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# TRANSGENIC MICE IN THE ANALYSIS OF METABOLIC REGULATION

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ABSTRACT

In normal animals, the extracellular concentration of glucose is maintained within a very narrow range by the matching of glucose flux into and out of the extracellular space through the tightly coordinated secretion of insulin and glucagon. Functional alterations in $\beta$-cells, liver, or skeletal muscle and adipose tissue may disrupt glucose homeostasis and lead to the development of non–insulin-dependent diabetes mellitus (type 2 diabetes). This review outlines the contribution of these organs and tissues to the control of glucose homeostasis. We discuss new insights obtained through studies of transgenic mice that overexpress or show decreased expression of putative key genes in the regulation of pancreatic $\beta$-cell function, in the control of hepatic glucose uptake and output, and in the regulation of glucose uptake and utilization by skeletal muscle and adipose tissue.

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## INTRODUCTION

One of the main goals of biomedical research is to understand the mechanisms that regulate metabolic interaction between the various organs and tissues in the whole animal. Although much has been learned, current knowledge remains incomplete. The elucidation of the metabolic pathways during the first 60 years of this century was a major step forward. In the second half of this century, studies centered on the control of the various processes and enzyme activities in different tissues of animals and humans. These were the years when the classical approach to metabolism was fruitful. However, by the middle 1970s, the expanding list of observations no longer provided major insights. At that time, research in molecular biology emerged, and the use of recombinant DNA techniques become popular. Many scientists left classical metabolism and started cloning genes of interest in the control of metabolic pathways (enzymes, transporters, receptors, etc). Progress in this area has moved fast, and regulation of the expression of hundreds of genes is now being studied. However, these studies cannot provide a full understanding of the temporal relations between multiple metabolic pathways and between different organs and tissues. During the last decade, major technological advances in the fields of molecular biology, molecular genetics, and embryo manipulation have provided tools for a new approach to the study of metabolic regulation, transgenic animals. The availability of genetically defined strains of mice, in which regulatory genes have been incorporated into or deleted from the germ line, is contributing to the knowledge of metabolic processes and their alterations in vivo. This review discusses the application of such techniques to the study of the control of glucose homeostasis.

### *Control of Glucose Homeostasis*

Glucose is a major energy source for all mammalian cells. In normal animals, the extracellular concentration of glucose is maintained within a very narrow range, despite large variations in the rate of utilization of glucose and in the availability of exogenous glucose. This homeostatic control is achieved by the matching of glucose flux into and out of the extracellular space through the tightly coordinated secretion of insulin and glucagon (16, 111). In the fasting state, most glucose uptake occurs in insulin-independent tissues, especially brain. In healthy animals, basal glucose uptake is precisely matched by the output of glucose from the liver, from glycogenolysis and gluconeogenesis. After a meal, this delicate balance is perturbed, and the maintenance of normal glucose homeostasis is dependent on three processes that must occur simultaneously in a coordinated and tightly integrated manner. (*a*) In response to the rise in plasma glucose concentration, insulin secretion by the pancreatic $\beta$-cells is stimulated.

(*b*) The combination of increased insulinemia and glycemia promotes glucose uptake by splanchnic (liver) and peripheral (muscle, fat) tissues. (*c*) Hepatic glucose output is suppressed through insulin-induced inhibition of glucagon secretion and blockage of the hepatic actions of glucagon. Thus, functional alterations at the level of $\beta$-cells, liver, or muscle and adipose tissue may lead to a disruption of glucose homeostasis and to the development of glucose intolerance or overt non–insulin-dependent diabetes mellitus (type 2 diabetes) (16).

Type 2 diabetes is the most common of all metabolic disorders. It is characterized by a decrease in the response of peripheral tissue to insulin (insulin resistance) and by inadequate compensatory insulin secretory response (16, 82). In populations with a high incidence of type 2 diabetes (e.g. Pima Indians, Mexican-Americans, and Pacific Islanders), insulin resistance (muscle and/or liver) occurs early in life and precedes evidence of glucose intolerance. When $\beta$-cells can no longer secrete enough insulin to compensate for insulin resistance, fasting hyperglycemia and overt diabetes develop. In spite of considerable research effort, the causes of insulin resistance are still not understood. Because type 2 diabetes is associated with multiple, complex pathophysiological alterations, it is difficult to determine which defects are primary and which are secondary consequences of the metabolic changes. Moreover, it is not clear where the process is initiated. Four candidate tissues are pancreatic $\beta$-cells, liver, skeletal muscle, and adipose tissue, and their relative roles in the development of the disease are the subject of extensive investigation. Although rodents such as male Zucker diabetic fatty rats (104) are being used to study these alterations, research on the expression of a selected foreign gene or the interruption of the expression of a specific endogenous gene by using transgenic animals is providing new insights into the etiology of type 2 diabetes and obesity. This review outlines the contribution of the various organs and tissues to the control of glucose homeostasis and to the development of type 2 diabetes.



## BASIC ASPECTS OF THE TRANSGENIC TECHNOLOGY APPROACH

The role of a specific gene in metabolism can be evaluated with two main strategies: expressing a foreign gene, or creating an alteration in the endogenous gene that modifies its expression. To express foreign genes in animals, the method used most widely and successfully is DNA microinjection directly into the pronucleus of a fertilized mouse oocyte, thus generating transgenic mice (38, 44). With this procedure, multiple DNA molecules integrate stably into the host genome arranged in a head-to-tail array and can be transmitted to the next generation. Tissue- or development-specific and inducible/regulated expression of foreign genes has been obtained in transgenic animals by selecting

a specific gene promoter [for example, by using the insulin promoter to direct expression of the transgene to $\beta$-cells (19, 116), the myosin light chain promoter to express genes of interest in skeletal muscle (61, 109), fragments of the phosphoenolpyruvate carboxykinase promoter to drive expression of foreign genes in the liver (64, 113), or the aP2 promoter to direct expression in adipose tissue (39, 96)].

In contrast to conventional transgenic mice, which have incorporated additional genes, an animal carrying a specific defect in an endogenous gene that modifies its expression may be generated by targeted mutagenesis (homologous recombination between DNA sequences residing in the chromosome and newly introduced DNA sequences) in pluripotent embryonic stem (ES) cells (5, 57). A targeting vector is created by using all or a fragment of the endogenous gene carrying an alteration of the sequence, which leads to either premature termination of translation or a frameshift mutation. Genes encoding selectable markers that confer antibiotic resistance or sensitivity are usually added. The vector is then introduced into ES cells, and the homologous recombination event is further enriched by selection for antibiotic resistance and sensitivity (positive/negative selection). Identified recombinant ES cell clones, in which the DNA vector replaces one copy of the native gene, are microinjected into mouse blastocysts or aggregate with morulae-stage embryos, which are then transferred to a recipient pseudopregnant mouse to produce chimeric mice. Offspring hetero- and homozygous for the specific defect are produced by mating germline chimeric animals.



