is reversed by the NAD^+-dependent histone deacetylase Sir2. Interestingly, it has recently been reported that SIRT1/Sir2 controls the hepatic gluconeogenic/glycolytic pathways in

response to fasting through the transcriptional coactivator PGC-1a (58). Given that SIRT1 protein levels and NAD^+ concentration increase in the fasted status, it is conceivable that the nutrient signaling including fasting or refeeding alters the levels of Foxo1 acetylation via Sir2/SIRT1-mediated deacetylation in the liver. Further studies are needed to clarify an alteration of the levels of acetylation as well as phosphorylation of Foxo1 in response to the nutritional statuses.

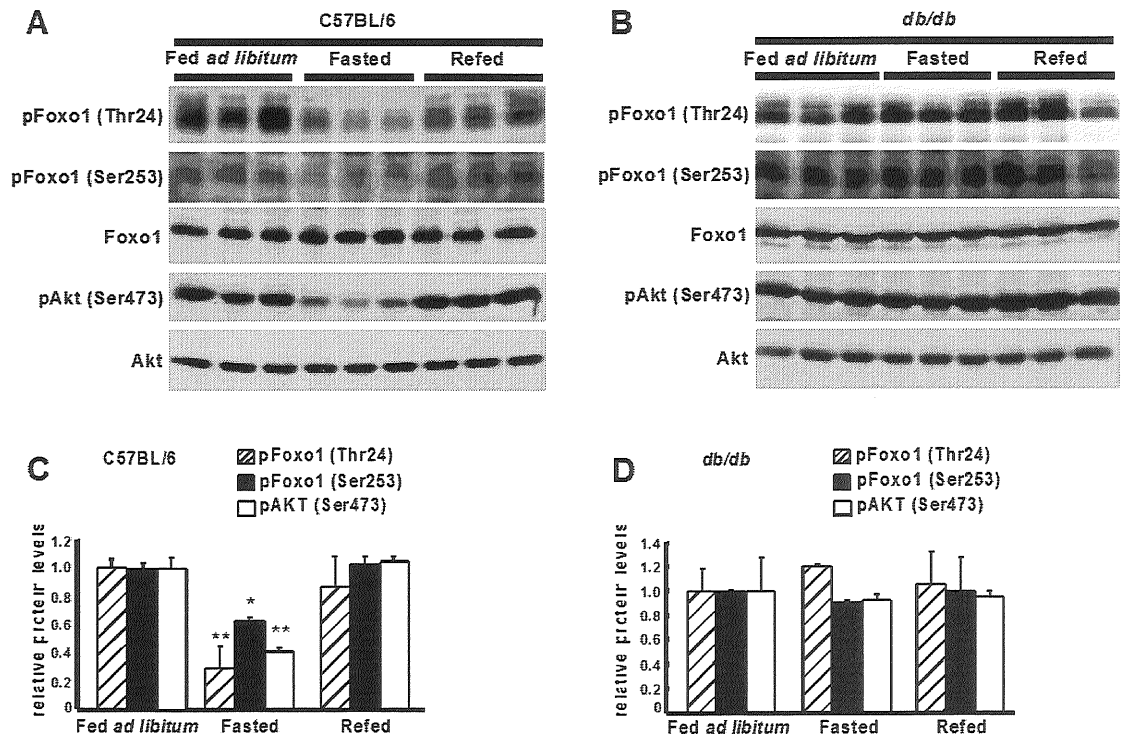

Fig. 6 Effect of nutritional statuses on Akt-mediated phosphorylation of Foxo1 in C57BL/6 and *db/db* mice. **A** and **B**, Liver extracts (50 μg) from C57BL/6 (A) or *db/db* (B) mice subjected to fed *ad libitum*, fasted or refed were immunoblotted by specific antibodies as indicated. **C** and **D**, Histograms show quantifications (relative value of fed *ad libitum* control) of phosphorylated Foxo1 (Thr-24, diagonal striping bar; Ser-253, black bar) and phosphorylated Akt (Ser-473, white bar) normalized to results from amount of Foxo1 and Akt, respectively. Significant difference from mice compared to fed *ad libitum* status is represented by asterisks. Each bar represents the mean ± S.E. (n = 3); *, $p < 0.05$, **, $p < 0.01$.

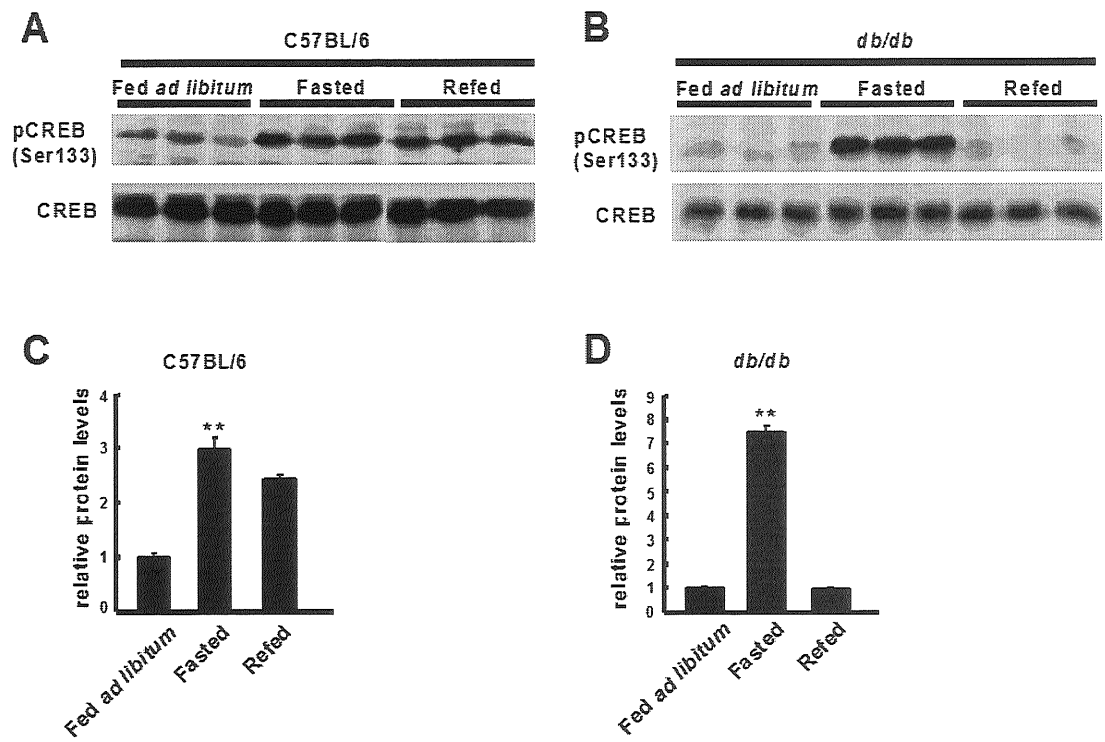

Fig. 7 Effect of nutritional statuses on CREB phosphorylation in C57BL/6 and *db/db* mice. **A** and **B**, Liver extracts (50 μg) from C57BL/6 (A) or *db/db* (B) mice subjected to fed *ad libitum*, fasted or refed were immunoblotted by specific antibodies for CREB and phospho-CREB (Ser-133) antibodies. **C** and **D**, Histograms show quantifications (relative value of fed *ad libitum* control) of phosphorylated CREB normalized to the amount of CREB. Significant difference from mice compared to fed *ad libitum* status is represented by asterisks. Each bar represents the mean ± S.E. (n = 3); **, $p < 0.01$.

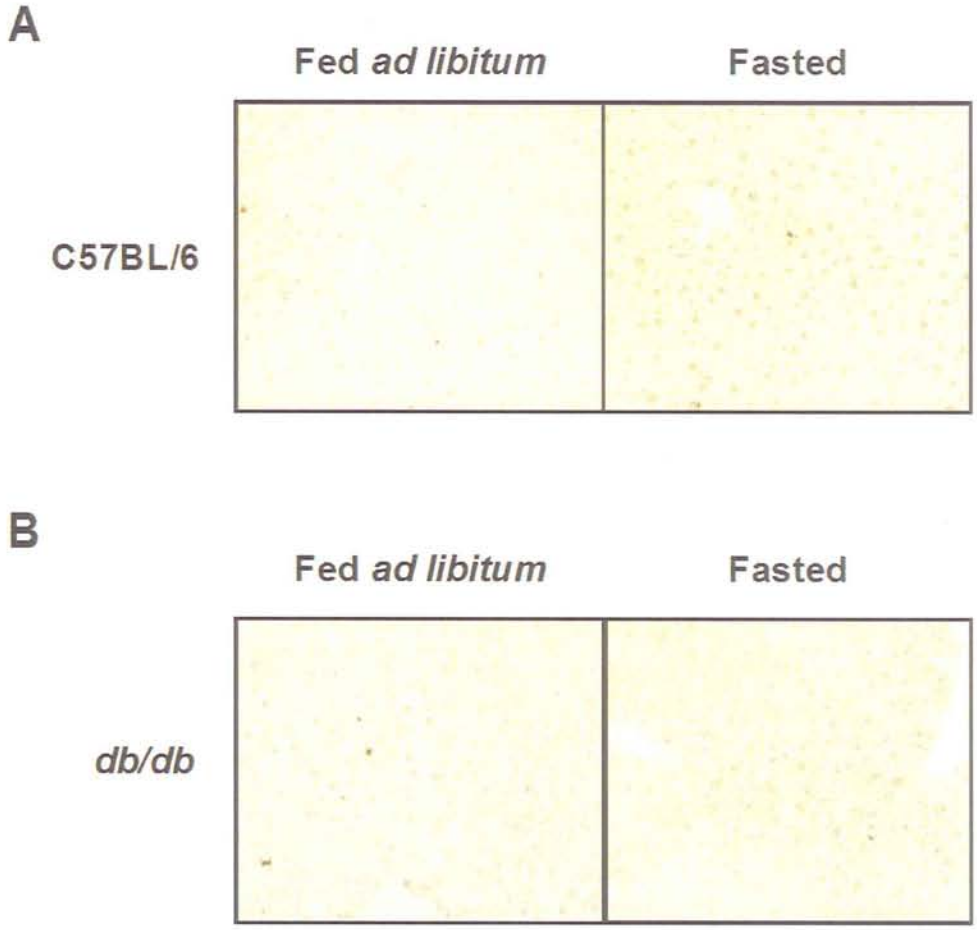

Fig. 8 Hepatic immunohistochemistry of Foxo1 in C57BL/6 and *db/db* mice. A and **B,** Paraffin sections were prepared for immunohistochemical staining with anti-Foxo1 antibody. Positive staining for Foxo1 is revealed by the brown color. Original magnification is x 40. The liver sections from C57BL/6 (A) and *db/db* (B) mice in fed *ad libitum* (*left panels*) and fasted (*right panels*) conditions.

