Insights into Molecular Pathogenesis of Type 2 Diabetes from Knockout Mouse Models

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I. Introduction

The pathogenesis of type 2 diabetes is characterized by two major features: peripheral insulin resistance and impaired insulin secretion from pancreatic β-cells [1–3]. These abnormalities are either genetically determined or augmented by environmental factors and hyperglycemia itself, and may interact in a complex manner to cause and sustain hyperglycemia.

Insulin plays a vital role in glucose homeostasis. Muscle, liver and adipose tissue represent the three major targets for the metabolic effects of insulin. Insulin action is mediated by phosphorylation of the insulin receptor (IR) tyrosine kinase present on the surface of most cells [4], followed by phosphorylation of the intermediate docking proteins such as insulin-receptor substrate (IRS)-1, -2, -3 or -4, Src homology/α-collagen protein (Shc) and Grb2-associated binder-1 (Gab1), which subsequently recruit various intracellular Src homology 2 (SH2)-domain-containing proteins such as the regulatory subunit for phosphoinositide 3-kinase (PI3-kinase). PI3-kinase is necessary for the action of insulin on glucose transport, glycogen synthesis, protein synthesis, antilipolysis, and suppression of phosphoenolpyruvate carboxykinase (PEPCK) [5–8]. Insulin-stimulated glucose transport in the skeletal muscles and adipocytes mediates increased glucose transporter (GLUT4) translocation to the plasma membrane. Atypical PKCs [9, 10] and PKB [11, 12], both of which are activated by the PI3-kinase/phosphoinositide dependent kinase (PDK) pathway in response to insulin [13, 14], have been proposed to mediate insulin-stimulated GLUT4 translocation. Which of the two serine threonine kinases downstream of PI3-kinase play a predominant role in GLUT4 translocation in the skeletal muscles and adipose tissue has yet to be established. IR phosphorylation also leads to the activation of downstream signaling cascades of the Ras/Raf/mitogen-activated protein (MAP) kinase pathway, which is not necessary for glucose transport but has a role in the mitogenic effects of insulin.

Glucose transporters represent a large family of proteins encompassing GLUT1–GLUT5 which facilitate glucose transport into the cells across the plasma membrane (PM). The genes encoding these proteins are differentially expressed and regulated in various tissues, and these transporters have different biochemical properties [15–17]. While GLUT1 is found in nearly every tissue type, GLUT4 is located in muscle and adipose tissues and serves as the major insulin-sensitive transporter. GLUT2 is present in liver, kidney, intestine and pancreatic β-cells. GLUT2 has a high Km for glucose and has been proposed to function together with glucokinase (GK) in glucose sensing by β-cells for the regulation of insulin secretion. A role for PI3-kinase activity in insulin-stimulated glucose transport and GLUT4 translocation has been suggested from in vitro experiments [8, 18].

Glucose metabolism through glycolysis in the cytosol and then through the tricarboxylic acid (TCA) cycle in the mitochondria promotes glucose-induced insulin secretion through the generation of metabolic signals such as adenosine triphosphate (ATP), or through an increase in the ratio of ATP to adenosine diphosphate (ADP) in pancreatic β-cells. GK, which converts glucose into glucose 6-phosphate and has a high Km for glucose, is thought to constitute a rate-limiting step in glucose metabolism in pancreatic β-cells [19, 20]. In addition, previous studies indicated that one or more factors derived

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from glycolysis other than pyruvate are required for the generation of mitochondrial signals that lead to insulin secretion. One candidate for these metabolic coupling factors is nicotinamide adenine dinucleotide (NADH). The cytosolic NADH is transferred to mitochondria through the NADH shuttle system composed of the glyceral phosphate shuttle and malate-aspartate shuttle. However, the precise role of the NADH shuttle system is unclear.

Insulin resistance in the skeletal muscles has been proposed to play a major role in the pathogenesis of type 2 diabetes and syndrome X. Skeletal muscle insulin resistance can be caused by either genetic mutation or obesity. The explosive increase in type 2 diabetes is closely linked to obesity due to high-fat diet and sedentary lifestyle in Western countries and Japan. The adipocyte plays a central role in energy balance [21]. Hypertrophic adipocytes produce an excess of hormones and nutrients such as tumor necrosis factor α (TNFα) and free fatty acids (FFA) which have been reported to cause insulin resistance in peripheral tissues such as skeletal muscles and liver [22, 23]. In fact, TNFα has been proposed to cause decreased tyrosine phosphorylation of IRS-1/decreased insulin receptor tyrosine kinase activity [24] and decreased GLUT4 expression [25]. FFA has been reported to cause decreased activation of IRS-1 associated PI3-kinase in response to insulin [26]. In principle, obesity, defined as increased adipose tissue mass, is caused by two distinct processes: the increased formation of new adipocytes from precursor cells (adipocyte differentiation), and an increase in adipocyte size due to fat storage (adipocyte hypertrophy) [27]. Peroxisome proliferator-activated receptor (PPARγ) is a ligand-activated transcription factor that belongs to the nuclear hormone receptor superfamily and forms heterodimers with the retinoid X receptor [21]. PPARγ has been proposed to play a central role in adipocyte differentiation. Thiazolidinedione (TZD), a synthetic ligand of PPARγ, can ameliorate obesity-induced insulin resistance and may be used clinically as an insulin sensitizer to type 2 diabetes [28]. TZD causes adipocyte differentiation to increase the number of small adipocytes and decrease the number of large hypertrophic adipocytes, thereby reducing production of TNFα and FFA leading to amelioration of insulin resistance [29]. However, the roles of endogenous PPARγ in adipocyte hypertrophy and regulation of insulin resistance remain largely unknown.