## USE OF TRANSGENIC MICE TO STUDY THE ROLE OF SPECIFIC TISSUES IN THE CONTROL OF GLUCOSE HOMEOSTASIS

### *Pancreatic Islet $\beta$-Cells*

Pancreatic islets regulate glucose homeostasis by secreting insulin from $\beta$-cells when blood glucose rises and by releasing glucagon from $\alpha$-cells when blood glucose is low, and by this, normoglycemia is established. The secretion of these hormones is controlled by nutritional, hormonal, and neural signals (53, 72). However, only glucose can initiate insulin release from $\beta$-cells by itself. Amino acids and fatty acids can also stimulate insulin secretion, but they require the presence of basal glucose levels. Similarly, nonnutrient secretagogues do not initiate, but merely modulate, the secretory response. Insulin secretion by pancreatic $\beta$-cells in response to postprandrial blood glucose levels of 5–10 mM requires both transport and metabolism of glucose (71, 72). Low-affinity glucose uptake in $\beta$-cells is mediated by the glucose

transporter GLUT2. A decrease in GLUT2 is noted in various animals with diabetes, which suggests it is required for normal glucose sensing (111). Furthermore, transfection of glucose-unresponsive insulinoma cells (RIN1046-38, RINm5F) or the insulin-producing anterior pituitary AtT-20ins cells with the GLUT2 cDNA confers glucose-stimulated insulin secretion (71). The glucose-phosphorylating enzyme glucokinase (GK) is a crucial step in the control of glucose metabolism in pancreatic $\beta$-cells (71, 72). GK is a member of the hexokinase gene family expressed in $\beta$-cells, liver, gastrointestinal cells, and anterior pituitary cells (43, 86). It has a high $K_m$ for glucose (about 8 mM) and is not inhibited by glucose 6-phosphate. The GK gene has two distinct promoters specific for the liver and pancreatic $\beta$-cells. The hepatic promoter is regulated by insulin, whereas the $\beta$-cell promoter is constitutively active. Thus, both GLUT2 and GK have a high $K_m$ for glucose, and this ensures that the uptake of glucose is proportional to extracellular glucose concentration when glucose is in the high physiological range.

ROLE OF GLUCOSE TRANSPORT To assess the role of glucose transporter GLUT2 in the regulation of insulin secretion and in the development of type 2 diabetes, we obtained transgenic mice expressing high levels of GLUT2 antisense RNA in $\beta$-cells (116). These animals show a chronic reduction in GLUT2 protein in the $\beta$-cells. Islets from these mice have reduced glucose-stimulated insulin secretion but have normal secretion in response to other secretagogues (aminoacids or forskolin). Transgenic mice have mild hyperglycemia, hypoinsulinemia, and altered glucose tolerance, which suggests that they have developed diabetes (116). Islets of these mice also show a large decrease of glucose transport, glucose phosphorylation by GK, and glucose utilization (G Solanes, F Bosch, unpublished results). A mutation in the GLUT2 gene that abolishes transport activity when the mutant protein is expressed in vivo, has been described in a type 2 diabetic patient (69). Nevertheless, the role of reduction in GLUT2 in humans with diabetes remains unclear (26).

ROLE OF GLUCOSE PHOSPHORYLATION Some patients with maturity-onset diabetes have heterozygous point mutations in the GK gene (MODY2) that show reduced enzymatic activity and decreased insulin secretion (27, 117). GK mutations also have been reported in a subset of patients with gestational diabetes and in a small number of Japanese patients with type 2 diabetes (23, 97). These mutations affect GK activity in both the $\beta$-cell and the liver. Several animal models have been developed to determine the contribution of alterations in $\beta$-cell GK activity to the MODY2 phenotype.

The role of glucose phosphorylation in regulating insulin secretion was analyzed by increasing hexokinase activity in the $\beta$-cells. Thus, transgenic mice

that expressed the yeast hexokinase B gene under control of the insulin promoter were developed (20). Yeast hexokinase B, like GK, is not inhibited by glucose 6-phosphate, but it has a higher affinity for glucose than mammalian GK does. The increase in hexokinase activity in the transgenic islets leads to an increase in glucose-stimulated insulin secretion, and to higher levels of insulin and lower concentration of glucose in the blood (20, 118).

GK activity in the $\beta$-cells of transgenic mice was reduced by expressing GK antisense RNA containing a ribozyme element. The antisense gene is under the control of the insulin promoter (19). These mice show a marked decrease in GK activity that leads to a decreased response to insulin secretion to glucose. However, these mice do not show changes in fasting plasma glucose levels or in glucose tolerance (19). Similar results were obtained when an antisense GK RNA was expressed in $\beta$-cells (42). However, in some genetic backgrounds, these mice showed alterations in glucose tolerance. Thus, transgenic C57BL/6 mice but not transgenic C3H mice had higher blood glucose levels than control animals had, following intraperitoneal glucose challenge. This suggests that a $\beta$-cell secretory defect, in combination with other undefined genetic factors, causes impaired homeostasis in mice.

The role of $\beta$-cell GK in the development of diabetes was also analyzed in mice carrying a $\beta$-cell–specific null mutation in the GK gene; this was obtained by disrupting the $\beta$-cell–specific exon (103). Heterozygous mutant mice showed mild early onset diabetes due to an impaired insulin-secretory response to glucose and normal insulin sensitivity, a phenotype that was similar to that found in mice expressing antisense GK mRNA. Homozygous mutants are severely diabetic shortly after birth and die within a week. Islets isolated from completely GK-deficient mice have defective insulin secretion in response to glucose, but they respond normally to other secretagogues.

To obtain a model of the MODY2 disease, GK deficiency was engineered in both $\beta$-cells and liver (4, 32). Heterozygous mice with only one normal GK allele have a phenotype similar to patients with MODY2, since blood glucose is elevated and insulin secretion is reduced (4, 32). The disruption of GK function in both $\beta$-cells and liver of heterozygous transgenic mice was associated with reduced insulin secretion, decreased tolerance to glucose, and abnormal liver glucose metabolism. Thus, these studies demonstrate a key role of GK in glucose homeostasis and implicate both liver and islets in the MODY2 disease. Mice completely deficient in GK (homozygous) have severe hyperglycemia and die perinatally (4, 32).