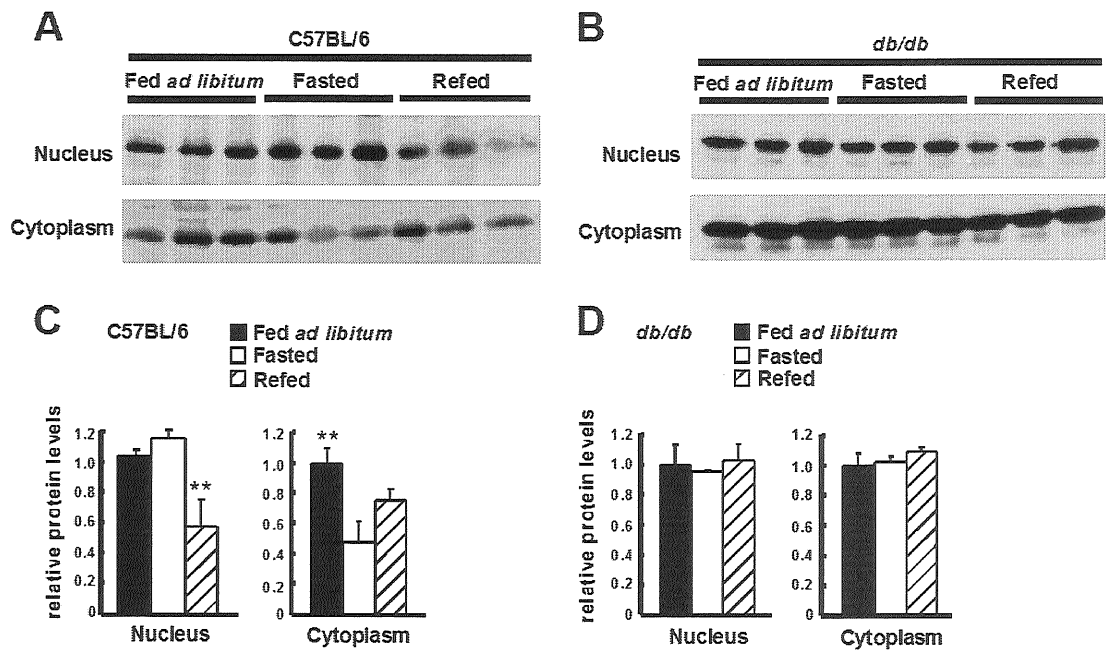

Fig. 9 Intracellular distribution of Foxo1 in response to nutritional statuses. **A** and **B**, Cytoplasmic and nuclear protein extracts (100 μg) from C57BL/6 (A) or *db/db* (B) mice subjected to fed *ad libitum*, fasted or refed were immunoblotted by anti-Foxo1 antibody. **C** and **D**, Histograms show quantifications (relative value of fed *ad libitum* control) of cytoplasmic or nuclear Foxo1 protein normalized to the amount of Foxo1 protein in whole liver extracts. Fed *ad libitum*, black bar; Fasted, white bar; Refed, diagonal striping bar. Significant difference from fasted mice in nutritional statuses is represented by asterisks (**, $p < 0.01$).

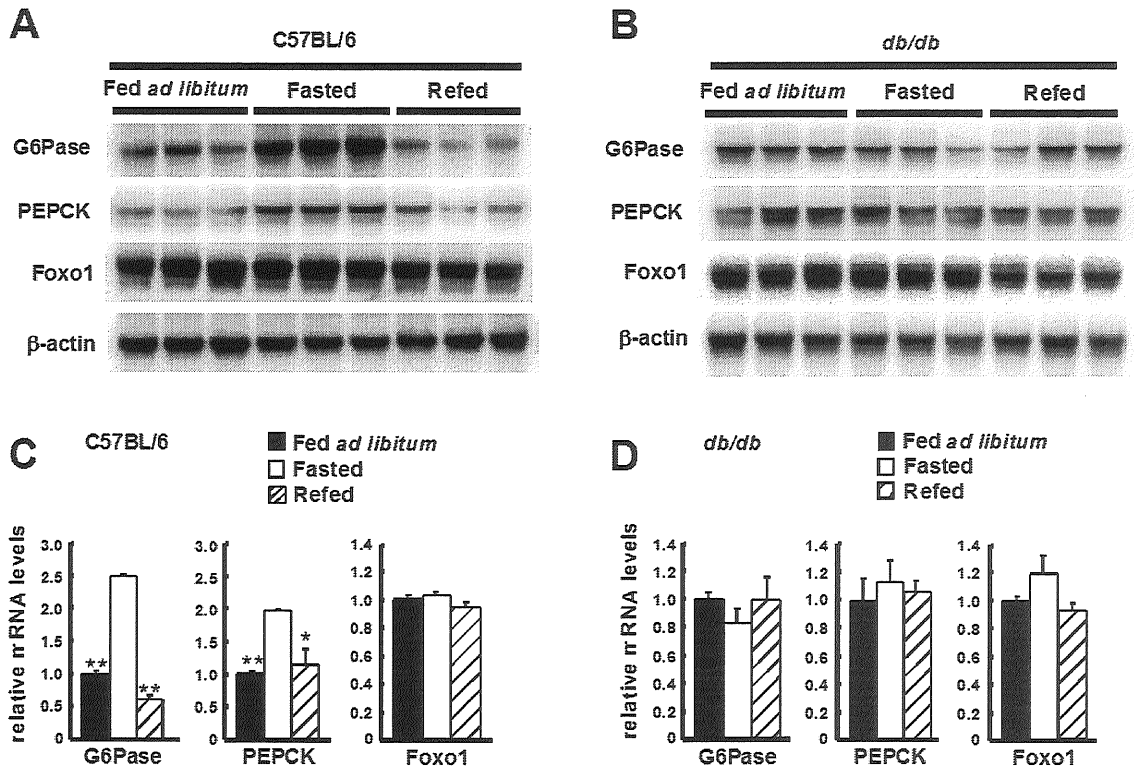

Fig. 10 Nutritional regulation of *G6Pase* and *PEPCK* genes expression in C57BL/6 and *db/db* mice. **A** and **B**, Northern blot analysis of G6Pase, PEPCK, Foxo1 and β-actin mRNA in the livers from C57BL/6 (A) or *db/db* (B) mice subjected to fed *ad libitum*, fasted or refed. **C** and **D,** Histograms show quantifications (relative value of fed *ad libitum* control) of G6Pase, PEPCK and Foxo1 genes expression normalized to β-actin in the indicated nutritional conditions of C57BL/6 (C) or *db/db* (D) mice. Fed *ad libitum*, black bar; Fasted, white bar; Refed, diagonal striping bar. Significant difference from fasted mice in nutritional statuses is represented by asterisks (*, $p < 0.05$, **, $p < 0.01$).

Chapter III

Foxo1 in adipose tissue regulates insulin sensitivity and energy expenditure

Summary

Adipose tissue plays important physiological roles including lipid storage and regulation of glucose homeostasis throughout secreting adipoocytokines. Increase in adipose mass or deregulation of signaling in adipocyte can result in the disruption of overall insulin sensitivity, which leads to obese and type-2 diabetes. In this study, I have identified Foxo1 as a key regulator of adipocyte metabolism *in vivo*. I generated transgenic mice (aP2-Foxo1 mice) overexpressing Foxo1 selectively in WAT and BAT. The aP2-Foxo1 mice exhibited adipocyte hypertrophy and increased adipose mass, but not hyperphagia and obese. Elevated concentrations of insulin and FFA suggested hyperinsulinemia and hyperlipodemia in aP2-Foxo1 mice. Notably, aP2-Foxo1 mice exhibited insulin resistance with normal glucose tolerance. This phenotype was associated with a marked reduced gene expression of PPARγ and GLUT4 in WAT of aP2-Foxo1 mice. More interestingly, aP2-Foxo1 mice exhibited impaired adaptive thermogenesis associated with decreased UCP1 expression in BAT, suggesting that low energy expenditure in these mice. Thus, these results revealed that Foxo1 in adipose tissue plays important roles in insulin sensitivity and energy expenditure by affecting expression of genes involved in metabolic regulators, secreting factors and energy expenditure.

Introduction

WAT and BAT play critical roles in energy homeostasis and endocrine function (7-11). In both animal models and human, obesity is characterized by an excess accumulation of adipose tissue and involved in the pathogenesis of multiple diseases. These associated pathologies including cardiovascular diseases, type-2 diabetes, dyslipidemia, hypertension and sleep apnea (59). Paradoxically, not only an excess of adipose tissue, but also the total absence of fat is associated with increased risk for these complications (60-62).

The role of adipose tissue in overall glucose metabolism is not clear. Although WAT accounts for less than 10% of whole-body glucose uptake (63), the adipose selective disruption of GLUT4 gene, which is the major glucose transporter regulated by insulin stimulation, causes glucose intolerance and hyperinsulinemia in mice (64). Accordingly, the downregulation of GLUT4 expression in human adipose tissue but not in skeletal muscle is common of insulin resistance status, including obesity and type-2 diabetes (65). Reduced glucose transport into adipocytes limits glycerol synthesis following impaired lipogenesis and re-esterification of fatty acids into triglyceride, resulting in increased serum FFA (so called nonesterified fatty acids, NEFA) (66). Thus, the glucose uptake in adipose tissue has important roles not only in attenuation of blood glucose concentration but also maintenance of FFA concentration.

Insulin is a critical regulator of various aspects of adipocyte physiology, and adipocytes are one of the most highly insulin-responsive cell types (67). Insulin promotes triglyceride stores in adipocyte via promoting the differentiation of preadipocyte into mature adipocyte, stimulating glucose transport and triglyceride

synthesis, as well as inhibiting lipolysis (68). Insulin also increases the uptake of FFA derived from circulating lipoprotein by stimulating lipoprotein lipase activity in adipose tissue (66). Metabolic effects of insulin are involved in rapid changes in protein phosphorylation and function, as well as changes in gene expression. However, the responsible factor for the effect of insulin in adipose tissue has been largely unknown.