The present review attempts to summarize the recent applications of knockout mouse models to answer questions on the physiological roles of these key molecules involved in insulin action/secretion and glucose homeostasis.

**Knockout animals as a tool to study in vivo function**

The analysis of genes by inactivation or modification, and the study of the consequences of the mutations in the organism are a central step to understanding the function of genes in vivo. In mammals, before the advent of transgenic technique, this approach was limited to the rare spontaneous mutations associated with obvious phenotypes, as in the case of inheritable diseases in humans. Moreover, it is often difficult to distinguish primary alterations from their consequences in animal models or human patients. The transgenic/knockout approach has brought a number of novel insights which directly or indirectly pertain to this question by altering genes in a primary fashion. The general strategy for overexpressing the product of a gene of interest or knocking it out by homologous recombination has been reviewed elsewhere [30–33]. Application of transgenic mouse technology in diabetology has been extensively reviewed [34, 35]. Gene knockout technology allows for the disruption or replacement of endogenous DNA sequences through the process of homologous recombination. In knockout mouse models, any resultant phenotypes should reflect physiological functions of the specific gene in vivo. In addition, researchers in the field of diabetology are now doing conditional, i.e. tissue-specific and/or inducible, knockouts using the Cre/loxP system for several reasons [36–38]. First, germline mutations may be lethal, in which case there is no mouse to study gene function. Second, genes may exert their function at several stages of ontogeny and in different cell types. Third, tissue-specific knockout enables us to understand the interplay among various organs involved in glucose homeostasis in vivo.

The conditional gene targeting approach as well as the classical approach have been used to analyze the pathophysiology of type 2 diabetes and to create animal models [39]. The significance of knockout
animals as a tool to study glucose homeostasis *in vivo* is summarized in Table 1.

**Insulin receptor (IR)**

The gene targeting approach was applied to directly inactivate the IR gene in mice [40, 41]. At birth, homozygous null mutant (*IR*⁻⁻) pups could not be distinguished from their other littermates. Human patients with mutations in *IR* including null alleles usually have severe intrauterine growth retardation [42, 43]. By contrast, IR deficiency in *IR*⁻⁻ led to a number of major metabolic alterations soon after suckling. The pups developed a severe form of diabetes with ketoacidosis and hepatic steatosis. The absence of insulin signaling in the liver resulted in reduced hepatic glycogen content. *IR*⁻⁻ pups had a marked postnatal growth retardation and developed skeletal-muscle hypotrophy. The number of fat cells appeared to be normal, and all stages of differentiation could be observed, but the cell fat content was markedly decreased [44]. All of these disorders led to the death of *IR*⁻⁻ pups within 1 week after birth. Interestingly, heterozygous null mutants (*IR*⁺⁻) showed increased insulin levels at the age of 4–6 months and 10% of *IR*⁺⁻ animals eventually developed type 2 diabetes, depending on the genetic background [45].

More recently, a muscle-specific IR gene knockout was achieved using the Cre/loxP system [46]. Mice carrying an altered IR gene with exon 4 flanked by loxP sites were generated and bred with transgenic mice that express the Cre recombinase gene under the control of the muscle creatine kinase gene promoter/enhancer. The resultant muscle-specific IR knockout (MIRKO) offspring showed a >95% reduction in IR expression specifically in skeletal muscle and a parallel decrease in insulin-stimulated IR and IRS-1 phosphorylation. Despite impaired insulin-stimulated glucose uptake in skeletal muscle, these mice showed elevated fat mass, serum triglycerides, and free fatty acids, but blood glucose, serum insulin, and glucose tolerance were normal. Thus, insulin resistance in muscle contributes to the altered fat metabolism associated with type 2 diabetes, but tissues other than muscle appear to be more involved in insulin-regulated glucose disposal than previously recognized. It is known that physical exercise promotes glucose uptake into skeletal muscle and makes the working muscles more sensitive to insulin. While MIRKO mice had normal resting 2-deoxy-glucose (2DG) uptake in soleus muscles but no significant response to insulin, they displayed normal exercise-stimulated 2DG uptake and a normal synergistic activation of muscle 2DG uptake with the combination of exercise plus insulin [47]. Glycogen content and glycogen synthase activity in resting muscle were normal in MIRKO mice, and exercise, but not insulin, increased glycogen synthase activity. Thus, normal expression of muscle insulin receptors is not needed for the exercise-mediated increase in glucose uptake and glycogen synthase activity *in vivo*.

Tissue-specific knockout of the IR gene in the pancreatic β-cell has also been achieved by breeding the same mice used to generate the MIRKO with transgenic mice expressing the Cre recombinase gene under the control of the rat insulin promoter [48]. The resultant mice (βMIRKO) exhibit a selective loss of insulin secretion in response to glucose and a progressive impairment of glucose tolerance. These data indicate an important functional role for IR in glucose sensing by the pancreatic β-cell and suggest that defects in insulin signaling at the level of the β-cell may contribute to the alterations in insulin secretion seen in type 2 diabetes. Another tissue-specific knock-out of the IR gene in the liver has been achieved by breeding the same mice used to generate the MIRKO

