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REVIEW

Knockout mouse models of insulin signaling: Relevance past and future

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Abstract

Insulin resistance is a hallmark of type 2 diabetes. In an effort to understand and treat this condition, researchers have used genetic manipulation of mice to uncover insulin signaling pathways and determine the effects of their perturbation. After decades of research, much has been learned, but the pathophysiology of insulin resistance in human diabetes remains controversial, and treating insulin resistance remains a challenge. This review will discuss limitations of mouse models lacking select insulin signaling molecule genes. In the most influential mouse models, glucose metabolism differs from that of humans at the cellular, organ, and whole-organism levels, and these differences limit the relevance and benefit of the mouse models both in terms of mechanistic investigations and therapeutic development. These differences are due partly to immutable differences in mouse and human biology, and partly to the failure of genetic modifications to produce an accurate model of human diabetes. Several factors often limit the mechanistic insights gained from experimental mice to the particular species and strain, including: developmental effects, unexpected metabolic adjustments, genetic background effects, and technical issues. We conclude that the limitations and weaknesses of genetically modified mouse models of insulin resistance underscore the need for redirection of research efforts toward methods that are more directly relevant to human physiology.

Key words: Insulin resistance; Mice; Knockout; Disease models, Animal; Glucose/metabolism; Signal transduction

Core tip: Insulin resistance is central to the pathophysiology of type 2 diabetes. The molecular origins of insulin resistance have been investigated using genetically modified mice. Much has been learned from this work, but new treatments for insulin resistance have not been forthcoming. Knockout mouse models of diabetes are limited by several factors including species differences in glucose metabolism. These are due partly to species differences in physiology, and partly to the failure of genetic modifications to produce an accurate model. Advancement may require a redirection of research efforts toward methods that are more directly relevant to human physiology.

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INTRODUCTION

Type 2 diabetes is a growing public health problem affecting approximately 26 million adults in the United States, with pre-diabetes affecting an additional 79 million[1]. The natural history of type 2 diabetes starts with insulin resistance, which develops over time and often

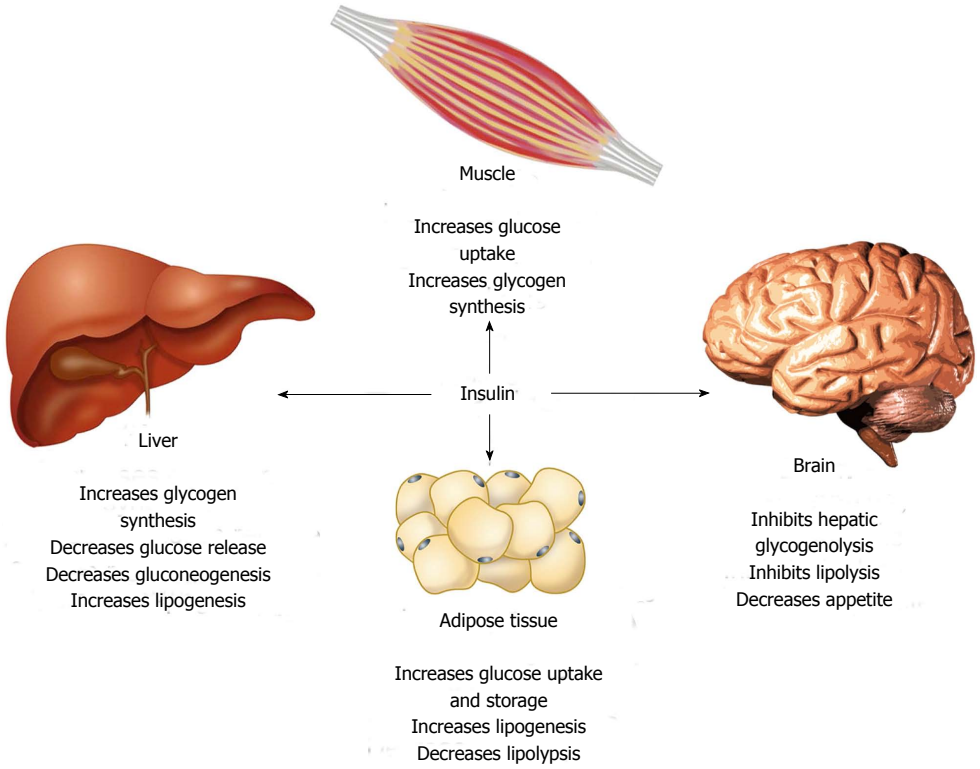

Figure 1 Insulin actions in main insulin-sensitive tissues. Insulin has different actions in each of the main insulin-sensitive tissues. In muscle, insulin promotes glucose uptake and glycogen synthesis. In liver, insulin promotes glycogen synthesis and lipogenesis and reduces gluconeogenesis and the release of stored glucose. In adipose tissue, insulin increases glucose uptake and lipogenesis and decreases lipolysis. In the brain, insulin Inhibits hepatic glycogenolysis and lipolysis and decreases appetite.

precedes a diagnosis by many years. The pancreas compensates for insulin resistance by increasing insulin secretion, often leading to hyperinsulinemia. For many insulin-resistant patients, the pancreas is unable to sustain a high level of insulin secretion. As the pancreas fails to meet the demand for insulin, plasma glucose rises. Patients are then at risk of morbidity and mortality associated with complications such as neuropathy, retinopathy, nephropathy, and increased risk of cardiovascular disease. Overall, type 2 diabetes decreases life expectancy at age 50 or older by about 8 years[2]. Aside from diabetes and the metabolic syndrome, insulin resistance is also associated with polycystic ovarian syndrome and other problems. Understanding the cellular and molecular causes of insulin resistance is an area of active research because of the need to discover new therapies to help patients.