Foxo1 is an important target for mediating the effects of insulin signals (31-33). Recent studies have suggested that Foxo1 functions as a negative regulator of adipocyte differentiation *in vitro* (46). In addition, Foxo1 haploinsufficiency prevented insulin resistance induced by a high-fat diet (46). Nevertheless, the physiological roles of Foxo1 in adipose tissue and regulation of insulin sensitivity have not been resolved. To investigate the physiological roles of Foxo1 in adipose tissue *in vivo*, I generated and analyzed transgenic mice (aP2-Foxo1 mice) overexpressing Foxo1 in their adipose tissues. The aP2-Foxo1 mice showed the adipocyte hypertrophy and increased fat mass. Remarkably, they developed hyperinsulinemia and hyperlipidemia and exhibited insulin resistance, even though a normal glucose tolerance. Furthermore, I identified down-regulated genes for insulin sensitivity and adipogenesis such as PPARγ and GLUT4 in WAT. Interestingly, aP2-Foxo1 mice exhibited impaired adaptive thermogenesis associated with decreased UCP1 expression in BAT, suggesting the low energy expenditure in these mice. Thus, my results suggest that Foxo1 in the adipose tissue has important roles in whole body insulin sensitivity and energy metabolism.

Material and Methods

Transgene construct and generation of transgenic mice

As indicated in Fig. 11A, the transgenic construct was designed for the two copies of chicken β-globin 5'HS4 insulator sequence in tandem form placed on either side of the aP2-Foxo1 chimeric gene, which comprises the 5.4-kb aP2 promoter/enhancer fragment, the mouse Foxo1 cDNA that added FLAG epitope tag on N-terminus and the 3'-untranslated region of a rabbit β-globin gene containing splice site, and the polyadenylation signal. The transgenic construct was generated as the following way. First, I cloned the 5.4-kb promoter/enhancer of mouse aP2 gene from C57BL/6 mice genomic library and subcloned into the pBluscript II KS+ vector (Stratagene) using the following strategy. The cloning gene was confirmed by sequencing. Second, I prepared the insulators-construct, which was modified pJC13-1 (kindly provided by Dr. G. Felsenfeld) by deleting γ-NEO reporter on the *Bam*HI site and mHS2 enhancer on the *Eco*RI instead of multiple cloning site containing fragments. Third, I generated the aP2-Foxo1 chimeric gene. The *Bam*HI -*Bgl*II fragment containing Kozak and FLAG peptide tag sequences generated by oligonucleotide synthesis was inserted into the *Bam*HI site of the pcDNA3 (invitrogen). The 1.2-kb *Bam*HI - *Xho*I fragment, the 3'-untranslated region of the rabbit β-globin gene was excised from the pSG2 vector (kindly gift of Y. Ogawa), and a 2.0-kb *Bam*HI fragment from Foxo1 expression vector (35) ligated into the *Bam*HI site of pcDNA3 vector containing Kozak and FLAG peptide tag sequences. The 3.2-kb *Kpn*I- *Xho*I fragment was excised from the resulting plasmid and made blunt with Klenow treatment and subsequent ligation into the *Sma*I site of pBluscript II KS+ vector containing the 5.4-kb promoter/enhancer of mouse aP2

gene. A 10.3-kb *Xho*I fragment containing the aP2 promoter/enhancer followed by the cDNA for FLAG-tagged Foxo1 was excised from the plasmid and ligated into *Xho*I site of the insulators -construct. The plasmid was sequenced over ligation sites. A 15.1-kb fragment flanked by the unique sites *Sfi*I at inserted multiple cloning sites, harboring the two copies of insulators placed on either side of aP2-Foxo1 chimeric gene, was used to generate transgenic mice. Following gel purification, the transgene was injected into the pronucleus of fertilized zygotes from C57BL/6J (Nippon Clea, Osaka Japan) mice and transferred to pseudopregnant female using standard techniques. Founders were mated with C57BL/6J mice, and their offspring were obtained. All studies were conducted using 3 months of age heterozygous mice. Transgenic mice and their wild type control littermates were fed *ad libtum* with a standard diet and maintained under a light/dark cycle of 12 h (lights on at 7:00 h). Care of experimental animals was within institutional guideline approved by the Laboratory Animal Resource Center at the University of Tsukuba.

Isolation of genomic DNA and Southern blot analysis

Genomic DNA was extracted from the tails of founders and their offspring. Tails were lysed with the buffer containing 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 20 mM EDTA (pH 8.0), 1% SDS and 0.3 mg/ml proteinase K (Wako, Osaka Japan). Lysates were treated with RNase and subsequent of ammumonium acetate for the purification of DNA. DNA was precipitated with ethanol and dissolved in TE (10 mM Tris-HCl ; pH 8.0, 1 mM EDTA ; pH 8.0). Two micrograms aliquots of sample DNA were digested with *Bam*HI, subjected to electrophoresis in 0.7% agarose gels, and transferred to nylon membranes (NEN Life Science Products) by the alkaline transfer method. The

membranes were hybridized with the ^{32}P-labeled 0.75-kb probe on the carboxyl-terminus of Foxo1 (shown as a bar-S in Fig. 11A) under stringent conditions. The membranes were washed in the buffer containing 1% SDS and 100 mM Tris-HCl (pH 8.0). Hybridization products were detected by an image analyzer (Tyhoon 8600, Amersham Biosciences) and computerized by using Image quant 6.1.

RNA preparation and Northern blot analysis

Total RNA was prepared from tissues with ISOGEN (NIPPON GENE) as previously described (53). I used cDNA probes for GLUT4, SREBP1, which were prepared by RT-PCR using first-strand cDNA from mouse epididymal fat total RNA as previously described (69). The Foxo1 probe was prepared as described in Chapter II. The cDNA probes of mouse UCP1, and PPARγ, and LPL were kindly gifted from Drs. H. Katagiri (University of Tohoku), S. Kato (University of Tokyo), Y. Kamei (University of Tokyo Medical and Dental), respectively. Equal aliquots of total RNA from adipose tissue of five mice were pooled (total 10μg), and northern blotting was performed as described previously (53).

Western blot analysis and immunoprepititation

Mice were killed, and WAT and BAT were removed and quickly frozen in liquid nitrogen. Total protein extracts from WAT and BAT in buffer containing 50 mM HEPES-KOH (pH 7.9), 1% Nonidet P-40, 250 mM KCl, 5 mM EGTA, 1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF and protease inhibitor. After centrifugation at 14,000 rpm for 20 min at 4°C, the supernatants were stored at –80°C until analysis. Western blot analysis was performed as described previously (51). Immunoprecipitation was

performed 3h at 4°C with antibody against FLAG (M2, Sigma). The rabbit polyclonal antibody specific for mouse Foxo1 was described previously (51). Anti-β-actin (AC-74) were purchased from Sigma.

Histology

Epididymal and parametrial WAT and interscapular BAT were isolated. Tissues were fixed for 1 week in 10% Fomalin Neutral Buffer Solution (Wako) at 4°C, dehydrated, embedded in paraffin, sectioned (5 μm), and stained with haematoxylin and eosin.

Blood tests

For measurements of blood glucose and serum insulin, samples were obtained from mice at the status fed ad libitum (18:00 h) and starved for overnight (10:00 h). Blood glucose was measured using an ONETOUCH Assist (LifeScan). Serum insulin concentration was measured by ELISA using mouse insulin as a standard (Morinaga). Serum triglyceride and cholesterol levels were measured using fasted animals by colorimetric enzyme assay using FUJI dry-chem system (Fujifilm, Tokyo, Japan). Serum-free fatty acid levels were measured using fasted animals by the NEFA-C test Wako kit (Wako, Osaka, Japan). Serum adiponectin was measured using the ELISA kit (R&D Systems). Serum leptin and resistin were measured using the ELISA kit (Linco Research).

Glucose and insulin tolerance tests

For glucose tolerance test, mice were fasted overnight (from 18:00 h to 10:00 h).

Mice were then injected intraperitoneally with D-glucose (1 g/kg of body weight), and subsequently blood was collected from tail vein. Blood glucose levels were measured at 0 (prior to injection), 30, 60, 90, and 120 min post-injection. For insulin tolerance tests, animals were fasted for 6 h, (from 10:00 h to 16:00 h). Mice were injected intraperitoneally with a 0.5 U/kg of recombinant human insulin, Humulin (Eli Lilly), and blood glucose levels were measured at 0 (prior to injection), 15, 30 and 60 min post-injection.

Physiological measurements

For measuring food consumption, mice were housed individually in metabolic cage and measured in mass of food intake for 6 days. Rectal temperature was monitored during early afternoon with a digital thermometer (CT-1310D; CUSTOM) equipped with a rectal probe. For cold exposure experiment, mice were treated the cold exposure, and rectal temperature of mice was measured 30 min after cold (4°C) exposure.

Results

Generation of transgenic mice overexpressing Foxo1 in adipose tissue

I generated transgenic mice overexpressing FLAG-Foxo1 under the control of the enhancer/promoter region of the adipocyte fatty acid binding protein (aP2) gene, known to promote a transcription in WAT and BAT *in vivo* (62,70-73). To distinguish between the endogenous and trangenic Foxo1 proteins, the FLAG epitope sequence was inserted on 5'-terminus of Foxo1 cDNA in my construction. Remarkably, to avoid suppression of the transgene expression due to its integration site dependent on a position effect, I attempted to employ the insulator elements at the 5' end of chicken β-globin locus, which can act as a barrier to chromosomal position effects when it surrounds a stably integrated reporter. They have been proven useful in construction of vectors for transgenic experiments in animals, resulting in uniform and high levels of expression (74-76) (Fig. 11A). Tail DNA from potential transgenic mice was screened for the presence of the aP2-Foxo1 chimeric gene by Southern blot analysis (data not shown). Two independent lines of aP2-Foxo1 transgenic mice were obtained and designated as line A and line B. Fig. 11B shows the result from Southern blot analysis of DNA derived from tails of the line A and line B mice. Line A and line B contain about 10 and 12 copies of the transgene, respectively. Fig. 11C shows that the transgene overexpression was limited to BAT and WAT in the line A of male and female mice. The level of expression was greater in BAT than WAT, and the similar results using line B were also seen (data not shown).

Endogenous and transgenic Foxo1 proteins were confirmed by immunoblot analysis using anti-Foxo1 antibody. Mobility shift of high molecular weight of FLAG

products was detected in lysates from transgenic mice, whereas only slight endogenous signal was seen from lysates from both transgenic and control mice (Fig. 11D *upper*). No differences were observed in the β-actin level throughout each lysate (Fig. 11D *middle*). The level of FLAG-Foxo1 protein was approximately more than ten fold greater in both of transgenic lines than in control mice. Furthermore, to confirm the transgenic expression, FLAG-Foxo1 fusion protein was immunoprecipitated using anti-FLAG antibody from tissue lysates and subjected to immunoblotting using anti-Foxo1 antibody. FLAG-Foxo1 fusion protein was detected from transgenic lysates, but not detected from nontransgenic lysates (Fig. 11D *lower*). These data clearly indicated that aP2-Foxo1 mice have overexpressed FLAG-Foxo1 protein in adipose tissues. I performed following physiological and pathological studies using two lines of transgenic mice as well as their control littermates.