Another strain of transgenic mice express a mouse $\beta$-cell GK cDNA from the human insulin promoter. They have been used to separate the effects of $\beta$-cell versus liver GK deficiency (32). These mice were mated with the GK-deficient mice. Expression of GK in $\beta$-cells in the complete absence of GK expression

in the liver is sufficient for survival. In addition, it is sufficient to normalize some (cholesterol and triglycerides as well as blood urea nitrogen, albumin, and total protein) but not all (fasting and fed glucose) abnormalities that are detected in 3-day-old GK-deficient mice (32). Although the mean glucose levels are higher in the transgenic homozygous mice than in wild-type mice, approximately 50% of these transgenic homozygous mice have glucose levels that are indistinguishable from wild-type mice. Furthermore, transgenic mice expressing GK in $\beta$-cells that are heterozygous for GK deficiency show fed and fasted glucose concentrations similar to wild-type mice (32). Thus, provided that $\beta$-cell GK levels are sufficient, it is possible to maintain normoglycemia in the presence of only one functional GK allele in the liver.



ROLE OF ION CHANNEL CONDUCTANCE AND INTRACELLULAR $Ca^{2+}$ The transduction of the glucose signal through metabolic pathways results in the generation of ATP that closes ATP-dependent $K^+$ channels in the plasma membrane (53, 72). This depolarizes the membrane and opens voltage-gated $Ca^{2+}$ channels, raising the intracellular $Ca^{2+}$ concentration and triggering exocytotic insulin secretion (53, 72). The molecular basis of the action of $Ca^{2+}$ on insulin secretion is not clear, although the intracellular $Ca^{2+}$-binding protein calmodulin (CaM) may have a role. Transgenic mice that overexpress CaM in $\beta$-cells have defective insulin secretion at an early age because metabolism of glucose, and the subsequent generation of ATP is impaired (21, 22, 91). Another mouse model expresses a mutant form of CaM (CaM-8) in the $\beta$-cells. These mutant CaM lack eight amino acids in its central helix and do not activate CaM-dependent proteins in vitro (45). The expression of the CaM-8 form of calmodulin causes a down-regulation of $Ca^{2+}$ channel currents, which reduces $Ca^{2+}$ entry and accumulation when glucose stimulates closure of the ATP-sensitive $K^+$ channels (45). The reduced intracellular $Ca^{2+}$ accumulation prevents sufficient amounts of insulin from being secreted from $\beta$-cells of CaM-8 mice. Similarly, transgenic mice overexpressing the voltage-dependent $K^+$ channel develop hyperglycemia and hypoinsulinemia, probably resulting from decreased intracellular $Ca^{2+}$ in the $\beta$-cell in response to glucose (81). These models emphasize the role of membrane potential in excitation-secretion coupling in the pancreatic $\beta$-cell.

## *Skeletal Muscle*

Skeletal muscle is postulated to be the most important tissue involved in disposal of a glucose load following oral ingestion. Skeletal muscle comprises about 40% of body mass and accounts for 75% of glucose uptake after a mixed meal (16). Skeletal muscle takes up glucose by facilitated diffusion in both insulin-dependent and insulin-independent manners and expresses relatively high levels

of GLUT4 (responsible for insulin-stimulated glucose transport) and low levels of GLUT1 (located primarily at the plasma membrane and responsible for basal, insulin-independent transport) (47, 76). The rapid conversion of glucose to glucose 6-phosphate is catalyzed by members of the hexokinase family and keeps the intracellular glucose at very low levels, ensuring continued entry of glucose into the cell. Hexokinases I and II (HKI and -II) are the predominant isoenzymes expressed in the skeletal muscle (59, 84, 85). HKI is widely and constitutively expressed in all organs and tissues, whereas HKII is the muscle- and fat-specific, insulin-stimulated isoform. Indeed, GLUT4 and HKII constitute the first step in insulin-mediated glucose utilization, and GLUT1 together with HKI is considered to be responsible for basal glucose uptake. Under basal conditions, GLUT4 is localized mainly in intracellular vesicles, and it is translocated to the cell membrane in response to insulin. This translocation is thought to be the primary mechanism by which insulin stimulates glucose uptake (88).



ROLE OF GLUCOSE TRANSPORT To determine the physiological role of GLUT4 in the regulation of glucose homeostasis, GLUT4 was overexpressed in the transgenic mice. Overexpression was either in all tissues where it is endogenously present (skeletal muscle, heart, and adipose tissue) (54, 75) or in only fast-twitch skeletal muscles (109). The increased expression of the GLUT4 gene results in a constitutively high level of cell surface–associated GLUT4 protein. These animals exhibit increased basal and insulin-stimulated glucose transport (40, 54, 75, 90, 108). GLUT4 overexpression also increases glucose transport stimulated by muscle contractions (34). In addition, glycolysis and glucose incorporation into glycogen were enhanced in muscle isolated from transgenic mice compared with controls. These findings are consistent with the role of GLUT4 as the primary mediator of transport stimulated by insulin or contraction. Furthermore, the expression of the GLUT4 gene in muscle and adipose tissue decreased plasma insulin and glucose levels and improved glucose tolerance (40, 54, 90, 108). Triglycerides, free fatty acids, and $\beta$-hydroxybutyrate were higher in the transgenic mice because of hypoinsulinemia-induced lipolysis (108). In fasted animals overexpressing GLUT4, the levels of free fatty acids and $\beta$-hydroxybutyrate increased further, and skeletal muscle glycogen levels decreased markedly compared with controls. Thus, high-level expression of the GLUT4 gene results in profound physiologic and metabolic alterations in vivo, including increased systemic glucose clearance and muscle glucose utilization and marked compensatory lipolysis. Because all these results suggest that skeletal muscle glucose transport is a major regulatory step for whole-body glucose disposal, the GLUT4 protein could be a potential target for pharmacological or genetic manipulation for treatment of type 2 diabetic patients. In this

regard, low-level expression of a GLUT4 minigene in transgenic mice prevents the impairment of glycemic control and accompanying hyperglycemia caused by ingestion of a high-fat diet (41). This fact was also demonstrated when GLUT4 was overexpressed in *db/db* mice (29), a classical genetic model of type 2 diabetes that displays many of the characteristics of the human disease, including hyperglycemia, insulin resistance, obesity, and a marked decrease in skeletal muscle glucose utilization. The decrease in muscle glucose utilization is due to a major defect in glucose transporter translocation without alterations in intracellular GLUT4 levels (50). Increased expression of GLUT4 in *db/db* mice overcomes the glucose transporter defect, as these mice show elevated plasma membrane-associated GLUT4 protein (29). Insulin resistance is alleviated in such mice, and they show improved glycemic control.