Animal models are often used to investigate mechanisms of insulin resistance and develop therapeutic agents. In the field of type 1 diabetes, serious limitations of animal models have become apparent[3]; we therefore sought to assess the utility of select mouse models used in type 2 diabetes research, specifically insulin signaling and resistance. We begin with a brief summary of insulin signaling, followed by a closer look at general limitations of mouse models and specific limitations of knockouts lacking select insulin signaling molecule genes.

Insulin resistance is defined as the failure of cells to respond normally to insulin, and most importantly, to insulin's glucose-lowering effects. It can be measured by a number of approaches, including the Homeostatic Model Assessment of Insulin Resistance, which is based on fasting glucose and insulin levels, and the gold standard approach, a hyperinsulinemic-euglycemic clamp test[4]. On a cellular level, insulin resistance manifests differently in different tissues (Figure 1). Insulin-resistant muscle cells fail to uptake glucose and other nutrients in response to insulin, whereas in adipose tissue, insulin resistance leads to greater hydrolysis of stored triglycerides in addition to decreased nutrient uptake. In the liver, insulin promotes glycogen synthesis and prevents the release of stored glucose, thereby raising blood glucose levels. In the brain, insulin decreases appetite and hepatic glucose production[5].

The molecular mechanisms of insulin resistance in type 2 diabetes have not been fully characterized, although many important biochemical, metabolic, and genetic features have been identified. Accumulated findings have highlighted several pathways to insulin resistance, including lipid accumulation, oxidative stress, and inflammation[6]. An important common feature of these mechanisms is the activation of stress-sensitive kinases including protein kinase C ζ (PKCζ) that cause a dampening of insulin signaling[6,7].

Insulin is involved in a number of cellular processes apart from nutrient metabolism, including protein synthesis, mitochondrial biogenesis, growth, autophagy, proliferation, differentiation, and migration[8-10]. As illustrated in Figure 2, the binding of insulin to its receptor triggers a cascade of cellular events that leads to nutrient uptake

