Abstract

The identification of insulin receptor substrate (IRS) proteins in the 1990s represents a key phase of diabetes research as it has enabled our present understanding of the molecular basis of insulin and insulin-like growth factor (IGF) action. The generation of mice with targeted deletions of the four major IRS proteins has revealed invaluable information about the biological functions of these signaling molecules and has provided novel insights into the role of defective insulin signaling in the development of diabetes and metabolic diseases. Irs1-deficiency in mice causes reduced body size, beta cell hyperplasia, and increased life-span. Disruption of Irs2 has demonstrated that this branch of the insulin/IGF signaling cascade has an important role in peripheral insulin action and pancreatic beta-cell growth and function. Global disruption of IRS2 signaling in mice causes diabetes due to failed beta cell compensation in the presence of peripheral insulin resistance. Gene targeting of Irs3 or Irs4 did not produce remarkable phenotypes suggesting that either they play very specific roles in limited tissues or that their absence may be compensated for by other signaling mechanisms. A complete understanding of the cellular events mediated by IRS1 and IRS2 will reveal new strategies to prevent or cure diabetes and other metabolic diseases.

Keywords: insulin, signal transduction, beta cell, gene targeting, animal models, insulin resistance, obesity.

Introduction

Data from health organizations and epidemiological studies indicate that the two most common forms of diabetes are increasing at alarming rates in developed countries, including Spain. Type 1 diabetics have partial or complete beta-cell deficiency due to destruction of insulin-producing cells by the immune system. Although peripheral insulin resistance was considered historically to be the principal cause of Type 2 diabetes, it has now become evident that early beta cell loss and/or defective insulin secretion also underlie this prevalent form of diabetes. However, the site of the primary defect in insulin action remains unclear as does the relationship between insulin resistance and impaired beta-cell function.

The current diabetes epidemic emphasizes the importance of understanding the combined defects that cause Type 2 diabetes. Elucidating the molecular basis of diabetes will certainly lead to the implementation of improved therapies for preventing or treating this insipid disease. Generation of mice with targeted mutations of the genes encoding insulin signaling molecules provides a unique approach to assess the contributions of impaired insulin action to the pathogenesis of insulin resistance and diabetes. Thus, in the present article, we will discuss mouse models where IRS proteins have been genetically targeted to generate loss-of-function or gain-of-function tools. Due to space limitations, we can only briefly discuss the phenotypes of these models but we refer the reader to the original articles and prior reviews.

The molecular basis of insulin action

Insulin binds to the alpha subunit of the insulin receptor in the plasma membrane which activates the intrinsic tyrosine kinase activity of the beta subunit. In contrast to most tyrosine kinase receptors which utilize autophosphorylation to create binding sites for SH2-containing signaling molecules, the IR interacts poorly with SH2 domains and therefore, relies on an alternative strategy of phosphorylating adaptor proteins to mediate intracellular signaling (figure 1). The molecular cloning of these proteins, including Spain. Type 1 diabetics have partial or complete beta-cell deficiency due to destruction of insulin-producing cells by the immune system. Although peripheral insulin resistance was considered historically to be the principal cause of Type 2 diabetes, it has now become evident that early beta cell loss and/or defective insulin secretion also underlie this prevalent form of diabetes. However, the site of the primary defect in insulin action remains unclear as does the relationship between insulin resistance and impaired beta-cell function.

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adaptors, named IRS proteins, in the 1990s provided a mechanistic and evolutionary explanation for the divergence of insulin signaling from oncogene and growth-factor signaling. Upon phosphorylation by activated IRs or IGF-I receptors, IRS proteins are capable of recruiting various signalling molecules including phosphoinositide-3-kinase (PI3K), Fyn kinase and Grb2.3,4 Thus, IRS proteins are the principal mediators of cellular responses to insulin and IGF-I. Products of PI3K activate a network of serine-threonine kinases, including AKT, implicated in the action of insulin on glucose transport, glycogen synthesis, cell proliferation and apoptosis (figure 1).