Because GLUT4 is shown to be dysregulated in type 2 diabetes and obesity (16), it was expected that genetic ablation of GLUT4 would result in abnormal glucose homeostasis. Surprisingly, mice with genetic disruption of both alleles of GLUT4 (GLUT4-null mice) did not show a diabetic phenotype, demonstrating that a functional GLUT4 protein is not required for maintaining nearly normal glycemia (49). However, cardiac hypertrophy, growth retardation, severe reduction of adipose tissue, and shortened life span resulted from deleting the gene for GLUT4. These animals also showed decreased insulin sensitivity, as measured by one kind of insulin-tolerance test. However, in an oral glucose–tolerance test, GLUT4-null mice cleared glucose as efficiently as did controls (49, 99).

The muscle-specific myosin light chain (MLC)-GLUT4 transgene was introduced into the GLUT4-null genetic background to study the relative role of muscle and adipose tissue in the etiology of the GLUT4 null phenotype. The resulting MLC-GLUT4-null mice express GLUT4 predominantly in the fast-twitch extensor digitorum longus muscle (110). In these mice, glucose uptake is normalized in skeletal muscle, and whole-body insulin action was restored, as indicated by normalization of insulin tolerance. In male mice heterozygous for a disruption in the GLUT4 gene, the deletion of one allele results in the development of a diabetic phenotype (100). Thus, most of the heterozygous males have normal glycemia and insulinemia at 2–4 months of age, but the majority of them are overtly diabetic 4–6 months later. Sexual dimorphism is significant in such animals, and diabetic progression has been reported only in males (100). Male mice heterozygous for the mutation have reduced GLUT4 expression in adipose tissue and skeletal muscle. The decrease in GLUT4 leads to a reduction in muscle glucose uptake, hypertension, and diabetic histopathologies in the heart and liver similar to those of type 2 diabetic patients.

Basal glucose transport in muscle is believed to be carried out by GLUT1. The importance of this transporter in the control of blood glucose homeostasis

in the postabsorptive state has been confirmed in transgenic mice overexpressing GLUT1 in skeletal muscle (61, 89). These mice showed reduced glycemia, in both fasted and fed states, normal insulinemia, an increase in basal glucose transport, and a dramatic rise in muscle glycogen content (61, 89). Intracellular free glucose accumulated in skeletal muscle from transgenic mice overexpressing GLUT1. Thus, glucose phosphorylation is saturated with markedly increased glucose flux into this tissue (33). Furthermore, muscle insulin resistance was also detected in these animals, suggesting that increased glucose flux into skeletal muscle results in resistance of GLUT4 to activation by insulin and various other stimuli (33).



ROLE OF GLUCOSE PHOSPHORYLATION Skeletal muscle HKII mRNA levels and enzymatic activity decrease when circulating insulin is low or when insulin signaling is impaired (59, 84, 85). Furthermore, glucose transport in muscle in response to euglycemic hyperinsulinemia is markedly impaired in type 2 diabetic patients (16). However, intracellular glucose phosphorylation is impaired to an even greater extent, and as a result, the intracellular free glucose concentration increases. This indicates that while both glucose transport and phosphorylation are resistant to the action of insulin, impaired glucose phosphorylation (HKII) is probably the rate-limiting step for insulin action (16). To determine the importance of HKII, i.e. muscle glucose phosphorylation, in the control of whole-body glucose homeostasis, transgenic mice overexpressing HKII specifically in skeletal muscle have been developed (11). These mice show that basal and insulin-mediated muscle glucose uptake can be increased by a selective increase in HKII expression. However, the effect on glucose homeostasis is much milder than that detected in transgenic mice overexpressing GLUT1 or 4 (11, 40, 54, 61, 108). Part of the limitation in glucose flux imposed by HKII may be due to feedback inhibition of the enzyme by its product, glucose 6-phosphate.

In contrast to HKII, the liver enzyme glucokinase (GK) has a high $K_m$ for glucose, and its activity is not inhibited by glucose 6-phosphate (43, 86). We have produced transgenic mice expressing GK specifically in skeletal muscle to determine whether GK expression in muscle might lead to increased glucose uptake. GK activity detected in the muscle of transgenic mice led to an increase in the intracellular concentration of glucose 6-phosphate and glycogen (P Oteagui, T Ferre, A Pujol, E Rui, F Bosch, unpublished results). These changes in muscle glucose metabolism led to a reduction in blood glucose levels. These transgenic mice also showed lower levels of blood glucose after an intraperitoneal glucose tolerance test, indicating that GK expression caused an increase in the disposal of blood glucose by the muscle. Moreover, lower levels of blood glucose were noted in transgenic mice in which diabetes had

been induced by streptozotocin treatment (P Oteagui, T Ferre, A Pujol, E Rui, F Bosch, unpublished results). These results indicate that the expression of GK activity in muscle increases glucose uptake and utilization and suggest that engineering skeletal muscle to express the liver enzyme might be a useful approach to counteracting diabetic hyperglycemia.

ROLE OF NONOXIDATIVE GLUCOSE DISPOSAL In skeletal muscle, the two major intracellular pathways of glucose disposal are oxidation and glycogen formation. However, most of the glucose that enters muscle fibers in response to insulin is converted to glycogen. Glycogen synthesis is severely impaired in type 2 diabetes and in insulin-resistant, normal-glucose-tolerant individuals who are predisposed to develop type 2 diabetes (16). Glycogen synthase (GS) is the key insulin-regulated enzyme controlling the rate of muscle glycogen formation (17, 52, 59). Although insulin activates both glucose transport and GS in skeletal muscle, controversy has arisen regarding the role of GS activation in insulin-stimulated glycogen synthesis. Manchester et al (58) constructed a strain of transgenic mice that overexpress a constitutively activated form of GS in skeletal muscle. In this mutant form of GS, Ser phosphorylation sites 2 and 3a are mutated to Ala. In these mice, glucose transport is not strictly rate limiting for glycogen synthesis (58). These animals have markedly increased muscle glycogen content and markedly decreased uridine diphosphoglucose concentrations, consistent with the increase in GS activity. The amount of GLUT4 either remains unchanged or decreases. Increased GS activity without a change in glucose transport was sufficient to increase glycogen accumulation (58). These results support the conclusion that the activation of GS, as well as increased glucose transport, contributes to the accumulation of glycogen in response to insulin in skeletal muscle.