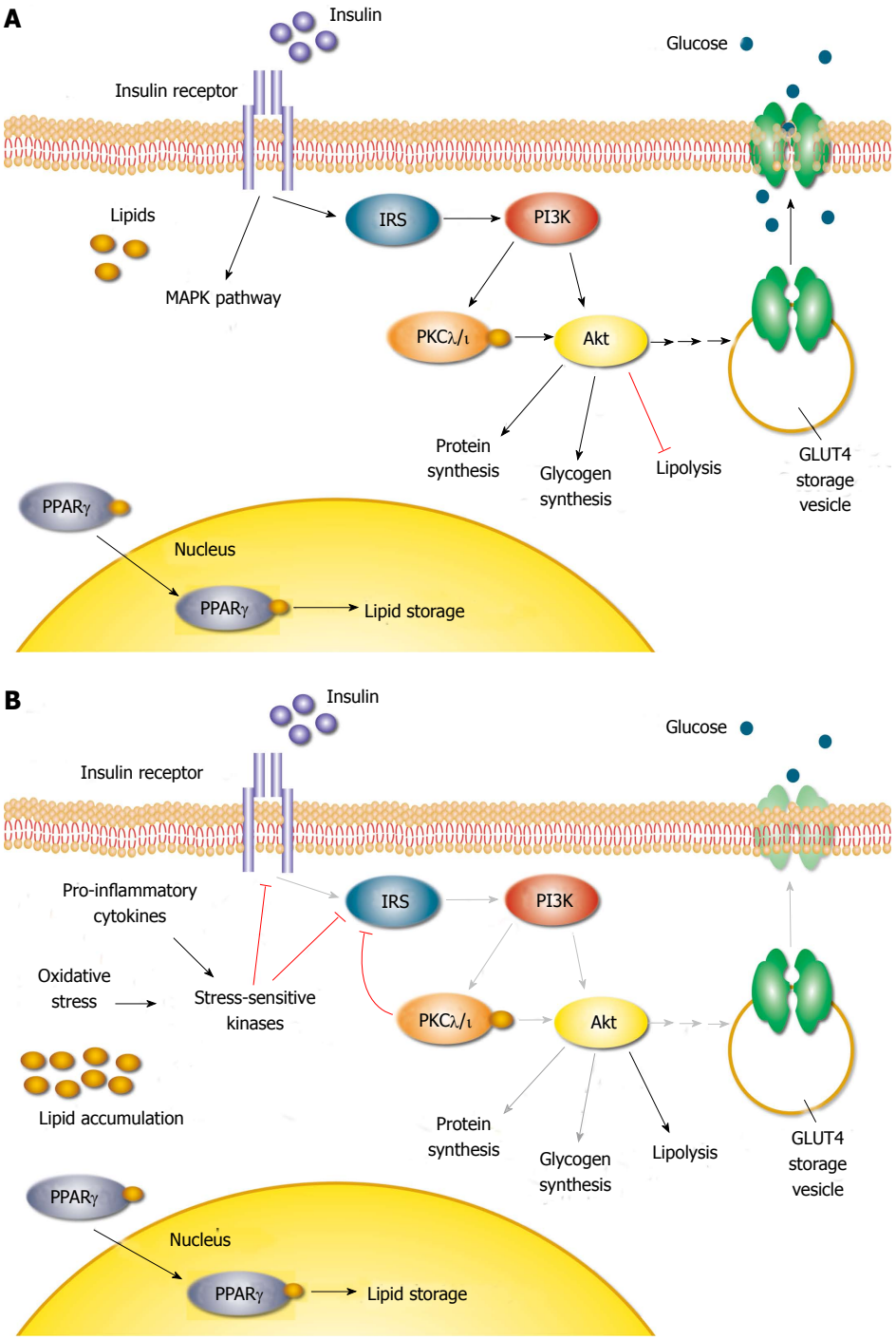

Figure 2 Insulin signaling in health and disease. Insulin signaling in health and disease. A: The binding of insulin to its receptor triggers a cascade of cellular events that lead to nutrient uptake and activation of various cellular programs. Insulin receptor substrate (IRS) activates phosphoinositide 3-kinase (PI3K) which produces a metabolite that activates protein kinase B (AKT) and protein kinase C λ/ι (PKCλ/ι). PKCλ/ι, which also depends on lipids for activation, can inhibit insulin signaling by a feedback mechanism. The nuclear receptor peroxisome proliferator-activated receptor gamma (PPARγ), is important in lipid metabolism, and is the target of insulin sensitizing thiazolidinedione drugs. PPARγ becomes activated upon binding of lipids and promotes expression of genes involved in fat storage; B: Under insulin-resistant conditions, accumulation of lipids, oxidative stress, and pro-inflammatory cytokines cause activation of stress-sensitive kinases such as protein kinase C θ (PKCθ), inhibitor of nuclear factor kappa-B kinase subunit β (IKKβ) and c-Jun N-terminal kinase 1 (JNK1), which inhibit insulin signaling.

and activation of these various cellular programs[8]. Under insulin-sensitive conditions, as shown in Figure 2A, insulin receptor substrate (IRS) activates phosphoinositide 3-kinase (PI3K), which produces a metabolite that activates protein kinase B (AKT) and PKCλ/ι. PKC λ/ι, which also depends on lipids for activation, can inhibit insulin signaling by a feedback mechanism. The nuclear receptor peroxisome proliferator-activated receptor gamma, or peroxisome proliferator-activated receptor γ (PPARγ), is important in lipid metabolism, and is the target of insulin sensitizing thiazolidinedione drugs (TZDs). PPARγ becomes activated upon binding of lipids and promotes expression of genes involved in fat storage. As shown in Figure 2B, under insulin-resistant conditions,

accumulation of lipids, oxidative stress, and pro-inflammatory cytokines cause activation of stress-sensitive kinases such as PKCθ, inhibitor of nuclear factor kappa-B kinase subunit β (IKK-β) and c-Jun N-terminal kinase 1 (JNK1), which inhibit insulin signaling[6,7].

Evidence for insulin signaling pathways and mechanisms of insulin resistance comes from human and animal cell and tissue studies, clinical studies, and whole animal experiments. While data from various models have been useful in formulating and testing hypotheses, some approaches are more promising than others. Rodent models have been used in the study of type 2 diabetes and insulin resistance for decades. Conditions relevant to the study of insulin resistance and diabetes are induced in rodents using several approaches, including genetic, pharmacological, surgical, and dietary inductions. A number of these approaches and models have been reviewed elsewhere[11-14]. Many researchers favor targeted genetic manipulation because it allows specific and complete or near-complete removal of target gene function in a whole organism or specific tissues[15]. In combination with pharmacological, cell-based and molecular studies, these knockout mouse studies have mapped the insulin signaling pathway in mice to a high level of detail. Other authors have described how pathway connections tested in humans have been shown to be conserved (*i.e.*,[16]). Many would argue that knockout mouse studies have been especially important in defining the function of genes for which no pharmacological or other molecular-based functional ablation is available[17]. In this respect, the genetic approach has become a central component of preclinical research in diabetes and other fields.

Despite this progress in our understanding of insulin action, the causative molecular basis for acquired human insulin resistance remains unclear and controversial. Furthermore, improved understanding of rodent cell signaling has not translated into improved human therapeutics. To wit, it has been almost 20 years since the first insulin signaling knockout mouse studies were published[18,19], but no new drugs targeting the insulin signaling phosphorylation cascade have emerged to treat insulin resistance in type 2 diabetes[9]. While much of this research is conducted for the purpose of hypothesis testing rather than drug development *per se*, the identification of drug targets is often a primary or secondary goal[20]. In light of this, we discuss the limitations of research on insulin resistance using knockout mice of select proteins important in the insulin signaling cascade (Figure 2). The following sections will focus mainly on peripheral insulin resistance and extrapancreatic insulin-sensitive tissues, since many therapeutic and research efforts are in this area. We first address physiological, cellular, and molecular differences in glucose metabolism between mice and humans that limit translatability. We then review select knockout mouse models of insulin signaling dysfunction, identifying cases with contradictory or untranslatable results. Finally, we briefly discuss the limitations of genetic manipulations of these targets in mice in regard to the search for safe and effective drugs for type 2 diabetes.

GLUCOSE DISPOSAL IN MICE AND HUMANS

A central aspect of glucose homeostasis is glucose disposal, meaning the facilitated transport of glucose from blood into storage tissues and organs. Insulin resistance in humans with type 2 diabetes involves defects in glucose sensing and disposal in a number of tissues, but the most significant effects on glucose homeostasis result from insulin resistance in the major glucose-disposing tissues: skeletal muscle, liver and adipose tissue.

Glucose disposal and glycogen storage patterns differ in mice and humans. In healthy humans, about one-third of glucose is taken up by the liver[21]. Estimates of skeletal muscle glucose uptake vary widely, in part because they are often based on indirect measurements and assumptions regarding muscle mass and blood flow. One report that measured muscle glucose more directly using nuclear magnetic resonance demonstrated muscle absorbing 64%-91% of infused glucose in a single male volunteer[22]. A follow-up study of 11 subjects reported muscle glucose uptake of 90% in normal subjects and 67% in diabetic subjects[23]. In a separate study of 10 healthy volunteers, muscle accounted for 38.3% of systemic glucose disposal, based on data from blood sampled from a forearm vein[24]. Overall, the data show greater glucose uptake in skeletal muscle than liver in humans. Genetic evidence underscores the importance of skeletal muscle to whole-body glucose tolerance in humans. Polymorphisms in the gene for the primary glucose transporter in muscle, glucose transporter isoform 4 (GLUT4), have been linked to type 2 diabetes and insulin resistance[25]. Overall, defects in skeletal muscle glucose disposal are a major component of insulin resistance in humans[26].