Foxo1 overexpression led to increase fat pad weight and lipid accumulation in the adipose tissues, and adipocyte hypertrophy

Both male and female aP2-Foxo1 mice appear normal in terms of growth, general behavior and reproduction. At 3 months after birth, despite no significant difference of body weight (Fig. 12A), gonadal WAT weight increased significantly in both males (1.4 fold) and females (1.5 fold) mice compared with control mice. Interscapular BAT weight also increased in both males (1.4 fold) and females (1.3 fold) mice compared with their control mice. There was no substantial difference in the weight of liver and kidney (Fig. 12B and C). Morphology shows that male transgenic mice exhibited enlarged interscapular BAT and gonadal WAT depots (Fig. 12D). Histological analyses of WAT and BAT were performed in transgenic, as well as control mice. A large amount

of lipid droplets in BAT and large cell size in gonadal WAT were observed in aP2-Foxo1 mice (Fig. 12E-H). Similar results were obtained from morphological and histrogical experiments using female mice (data not shown). These data suggested that the higher lipid accumulation in adipose tissue caused by the hypertrophy of adipocyte, probably induced by overexpression of Foxo1. Livers from control and aP2-Foxo1 mice appear normal in terms of gross morphology (data not shown).

Metabolic disorders in aP2-Foxo1 mice

Since it is well known that the adipose tissue regulates insulin action on glucose and lipid metabolism (8), I measured glucose and insulin concentrations of aP2-Foxo1 and control mice in fasted and fed statuses. Glucose concentration was indistinguishable between aP2-Foxo1 mice and their littermates (Fig. 13A). Although there was no difference in glucose concentrations, female transgenic mice showed significantly higher insulin concentrations in both fasted and fed statuses compared to control mice. Male transgenic mice also showed higher but not significant changes (Fig. 13B). The homeostasis model assessment on insulin resistance (HOMA-R, mM glucose x pg/ml insulin) was about 2.5 fold higher in aP2-Foxo1 than control mice, indicating the peripheral insulin resistance.

Since lipid metabolism strongly affects insulin sensitivity, I measured triglycerids, cholesterol and FFA from mice serum. Serum triglycerids and FFA concentration significant increased, whereas serum cholesterol concentration decreased in aP2-Foxo1 male mice as compared with control mice (Fig. 13D). There was no significant difference in serum triglycerids and serum cholesterol concentration in each genotype, while serum FFA concentration in female transgenic mice were notably increased (Fig.

13E). Increase in serum FFA level in both male and female suggests the development of lipotoxicity in aP2-Foxo1 mice, which links to insulin resistance (77).

aP2-Foxo1 mice exhibited insulin resistance with normal glucose tolerance

To investigate the possibility of insulin resistance in aP2-Foxo1 mice, I performed intraperitoneal glucose and insulin tolerance tests (GTT and ITT, respectively). In GTT, male aP2-Foxo1 mice were normal glucose tolerance, while, surprisingly, female transgenic mice show more sensitive to glucose (significantly lower than control at 60 and 90 min; $p < 0.01$), indicating that aP2-Foxo1 mice had entirely normal glucose tolerance (Fig. 14A and B). To examine the pancreatic function involving insulin release in response to an intraperitoneal glucose, I examined serum insulin concentration during glucose tolerance tests. Remarkably, hyperinsulinemia was detected in aP2-Foxo1 female mice throughout the time course of the experiment (Fig. 14C and D). Male aP2-Foxo1 mice also exhibit higher insulin concentration (significantly higher than control at 60 and 90 min; $p < 0.05$). In ITT, the glucose-lowering effect of exogenous insulin was attenuated in male and female aP2-Foxo1 mice as compared with control mice, indicating that whole-body insulin sensitivity was markedly impaired in aP2-Foxo1 mice (Fig. 14E and F). Similar results of GTT and ITT were obtained using the line B of transgenic mice and their control mice (data not shown).

Adipocytokines in aP2-Foxo1 mice

Adipose tissue has a substantial influence on systemic glucose homeostasis through secretion of adipocytokines (7-13). Recent studies have shown the roles for leptin and adiponectin as mediators of insulin-sensitizing, but for resistin and TNFα as

inducers of insulin resistance (7). The leptin concentration in aP2-Foxo1 mice was significantly lower than that control mice, whereas serum adiponectin concentration in aP2-Foxo1 mice was 15% higher increased as compared with control mice (Fig. 15A and B). Serum concentration of TNFα was below the level of detection of the mouse enzyme-linked immunosorbent assay (ELISA) in control and aP2-Foxo1 mice. However, I found elevation of TNFα expression in WAT of aP2-Foxo1 mice by microarray analysis (data not shown). Serum resistin concentration in aP2-Foxo1 mice was 20% lower than that in control mice (Fig. 15C), paralleling a slight decrease in its mRNA level, revealed by Northern blot analysis (data not shown).

Impaired adaptive thermogenesis and decreased UCP1 expression in aP2-Foxo1 mice

Regulation of energy balance is determined by the food intake and energy expenditure, and the impaired regulation could account for causes of obesity and insulin resistance (59). Energy expenditure represents basal body temperature and adaptive thermogenesis in response to diet or environment temperature. Thus, I examined food consumption and the rectal temperature under the room temperature (20°C) and cold (4°C) conditions. Notably, although aP2-Foxo1 mice had increased fat mass (Fig. 12B and 12C), food intake of transgenic mice was similar to that of control mice (Fig. 16A). Rectal temperature of both male and female aP2-Foxo1 mice was significantly lower than that of control, indicating that energy expenditure in basal condition could be decreased in aP2-Foxo1 mice (Fig. 16B). When aP2-Foxo1 and control mice were exposed to cold at 4°C for 30 min, the rectal temperature of aP2-Foxo1 mice dropped (4.4% decrease), whereas that of control mice only slightly dropped (1.5% decrease) (Fig. 16C). To determine whether the failure to induce thermogenic mechanisms due to

decreased in the expression level of the uncoupling protein 1 (UCP1), the most important thermogenic molecule, I performed Northern blot analysis. The levels of UCP1 expression in BAT were lower in aP2-Foxo1 than in control female mice (Fig. 16D). These results suggest that impaired energy expenditure and subsequent hypometabolism may be revealed in aP2-Foxo1 mice.

Foxo1 overexpression in adipose tissue has pleiotropic effect on gene expression

To investigate the mechanism of the adipocyte hypertrophy and attenuated insulin sensitivity in aP2-Foxo1 mice, I analyzed the expression level of genes involved in adipocyte differentiation, glucose and lipid metabolism by Northern blot analysis (Fig. 17). Expression of peroxisome proliferator-activated receptor gamma (PPARγ), the most important molecule for adipogenesis (78,79), was decreased in aP2-Foxo1 mice as compared with control. Interestingly, GLUT4 gene expression also dramatically decreased in aP2-Foxo1 mice. On the other hand, GLUT4 expression level in skeletal muscle was not significantly different in each genotype (data not shown). The genes involved in lipid metabolism such as SREBP1, lipoprotein lipase (LPL) and PEPCK were also decreased in WAT of aP2-Foxo1 mice. The above results were consistent with decrease in insulin sensitivity of aP2-Foxo1 mice.

Discussion

Using a transgenic approach, I examined the metabolic role of Foxo1 in white and brown adipose tissue (WAT and BAT) *in vivo*. The Foxo1 transgene was dominantly overexpressed in both WAT and BAT (Fig. 11). The aP2-Foxo1 mice exhibited the distinct phenotype from four prominent features; hypertrophic adipocyte in WAT and BAT (Fig. 12); hyperinsulinemia and hyperlipomia (Fig. 13 and Fig. 14); insulin resistance with normal glucose homeostasis (Fig. 13 and Fig. 14); decreased energy expenditure (Fig. 16). In contrast to other mouse models of insulin resistance (80,81), it should be noted that the aP2-Foxo1 mice were not obese (Fig. 12a and 12b). These results led me to conclude that Foxo1 in adipose tissue has an important role in terms of the regulation of insulin sensitivity and energy metabolism. How does Foxo1 regulate fat mass and insulin sensitivity associated with the pleiotropic effect on gene expression? In this point, I would like to discuss the causal relationship between the effect on various genes by overexpressed Foxo1 in adipose tissue and phenotype of transgenic mice. I present some possibilities of the molecular pathogenesis of insulin resistance in aP2-Foxo1 mice in below text and Fig. 18.

First possibility might relay on suppression of GLUT4 gene expression in WAT of aP2-Foxo1 mice (Fig. 17A and 17B). GLUT4 in muscle and adipose cells is a predominant glucose transporter and is essential for maintenance of insulin sensitivity and glucose homeostasis (82). Disruption of GLUT4 gene selectively in the adipose tissue resulted in insulin resistance of muscle and liver (64). Conversely, overexpression of GLUT4 gene selectively in adipose tissue (aP2-GLUT4 mice) exhibited increased insulin sensitivity (72). Thus, the insulin resistance of aP2-Foxo1 mice may be

accounted for dominant suppression of GLUT4 expression.

Second possibility might relay on low PPARγ expression in both WAT and BAT (Fig. 16D and Fig. 17A). PPARγ, a member of the nuclear receptor, has been considered to be a key regulator in the adipocyte differentiation, glucose and lipid metabolism in WAT and energy expenditure in BAT. It is well known that activation of PPARγ by antidiabetic thiazolidinediones (TZDs) drugs leads to an improvement in insulin sensitivity (83). Moreover, PPARγ promotes the expression of adipocyte specific genes such as aP2, LPL and PEPCK, to promote normal adipose differentiation and subsequent regulate insulin sensitivity (84-86). Interestingly, all of these adipocyte specific genes as well as PPARγ expression are downregulated in aP2-Foxo1 mice (Fig. 17A and B), suggesting that Foxo1 negatively regulates activity and/or expression of PPARγ. A recent molecular investigation has demonstrated that Foxo1 antagonizes PPARγ activity (87), which could disrupt DNA binding activity of PPARγ/retinoid X receptor α (RXRα) heterodimeric complex by Foxo1. Accordingly, Foxo1 haploinsufficiency exhibits increased PPARγ expression (43), strongly suggesting that Foxo1 is a negative regulator of PPARγ. Although I did not verify the molecular mechanism of suppression in PPARγ activity by its expression or activity in this study, my results suggest that the antiadipogenic effect of Foxo1 through suppression of PPARγ activity *in vivo* may result in insulin resistance and abnormal adipogenesis following adipocyte hypertrophy.