ROLE OF THE HEXOSAMINE BIOSYNTHETIC PATHWAY Glucose is an important regulator of normal metabolism. However, sustained hyperglycemia causes insulin resistance in humans and rodents; a major site of glucose-induced insulin resistance is skeletal muscle (16, 63). Some of the adverse results of hyperglycemia might be caused by normally functioning regulatory pathways (63). Excessive glucose flux leads to insulin resistance in mice overexpressing GLUT1 specifically in muscle, despite the fact that their serum glucose levels are lower than normal (33). The hexosamine biosynthetic pathway may be involved in some of the adverse regulatory effects of excessive glucose flux (63). Glutamine:fructose-6-phosphate amidotransferase (GFAT) is the first, as well as the regulatory, enzyme in the hexosamine pathway (63). Interestingly, chronically increased glucose flux increases muscle GFAT activity posttranscriptionally in mice that overexpress GLUT1 (8). Furthermore, glucose flux

via the hexosamine biosynthetic pathway is increased in muscles of these mice but not in muscles of GLUT4-overexpressing mice (which do not develop insulin resistance) (8).

Transgenic animals overexpressing GFAT in muscle and adipose tissue have been used to test the hypothesis that hexosamine metabolism is involved in insulin resistance (37). A 2.4-fold increase in GFAT activity in muscles of these mice led to weight-dependent hyperinsulinemia. Using the hyperinsulinemic-euglycemic clamp technique, these investigators confirmed that transgenic mice developed insulin resistance and had a decreased glucose disposal rate (37). Thus, the results obtained from both types of mice support the notion that excessive flux through the hexosamine biosynthetic pathway mediates adverse regulatory and metabolic effects of hyperglycemia.



ROLE OF INSULIN-MEDIATED SIGNAL TRANSDUCTION The pathway by which insulin enhances glucose uptake and utilization begins with binding of the hormone to specific tyrosine kinase receptors on the cell surface of insulin target tissues (70, 119). Binding of insulin causes activation and autophosphorylation of the receptor, which in turn leads to tyrosine phosphorylation of a variety of intracellular substrates, including the insulin receptor substrate proteins (IRS-1 and -2). Then phosphorylations cause a cascade of intracellular phosphorylation-dephosphorylation reactions (70, 119). Two independent groups (1, 46) have studied the role of the insulin receptor (IR) in glucose metabolism in mice lacking the IR gene. In mice containing genetic disruptions of both alleles of IR (IR-null mice), intrauterine development, growth, and metabolism are normal, indicating that IR is not needed for these processes. However, these mice rapidly develop severe diabetic ketoacidosis and hypertriglyceridemia, despite hyperinsulinemia, and die within 7 days of birth (1, 46). Treatment of IR-null mice with insulin-like growth factor-1 (IGF-1) caused a marked decrease in plasma glucose levels because of increased peripheral glucose uptake and inhibition of hepatic glucose production (14). These results indicate that IGF-1 can mimic insulin effects on glucose metabolism through its own receptor. However, plasma-free fatty acids were unaffected, and despite decreased glucose levels, IR-null mice treated with IGF-1 also died within 7 days of birth (14). Mice with one intact copy of an IR gene, a 50% reduction in IR protein, had normal glucose tolerance and compensated insulin resistance (1, 46). This normal phenotype is also found in transgenic animals with muscle-specific overexpression of a dominant negative form of kinase-deficient IR (Ala1134 $\longrightarrow$ Thr) (10). In these animals, IR kinase activity was decreased and insulin sensitivity slightly decreased, but glucose tolerance was not impaired. These results indicate that both types of mice compensate effectively for a partial decrease in active IR.

IRS-1 is the major cytoplasmic protein substrate for activated IR. Mutations in the IRS-1 gene have been described in 10–20% of type 2 diabetic patients in a variety of populations (2, 9, 13, 51, 66). Two independent groups (3, 101) have confirmed the critical role of IRS-1 in glucose metabolism by generating knockout mice. Mice with one intact copy of the IRS-1 gene show normal glucose disposal during insulin and IGF-1 tolerance tests. In contrast, mice with genetic disruption of both alleles of IRS-1 (IRS-1-null mice) exhibited both insulin and IGF-1 resistance, marked hyperinsulinemia, impaired glucose tolerance, a 40% reduction in embryonic and postnatal growth, and a 50% reduction in glucose transport in isolated adipocytes in vitro (3, 101). However, despite marked insulin resistance, because of significant residual insulin action through a second insulin receptor substrate, IRS-2, these mice were not overtly diabetic (78). The insulin resistance of IRS-1-null mice is localized mainly in muscle, as the liver, but not the muscles, of these mice contains an amount of phosphorylated IRS-2 similar to that for IRS-1 in normal mice (120). Although it indicates the key role of IRS-1 in insulin signaling, this model demonstrates the importance of alternative signal pathways for insulin action.

To explore the role of combined receptor and postreceptor defects, mice with only one intact copy of both IR and IRS-1 have been generated (6). These mice exhibit the expected reduction in expression in these two proteins, but there is synergism at the level of insulin resistance with dramatic hyperinsulinemia and $\beta$-cell hyperplasia. Interestingly, 40% of these mice developed overt diabetes at 4–6 months of age. Similarly, mice with only one intact copy of both IR and $\beta$-cell GK have been generated (102). These mice developed overt diabetes when older. At 10 months of age they had fasting hyperglycemia and hyperinsulinemia and showed impaired glucose-stimulated insulin secretion and $\beta$-cell hyperplasia. These double heterozygous mutant mice have become novel polygenic models of type 2 diabetes mellitus in which diabetes arises in an age-dependent manner from the interaction between two genetically determined, subclinical defects in the insulin signaling pathway.

### *Adipose Tissue*

Although adipose tissue is responsive to insulin, it is responsible for the disposal of less than 5% of an ingested or infused glucose load (16). However, marked alteration of GLUT4 gene expression is seen in adipocytes, but not in muscles, of humans and rodents in such insulin-resistant states as high-fat feeding and diabetes (15, 28, 50, 79). To study the role of nutrient partitioning in the development of obesity, transgenic animals overexpressing GLUT4 selectively in adipose tissue have been produced by using the aP2 promoter (96). These mice have enhanced glucose tolerance, improved insulin sensitivity, and increased basal and insulin-stimulated glucose transport in vitro (96). However,

total body lipid increases two- to threefold in these transgenic mice, resulting solely from adipocyte hyperplasia. The overexpression of GLUT4 in adipose tissue does not reverse insulin resistance induced by high-fat feeding (31). The increased glucose uptake results in a general activation of glucose metabolism in all major pathways, although de novo fatty acid synthesis is the most strongly activated (107). Even when expression of GLUT4 is 20-fold higher than that in adipocytes of control animals, the transporter is still specifically targeted to the same unique structurally defined, insulin-sensitive vesicles observed in normal cells (106).