By contrast, the liver is much more important for glucose disposal in mice. Interfering with glucose uptake in mouse liver causes whole-body insulin resistance and glucose intolerance, but similar manipulations in muscle usually do not. The muscle-specific insulin receptor knockout mouse has normal glucose tolerance, insulin sensitivity, and glucose and insulin levels, with only mild dyslipidemia[27]. Muscle-specific deletion of IRS1 and IRS2 also does not produce a diabetic phenotype, nor does a whole-body knockout of the major muscle glucose transporter, GLUT4[28,29]. One exception to this pattern may be a muscle-specific GLUT4 knockout strain that developed a diabetic phenotype in one study[30], a result that has not been replicated by others[31,32]. In contrast to the above strains deficient in muscle insulin signaling, a liver-specific insulin receptor knockout mouse strain was insulin resistant and severely hyperinsulinemic, and developed hyperglycemia and glucose intolerance at an early age (2 mo)[33]. Liver-specific deletion of IRS1 and IRS2 also cause insulin resistance under certain conditions[34]. Mice with a deletion of the primary glucose transporter in the liver, GLUT2, are hyperglycemic and die at 2-3 wk of age[35].

Glycogen storage is a major destination for glucose in mammals. In mice, approximately 8 times more glyco-

gen is stored in the liver than skeletal muscle[36], but the reverse is true in humans, where 3-8 times more glycogen is found in skeletal muscle[37]. These physiological differences in glucose disposal and storage have implications for modeling insulin resistance, since muscle and liver have different roles and different metabolic and signaling pathways.

There are two important differences in glucose transport between liver, the primary glucose disposal organ in mice, and skeletal muscle, the primary glucose disposal organ in humans. First, skeletal muscle cells have multiple pathways for glucose transport. Contraction-stimulated glucose transport in skeletal muscle is insulin-independent, mediated through 5' adenosine monophosphate activated protein kinase-mediated signaling mechanisms[38]. In contrast, liver has no such activity-stimulated transport method. Second, the transporters involved in glucose uptake are different in the two tissues. In liver, the low-affinity GLUT2 is present at high levels on cell membranes independent of insulin or other signaling[39], and glucose transport rates vary with the extracellular concentration of glucose[40]. In contrast, in skeletal muscle cells, the high-affinity glucose transporter GLUT4 is translocated from internal vesicles to the plasma membrane in response to glucose uptake signals[41]. In human skeletal muscle cells, this transport is facilitated by clathrin isoform CHC22, which is not present in the mouse[42]. The rate-limiting step in glucose metabolism in liver is phosphorylation, while in skeletal muscle it is transport through GLUT4[43]. The divergent features of cells in these organs, combined with the divergent physiology of rodents and humans, means that glucose disposal is affected very differently in the different species.

Because mice rely principally on the liver for glucose homeostasis, while humans rely on skeletal muscle where transport mechanisms and biochemical pathways differ, mice may not be expected to be analogous to type 2 diabetes patients in regards to mechanisms of glucose metabolism or its dysfunction.

Mice and humans have a number of other metabolic differences. The small size and fast metabolism of mice enables heart rates in the range of 350-550 beats per minute, while in humans, normal heart rate is about 70 beats per minute[44]. Mice are capable of the physiological state of torpor, a state of reduced metabolic rate, while humans are not[45]. Prolonged fasting in humans impairs insulin-stimulated glucose utilization, but causes enhancement in mice[46]. In regards to eating patterns, mice consume most of their food at night[45], and an overnight fast of 14-18 h, typical for laboratory experiments, induces a state akin to starvation[47]. In addition, circulating lipids have an inverted composition in mice, with high-density lipoprotein (HDL) being typically higher than low-density lipoprotein (LDL), while HDL is lower in humans[48]. The thermoneutrality point, that is, the temperature at which an organism expends minimal energy for temperature regulation, is higher in mice[49]. This last difference could be compensated for if mice were housed above room temperature, but that is not standard practice.

Finally, experiments investigating mouse metabolism present technical challenges. Insulin sensitivity is often measured using a hyperinsulinemic-euglycemic clamp test, which involves either implanted arterial catheters or repeated blood sampling. The results of this test are dependent on a number of experimental factors which are not standardized between laboratories, including fasting time, anesthesia use, and blood sampling site[46]. Fasting glucose, insulin, and lipid levels are often measured after 14-18 h overnight fasts, but this induces a catabolic state in mice, who normally eat mostly at night. Data shows that a 6 h fast is best to assess glucose tolerance in mice[50].

KNOCKOUT MODELS OF INSULIN SIGNALING

Mouse models of diabetes are often used to explore signaling pathways[13]. The following sections highlight cases relevant to insulin signaling dysfunction where similar or identical genetic manipulations produced disparate results. These cases are consistent with other results showing differences in insulin action, secretion, and responses to hypoglycemia in different inbred mouse strains[51]. Previous reviewers have also noted the strong effect of genetic background in knockout mouse experiments[52]. Other factors influencing disparate findings include compensatory metabolic adjustments and technical challenges associated with evaluating mouse metabolism. Later, we will focus on the challenges of translating mouse knockout results to humans.