Third possibility might relay on the changes adipocytokines such as leptin, adiponectin and resistin (Fig. 15). Reduced serum leptin level in aP2-Foxo1 mice may cause insulin resistance. Leptin, involved in the control of food intake, also enhances whole-body glucose utilization and reduces β-cell insulin secretion (88,89). Accordingly,

glucose uptake in rat adipocyte *in vitro* has been suggested to regulate leptin secretion and reduced blood leptin level may results in insulin resistance (90). Reduced adiponectin is another possible mechanism for developing insulin resistance (91). Surprisingly, however, I found that serum adiponectin concentration was elevated in aP2-Foxo1 mice compared to control mice. Another surprising thing is that serum resistin concentration was reduced in aP2-Foxo1 mice. Resistin has shown to be a key endocrine factor for the induction of G6P gene expression, following promoting hepatic gluconeogenesis in the liver (92). In my study, there was no difference in hepatic G6P expression, comparing fasted aP2-Foxo1 with control mice (data not shown). Elevated adiponectin and reduced resistin concentration may account for that aP2-Foxo1 mice do not affect glucose tolerance even though serve insulin resistance and hyperlipomia.

Fourth possibility might relay on the attenuation of fatty acids re-esterification in adipose tissue and subsequent increase in FFA concentration in aP2-Foxo1 mice. Adipose tissue modulates an overall glucose metabolism by regulating levels of FFA (75). Furthermore, there is also a clear evidence from clinical and biochemical studies that circulating FFA could impair insulin sensitivity in muscle (77). Serum FFA concentration in the fasted state was increased in aP2-Foxo1 mice as compared with control mice (Fig. 13D and E). Elevation of circulating FFA may cause insulin resistance by impairing insulin signaling in peripheral tissues of aP2-Foxo1 mice. Interestingly, reduce in glucose transport into adipocytes limits glycerol synthesis (glyceroneogenesis) following impaired re-esterification of fatty acids into triglyceride, resulting in increased serum FFA (5). Accordingly, the expression level of PEPCK, the enzyme catalyzes the rate-limiting step in adipose glyceroneogenesis, was attenuated in WAT of aP2-Foxo1 mice (Fig. 17A). Thus, the biochemical evidences such as the

impaired glyceroneogenesis and re-esterification may account for the mechanism of increasing FFA concentration in aP2-Foxo1 mice.

The insulin resistance following the obesity and type-2 diabetes is largely linked to excessive caloric intake and impaired energy expenditure (59). Although I found that food intake was unchanged between aP2-Foxo1 and control mice, rectal temperature was decreased in aP2-Foxo1 compared with control mice (Fig. 16). Furthermore, aP2-Foxo1 mice exhibit failure to induce thermogenic mechanisms for cold sensitivity, probably due to impaired energy expenditure. Regulation of thermogenesis is associated with gene expression linked to mitochondrial UCP1 and its mediated energy expenditure (93). Accordingly, decreased UCP1 expression was thought to due to low expression of PPARγ by overload of Foxo1 in BAT of transgenic mice (Fig. 16D). Thus, I propose that the phenotypes of adipocyte hypertorophy in aP2-Foxo1 mice due to, at least in part, defective energy expenditure in response to food intake.

Nakae *et al.* (46) have reported that Foxo1 prevents differentiation of preadipocytes, suggesting that Foxo1 functions as a negative regulator of adipocyte differentiation *in vitro*. In addition, Foxo1 haploinsufficiency prevents insulin resistance and diabetes induced by a high-fat diet, possibly by preventing adipocyte hypertrophy. Interestingly, aP2-Foxo1 mice exhibit adipocyte hypertrophy and insulin resistance. My result with overexpression of Foxo1 in the adipose tissue is consistent with an *in vivo* role from Foxo1 haploinsufficiency. Together, these findings indicate that the regulation of Foxo1 in adipose tissue may critically affect insulin sensitivity, probably accompanied by modulating adipocyte differentiation (94).

In conclusion, the data presented here lead me to suggest that Foxo1 in adipose tissue has an important physiological role in insulin sensitivity and energy expenditure.

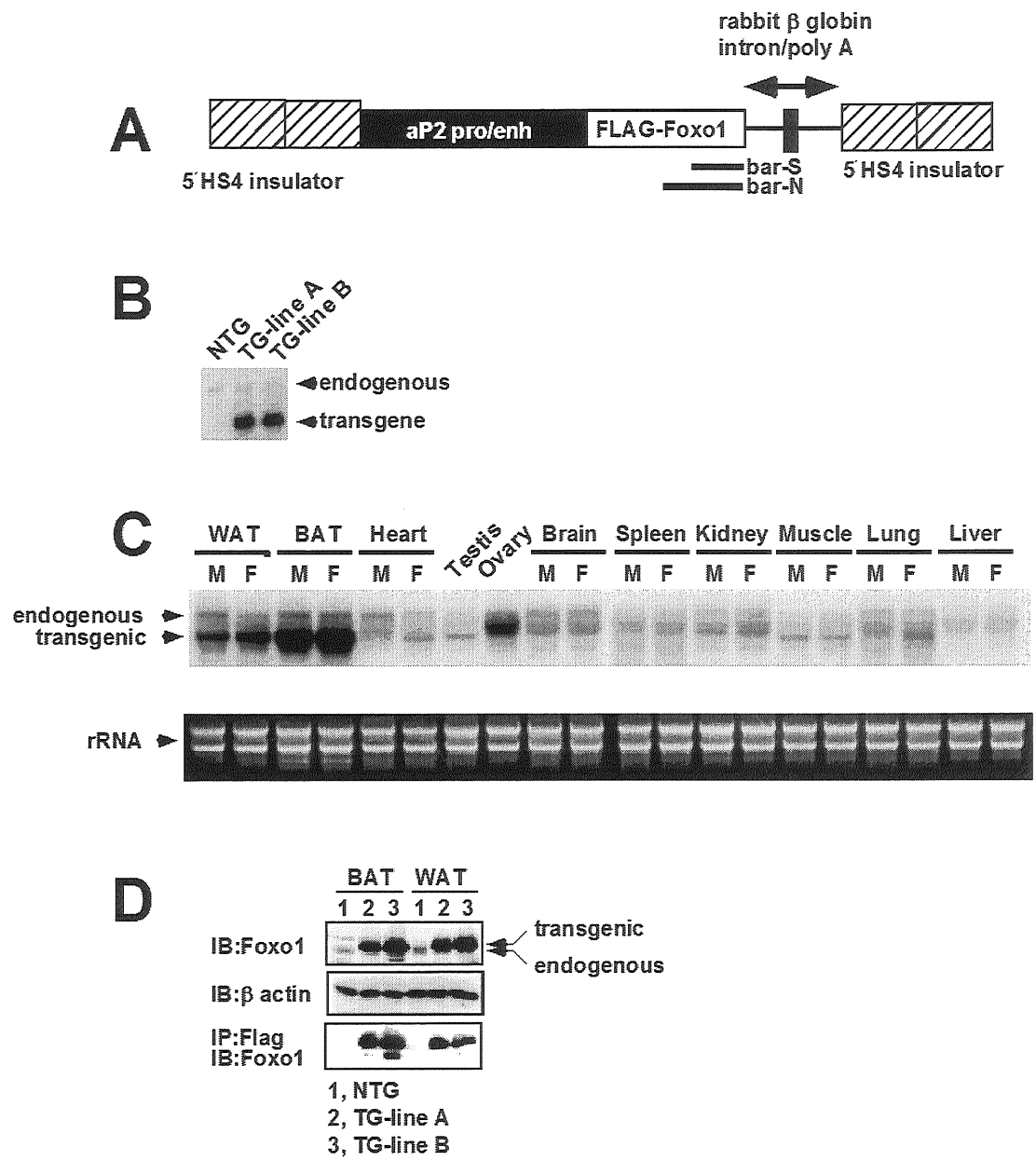

Fig. 11 Generation of transgenic mice overexpressing FLAG-Foxo1 gene driven by aP2 enhancer/promoter (aP2-Foxo1 mice). A, a schematic of the aP2-Foxo1 construct. The diagonal striping boxes indicate the insulators elements at the 5' end of chicken β-globin locus. The FLAG-Foxo1 transgene (open box) was under the control the mouse aP2 enhancer/promoter region (solid box). The 3'-untranslated region of rabbit

β-globin gene containing intron region was employed for polyadenylation signal to express stably transgene. The bar-S and bar-N indicates cDNA probes using Southern and Northern hybridization, respectively. **B**, Southern blot analysis of genomic DNA from the tail of nontransgenic (NTG) and aP2-Foxo1 mice. Arrows indicate the endogenous (*upper*) and transgenic (*lower*). **C**, Northern blot analysis of endogenous and transgenic expression in various tissues from male and female aP2-Foxo1 mice. *Upper panel*, Total RNA from white adipose tissue (WAT), brown adipose tissue (BAT), heart, testis, ovary, brain, spleen, kidney, skeletal muscle (gastrocnemius), lung and liver were analyzed. Arrows indicate the endogenous (*upper*) and transgenic (*lower*) RNA bands. M and F indicate RNA samples from male and female mice, respectively. *Lower panel*, The 28S and 18S ribosomal RNAs for the present analysis are shown. **D**, Immunoblot analysis of protein extracts (30 μg) in parametrial WAT and intracepter BAT from female aP2-Foxo1 as well as control (NTG) mice. *Upper panel*, The broad signals from FLAG-Foxo1 were described as slightly separated from the signal from endogenous Foxo1 using by 8% SDS-PAGE gel. Arrows indicate the transgenic (*upper*) and endogenous (*lower*) protein bands. *Middle panel*, β-actin was shown as an internal control. Protein expressions of FLAG-Foxo1 in WAT and BAT were detected by immnunoprecipitation with anti-FLAG antibody followed by immunoblotting with anti-Foxo1 antibody. Equal amounts of protein were used for immnunoprecipitation.