Insulin receptor substrate 1 (IRS1) was the first major substrate of the insulin receptor to be cloned;5 deletion of this gene in mice provided researchers with an unexpected surprise as it revealed the existence of other IRS proteins.6,7 The IRS-protein family contains at least four principal members, IRS1-4. IRS1 appears to be ubiquitously expressed.5 IRS2 was initially identified as a component of the interleukin-4 signaling pathway, but it is now known to be expressed in nearly all cells and tissues.7,8 IRS3 is predominantly expressed in adipose tissue, and was purified and cloned from rat fat cells;9 IRS4 was purified and cloned from HEK293 cells, where it is the major IRS-protein.10,11 IRS4 is expressed predominantly in the pituitary, thymus and brain.

### The basic technology of gene targeting

Animal models are indispensable tools for studying the molecular basis of disease as well as the physiological role of a specific gene product. Experimental models can be categorized as natural or induced. Natural models are those in which a condition occurs spontaneously such as the db/db mutation which produces obesity in mice.12 In 2007, the Nobel Prize in medicine was awarded to Mario R. Capecchi, Martin J. Evans and Oliver Smithies for their discoveries related to embryonic stem cells and DNA recombination in mammals. These discoveries provided the basis for the development of the immensely powerful technology referred to as gene targeting for the production of induced models in mice.

Knockouts are used to study the function of specific genes, detect their protein products, and link them to diseases that arise when their function is inadequate. Gene targeting experiments have elucidated the roles of numerous genes in embryonic development, adult physiology, aging and disease. To date, more than ten thousand mouse genes (approximately half of the genes in the mammalian genome) have been knocked out.13,14 Although mice are especially attractive for gene targeting given that their physiology is similar to humans, *Drosophila* and *C. elegans* are also useful for producing transgenics to study insulin signaling.

In order to create genetically modified animals, it is necessary to modify the DNA of germ-line cells so that the modified DNA is transmitted from generation to generation. When an investigator wants to accomplish this, the method of choice is homologous recombination.13,14 To perform homologous recombination, the DNA sequence of the gene of interest must be known. With this information, it is possible to replace any gene with a DNA construct of your choosing. The first step involves the design and production of the DNA sequence you want to insert into the chromosome in place of the wild-type allele. Regardless of what is inserted, one must include some flanking DNA that is identical in sequence to the targeted locus. In addition to the positive selection marker (e.g. antibiotic resistance), a negative selection marker (e.g. thymidine kinase) is often incorporated in the replacement vector. The DNA construct that has been engineered to contain a mutant copy of the
gene is introduced into special embryonic stem cells (ES cells) that are grown in tissue culture. Cells that take up the foreign DNA are screened to find those in which the mutant copy has replaced one good copy of the gene. ES cells with one mutant copy are introduced into an early embryo (blastocyst) that is subsequently implanted in a foster mother. Mice that are born from this manipulation are mated to each other. One in four mice from this mating will contain two mutant copies of the gene. Now begins the work of establishing a knockout colony and characterizing the phenotypes (if any) produced by targeted deletion of the gene of interest.

Constitutive deletion of Irs1 in mice

Irs1 knockout mice are IGF-1 resistant and are growth retarded both prenatally and postnatally.\(^6,15,16\) They exhibit birth weights between 40-60% of wild-type mice, and this persists throughout adult life. Disruption of Irs1 also causes insulin resistance, mainly in skeletal muscle, and abnormal glucose tolerance. However, these mice do not develop diabetes due to the presence of β-cell hyperplasia. Irs1-deficiency has also been observed to produce hypertension and hypertryglyceridemia.\(^13\) Isolated islets from Irs1 knockout mice manifest a secretory defect and reduced insulin synthesis, suggesting a role for IRS1 in islet function.\(^18\) Recently, Selman et al have reported that deletion of Irs1 but not Irs2 extends lifespan in female mice.\(^19\) Irs1-deficient females displayed resistance to age-sensitive markers of aging including skin, bone, immune, and motor dysfunction. Thus, these findings reinforce observations from other long-lived mouse models which suggest that longevity is governed by an endocrine-signaling axis involving IGF1, IGF1R, and IRS1.