INSULIN RECEPTOR AND INSULIN RECEPTOR SUBSTRATE

Binding of insulin to the insulin receptor is the first step in the insulin signaling pathway. Mice with complete deletion of the insulin receptor are about 10% underweight and suffer from chronic hyperglycemia[53,54]. They die within several days of birth due to diabetic ketoacidosis. In humans, donohue syndrome is a rare monogenic disease resulting from mutation of the insulin receptor. Individuals with this disease suffer from severe pre-natal and post-natal growth retardation, fasting hypoglycemia, and post-prandial hyperglycemia[55]. They generally die before adulthood. The difference between the glucose homeostasis in mice and humans with this mutation may be attributable to the fact that the human pancreas develops earlier in gestation, hence better enables the compensatory hyperinsulinemia[55].

The pancreatic beta-cell specific insulin receptor knockout mouse strain (called BIRKO) has impaired insulin response to glucose challenge and develops impaired glucose tolerance and high insulin levels[56]. In the initial description of this mutant strain, glucose levels and body weight were normal, however, a follow-up report from the same laboratory described consistent hyperglycemia and sporadic obesity[57]. In the same report, a muscle

Table 1 Knockout mouse reproducibility

Model	Ref.	Genetic background	Observed discrepancy
IRS1 knockout	Tamemoto *et al*[19]	C57BL/6 × CBA	Growth defect twice as severe in Araki 1994
	Araki *et al*[18]	C57BL/6	
IRS2 knockout	Withers *et al*[60]	C57BL6 × 129Sv	Growth defect observed only in Withers *et al*[60]. Much more severe glucose dysregulation in Withers *et al*[60]
	Kubota *et al*[61]	C57BL/6 × CBA mixed	
IR and IRS1 double heterozygous knockout	Kulkarni *et al*[62]	C57BL/6 129/Sv DBA/2	Diabetes not observed in 129/Sv mice, observed in 85% of C57BL/6 mice and 64% of DBA/2 mice. Glucose intolerance only in C57BL/6 strain
AKT2 knockout	Cho *et al*[64]	C57BL/6	More severe hyperglycemia and hyperinsulinemia in Garofalo *et al*[63]. Growth defect only in Garofalo *et al*[63]
	Garofalo *et al*[63]	DBA/1lacJ	
AKT1 knockout	Chen *et al*[65]	C57BL/6 × 129R1	High neonatal mortality only in Cho *et al*[64]. Improved glucose tolerance and insulin sensitivity only in Buzzi *et al*[68]
	Cho *et al*[66]	C57BL/6	
	Buzzi *et al*[68]	129/Ola, C57BL/6 mixed	
Pik3r1 heterozygote	Mauvais-Jarvis *et al*[72]	129Sv, C57BL/6 mixed	Improved glucose tolerance and insulin sensitivity and low glucose and insulin levels on normal diet only in Mauvais-Jarvis *et al*[72]
	McCurdy *et al*[73]	C57BL/6SVJ	
Liver-specific *Pik3ca*	Sopasakis *et al*[74]	129Sv, C57BL/6, FVB mixed	Insulin resistance and glucose intolerance on normal diet in Sopasakis *et al*[74] only
	Chattopadhyay *et al*[75]	129, C57BL/6J mixed	
GLUT4 heterozygous knockout	Stenbit *et al*[76]	CD1, C57BL/6 mixed	Unexpected more severe phenotype in heterozygous knockout than homozygous
PKCλ heterozygous knockout	Farese *et al*[79]	C57BL/6, 129P2/Sv, FVB mixed	Unexpected more severe hepatic steatosis in heterozygous knockout than homozygous
PKCδ knockout	Leitges *et al*[81]	129/SV × Ola	High neonatal mortality observed only in Bezy *et al*[82]
	Bezy *et al*[82]	C57BL6/J	
PPARγ	He *et al*[86]	C57BL/6J	Resistance to diet-induced insulin resistance only in Jones *et al*[85] study
	Jones *et al*[85]	C57BL/6J, FVB mixed	
Muscle-specific PPARγ	Norris *et al*[87]	129/sv, C57BL/6, FVB mixed	Insulin resistance and glucose intolerance on normal diet in Hevener *et al*[88] only. Improvement with rosiglitazone in Norris *et al*[87] only
	Hevener *et al*[88]	C57BL6/J	

Reproducibility problems in knockout mouse studies. Some variant results can be explained by differences in genetic background. IRS: Insulin receptor substrate 1; IR: Insulin receptor; AKT2: Protein kinase B isoform 2; GLUT4: Glucose transporter isoform 4; PKCλ: Protein kinase C λ; PPARγ: Peroxisome proliferator-activated receptor γ.

and beta-cell double insulin receptor knockout (BIRKO-MIRKO) mouse strain had an unexpectedly mild condition. This strain had impaired glucose tolerance, mild hyperglycemia, high triglycerides and free fatty acids, and extra fat pad mass. These findings would seem to indicate that muscle-mediated glucose disposal is dispensable for normal glucose homeostasis in mice, but 2-deoxyglucose uptake studies showed that both muscle-specific insulin receptor knockout (MIRKO) and BIRKO had normal muscle glucose uptake, suggesting most muscle glucose uptake under these conditions is insulin-independent[57]. Studies of liver glycogen synthesis and liver glycogen content confirm that mice with insulin insensitive muscle shifted glucose utilization away from muscle and towards liver[57].

Mouse strains lacking insulin receptor in other tissues have been developed. A knockout of insulin receptor in neuronal tissue (NIRKO) demonstrated elevated body weight, white adipose tissue, serum triglycerides, and circulating leptin, with most of these changes being more pronounced in the females[58]. In addition, both sexes of NIRKO mice had reduced fertility, demonstrating the importance of insulin in reproduction. A knockout of insulin receptor in adipose tissue (FIRKO) had low fat mass, and the normal relationship between leptin levels and fat mass was disrupted[59]. These mice were protected against age-related glucose intolerance.