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with transgenic mice expressing the Cre recombinase gene under the control of the rat albumin promoter [49]. The resultant mice (LIRKO) exhibit insulin resistance associated with glucose intolerance at the age of 2 months. However, by 4 months of age, the fasting hyperglycemia is normalized. Therefore, isolated liver insulin resistance is sufficient to cause severe defects in glucose and lipid homeostasis, but not uncontrolled fasting hyperglycemia or diabetes. Recently, a double tissue-specific insulin receptor knockout in the muscles and β-cells (βIRKO-MIRKO) was generated [50]. Surprisingly, βIRKO-MIRKO showed an improvement rather than a deterioration of glucose tolerance when compared to βIRKO mice. This is due to improved glucose-stimulated acute insulin release and redistribution of substrates with increased glucose uptake in adipose tissue and liver in vivo, without a significant decrease in muscle glucose uptake. Thus, insulin resistance in muscle led to improved glucose-stimulated first-phase insulin secretion from β-cells and shunting of substrates to nonmuscle tissues, collectively leading to improved glucose tolerance. These data suggest that muscle, either via changes in substrate availability or by acting as an endocrine tissue, communicates with and regulates insulin sensitivity in other tissues.

The insulin receptor-related receptor (IRR) is an orphan receptor of the IR gene subfamily. IRR was highly expressed in the islets as well as in several highly differentiated β-cell lines derived from transgenic mice [51]. Very recently, mice lacking IRR and a combined knockout of IR and IRR have been generated [52]. Islet morphology, β-cell mass, and secretory function were, however, unaffected in IRR-deficient mice. Moreover, lack of IRR did not impair compensatory β-cell hyperplasia in insulin-resistant IR+/− mice, nor did it affect β-cell development and function in IR−/− mice. Thus, IRR does not play a physiologic role in embryonic β-cell development and regulation of insulin secretion.

Changes in insulin action in various tissues and the resultant phenotypes of IR-knockout mice [40, 41] and tissue-specific IR-knockout mice [46–49] are summarized in Table 2.

### Insulin receptor substrates (IRSs)

IRS-1 is the major substrate of IR and IGF-1 receptor tyrosine kinases. We [53] and others [54] made mice with a targeted disruption of the IRS-1 gene locus. Homozygous IRS-1-deficient mice (IRS-1−/−) were born alive but had retarded embryonal and postnatal growth [53, 54], indicating that IRS-1 plays an important role in the growth-promoting function of IGFs. IRS-1−/− also had resistance to the glucose-lowering effects of insulin, IGF-1 and IGF-2. Despite the insulin resistance, IRS-1−/− showed normal fasting glycemia and normal glucose tolerance due to compensatory hyperinsulinemia from pancreatic β-cells. This compensatory hyperinsulinemia was caused by selective β-cell

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hyperplasia which will be discussed later in more detail [55]. Importantly, comparison of mild phenotypes of IRS-1\(^{-/-}\) with very severe IR\(^{-/-}\) phenotypes strongly suggested the existence of both IRS-1-dependent and IRS-1-independent pathways for signal transduction of insulin and IGFs [53, 54].

To identify IRS-1-independent pathways, Kahn's group and ourselves examined the insulin-stimulated tyrosine-phosphorylated proteins in livers of wild type and IRS-1\(^{-/-}\) [53, 54, 56, 57]. Patti et al. [56] first demonstrated that the insulin-stimulated tyrosine-phosphorylated protein in the liver of IRS-1\(^{-/-}\) was in fact IRS-2/4-PS [58]. Tobe et al. [57] demonstrated that pp190, which is immunologically distinct from IRS-1, was strongly tyrosine phosphorylated in response to insulin in the liver of IRS-1\(^{-/-}\). Pp190 as IRS-1 was able to bind to both the 85-kDa subunit of PI3-kinase and the ASH/Grb2. These data suggest that IRS-2 plays some physiological role in insulin's signal transduction and compensates for IRS-1 in IRS-1\(^{-/-}\).

Our two groups investigated the roles of IRS-1 in the biological actions in physiological target organs such as skeletal muscle, liver, adipose tissue, and the roles of insulin by comparing the effects of insulin in wild-type and IRS-1\(^{-/-}\). In muscles from IRS-1\(^{-/-}\), insulin-induced PI3-kinase activation, glucose transport, p70 S6 kinase and MAP kinase activation were significantly impaired compared with those in wild-type mice [59]. In contrast, insulin-induced PI3-kinase and MAP kinase activation were essentially normal in the liver. The amount of tyrosine-phosphorylated IRS-2 in IRS-1\(^{-/-}\) was roughly equal to that of IRS-1 in wild-type mice in the liver, whereas it was only 20 to 30% of that of IRS-1 in the muscles. These data suggested that IRS-1 plays central roles in two major biological actions of insulin in muscles, glucose transport and protein synthesis; that the insulin resistance of IRS-1\(^{-/-}\) is mainly due to resistance in the muscles; and that the degree of compensation for IRS-1-deficiency appears to be correlated with the amount of tyrosine-phosphorylated IRS-2 in IRS-1\(^{-/-}\) relative to that of IRS-1 in wild-type mice [59] (Fig. 1). Very similar conclusions were drawn by Brüning et al. [60]. In adipose tissues from IRS-1\(^{-/-}\) mice, insulin-induced PI3-kinase activity in the antiphosphotyrosine immunoprecipitates was decreased to 54% of wild-type mice, and pp60 was the major tyrosine-phosphorylated protein associated with PI3-kinase, whereas tyrosine phosphorylation of IRS-2 as well as its association with PI3-kinase was almost undetectable [61]. Pp60 was cloned and