Insulin binding to its receptor causes an increase in the amount of GTP bound to the GTP-binding protein Ras (7, 77, 83). However, the role of Ras signaling pathways in the regulation of insulin-stimulated glucose transport remains controversial. To address this question in vivo, transgenic mice with adipose-specific overexpression of Ha-*ras* have been developed (39). In isolated adipocytes from these mice, transport under basal conditions at submaximally effective insulin levels was increased; at maximally effective insulin level glucose transport was not stimulated. These adipocytes also had twofold more cell-surface GLUT4 both in the absence of insulin and at half-maximal insulin concentration. The consequences of increased expression of wild-type Ras in vivo included increased glucose tolerance, decreased plasma insulin levels, increased insulin sensitivity, and decreased adipose mass (39).



## *Liver*

The liver has a central role in glucose homeostasis. When plasma glucose is high, the liver takes it up and stores it as glycogen (80). During starvation, hepatic glucose derived from glycogenolysis and gluconeogenesis is released to blood.

ROLE OF HEPATIC GLUCOSE UPTAKE Glucose transport and phosphorylation are the first steps in glucose utilization. In the liver, glucose is mainly transported by GLUT2 (76). In nondiabetic animals, the transport capacity of GLUT2 is high while the capacity of phosphorylation is relatively low. Phosphorylation probably plays a major regulatory role. Thus, glucose phosphorylation by GK seems to be key in the regulation of glucose utilization by hepatocytes. Regulation of hepatic GK activity is mainly due to changes in the transcription of its gene. Insulin increases, whereas glucagon inhibits, hepatic GK gene transcription. During diabetes, GK gene expression and GK activity are very low, and thus the liver is unable to metabolize blood glucose (43, 86). The expression of GK in rat hepatoma cell lines that lack endogenous GK causes increased glucose uptake, glycolysis, and glycogen synthesis (112). The promoter of genes coding for some of the enzymes of glycolysis and lipogenesis

contains glucose/carbohydrate regulatory elements (30, 105). The insulin effect on glucose-dependent activation of the L-pyruvate kinase gene promoter in hepatocytes can be mimicked by overexpression of GK expression vector (18).

To determine the contribution of GK to hepatic control of whole-body glucose homeostasis, we have created transgenic mice that overexpress GK. A 450-bp fragment of the phosphoenolpyruvate carboxykinase (PEPCK) promoter was used to direct the expression of GK to the liver of transgenic mice (24, 25). In the liver of transgenic mice, this fragment of the PEPCK promoter regulates expression of chimeric genes in a manner similar to that for the endogenous PEPCK gene, (64). Glucagon and glucocorticoids increase transcription from the PEPCK promoter, whereas insulin has the opposite effect (35). These mice do not overexpress the transgene in the $\beta$-cells. The activation of hepatic GK led to an increase in the intracellular concentration of glucose 6-phosphate and glycogen, and to an increase in L-pyruvate kinase activity, indicating that in vivo the activation of GK can control the induction of glycolysis and glycogen synthesis (25). These changes in liver glucose metabolism led to a marked reduction in blood glucose and insulin concentrations. Transgenic mice also had lower levels of blood glucose after an intraperitoneal glucose tolerance test; this suggests that GK overexpression resulted in an increase in blood glucose disposal by the liver (25). Similar results have been obtained using transgenic mice expressing the human GK gene in the liver (36), or in mice overexpressing one or more extra copies of the entire mouse GK gene locus (73, 74). In contrast, heterozygous mice for a targeted mutation in the GK gene (4, 32), with only one normal GK allele, show diminished liver GK activity and increased fasting glucose, consistent with an increase in hepatic glucose production in these mice. In hyperglycemic clamp studies, these mice have decreased glucose tolerance and abnormal liver glucose metabolism (4). In these heterozygous mice, GK is expressed in both liver and $\beta$-cells. Fed and fasting glucose levels are not distinguishable from those of wild-type mice (32). These studies suggest that both liver and islets are involved in MODY2 disease.

In transgenic mice that express a PEPCK/GK chimeric gene, increased expression of GK in the liver of diabetic mice prevented metabolic alterations (24). In contrast to control mice, transgenic mice treated with streptozotocin had high levels of both glucokinase mRNA and enzyme activity in the liver; this led to an increase in intracellular levels of glucose 6-phosphate and glycogen. Furthermore, glycolysis was induced while gluconeogenesis and ketogenesis were blocked in the livers of diabetic mice expressing GK. This was associated with normal levels of blood glucose, ketone bodies, triglycerides, and free fatty acids, even in the absence of insulin (24). These results suggest that engineering a diabetic liver to express glucokinase might counteract diabetic hyperglycemia.

c-*myc* is a member of a gene family encoding nuclear phosphoproteins that act as transcription factors; these factors recognize "E-box" motifs with the central consensus sequence CACGTG (48, 55, 60). This sequence is also contained in the glucose/carbohydrate regulatory elements located in the promoters of genes coding for some of the liver enzymes of glycolysis and lipogenesis (30, 105). An increase in c-Myc protein in the liver of transgenic animals stimulates hepatic glycolysis by increasing expression of genes coding for enzymes that control the glycolytic pathway and increases glycogen synthesis in the absence of cell proliferation and transformation (115). These changes in liver glucose metabolism caused a reduction of blood glucose and insulin concentrations. Thus, c-Myc is involved in the control of liver carbohydrate metabolism in vivo. After an intraperitoneal glucose tolerance test, c-Myc mice had lower levels of blood glucose than did control mice, which indicates that the overexpression of c-Myc stimulated blood glucose disposal by the liver. Furthermore, the overexpression of c-Myc counteracted the development of the diabetic state by increasing expression of GK, and thus hepatic glucose uptake and utilization, and blocking the activation of gluconeogenesis and ketogenesis (92). Thus, c-Myc may counteract diabetic alterations after the destruction of insulin-producing cells. Results of experiments using all these transgenic models reinforce the key role of the liver in maintaining normoglycemia and suggest that engineering the liver to increase glucose uptake and utilization may be a useful approach to the treatment of diabetes mellitus.