Complete deletion of Irs2 reveals its importance for beta cell function

Mice lacking Irs2 develop diabetes due to insulin resistance and pancreatic beta cell dysfunction.\(^16,20,21\) As early as 4 weeks of age, these animals have markedly abnormal glucose tolerance. By 8 weeks, male Irs2 knockout mice have reduced insulin-stimulated whole-body glucose disposal and a partial reduction in insulin suppression of hepatic glucose production, suggesting profound insulin resistance in liver and skeletal muscle. By 12-16 weeks, male Irs2-deficient mice exhibit severe hyperglycemia, polydipsia, and polyuria and die from dehydration and

| Table 1. Phenotypes resulting from the targeting of IRS genes in mice |
|---------------------------------|--------|----------------|----------------|
| Genetic manipulation           | Diabetes | Insulin resistance | Obesity |
| Complete deletion of Irs1      | No      | Yes             | No             | Body size reduced by 50%. Beta cell hyperplasia. Increased life-span | 5-6, 15-16,19 |
| Complete deletion of Irs2      | Yes     | Yes             | Females develop moderate obesity and hyperleptinemia | Female infertility; Small brain. Loss of photoreceptors. Enhanced neointima formation | 16, 20, 21-22, 29 |
| Complete deletion of Irs3      | No      | No              | No             |                             | 33 |
| Complete deletion of Irs4      | No      | No              | No             | Mild growth retardation. Reduced fertility | 35 |
| Complete disruption of Irs1 and Irs2 | Not viable |                |                |                             | 16, 21 |
| Conditional deletion of Irs2 using RIP-Cre | Yes, initially but recover due to re-population of endocrine pancreas | No | Yes |                             | 30-32 |
| Conditional deletion of Irs2 using NestinCre | Yes | No | Yes |                             | 32 |
| Conditional deletion of Irs2 using POMCre | No | No | No |                             | 32 |
| Conditional overexpression of Irs2 in beta cells using RIP | No | No | No | Beta cell hyperplasia. Rescues diabetes in diabetic models | 24 |
hyperosmolar coma. Female IRS2 knockouts develop diabetes less severely than males.22 Morphometric analysis of pancreas sections from Irs2 null mice at 4 weeks of age revealed a significant reduction in the beta-cell mass of Irs2 knockout mice; the number of islets in these animals is decreased by ~50%. Additionally, islet insulin content is reduced in the Irs2 knockout mouse, most likely owing to the severe reduction in the expression of the transcription factor pancreatic and duodenal homeobox 1 (PDX1). PDX1 is critical for development of the pancreas in mice and humans, and its complete disruption blocks pancreas development. Reconstitution of PDX1 expression in the Irs2 knockout pancreas rescues diabetes in this model.21

Various lines of evidence suggest that Irs2 null mice do not possess mechanisms to generate new beta-cells nor can they sustain survival of existing insulin-producing cells. Conversely, overexpression of IRS2 specifically in the endocrine pancreas via the rat insulin promoter (RIP) causes beta cell hyperplasia.23 RIP-IRS2 islets can rescue the endocrine pancreas via the rat insulin promoter (RIP) cells. Conversely, overexpression of IRS2 specifically in the beta cells is through regulation of cell-cycle machinery. Indeed, the cell-cycle inhibitor p27KIP1 progressively accumulates in the nucleus of pancreatic beta-cells in mice that lack IRS2.25 Deletion of the gene for p27KIP1 in the IRS2 model (double knockouts of p27 and IRS2) prevents the development of diabetes by increasing islet mass and maintaining compensatory hyperinsulinemia, effects that were attributable predominantly to stimulation of pancreatic beta-cell proliferation. Consistent with this, when either rats or mice are subjected to a 60% pancreatectomy, regeneration of the pancreas is associated with up-regulation of IRS2 expression.26, 27