![Fig. 1. Insulin action in the liver, skeletal muscles, and adipocytes in IRS-1\(^{-/-}\) mice](image)

The insulin signaling system was essentially preserved in the liver from IRS-1\(^{-/-}\), due to compensatory phosphorylation of IRS-2. On the other hand, in the skeletal muscles from IRS-1\(^{-/-}\) mice, PI3-kinase activity or MAP kinase activity was impaired because of insufficient phosphorylation of IRS-2. In adipocytes from IRS-1\(^{-/-}\) mice, PI3-kinase activity was impaired because of insufficient phosphorylation of IRS-3. The schematic representation is based upon the experimental results reported in references 57, 59, 61.
designated as IRS-3 by Lienhard’s group [62]. These data suggest that both IRS-1 and IRS-3 play a major role in insulin-induced glucose transport in adipocytes, and that IRS-3 is predominantly involved in regulating this process in the absence of IRS-1 [61] (Fig. 1).

Abe et al. [63] reported that IRS-1−/− showed features of syndrome X such as hypertriglyceridemia and hypertension. Insulin resistance in the adipose tissue caused decreased lipoprotein lipase activity sufficient to cause dyslipidemia. Moreover, hypertension in IRS-1−/− was in part due to insulin resistance in endothelium-dependent vascular relaxation. Thus, insulin resistance due to IRS-1 deficiency per se was sufficient to cause syndrome X but not to cause type 2 diabetes, which, as described below, requires a simultaneous defect in insulin secretion [55].

Targeted disruption of the IRS-2 gene was achieved by White’s group [64] and ourselves [65]. The phenotype obtained was strikingly different from IRS-1−/−. IRS-2−/− progressively developed a marked glucose intolerance and had full-blown diabetes at 10 weeks [64]. The phenotype was milder in our IRS-2−/− animals than those studied by White’s group. The severity of diabetes in two IRS-2−/− animals was presumably due to the difference in genetic background [66]. The most striking finding was that IRS-2 deficiency not only caused peripheral insulin resistance, as did IRS-1 deficiency, but also prevented β-cell compensation for insulin resistance. Histological analysis revealed that, as early as 4 weeks, β-cell mass was reduced by 17% compared with wild-type, in marked contrast to the 85% increase in β-cell mass in IRS-1−/− islets [65]. Thus, a single gene mutation in this animal model was capable of inducing both the peripheral insulin resistance and the β-cell deficiency seen in typical type 2 diabetes.

Ablation of IRS-1 caused selective β-cell hyperplasia to compensate for insulin resistance [55]. In addition, ablation of IRS-1 caused decreased insulin content and decreased insulin response to glucose [65, 67]. In fact, first phase insulin secretion in response to glucose was impaired in islets from IRS-1−/− resembling βIRKO [48]. Interestingly, even though β-cell hyperplasia was defective in IRS-2−/−, individual β-cells showed normal or increased insulin secretion to glucose [65]. Thus, IRS-1 and IRS-2 play distinct roles in maintenance of glucose-induced insulin secretion and β-cell mass.

With regard to insulin signaling, Withers et al. [64] reported that the degree of stimulation of PI3-kinase activity associated with IRS-1 in liver and muscle of IRS-2−/− was reduced by more than 50%, due in part to an increased basal activity, in contrast with the marked enhancement of insulin-stimulated PI3-kinase associated with IRS-2 seen in IRS-1−/−. The authors suggested this functional defect in IRS-1−/− to be the major factor underlying defective glucose metabolism. We noted, however, insulin-induced PI3-kinase activation was significantly impaired in the liver of IRS-2−/−, while its activation in the skeletal muscles was essentially normal [65]. Moreover, IRS-1-mediated PI3-kinase activity was essentially normal in both these tissues in IRS-2−/−.

IRS-1 and IRS-2 mediate the pleiotropic effects of insulin and IGF-1, including regulation of glucose homeostasis and cell growth and survival. Withers et al. intercrossed mice heterozygous for two null alleles (IRS-1+/− and IRS-2+/−) and investigated growth and glucose metabolism in mice with viable genotypes [68]. They showed that IFS-1 and IRS-2 are critical for embryonic and postnatal growth, with IRS-1 having the predominant role. Although both IRS-1 and IRS-2 were involved in peripheral carbohydrate metabolism, IRS-2 had the major role in β-cell development and compensation for peripheral insulin resistance. The authors also intercrossed mice heterozygous for null alleles of IGF-1 receptor and IRS-2 [68] and found that IGF-I receptors promoted β-cell development and survival through the IRS-2 signaling pathway, thus proposing a role for the IGF-I receptor in pancreatic β-cells.

Targeted disruption of the IRS-3 gene was achieved by Lienhard’s group [69]. Homozygous IRS-3-deficient mice showed normal body weight throughout development, normal blood glucose and insulin levels, and normal glucose transport in adipocytes. It should be noted, however, that the important roles of IRS-3 in adipocytes and potentially in β-cells may be masked by compensation by either IRS-1 or IRS-2 in these tissues.