The IRS proteins transmit signals from the insulin and IGF1 (insulin-like growth factor 1) receptors. Two groups independently showed a significant pre-natal and post-natal growth defect in IRS1 knockout mice[18,19] (Table 1). Despite having similar genetic backgrounds, only one of the strains exhibited glucose intolerance as measured by a glucose tolerance test[18]. In addition, the two strains had significantly different growth defect severities, with a 40%-60% decrease in weight at various life stages observed in one study[18], and a 20%-30% decrease in the other[19]. These differences could have been due to the genetic manipulation approaches or the genetic backgrounds.

Two independent groups described IRS2 knockout mouse models, and the phenotypes were different despite similar genetic backgrounds. Withers *et al*[60] observed a 10% decrease in body weight throughout all life stages for the IRS2 knockout mice in a C57BL6 × 129Sv background, while Kubota *et al*[61] observed the IRS knockouts to be of normal size in a C57BL/6 × CBA mixed background. Fasting hyperglycemia was observed at age 6 wk in Withers *et al*[60], but average glucose levels did not reach hyperglycemic levels in Kubota *et al*[61]. Hyperinsulinemia and glucose tolerance showed a similar pattern: more severe, earlier phenotypes observed in Withers *et al*[60] than in Kubota *et al*[61]. Reduced β-cell mass was observed by both groups.

Kubota *et al*[61] suggested that the difference in glucose and insulin levels between the two reports was likely due to low β-cell mass in their strain, caused either by β-cell death or by the failure of insulin-resistance induced hyperplasia, and acknowledge that genetic differences other than the intended manipulation may influence the results. The authors concluded based on their data and data from a related study that both β-cell dysfunction and reduced β-cell mass can contribute to the murine diabetic state, but only studies of human patients can validate whether one or both mechanisms are more important in the pathogenesis of type 2 diabetes in humans.

Double heterozygous knockout of IR and IRS1 were generated in three different genetic backgrounds: C57BL/6, 129/Sv and DBA/2[62]. While all three strains had mild growth retardation, the results in regards to glucose homeostasis were drastically different. In C57BL/6 mice, the double heterozygous knockout caused severe hyperglycemia and hyperinsulinemia in the vast majority of cases, whereas the glucose levels of 129Sv mice were not significantly different from control littermates. In DBA mice, more than half of the mice were hyperglycemic but maintained normal glucose tolerance. Triglycerides were significantly reduced in the double heterozygous knockouts of the B6 and DBA strains, and the wild type DBA strain had significantly elevated triglycerides as compared to the other wild type strains[62].

AKT/PROTEIN KINASE B

The metabolite phosphatidylinositol 3,4,5-trisphosphate (PIP3) activates AKT/protein kinase B and atypical protein kinase C. AKT has three isoforms in mammals, of which AKT1 and AKT2 are most important for metabolism. Two independently developed AKT2 knockout mouse strains in different backgrounds developed hyperglycemia, glucose intolerance, and insulin resistance[63,64]. Garofalo *et al*[63] observed hypoinsulinemia due to pancreatic β-cell death in a subset of male mice, and hyperinsulinemia with no pancreatic changes in the remainder, while Cho *et al*[64] observed hyperinsulinemia and associated pancreatic hyperplasia. In Garofalo *et al*[63], both hyperglycemia and hyperinsulinemia were more severe than in Cho *et al*[64], with average fed insulin measurements five times higher. Also, Cho *et al*[64] observed normal growth in the AKT2 knockout, but Garofalo *et al*[63] observed a mild growth deficiency evident at all life stages. Only Garofalo *et al*[63] observed lipoatrophy and high levels of serum triglycerides. The control mice in Garofalo *et al*[63] had near-diabetic random fed glucose levels that were almost as high as the knockout mice in Cho *et al*[64] Neither of these knockout strains were obese.

The characteristics of AKT1 knockout mouse strains are also sensitive to genetic background and environmental factors. Two labs independently reported that AKT1 knockout mice with different genetic backgrounds had a growth defect causing 15%-20% reduced body weight[65,66]. One of the studies observed high neonatal mortality among the knockout mice[66], while the other observed high mortality with γ-radiation[65]. Glucose tolerance in Chen *et al*[65] appeared normal, but the glucose tolerance test was performed using a longer fasting time and lower glucose dose than is optimal[50]. One study demonstrated a non-significant improvement in glucose tolerance and insulin sensitivity in males. A similar strain was later shown to be resistant to diet-induced obesity[67]. Later data on a third, independently developed AKT1 knockout strain showed dramatic improvement in glucose tolerance and insulin sensitivity[68].

Studies of spontaneous human genetic variants in AKT1 and AKT2 have confirmed the importance of these proteins in growth and glucose homeostasis, mostly respectively, although the manifestations of the mutations differ between humans and mice[16]. For example, the human patients with a specific AKT2 mutation display asymmetric hypertrophy[69], while the above-described AKT2 knockout mouse models have normal growth[64] or a growth deficiency[63].

PHOSPHOINOSITIDE 3-KINASE

PI3K, an enzyme complex composed of a regulatory subunit and a catalytic subunit that produces the metabolite PIP3. PI3K is activated by IRS proteins in the insulin signaling cascade (Figure 2). In humans, *PI3K* gene polymorphisms are associated with cancer risk[70] but not diabetes, to our knowledge.