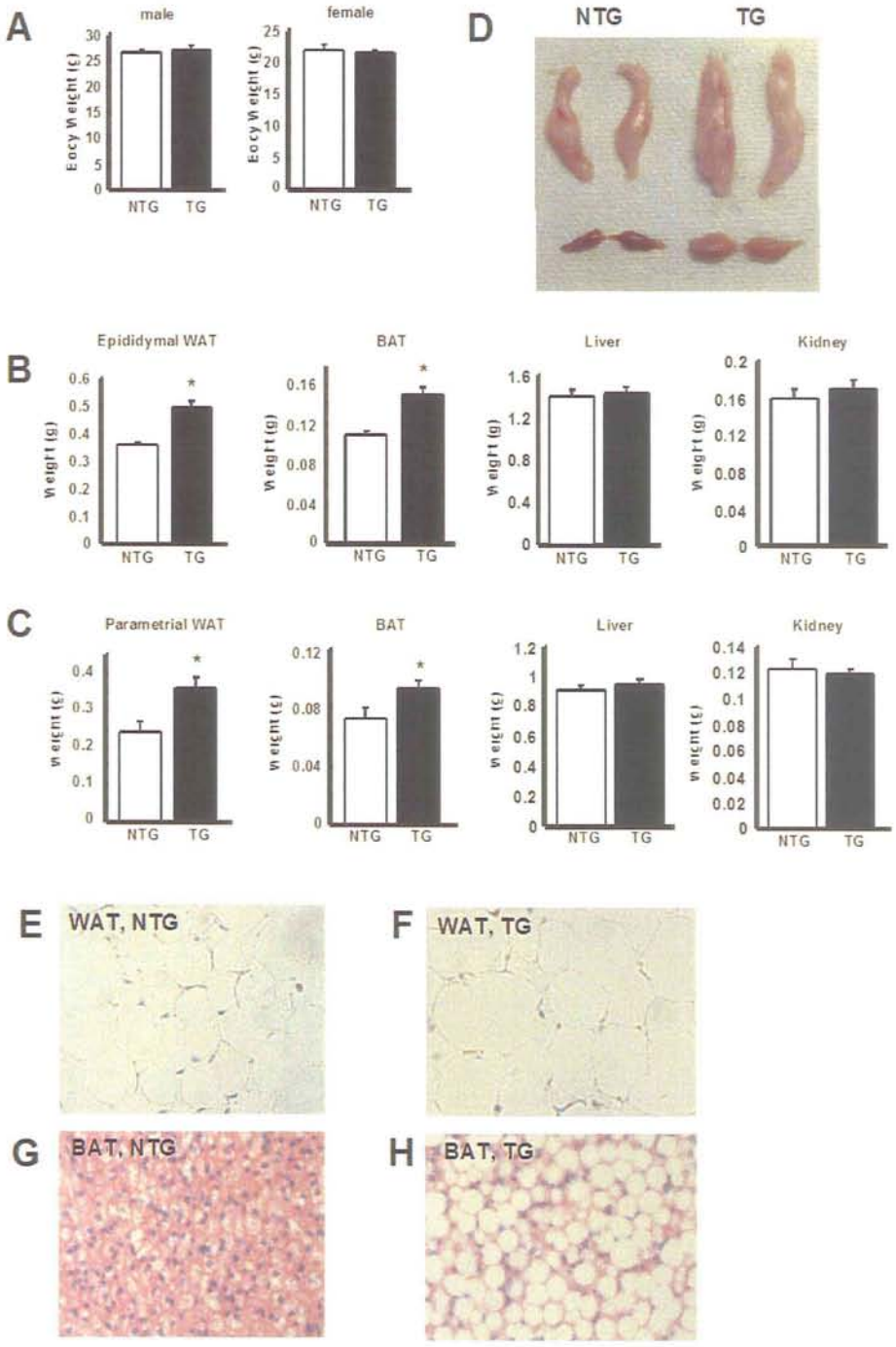

Fig. 12 Increased fat mass and adipocyte hypertrophy of aP2-Foxo1 mice. A-C, Body and tissue weight of aP2-Foxo1 (TG) and control (NTG) mice (each group n=12). Body weight of male and female mice. (A), and WAT, BAT, liver and kidney weight of male mice (B) and female mice (C). **D-H**, Morphological and histological analysis of epididymal WAT and interscapular BAT in aP2-Foxo1 (TG) and control (NTG) male mice. Data are mean S.E. *, $p < 0.05$.

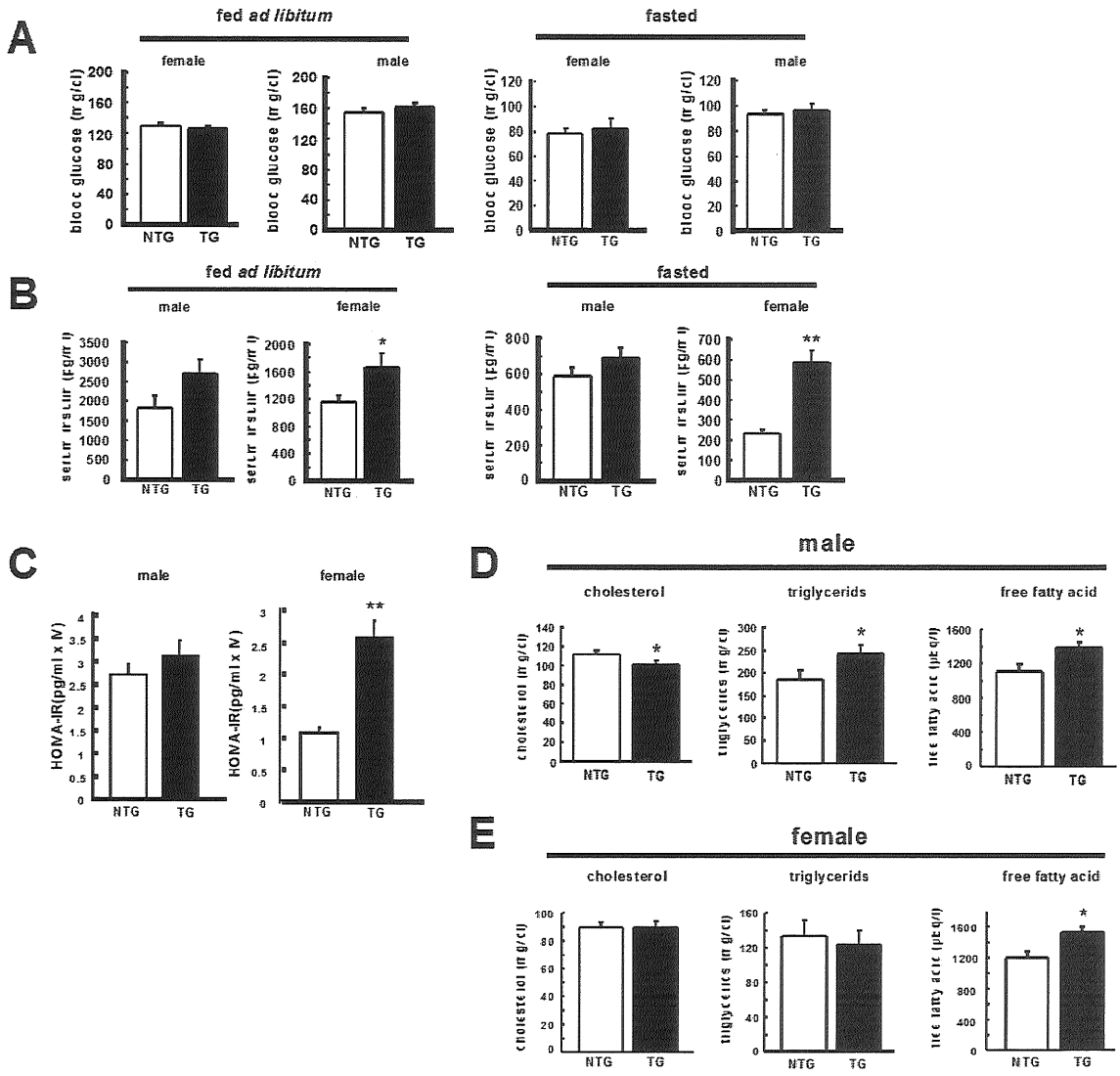

Fig. 13 Metabolic parameter in aP2-Foxo1 mice. **A** and **B**, Blood glucose (A) and serum insulin (B) levels of aP2-Foxo1 (TG) and control (NTG) mice in fed *ad libitum* and fasted status were determined. (each group n=10-14) **C**, HOMA-R index of insulin sensitivity (mM glucose x pg/ml insulin) was calculated using results from fasted condition described as Fig. 13A and 13B. **D** and **E**, Concentration of serum cholesterol, triglycerids and FFA of aP2-Foxo1 and control male (D) and female (E) mice (n=10, each group). All of lipid parameters were analyzed mice serum in fasted status. Data are mean S.E. *, $p < 0.05$, **, $p < 0.01$.