Changes in insulin action in various tissues and the resultant phenotypes of IRS-1/2/3-knockout mice are summarized in Table 2.
P13-kinase

A role for P13-kinase activity in insulin-stimulated glucose transport and GLUT4 translocation has been suggested in vitro [8], but its role in vivo and the molecular link between activation of P13-kinase and GLUT4 translocation has yet to be elucidated. The p85 regulatory subunit associates with IRS molecules through its SH2 domain, which results in P13-kinase activation. To determine the role of P13-kinase in glucose homeostasis, we [70] generated mice with a targeted disruption of the gene encoding the p85α regulatory subunit of P13-kinase (Pik3r1) [71–73]. To this end, we specifically deleted the first exon of Pik3r1 in mice (exon 1A). Because this exon contains the initiation codon for p85α, we were able to selectively abolish the expression of full-length p85α mRNA without disrupting the p55α and p50α [74, 75] splicing variants. Mice deficient in p85α were born and showed no apparent growth abnormalities, presumably due to the redundant P13-kinase activities [70]. In fact, mice lacking all three isoforms of the p85α gene were reported to be lethal in the perinatal period [76]. Pik3r1−/− mice showed increased insulin sensitivity and hypoglycemia due to increased glucose transport in skeletal muscle and adipocytes. Insulin-stimulated P13-kinase activity associated with IRSs was mediated via full-length p85α in wild-type mice, but via the p50α isoform in Pik3r1−/−. P13-kinase activity associated with p50α has been shown to be greater than that associated with p85α or p55α [75, 77]. Thus, the phenotype of p85α-deficient mice can be explained by an isoform switch from p85α to p50α in peripheral tissues, leading to an increase in insulin-induced generation of phosphatidylinositol(3,4,5)triphosphate and facilitation of GLUT4 translocation from the low-density microsome fraction to the plasma membrane. IRS-1 became readily dissociated from the p50α/p110α heterodimer in the LDM fraction once P13-kinase was activated by the association of p50α/p110α heterodimer with IRS-1 in response to insulin in the PM and cytosolic fractions of Pik3r1−/−. These results provide the first direct evidence that P13-kinase and its regulatory subunit play a role in glucose homeostasis in vivo [70]. Pik3r1−/− also showed X-linked immunodeficiency-like immunodeficiency due to a selective decrease in the number of mature B lymphocytes [78]. Similar results were reported by Cantley's laboratory [76].

Glucose transporters

Gene targeting was used to generate mice carrying a null mutation in the GLUT4 gene [79]. Because GLUT4 is dysregulated in diabetes and obesity, it was expected that genetic ablation of GLUT4 would result in abnormal glucose homeostasis. Homozygous null mutants (GLUT4−/−), however, did not show a diabetic phenotype. Indeed, blood glucose levels in females were not significantly elevated under either fasting or fed conditions, and males showed rather lower glucose levels under fasting conditions. Insulin resistance was apparently present in both sexes, as evidenced by 5–6-fold higher postprandial hyperinsulinemia and impaired glucose response to insulin tolerance test. GLUT4 deficiency resulted in decreased levels of lactate and FFAs under both fasting and fed conditions, and of β-hydroxybutyrate under fasting conditions. There was a marked reduction in fat tissue deposition in GLUT4−/−, contrasting with the increased adiposity of mice overexpressing GLUT4 in adipocytes [80]. GLUT4−/− showed growth-retardation and significant cardiac hypertrophy, which may be related to hyperinsulinemia and decreased supply of FFAs. Possibly as a result of abnormal cardiac function, GLUT4−/− had reduced longevity (5–7 months). Increased expression of other glucose-transporter genes was detected in liver (GLUT2) and heart (GLUT1), but not in skeletal muscle.

Heterozygous GLUT4+/− mice were born with normal phenotype and were fertile. Interestingly, although the male mice did not become obese as they aged, they developed hyperinsulinemia and subsequently hyperglycemia and hypertension despite no evidence of β-cell deficiency [81]. GLUT4 content and glucose uptake in muscle was markedly reduced. There was a drastic decrease in GLUT4 expression in adipose tissue as well, with no change in total mass, but average cell volume increased by 35%. The mice also showed diabetic histopathologies (diabetic cardiomyopathy and liver steatosis) in the heart and liver similar to those of humans with type 2 diabetes [81]. Thus, male GLUT4+/− represent a good model for studying the development of type 2 diabetes without the complications associated with obesity.
A muscle-specific GLUT4 gene knockout (G4M−/−) was achieved using the Cre/loxP system [82]. G4M−/− showed 90–95% reduction in GLUT4 expression in skeletal muscle, with no compensatory increase in GLUT1. In contrast to MIRKO [46], G4M−/− had more severe insulin resistance, fasting hyperglycemia and glucose intolerance. This difference may be due to the fact that contraction-induced glucose uptake in muscle is intact in MIRKO, while it is abolished in G4M−/−. Recently, an adipose-specific GLUT4 gene knockout (G4A−/−) was generated [83]. G4A−/− mice had normal growth and adipose mass despite markedly impaired insulin-stimulated glucose uptake in adipocytes. Although GLUT4 expression was preserved in muscle, these mice developed insulin resistance in muscle and liver. Insulin resistance occurred secondarily in muscle and liver as evident from defective proximal signaling and reduced biological responses. The insulin resistance cannot be accounted for by changes in circulating FFAs, triglycerides or leptin, or changes in TNFα expression in adipose tissue. The degree of glucose intolerance and insulin resistance in G4A−/− mice is similar to that in G4M−/−, suggesting distinct and complementary roles for adipose tissue and skeletal muscle in mediating glucose disposal in vivo. Thus, glucose transport in adipose tissue plays a critical role in glucose homeostasis and the adipose-selective downregulation of GLUT4 seen in human obesity and type 2 diabetes may contribute to insulin resistance and to the risk of developing type 2 diabetes.