Complete loss of the *Pik3r1* gene, which encodes isoforms of the regulatory subunit of PI3K, results in perinatal lethality in mice, perhaps due to impaired B cell development[71]. Mice heterozygous for *Pik3r1* deletion, having attenuated expression of all isoforms of the regulatory subunit, had improved glucose tolerance and insulin sensitivity and low glucose and insulin levels[72]. Lipid metabolism was unchanged except for a modest increase in serum free fatty acids, indicating that the observed insulin sensitivity was not due to indirect effects *via* changes in lipid metabolism. A minor increase in basal muscle glucose uptake was observed, but the authors note that changes in liver were likely most responsible for the increased insulin sensitivity[72]. A later, independent study observed that the heterozygous knockout mice were essentially indistinguishable from control mice on a normal diet[73]. On a high-fat diet, these mice showed lower fasting insulin levels, improved overall insulin sensitivity, and improved glucose uptake in fat and muscle[73]. Macrophage accumulation was reduced in the adipose tissue of these heterozygous knockout mice, but results from bone marrow transplant experiments suggested the improved insulin sensitivity did not occur solely *via* PI3K's role in inflammation.

The catalytic subunits of PI3K have also been studied using knockout mouse strains. Liver-specific deletion of *Pik3ca* caused mild obesity, insulin resistance, glucose intolerance, and high glucose and insulin levels[74]. The same genetic manipulation in a second laboratory produced a strain with normal glucose and insulin levels and body weight[75]. The *Pik3ca* knockout mice in the second

study were resistant to high-fat diet induced hepatic steatosis and somewhat resistant to diet-induced glucose intolerance as well[75]. For this gene, liver-specific deletion produced diabetes-like symptoms in one laboratory, but in another laboratory, glucose homeostasis was identical in control and knockout mice[74,75].

GLUT4

As described above, GLUT4 is the major glucose transporter in muscle, the most important tissue type for glucose disposal in humans. Unexpectedly, in GLUT4 knockout mice, glucose levels are normal except for mild fed hyperglycemia and fasted hypoglycemia observed only in males[29]. Consistent with results regarding insulin signaling and growth[18], these animals display significant growth retardation, shortened life spans, cardiac hypertrophy, and reduced adipose tissue[29]. Somewhat surprisingly, mice heterozygous for the GLUT4 knockout have a more severe phenotype. A diabetes-like condition developed at varying ages, with a majority of males both hyperinsulinemic and hyperglycemic by age 5-7 mo[76].

The authors pointed out that the unexpectedly mild condition of the homozygous GLUT4 knockout and more severe condition in the GLUT4 knockout heterozygote were likely due to compensatory metabolic adjustments that occur during development. These could include the transfer of glucose disposal from tissues that primarily use GLUT4 to tissues that primarily use GLUT2, as observed in the muscle-specific GLUT4 knockout[30], or the upregulation of alternative glucose transporters[52].

PROTEIN KINASE C

Protein kinase C enzymes (PKCs) are involved in regulating a variety of cellular functions in mammals, including insulin signaling[77]. Atypical PKCs include the isoforms PKCλ/ι and ζ (PKCλ refers to the mouse isoform of PKCι)[78]. Activated PKCs can inhibit insulin signaling by a feedback mechanism that prevents signal transduction between insulin receptor and IRS[7,78].

Atypical protein kinase C family member PKCλ was knocked out specifically in mouse muscle, resulting in diabetic symptoms including glucose intolerance, insulin resistance, hyperglycemia, and high insulin levels[79]. Altered fat metabolism was also observed: high triglycerides, and mildly elevated free fatty acids and liver triglycerides. While some symptoms were observed in both the heterozygous and homozygous muscle-specific knockout of PKCλ, the heterozygotes were as insulin resistant and glucose intolerant as the homozygous knockouts, and had more abdominal obesity and hepatic steatosis[79]. This is unexpected, since the heterozygous knockout had reduced, but not ablated, expression of PKCλ.

Differential expression of PKCδ has been identified as one factor in the different vulnerability of common laboratory mouse strains to diabetes[80]. One study of a PKCδ knockout mouse strain in a 129/Sv × Ola genetic background had normal growth and development[81]. Surprisingly, the same deletion in the C57BL6/J strain caused a high mortality rate, with survivors being 14% underweight[82]. The C57BL6/J PKCδ knockout mouse had better glucose tolerance than control mice[82], but glucose tolerance was not tested in the original knockout. The authors noted that improved glucose tolerance may have been due to decreased inflammation in adipose tissue[82]. In humans, PKCδ deficiency can cause B-cell deficiency with severe autoimmunity[83].

PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR γ

The nuclear receptor PPARγ, becomes activated upon binding of lipids and is important for lipid metabolism and storage, adipogenesis, and insulin sensitivity. This nuclear receptor is the target of insulin-sensitizing TZDs[84].

Two independently generated adipose tissue-specific PPARγ knockout strains showed important differences in glucose homeostasis under high-fat diet conditions. On normal chow, both these strains had reduced adipose tissue mass, high blood lipid levels, and hepatic steatosis, but glucose tolerance was normal[85,86]. On high-fat diet with 40% of calories from fat, He *et al*[86] observed hyperinsulinemia and insulin resistance in both the knockout and control mice, although these traits were more severe in the knockout. The knockout strain studied by Jones *et al*[85] was resistant to diet-induced hyperinsulinemia and insulin resistance despite being subjected to a more extreme high-fat diet, with 60% of calories from fat. The knockout strains in both studies were more prone to high-fat diet induced hepatic steatosis.

Two studies on independently developed muscle-specific PPARγ knockout models have provided contradictory findings regarding the mechanism of action of TZDs. The first strain was more susceptible to diet-induced obesity, glucose intolerance, and insulin resistance but was indistinguishable from controls on a normal diet[87]. Rosiglitazone reduced the hyperinsulinemia and impaired glucose homeostasis observed in this strain on high-fat diet, therefore the authors suggested that muscle PPARγ is not required for the positive effects of this TZD[87]. In contrast, the second strain developed insulin resistance and glucose intolerance on a normal diet[88]. Glucose disposal in a hyperinsulinemic-euglycemic clamp experiment was not improved with rosiglitazone treatment, suggesting that the insulin sensitizing effect of TZDs is dependent on muscle PPARγ. In this case, two mouse models have provided conflicting data not just on the role of a gene, but also on a drug mechanism of action.