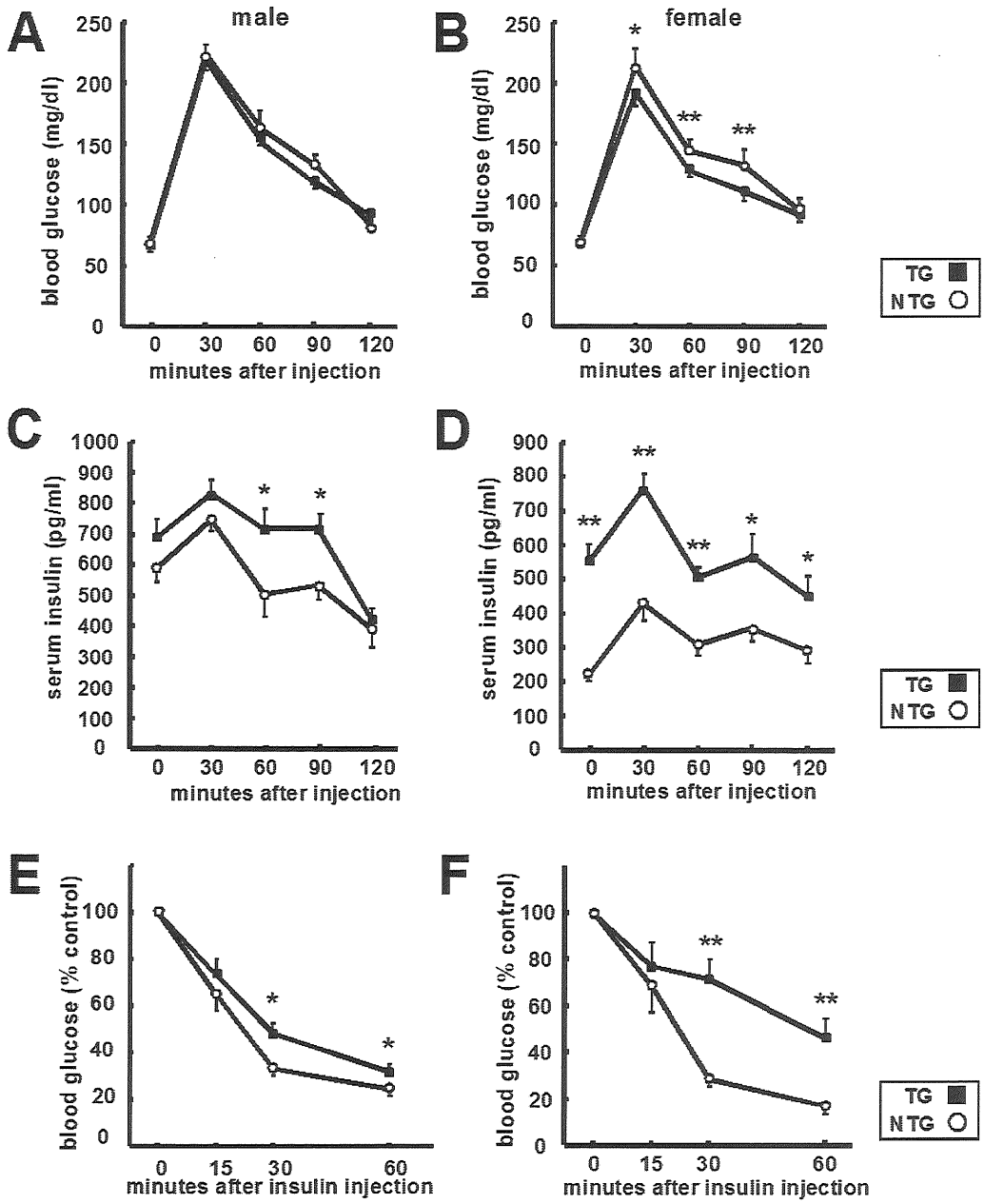

Fig. 14 Glucose and insulin tolerance test in aP2-Foxo1 and control mice. The solid squares and the empty circles represent aP2-Foxo1 (TG) and control (NTG) mice, respectively. **A** and **B**, Glucose tolerance test using control male (A) and female (B) mice (n=8-11). **C** and **D**, Comparison of insulin release in response to an intraperitoneal glucose in male (C) and female (D) mice (n=7). **E** and **F**, Insulin tolerance test of male (E) and female (F) mice (n=8-12). Data are mean S.E. *, $p < 0.05$, **, $p < 0.01$.

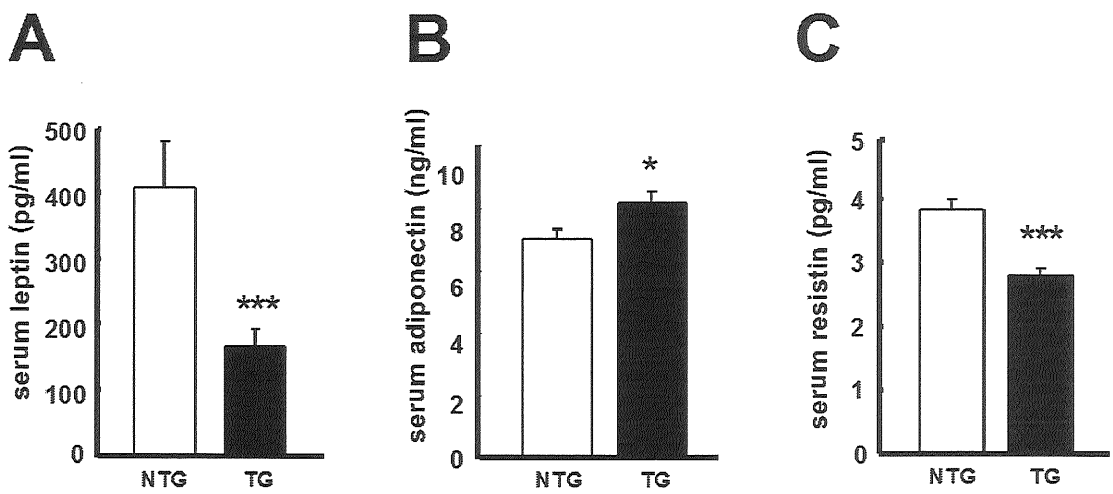

Fig. 15 The concentration of adipocytokine in aP2-Foxo1 mice. A-C, Serum leptin (A), adiponectin (B) and resistin (C) concentration in aP2-Foxo1 (TG) and control (NTG) female mice in fasted state. All of them were determined by the ELISA assay (n=9-14 in each parameter). Data are mean S.E. *, $p < 0.05$, ***, $p < 0.001$.

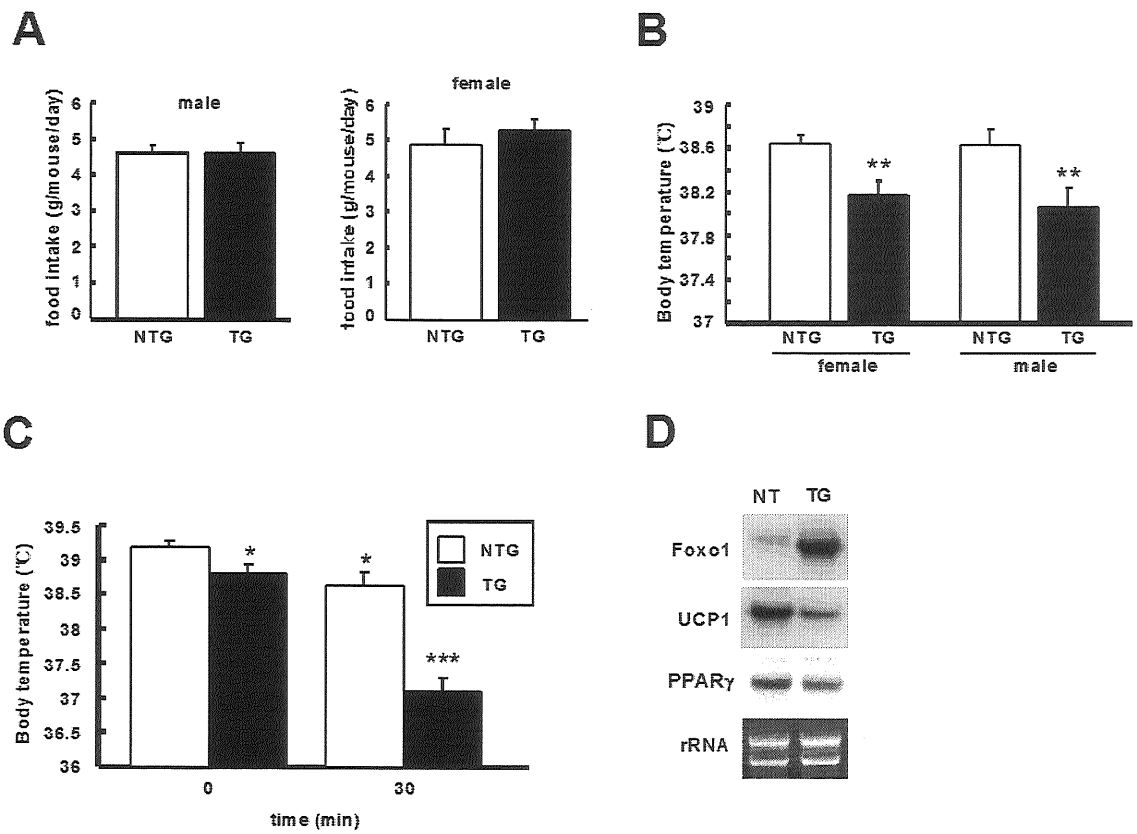

Fig. 16 Food intake and energy expenditure in aP2-Foxo1 mice. **A**, Food intake in aP2-Foxo1 (TG) and control (NTG) mice (n=7 each group). Results are shown as food intake per day. **B**, Rectal temperature at early afternoon (13:30 h) in male and female mice (n=12 each group). **C**, Rectal temperature during cold exposure in aP2-Foxo1 (TG) and control (NTG) male mice (n=4 each group). **D**, Northern blot analysis of UCP1 and PPARγ expression in BAT from aP2-Foxo1 (TG) and control (NTG) female mice under fasted status. β-actin was used for internal control. Data are mean S.E. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.

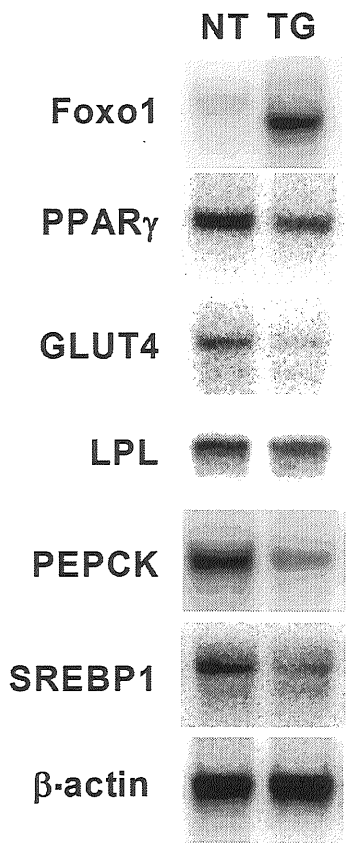

Fig. 17 Northern blot analysis of WAT in aP2-Foxo1 mice. Northern blot analysis of total RNA from parametrial WAT in aP2-Foxo1 mice. The expression levels of Foxo1, PPARγ, GLUT4, LPL, PEPCK, SREBP1 and β-actin in aP2-Foxo1 (TG-A; line A, TG-B; line B) and control (NTG) mice were compared. Full name of probes are shown in text.