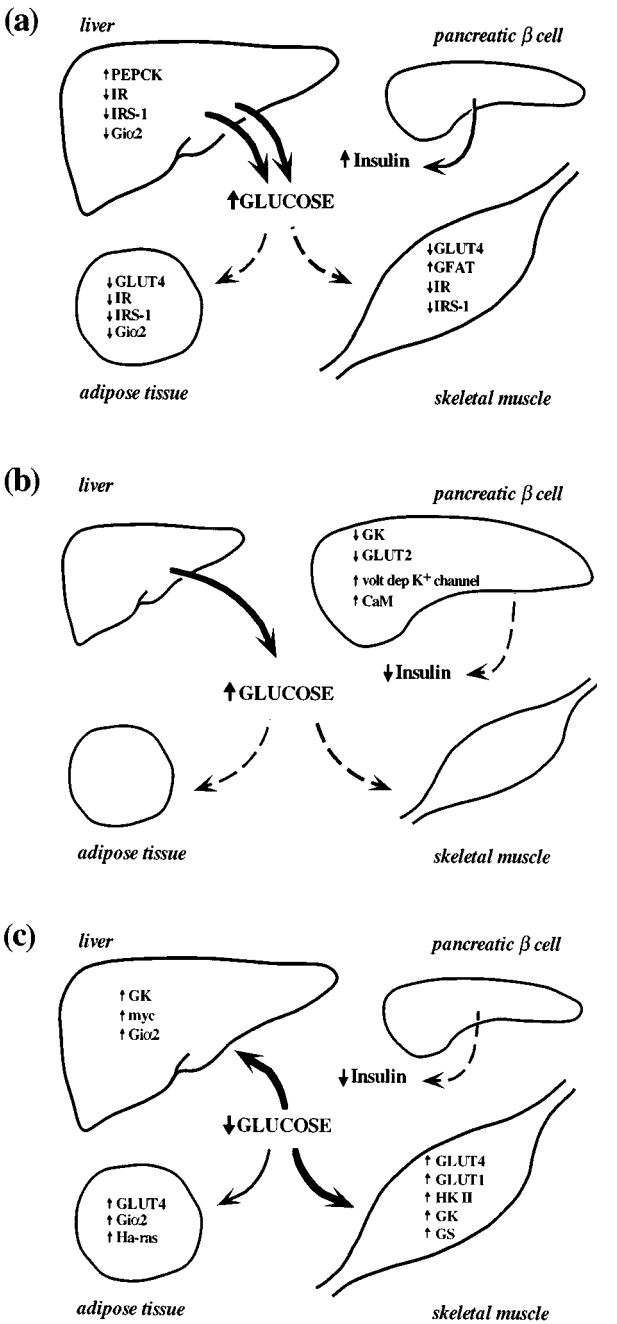
ROLE OF HEPATIC GLUCOSE OUTPUT In the basal postabsorptive state, the liver produces glucose, thus preventing hypoglycemia (16). This glucose efflux is critical to the needs of the brain and other neural tissues. In type 2 diabetics with overt fasting hyperglycemia, excessive hepatic glucose output is a major factor responsible for the elevated plasma glucose concentration. An increase in hepatic gluconeogenesis is believed to be responsible for the fasting hyperglycemia detected in these patients (16). The cytosolic form of the PEPCK is a regulatory enzyme of gluconeogenesis (35). PEPCK activity is regulated by the transcription rate of its gene. To determine whether the overexpression of PEPCK leads to a rise in the rate of gluconeogenesis and, if so, to assess the contribution of the liver to the development of type 2 diabetes, we produced lines of transgenic mice expressing a PEPCK minigene under the control of 500 bp of its own promoter (114). Overexpression of the PEPCK gene led to an increase in glucose production from pyruvate in hepatocytes in primary culture. Transgenic mice were hyperglycemic and had a higher serum insulin concentration. When intraperitoneal glucose tolerance tests were performed, blood glucose levels were higher than those detected in normal mice. This shows that primary alterations in the rate of hepatic glucose production

may induce insulin resistance (114). Similarly, transgenic rats overexpressing PEPCK in the liver under control of the metallothionein promoter were hyperinsulinemic and showed impaired glucose tolerance (93). Furthermore, older mice showed impaired glucose- and amino acid–stimulated insulin secretion in pancreatic islets and alterations in glucose transport, phosphorylation, and utilization (A Pujol, A Arbos, A Valera, F Bosch, unpublished results). Thus, primary defects in hepatic glucose production cause alterations in pancreatic $\beta$-cells. This indicates a key role for the liver in control of whole-body glucose homeostasis.

Older PEPCK transgenic mice exhibited increased adiposity, particularly in abdominal depots; this was more evident in females. An increase in body weight was also observed in the PEPCK transgenic rats (93). Therefore, these animals develop a clinical phenotype that closely resembles type 2 diabetes in humans. Another model developed to study the effects of chronic hyperglycemia has been obtained by expressing a soluble secreted derivative of the ectodomain of the human insulin receptor in transgenic mice (94). These mice accumulated high-affinity insulin-binding protein in the plasma, which binds insulin in the circulation and therefore limits its availability to tissues. These mice develop fasting hyperglycemia concomitant with increased hepatic glucose production and hyperinsulinemia, indicating that they were insulin resistant.

ROLE OF ALTERATIONS IN INSULIN ACTION After a mixed meal or a glucose load has been ingested, insulin is released to the portal vein and carried to the liver, where it binds to specific receptors on the hepatocyte and suppresses hepatic glucose output and induces glucose uptake (16). In addition to alterations in muscle insulin signaling, heterozygous mice carrying targeted disruption of the insulin receptor (*IR*) gene show a reduction (about 60%) in *IR* expression and a 40% decrease in IRS-2 phosphorylation in the liver (6). Similarly, inactivation of one allele of the *IRS-1* gene results in a 60% reduction in liver IRS-1 expression, which leads to a 20% increase in IRS-2 phosphorylation. All these animals are normoglycemic and have normal glucose and insulin-tolerance tests (6). Mice with combined heterozygous deficiencies in the *IR* and *IRS-1* genes show a 70% reduction of insulin-stimulated tyrosine phosphorylation of IRS-1 and 40% impairment of IRS-2 phosphorylation in the liver (6). These mice are markedly insulin resistant; alterations in the liver insulin signaling pathway contribute to this phenotype.