In conclusion, we above described several cases where genetic modification of insulin signaling genes produced significantly, sometimes dramatically, different results in separate studies or varied genetic backgrounds (Table 1). We also described two cases where heterozygous knockouts had unexpectedly severe phenotypes: GLUT4 and

PKCλ. Although the mechanisms behind the unexpected observations are unknown, it is known that organisms respond unpredictably to the absence of gene products during development. Compensatory metabolic adjustments that may occur during development constitute a general limitation of knockout mouse models. These concerns are mitigated by the use of conditional knockouts, however, those strains require injection or gavage of an inducing drug, which can produce artifacts[89]. These examples illustrate the challenges associated with producing reliable, reproducible, and translatable results in mice.

CLINICAL TRANSLATION

In the following section, we will address factors which limit the applicability of mouse models to human therapeutic treatment development. As described above, insulin signaling gene knockout mice often have phenotypes unrelated to type 2 diabetes including growth defects[18,33,60,63], neonatal mortality[66], and others, including resistance to tumor formation[90]. These phenotypes are a result of the loss of diverse non-metabolic insulin functions, and these studies have yielded information about those biological processes in mice. At this juncture, it is worth examining whether these mouse models of insulin resistance are contributing positively to the development of new, unique, safe, and effective type 2 diabetes treatments. Here we focus on select pharmaceuticals targeting the signaling proteins discussed above.

As might be predicted based on the importance of insulin to growth, several drugs targeting insulin signaling molecules PI3K and AKT are under investigation as therapeutics for cancer[91,92]. Unsurprisingly, some PI3K inhibitors have been shown to induce insulin resistance[93].

The nuclear receptor PPARγ is an important drug target, and is genetically linked to insulin sensitivity and type 2 diabetes risk[94,95]. However, PPARγ-activating TZD drugs are associated with a number of side effects and risks, including congestive heart failure[96]. Although some studies have been inconclusive in regards to certain risks associated with the TZD rosiglitazone[97], one meta-analysis of 42 studies found that the risk of cardiovascular death increased 64%[98]. Rodent studies did not predict these deaths, and in fact have provided conflicting evidence regarding cardioprotective and cardiotoxic effects of TZDs. The TZD pioglitazone was shown to limit myocardial infarct size after coronary occlusion in mice[99]. Similar results have been seen for rosiglitazone after ischemia/reperfusion injury[100]. TZDs have been shown to have both positive and negative effects on cardiac hypertrophy in rodents[101,102].

An inhibitor of PKCβ, LY333531, or ruboxistaurin, has been investigated as a potential treatment for diabetic microvascular complications[103]. Although initially promising results were observed in a trial for diabetic neuropathy, the drug was not shown to be effective in a larger, placebo-controlled study[104]. Promising results were also seen in a small trial for diabetic kidney disease[105], but these have not been replicated at a larger scale. Eli Lilly withdrew the marketing authorization application for ruboxistaurin as a treatment for diabetic retinopathy. Rather than diabetes or its complications, PKC inhibitors are now being investigated as potential treatments for cancer[106] and conditions requiring immunosuppressive therapy[107].

CONCLUSION

The limitations of these mouse models of insulin signaling dysfunction arise from a number of sources. Described above are physiological and molecular-level differences between mice and humans, reproducibility problems in mouse experiments, and complicating factors in drug discovery efforts that interfere with translating mouse results to human patients.

Researchers in a variety of fields have commented on the limitations of mouse models of human disease[108,109]. No single mouse model can accurately represent the spectrum of symptoms and complications associated with type 2 diabetes[11]. The translation of results from mice is further complicated by a plethora of immutable species differences at every level of glucose regulation from the molecular to the population level[110-113]. In addition, mice are not prone to hypertension, high LDL cholesterol, atherosclerosis, sedentary behavior, obesity, insulin resistance, or many other features common to human type 2 diabetes patients. Although all laboratory mice are more insulin resistant and have more fat tissue than their free-living counterparts[114], the risk for mice developing these symptoms varies widely depending on the specific inbred strain[62,80]. Genetic background, housing conditions, and diet can dramatically affect results. Examples highlighted here have shown that different studies even from the same laboratory often obtain different results with identical genetic modifications.

The idea that the limitations of genetically modified mouse models of human disease, and rodent models in general, are severe enough to warrant a shift in research approaches is controversial, and will likely continue to be for the next decade. Nonetheless, science in many medical fields has been progressing away from crude, animal-based experiments and towards more high-tech and human-based research methods, and that trend will continue. For example, one area of active research is additional uncharacterized insulin signaling cofactors, which could be identified using phosphoproteomics[115], protein array techniques, or protein interaction-based techniques[116] including yeast two-hybrid and computational approaches. Similar approaches could be used to identify gene products involved in acquired insulin resistance. In addition, insulin resistance can be investigated in human cells by gene silencing[117], metabolomics[118], and microarray technology. Remaining questions about the role of inflammation and accumulated intracellular lipids can be studied using tissue biopsy samples from various patient populations[119]. Many more *in vitro*[120], in silico[121], non-invasive[122], and minimally invasive[123] approaches are available and in development.

In the last 20 years, the use of genetically modified mice to investigate diabetes has become routine. While some findings have borne out in humans, investigations of insulin resistance using knockout mouse models are inherently limited by physiological, genetic, and metabolic differences between mice and humans. Researchers and patients would benefit from a transition towards human-based research methods.

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