Gene targeting approach was applied to generate mice carrying a null mutation in the GLUT2 gene [84]. Homozygous null mutants (GLUT2−/−) were normal at birth, but developed postnatal growth retardation and usually died at 2–3 weeks of age. Animals 10–15 days old showed moderate hyperglycemia, slight hypoinsulinemia, markedly elevated glucagon, FFA and β-hydroxybutyrate. They also had extreme glycosuria, presumably due to lack of GLUT2 in kidney proximal tubules, and resultant blood hyperosmolarity. Isolated islets from 12–15-day-old mice had a complete absence of insulin secretion in response to stimulatory concentrations of glucose, and a lack of rapid onset response in the presence of isobutylmethyl xanthine, but a normal response to non-glucidic secretagogues. Insulin gene transcription was also affected. Thus, GLUT2 is essential for glucose-stimulated insulin secretion from the β-cells.

**Glucokinase (GK)**

GK, which is mainly expressed in pancreatic β-cells and the liver, is thought to constitute a rate-limiting step in glucose metabolism in these tissues [85]. Since insulin secretion parallels glucose metabolism and the high Km of GK (5 to 8 mM) ensures that it can change its enzymatic activity within the physiological range of glucose concentrations, GK has been proposed to act as a glucose-sensor in the pancreatic β-cell [20, 85]. We generated mice carrying a null mutation in the GK gene in pancreatic β-cells, but not in the liver, by disrupting exon 1β expression and thereby selectively eliminating expression of the pancreatic β-cell GK isoform without affecting expression of the liver isoform [86]. The heterozygous mutant mice had normal GK and hexokinase activities in the liver and approximately 50% reduced GK activity in the islets. At the age of 10 weeks, these mice showed mild diabetes due to impaired insulin secretion to glucose. Homozygous null mutants developed postnatal metabolic disorders and died within a week after birth. In in vitro experiments, glucose-induced insulin secretion from their islets was defective, while insulin secretion to arginine was essentially preserved.

The GK gene was inactivated in mice in both β-cells and liver using two strategies that consisted of inserting a neo cassette between exons 3 and 5 [87] or into exon 2 [88]. The heterozygous null mutants (GK+/−) were of normal size and were fertile. Although the circulating insulin levels in these mice were comparable with those in control animals, glucose-stimulated insulin secretion during a hyperglycemic clamp was moderately decreased [88]. The lipid metabolism in GK+/− mice was also affected. Overall, the phenotype was quite similar to that of MODY patients. While one study found that GK−/− had normal fed glucose levels [88], another study [87] reported consistently elevated fed glucose levels from birth in GK−/−. Although the second group was unable to generate homozygous null mutants due to embryonic lethality [88], the first group was able to obtain viable GK−/− pups, which developed severe diabetes with ketoacidosis and died within a week after birth [87], like our β-cell-specific GK−/− pups [86].
When $GK^{++}$ mutants and transgenic mice expressing the rat $GK$ cDNA in β-cells under the control of the human insulin promoter were crossed, homozygous null mutants expressing the transgene in β-cells became viable, because reconstitution of GK activity in β-cells in a GK-deficient background in part normalized the blood glucose levels [87].

These three studies pointed to β-cell GK having a greater impact on glucose homeostasis than liver GK and provided strong support for the concept that GK is important for glucose sensing [86–88]. More recently, a β-cell-specific or liver-specific GK gene knockout was achieved using the Cre/loxP system [89]. Animals either globally deficient in GK, or lacking GK just in β-cells, died within a few days of birth from severe diabetes. Mice that are heterozygous null for GK, either globally or just in the β-cells, survived but were moderately hyperglycemic, similar to our β-cell-specific GK-deficient mice [86] and mice lacking GK in both β-cells and liver [87]. In contrast, mice lacking GK only in the liver were only mildly hyperglycemic but displayed pronounced defects in both glycogen synthesis and glucose turnover rates during a hyperglycemic clamp. Interestingly, hepatic GK-knockout mice also had impaired insulin secretion in response to glucose. These studies indicated that deficiencies in both β-cells and hepatic GK contribute to the hyperglycemia of MODY-2 patients.

mGPDH

Glucose metabolism in glycolysis and in mitochondria is pivotal to glucose-induced insulin secretion from pancreatic β cells. The electrons of the glycolysis-derived reduced form of nicotinamide adenine dinucleotide (NADH) are transferred to mitochondria through the NADH shuttle system, which is composed of the glycerol phosphate shuttle and malate-aspartate shuttle. Eto et al. [90] generated mice which lack mitochondrial glycerol-3 phosphate dehydrogenase (mGPDH), a rate-limiting enzyme of the glycerol phosphate shuttle. When both shuttles were halted in mGPDH-deficient islets treated with aminooxyacetate, an inhibitor of the malate-aspartate shuttle, glucose-induced insulin secretion was almost completely abrogated. Under these conditions, although the flux of glycolysis and supply of glucose-derived pyruvate into mitochondria were unaffected, glucose-induced increases in NAD(P)H autofluorescence, mitochondrial membrane potential, $Ca^{2+}$ entry into mitochondria, and ATP content were severely attenuated. Thus, the NADH shuttle system is essential for coupling glycolysis with the activation of mitochondrial energy metabolism to trigger glucose-induced insulin secretion, and thus revises the classical model of metabolic signaling in glucose-induced insulin secretion.