Additionally, it should be remembered that Irs1 knockout mice are characterized by beta cell hyperplasia. Studies of this phenomenon have demonstrated that even in the absence of insulin resistance, Irs1-deficient islets display increased proliferation and decreased apoptosis which are mediated by IRS2 signals.18 These observations provide strong evidence for the positive role of IRS2 in promoting cell-cycle progression in pancreatic beta cells. Kadowaki and co-workers have recently provided yet another proof-of-concept that IRS2 is required for beta cell compensation in response to insulin resistance. When fed a high-fat diet, mice heterozygous for glucokinase (GCK) displayed decreased beta cell replication and insufficient beta cell compensation despite a similar degree of insulin resistance as WT controls.28 Microarray analysis of these islets revealed a significant reduction of IRS2 expression in the high fat diet-fed Gck(+/–) mouse islets compared with WT islets, demonstrating that without intact IRS2 signaling, beta cells are unable to expand to meet the demands imposed by insulin resistance.

Increased expression of the pro-apoptotic protein BAD has been detected in islets of Irs2 knockouts.21 Moreover, isolated islets from these animals display higher levels of active caspase-3 which can be corrected by re-introduction of IRS2 via adenovirus infection.22 In isolated WT murine islets, IGF1 stimulates phosphorylation of Erk1/2, Akt, and the Akt target Foxo1. By contrast, in islets of Irs2 null mice the phosphorylation of these targets is reduced, and cleaved/activated caspase-3 is insensitive to IGF1 stimulation, which is consistent with decreased growth and survival of Irs2-deficient beta cells.24

Additional phenotypes in the Irs2 knockout include that female Irs2 null mice are infertile, hyperphagic, and develop obesity.22 These were the first clues that IRS2 signaling might also play a critical role in the regulation of appetite and body weight. Irs2 null mice are resistant to the effects of leptin in the hypothalamus,20,22 suggesting that IRS2 acts as a point of convergence for leptin and insulin signaling. Detailed analysis of the infertility of Irs2 null females has revealed reduced follicle size, increased numbers of atretic follicles, and impaired oocyte growth and antral cavity development.29 Granulosa cell proliferation is as well defective in -deficient ovaries. These abnormalities were associated with reduced expression of cyclin D2 and increased p27KIP1 levels, indicative of cell-cycle dysregulation. These findings suggest that ovarian rather than central nervous system IRS2 signaling is important in the regulation of female reproductive function. Thus, the Irs2 knockout model may have relevance for the pathophysiology of polycystic ovary disease which is associated with insulin resistance.

**Tissue-specific targeting of Irs2**

The multiple phenotypes resulting from the whole-body deletion of Irs2 have complicated studies aimed at defining the relevance of this signaling molecule in specific tissues. Since insulin resistance caused by the absence of IRS2 in peripheral tissues may alter various metabolic pathways and beta cell function, conditional deletion of the
Irs2 gene specifically in β cells and the hypothalamus has generated appropriate tools to more precisely determine the roles of IRS2 at these sites. Three different laboratories have produced these tissue-specific transgenes and have all obtained results, though varying to some degree, that support fundamental roles for IRS2 in beta cell compensation and hypothalamic regulation of obesity.