G proteins are key elements in transmembrane signaling and have been implicated as regulators of complex biological processes such as differentiation and development. The G protein $G_{i\alpha 2}$ mediates inhibition of adenylate cyclase (56, 65, 95). Moxham et al (67) created transgenic mice expressing an antisense RNA for the G protein $G_{i\alpha 2}$; the transgene is under control of a 2000-bp



fragment of the PEPCK promoter. Experiments with these mice demonstrated that $G_{i\alpha 2}$ was essential for the development of liver and fat (67). $G_{i\alpha 2}$ deficiency in liver and adipose tissue of transgenic mice led to hyperinsulinemia, impaired glucose tolerance, and resistance to insulin in vivo (67, 68). In addition, $G_{i\alpha 2}$ deficiency increased protein-tyrosine phosphatase activity and attenuates insulin-stimulated tyrosine phosphorylation of IRS-1 in vivo (68). In contrast, transgenic mice expressing a constitutively active mutant form (Q205L) of $G_{i\alpha 2}$ displayed enhanced glucose tolerance (12), mimicking insulin action in vivo. Therefore, these animals indicate that $G_{i\alpha 2}$ functions in vivo not only as a mediator of inhibitory adenylyl cyclase, but also as a critical, positive regulator of insulin action, providing a link between the G-protein and tyrosine kinase signaling cascades.



## CONCLUSIONS

After analyzing all these results, obtained from a wide range of models, what first emerges is the notion that changes in the expression of key genes of glucose metabolism in specific tissues can cause compensatory responses in the whole body. The engineered animal allows us to determine the contribution of the other tissues to the new, altered phenotype in great detail. Second, the relative role of a specific gene can be dissected from the whole genetic background, and therefore its contribution to a specific function can be reevaluated. Finally, these transgenic animals also provide information on the role specific genes may play in the complex pathogenesis of type 2 diabetes.

With regard to glucose homeostasis, the animals can be grouped in three major phenotypes. (*a*) One phenotype is observed in those animals in which a genetic alteration in peripheral tissues or in the liver leads to hyperglycemia as well as to compensatory hyperinsulinemia (Figure 1*a*). Thus, a decrease in insulin signaling in skeletal muscle, adipose tissue, or liver, as noted in IR and IRS-1 knockout mice (1, 3, 6, 46, 101) or in the $G_{i\alpha 2}$ antisense transgenic

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*Figure 1* Schematic representation of interorgan glucose transfer in animals expressing several different transgenes. (*a*) Transgenic mice in which a genetic alteration in peripheral tissues or in the liver leads to hyperglycemia as well as compensatory hyperinsulinemia. (*b*) Transgenic animals in which a genetic alteration in pancreatic $\beta$-cells causes a decrease in insulin secretion, resulting in hyperglycemia secondary to both the decrease in peripheral glucose uptake and the increase in hepatic glucose output. (*c*) Transgenic mice in which a genetic alteration in the liver or peripheral tissues reduces glycemia as the result of increased glucose uptake. GFAT, glutamine:fructose-6-phosphate amidotransferase; $G_{i\alpha 2}$, G protein; GK, glucokinase; GLUT, glucose transporter; GS, glycogen synthase; IR, insulin receptor; IRS-1, insulin receptor substrate 1; PEPCK, phosphoenolpyruvate carboxykinase; CaM, calmodulin; HKII, hexokinase II.

mice (67, 68), causes a decrease in glucose uptake by peripheral tissues and an increase in hepatic glucose production. A decrease in GLUT4 in muscle and adipose tissue of heterozygous GLUT4 knockout mice (100) or an excessive glucose flux through the hexosamine biosynthetic pathway in skeletal muscle [in transgenic mice overexpressing GLUT1 (8, 33) or GFAT (37)] also results in insulin resistance and in compensatory hyperinsulinemia. In addition, chronic hyperglycemia—caused by increased hepatic gluconeogenesis in transgenic mice overexpressing the PEPCK gene (93, 114) or by reduced glucose uptake by peripheral tissues and increased hepatic glucose production in mice expressing a secreted ectodomain of the insulin receptor (94)—leads to hyperinsulinemia and insulin resistance. In all these models, primary alterations in glucose metabolism in peripheral tissues and in the liver may lead to insulin resistance and to compensatory increased insulin secretion by the pancreatic $\beta$-cells. The results suggest that type 2 diabetes may be initiated in these tissues. (*b*) A second phenotype is observed in animals in which a genetic alteration in pancreatic $\beta$-cells causes a decrease in insulin secretion, resulting in hyperglycemia secondary to both the decrease in peripheral glucose uptake and the increase in hepatic glucose output (Figure 1*b*). Decreased expression of the glucose phosphorylating enzyme GK (4, 32, 103) or of the glucose transporter GLUT2 (116) alters the glucose signal that regulates insulin secretion, leading to decreased insulin release. Similarly, an increase in the expression of either CaM (21, 22, 45, 91) or the voltage-dependent $K^+$ channel (81) inhibits insulin secretion. Results with these animals show that primary defects in $\beta$-cell function may also initiate a process that leads to type 2 diabetes. (*c*) The third phenotype is observed in those animals in which a genetic alteration in the liver or peripheral tissues reduces glycemia as the result of increased glucose uptake (Figure 1*c*). The lower blood glucose levels may cause a compensatory decrease in insulin secretion by the $\beta$-cells. Thus, engineering the skeletal muscle to overexpress GLUT4 (29, 34, 40, 41, 54, 90, 108, 109), GLUT1 (33, 61, 89), HKII (11), GK, or GS (58) increases uptake and utilization of glucose. Similarly, overexpression of GLUT4 (31), $G_{i\alpha 2}$ (12), or Ha-*ras* (39) in adipose tissue increases glucose uptake. Furthermore, an increase in the expression of the GK (24, 25, 36, 73, 74), *c-myc* (92, 115), and $G_{i\alpha 2}$ (12) genes in the liver results in higher glucose uptake and utilization. Therefore, the results reported in all these transgenic models reinforce the key role of both the liver and skeletal muscle in the control of glucose homeostasis and suggest that engineering these tissues to take up glucose under diabetic conditions might prevent hyperglycemia.

Although much has been learned from these transgenic lines, new information is expected to accrue with the development of new models, in which the effects of alteration of the expression of other genes will be determined. Furthermore,

the development of mice with two or more genetic alterations in the same or in different tissues, after breeding different transgenic lines, may also significantly contribute to our understanding of metabolic regulation. Moreover, the identification of new putative key genes as well as new advances in recombinant DNA techniques, gene transfer approaches, and embryo manipulation will provide new tools to be used in the analysis of metabolic interactions in the whole animal. In this regard, the development of transgenic mice with tissue-specific and inducible gene knockouts [e.g. by using the Cre recombinase/*lox*P sites system (62, 87) together with inducible promoters like the tetracycline system (98)] may allow the design of more sophisticated gene alterations to address key questions related to the control of glucose homeostasis and the development of type 2 diabetes. Thus, transgenic analysis of metabolic regulation is expected to become a more active field of research in the future, and major advances in our understanding of the control of whole-body glucose homeostasis may be anticipated.



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