PPARγ

Agonist-induced activation of PPARγ is known to cause adipocyte differentiation and insulin sensitivity. To investigate the biological role of PPARγ, we [91] and others [92, 93] generated $PPAR\gamma^{-/-}$ deficient mice by gene targeting. Homozygous $PPAR\gamma^{-/-}$ deficient embryos died at 10.5–11.5 d.p.c. due to placental dysfunction. Heterozygous $PPAR\gamma^{-/-}$ deficient mice ($PPAR\gamma^{+/+}$) were protected from an increase in white adipose tissue mass due to adipocyte hypertrophy. Development of insulin resistance under a high-fat diet was also prevented in $PPAR\gamma^{+/+}$ [91]. In fact, a high-fat diet-induced reduction of GLUT4 expression in white adipose tissues was in part prevented in $PPAR\gamma^{+/+}$. Despite reduced adipose tissue mass and adipocyte size, leptin expression was increased due to derepression of the leptin gene by a loss of one $PPAR\gamma$ allele. Since leptin has been shown to decrease adipose tissue mass and increase insulin sensitivity, it seems likely that protection from high-fat diet-induced adipocyte hypertrophy and insulin resistance was at least in part mediated by increased leptin expression. Similar results were also reported by another group [94].

We hypothesize that PPARγ also has a role in the regulation of adipocyte hypertrophy in relation to insulin sensitivity (Fig. 2). PPARγ promotes the differentiation of pre-adipocytes (P) to normal, insulin-sensitive small adipocytes (S), which can be activated by artificial PPARγ ligands such as TZD. High-fat diet promotes adipocyte hypertrophy which converts small adipocytes (S) into large adipocytes (L), which in turn induces factors such as TNFα and FFA, thereby causing insulin resistance. Since heterozygous $PPAR\gamma$ deficiency prevented adipocyte hypertrophy and development of insulin resistance under a
high-fat diet, it appears that the amount of PPARγ plays a critical role in adipocyte hypertrophy and development of insulin resistance under a high-fat diet. Thus, it seems likely that PPARγ plays dual roles in the regulation of insulin sensitivity. In adults under a high-fat diet, moderate activation of PPARγ by endogenous ligands can promote adipocyte hypertrophy and hence effect the size of adipocytes, but is not sufficient to promote differentiation of preadipocytes [28] and apoptosis of large adipocytes [29], leading to insulin resistance. In the presence of potent synthetic ligands such as TZD (presumably in childhood), however, PPARγ stimulation results in adipocyte differentiation to generate small adipocytes and insulin sensitivity [95, 96].

Recently, we generated transgenic mice overexpressing constitutively-active PPARγ2 (Ser112Ala) in white and brown adipose tissues [97]. Under a normal diet, S112A transgenic mice showed similar body weight, fat mass, glucose tolerance/insulin sensitivity to wild-type mice. Under a high-fat diet, however, S112A mice became obese and developed insulin resistance. These results support our hypothesis that PPARγ has a role in high-fat diet-induced obesity and insulin resistance (Fig. 2).

PPARγ appears to facilitate energy storage under a high-fat diet in part by inhibiting expression of the leptin gene in adipocytes. It seems that PPARγ serves as a typical thrifty gene [91, 98]. Consistent with this possibility, human subjects with the Pro12Ala variant of the PPARγ2 gene, which causes a modest reduction of PPARγ activity, have a decreased body mass index and decreased insulin resistance as well as protection from type 2 diabetes [99, 100]. The identification of a novel role for PPARγ in obesity and insulin resistance may lead to new therapeutic strategies such as a PPARγ antagonist for obesity and obesity-linked insulin resistance.

**Interplay between genetic defects on the development of type 2 diabetes**

The peripheral insulin resistance and impaired insulin secretion from pancreatic β cells seen in type 2 diabetes [1-3] are either genetically defined or aug-
mented by environmental factors and hyperglycemia itself. These factors may interact in a complex manner to cause and sustain hyperglycemia. Previous prospective studies demonstrated that both insulin resistance (often accompanied by hyperinsulinemia) and low insulin response to glucose are predictors of human type 2 diabetes [1–3]. To investigate the interactions between insulin resistance and insulin secretory defects in the development of diabetes, we generated an animal model with these two genetic defects by crossing IRS-1 deficient mice and β-cell GK-deficient mice [55]. As described above, IRS-1−/− showed insulin resistance but normal glucose tolerance with compensatory hyperinsulinemia [53–55], and heterozygous β-cell GK−/− showed mild glucose intolerance due to an impaired insulin response to glucose [86]. When these two were crossed with each other, the double knockout mice kept the features of both lines showing insulin resistance and impaired insulin secretion, and exhibited sustained hyperglycemia [55]. Thus, genetic predispositions to diabetes can interact in a cooperative manner when they coexist, and cause overt diabetes. In this case, mild insulin resistance and a slight defect in insulin secretion, when combined, lead to the development of diabetes. Another example of this principle comes from the study of mice doubly heterozygous for a null allele of IR or IRS-1 [45]. Mice heterozygous for a null allele of IR or IRS-1 had no obvious clinical phenotype. Mice doubly heterozygous for a null allele of IR or IRS-1 were generated by crossing the single mutants. Such animals were indistinguishable from other littersmates at birth. The expression of IR and IRS-1 in liver and muscle of these mice was diminished by 60%. Consequently, insulin-stimulated autophosphorylation of IR, tyrosine-phosphorylation of IRS-1 and IRS-2 and association of the p85 subunit of PI3-kinase with IRS-1 were all reduced. Blood glucose was normal and remained so up to the age of 4–6 months, but insulin levels were already higher at 2 months in the double heterozygotes than in each single mutant. Interestingly, at the age of 4–6 months, these animals developed a marked insulin resistance. At 6 months, 40% of double heterozygous mice developed severe diabetes. It therefore appears that the combination of minor defects in the insulin signaling cascade can act synergistically to cause insulin resistance and type 2 diabetes in an age-dependent manner.