Using the cre-loxP system and the rat insulin promoter, the groups of Morris White and Takashi Kadowaki generated mice with deficiency for Irs2 in the beta cell and the hypothalamus owing to the expression of the RIP promoter in certain neuronal populations. Both studies concur that this form of conditional deletion of Irs2 causes increased appetite, obesity and insulin resistance that progressed to diabetes at around 8-10 weeks of age. Both beta cell mass and proliferation were significantly diminished in young transgenic animals. However, the White group studied these diabetic animals for longer periods of time and made the astute observation that the diabetes in these animals was corrected between 6 and 10 months of age due to the re-population of the endocrine pancreas by functional beta cells. These observations were confirmed by the elegant work published subsequently by the laboratory of Dominic Withers where tissue-specific knockouts of Irs2 were produced not only with the RIP promoter but also using the nestin promoter to delete Irs2 generally in neurons and the proopiomelanocortin (POMC) promoter to restrict the conditional inactivation to a specific population of hypothalamic neurons. This strategy allowed these researchers to address important questions that were not answered by the former studies which relied solely on the RIP promoter to restrict the deletion of Irs2. Their studies have demonstrated that IRS2 pathways acting in a neuronal population distinct from POMC and neuropeptide-Y (NPY) neurons regulate energy homeostasis and growth. The Withers’s study also concludes that IRS2 in β cells is required for the maintenance of β cell mass, as β cells that escape Cre-mediated recombination are able to repopulate islets with time. Taken all together, these observations clarify the role of IRS2 in β cell function and energy homeostasis and suggest that modulation of IRS2 function is a valid target for the treatment of diabetes and obesity.

**Deletion of IRS3 and IRS4**

Targeting of Irs3 or Irs4 in mice did not produce remarkable phenotypes. Growth and glucose homeostasis were completely normal in Irs3 knockout mice. However, when Irs1-knockout mice and Irs3-knockout mice were inter-crossed, the resulting double mutants displayed lipo-atrophy with insulin resistance, but without intrahepatic and intramuscular deposits of triglycerides. Male Irs4 knockouts were slightly smaller than WT controls but female null mice were of normal size. Additionally, the breeding of Irs4 knockouts revealed reduced rates of reproduction. Although glucose levels were slightly lower in Irs4 knockout mice, insulin values were normal. Thus, deficiency of Irs4 causes mild defects in growth, reproduction, and glucose homeostasis.

**Conclusions:**

**The clinical impact of IRS models**

More than a decade has passed since the creation of the first Irs knockout. Clearly, the labor of various laboratories to generate and characterize total and conditional knockouts of IRS proteins has provided many experimental rewards. Although we continue to make new observations in these animals, the IRS knockout models have demonstrated that these proteins exert unique and complementary signals in mediating insulin/IGF-I action.

From the conditional knockouts, we have learned that different tissues contribute uniquely to the pathogenesis of type 2 diabetes. Although peripheral insulin resistance is a well known component of type 2 diabetes, it is clearly not sufficient to provoke diabetes, based on observations from IRS transgenic models. Rather, the Irs2 knockout model emphasizes beta cell insufficiency as a key factor in the development of diabetes. Irs2-deficient mice display peripheral insulin resistance but the real trigger for diabetes in this model seems to be the inability of beta cells to compensate due to a reduction in their number and function. Thus, failure of the IRS-2 branch of insulin/IGF signaling is likely to be an important component of human diabetes. Recently, microarray analysis has revealed that IRS2 expression is significantly reduced in pancreatic islets from humans with Type 2 diabetes, consistent with a critical role for IRS2 in maintaining glucose homeostasis in humans. This study coincides with findings from mouse models where hyperglycemia and dyslipidemia are correlated with a reduction in the expression levels of IRS2.28,37

**Conflict of interest**

The authors declare that they have no conflict of interest in relation to the content of this review.
Practical considerations

- The generation of mouse knockouts by gene targeting provides optimal models for studying the physiological function of specific genes and for linking them to human diseases.
- Targeted deletion of the individual Irs genes in mice has provided proof-of-concept that IRS proteins have distinct physiological roles in different tissues.
- These models have demonstrated that the IRS2-branch of the insulin/IGF signaling cascade has an important role in both peripheral insulin action and pancreatic beta-cell growth and function.

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