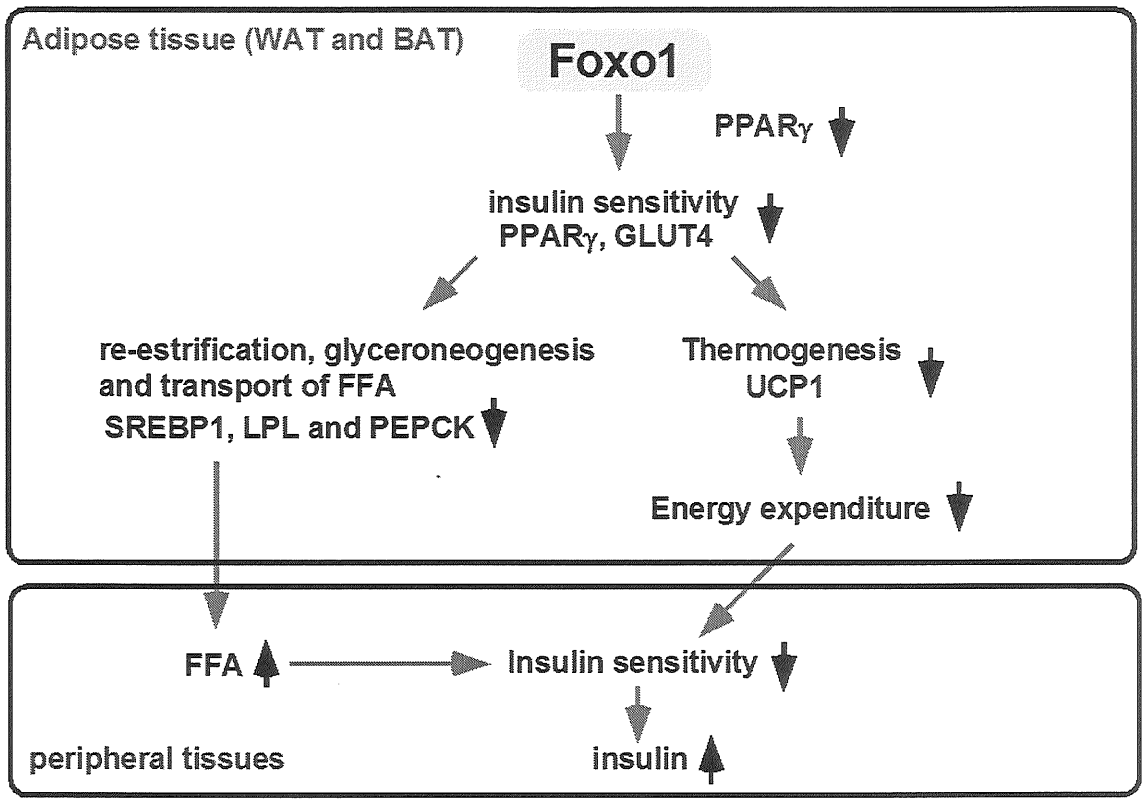

Fig. 18 A molecular hypothetical representation of pathophysiology of insulin resistance in aP2-Foxo1 mice. Overload Foxo1 in adipose tissue including both WAT and BAT could negatively regulate the activity or expression of PPARγ, which is a key transcriptional activator of genes, involving in glyceroneogenesis, adipogenesis, fatty acid re-esterification, adaptive thermogenesis and glucose uptake in adipose tissue. UCP1 expression is suppressed in aP2-Foxo1 mice because PPARγ also activates UCP1 expression. Therefore, aP2-Foxo1 mice may exhibit impaired energy expenditure. Decreased fatty acid re-esterification results in increasing concentration of free fatty acid, and subsequent impairment of insulin sensitivity in peripheral tissues such as muscle. Lower insulin sensitivity in muscle could lead to compensatory supply of insulin from the pancreatic β cells.

Chapter IV
Concluding Remarks

Liver and adipose tissue are key organs targeted by the nutrient signal, and they have important roles to keep energy (glucose and fatty acids) concentration even in fasted or fed conditions. I elucidated the two potentially important aspects of whole body energy metabolism in the liver and the adipose tissue with respect to biochemical and physiological analyses of Foxo1. First, in Chapter II, Foxo1 in the liver plays an important role in gluconeogenic gene expression through the nutrient responses due to fasting or feeding. Second, in Chapter III, Foxo1 in the adipose tissue plays an important role in control of overall lipid metabolism, insulin sensitivity and energy expenditure. These observations prompted me to speculate an integrative physiological function of Foxo1 in energy metabolism throughout nutrient status (Fig. 19). Namely, during fasted conditions, Foxo1 can control gluconeogenesis in the liver, and glyceroneogenesis and fatty acids re-esterification in the adipose tissue. These physiological activities of Foxo1 could result in an overall energy supply in order to keep whole-body energy condition both for short- and long-term starvation. In the short-term starvation, hepatic gluconeogenesis is required for the basic glucose metabolism because mammalian cells such as red blood cells and neurons use glucose as a major energy source. On the other hand, adipose tissue promotes not only lipolysis of triglyceride but also an attenuation of fatty acids re-esterification, thereby increasing fatty acids concentration in the long-term starvation. These can bring β-oxidation in the muscle and the liver, and promoting ketogenesis in the liver. Ketone bodies are certain chemicals produced by catabolizing fatty acids mainly in the liver and particularly important for energy source of the brain (95). Thus, I represent here that Foxo1 is

precisely required for supply of overall energy source.

Other insulin responsible tissues may be required for Foxo1 activity in response to nutrient statuses. One of the physiological roles of insulin is to postprandially clear circulating glucose by enhancing glucose uptake in the skeletal muscle (96). The skeletal muscle Foxo1 regulates skeletal muscle mass and myoblast differentiation (97). Moreover, Foxo1 overexpression in skeletal muscle in mice exhibited impaired glucose tolerance and insulin sensitivity (98). On the other hand, Foxo1 negatively controls the proliferation of β cells in the pancreas (99). Transgenic mice overexpressing the consititutive active Foxo1 mutant in the liver and the β cells develop impaired glucose tolerance and insulin resistance because of deficiency of β cells and abnormal increase of gluconeogenesis in the liver (43). Conversely, the haploinsufficiency of the Foxo1 restores insulin sensitivity and rescues the diabetic phenotype in insulin-resistant mice by reducing hepatic expression of glucogenetic genes and increasing insulin-sensitizing genes in adipose tissue (43,46). Considering these finding along with my present results, Foxo1 may be a gatekeeper of energy homeostasis and insulin sensitivity in response to nutritional signaling.

A number of genetic studies have demonstrated that *daf-16*, the ortholog of mammalian FOXO transcriptional factor, controls lifespan and fat metabolism in *C. elegance* in response to nutrient conditions (20-23). Based on my result, Foxo1 can be regulated in response to nutrient statuses in mice. Taken together, the nutrient control of Foxo1 may be largely conserved in evolution from *C. elegance* to mammals. If the speculation is right, the result of this work has led me to develop a new hypothesis that the Foxo1 regulates life span in animals. Many genetic data demonstrated that inhibited insulin/IGF-1 signaling extends life span in animals (100). I speculate that the longevity

is necessary for whole-body insulin resistance unless impaired glycemic control. Accordingly, the similar results of my study in terms of insulin resistance with normal glucose tolerance and normal body weight were shown in longevity of *Klotho* transgenic mice (101). However, it is unclear whether the genetic interaction of *Klotho* and insulin/IGF-1 signaling participates in life span. Further studies would be required to elucidate the longevity mechanism.

The pathophysiologic consequence of altered posttranslational modification of Foxo1 in response to nutrient status could account for overall insulin sensitivity and glucose metabolism. As demonstrated in Chapter II, impaired nutritional regulation of Akt-mediated Foxo1 phosphorylation and translocation implicates defective suppression of gluconeogenic gene expression in diabetic (*db/db*) mice. The result suggested that a posttranslational modification of Foxo1 may be significant in pathogenesis of type-2 diabetes. Considering in this result, the various growth factors including insulin/IGF-1, an oxidative stress and various cytokines could affect the transcriptional activity of Foxo1, resulting in regulation of numerous genes involved in lipid and glucose metabolism and subsequent developing insulin resistance. Further studies are needed to clarify the relationship between the posttranslational modification of Foxo1 and the pathogenesis of insulin resistance.

I represented on this thesis that Foxo1 strongly participates in overall energy metabolism. Finally, I'd like to gain insight of the new perspective into the molecular therapeutic of insulin resistance via modulation of Foxo1 function. The suppression of gluconeogenesis and hepatic glucose output remains a very attractive therapeutic target in diabetes (102). Based on my result, attenuation in the protein levels of Foxo1 in the nucleus could compensate for insulin resistance in the liver. Thus, drugs that sequester

Foxo1 in the cytosol or lower its levels might be effective in treating diabetes. The exiting speculation of drug discovery targeted Foxo1 is buttressed by my observation that metformin, which is now world widely used for anti-diabetic drug, lower the protein levels of Foxo1 in hepatoma cell line (unpublished observations). Thus, I sought to that Foxo1 is a possible therapeutic target to diabetes.

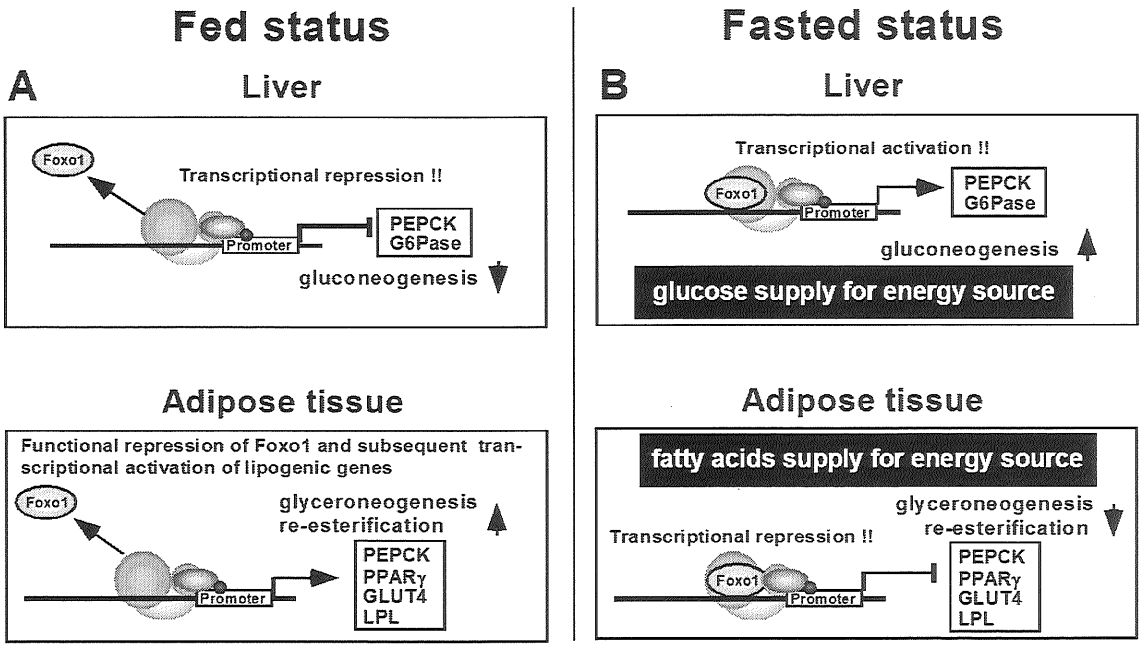

Fig. 19 Control of energy metabolism through Foxo1 in liver and adipose tissue during nutrient status. A, Foxo1 in the fed status. In the liver, feeding promotes attenuation in Foxo1 transcriptional activity, and thereby repressing the expression levels of gluconeogenic genes (G6Pase and PEPCK). In the adipose tissue, feeding could also promote a functional repression of Foxo1 (possibly phosphorylation and nuclear exclusion of Foxo1), following transcriptional activation of genes (*i.e.* GLUT4, PEPCK, PPARγ and LPL). **B,** Foxo1 in the fasted status. In the liver, fasting promotes Foxo1 activation to promote expression of gluconeogenic genes, while in the adipose tissue, to repress the genes involving in glucose and lipid metabolism. Suppression of these directly or indirectly results in impaired glyceroneogenesis and fatty acid re-esterification in the adipose tissue. Consistent with the roles of Foxo1 in different tissues during fasted status, Foxo1 can lead to supply overall energy source, namely glucose from the liver and fatty acid from the adipose tissue.

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