Human type 2 diabetes is a polygenic disease requiring the interactions of multiple genetic factors and environmental factors to become manifest. However, genetic dissection of type 2 diabetes in humans has been limited for both practical and ethical reasons. In this respect, use of inbred animal models that spontaneously reproduce the main features of type 2 diabetes is an important strategy for genetic investigations. We [55] and others [45] employed a novel strategy to reconstitute a polygenic model of type 2 diabetes by crossing individually defined genetic defects. Since whole genome mapping and identification of human type 2 diabetes genes are progressing rapidly [101–103], the genetic reconstitution of defects in type 2 diabetes genes in mice should shed light on the molecular mechanism and actual biochemical pathways of human type 2 diabetes.

The potential roles of insulin, glucose, IR/IGF-I receptor family, and IRSs in pancreatic β-cells deduced from the phenotypes of knockout animals involved in insulin secretion and β-cell mass regulation

In general, β-cell mass is determined by the differentiation/proliferation of β-cells and the β-cell death including apoptosis. The insulin receptor (IR) is structurally related to the insulin-like growth factor-1 (IGF-1) receptor and the insulin receptor-related receptor (IRR), an orphan receptor for which no ligand has been identified. While IRS-1−/− showed normal glucose tolerance due to compensatory β-cell hyperplasia and hyperinsulinemia [55], IRS-2−/− developed diabetes due to failure of β-cell hyperplasia [64, 65]. Apoptotic changes were observed in islets from IRS-2−/− [64]. Ablation of IRS-1 caused decreased insulin content and decreased insulin response to glucose [65, 67]. Interestingly, even though β-cell hyperplasia was defective in IRS-2 knockout mice, individual β-cells showed normal or increased insulin secretion to glucose [65]. Mice lacking IR in pancreatic β-cells exhibited a selective loss of insulin secretion in response to glucose, although they showed normal or slightly decreased β-cell mass [48]. IRR-deficient mice showed normal β-cell mass and insulin secretion [52]. In addition, it was reported that secreted insulin acts via β-cell insulin receptors and up-regulates insulin gene transcrip-
tion by signaling through the IRS-2/PI3-kinase/p70 S6 kinase and CaM kinase pathways [104]. These results indicate important functional roles for IR /IGF-I receptor and IRSs as well as associating PI3-kinase in regulation of β-cell mass and glucose sensing by β-cells (Fig. 3), and suggest that defects in insulin signaling at the level of the β-cells may contribute to the alterations in β-cell mass and/or insulin secretion.

Is it right to extrapolate insights from mouse models to explain the pathophysiology of human diabetes?

There is a natural temptation to extrapolate insights from animal models to explain the pathophysiology of human diabetes. In some cases, the mouse model closely resembles the human diseases, as is the case with GCK−/− [86] and MODY-2 [85, 105]. By contrast, while human patients with mutations in IR including null alleles usually had severe intrauterine growth retardation [42, 43], IR−/− pups did not exhibit intrauterine growth retardation but developed a severe form of diabetes with ketoacidosis shortly after birth [40, 41]. Why did the same mutation cause different phenotypes in different species? There may be differences in the genetic background. For example, redundant pathways may vary in importance in different species. Moreover, there can be striking differences in phenotypes even when a specific mutation is introduced into different strains of mice, as described above [66]. Mapping and ultimately cloning modifier gene(s) that account for variations in phenotypes should advance our understanding of the pathophysiology of diabetes. Nevertheless, because human type 2 diabetes is a complex heterogeneous disease with multiple genes contributing to the cause of this disorder and genetic dissection/reconstruction of type 2 diabetes in humans is limited for both practical and ethical reasons, insights from mouse models should help to explain, at least in part, the pathophysiology of human diabetes.

Concluding remarks

The application of knockout approaches to the field of insulin action/secreetion and glucose homeostasis has provided a wealth of novel information and a means to examine the relevance of key concepts often drawn from in vitro experiments. We
are able to understand the precise roles of each molecule involved in glucose homeostasis in vivo. Establishment of animal models with well-specified phenotypes should open new avenues for therapeutic trials. Genetic engineering in mice to perform tissue-specific gene disruptions, to reconstitute expression of a given gene in selected tissues in a null background using transgenic mice, or to replace specific genes with mutated genes should provide new investigative tools for further studies. In addition, cell lines derived from the various animal models should allow more in-depth investigation. Both conditional gene targeting approaches and classical approaches will be applied to the analysis of the molecular pathogenesis of type 2 diabetes and establishment of therapeutic methods to improve insulin sensitivity and insulin secretion.

Acknowledgments

We are grateful to Drs. Kazuyuki Tobe, Mitsuhiko Noda, Kazuhiro Eto, Toshimasa Yamauchi, Naoto Kubota, Kajuro Komeda, and Shinichi Aizawa, who played major roles in the works cited in this review. This work was supported by a Grant-in-Aid for Creative Scientific Research (10NP0201) from the Japan Society for the Promotion of Science, a Grant-in-Aid for the Development of Innovative Technology from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and Health Science Research Grants (Research on Human Genome and Gene Therapy) from the Ministry of Health and Welfare.

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