Dysregulation of insulin receptor substrate 2 in β cells and brain causes obesity and diabetes

Xueying Lin, Akiko Taguchi, Sunmin Park, Jake A. Kushner, Fan Li, Yedan Li, and Morris F. White

Howard Hughes Medical Institute Children’s Hospital, Division of Endocrinology, Harvard Medical School, Boston, Massachusetts, USA.

The molecular link between obesity and β cell failure that causes diabetes is difficult to establish. Here we show that a conditional knockout of insulin receptor substrate 2 (Irs2) in mouse pancreas β cells and parts of the brain — including the hypothalamus — increased appetite, lean and fat body mass, linear growth, and insulin resistance that progressed to diabetes. Diabetes resolved when the mice were between 6 and 10 months of age: functional β cells expressing Irs2 repopulated the pancreas, restoring sufficient β cell function to compensate for insulin resistance in the obese mice. Thus, Irs2 signaling promotes regeneration of adult β cells and central control of nutrient homeostasis, which can prevent obesity and diabetes in mice.

Introduction

Type 2 diabetes affects 18.2 million people in the United States and is increasingly common among young people (1, 2). Most disease models point to obesity as an important cause of type 2 diabetes, because of its association with skeletal muscle insulin resistance and pancreatic β cell failure (3, 4). However, dysregulated peripheral and central signaling — owing to many different causes — might initiate and sustain the cascade of events that progress to obesity and diabetes. Most if not all insulin signals are produced or modulated through tyrosine phosphorylation of insulin receptor substrate 1 (IRS1); IRS2 or its homologs; or other scaffold proteins including Src-homology-2 (SH2) domains including SH2 domain containing Shc, Casitas B-lineage lymphoma proto-oncogene (c-CBL), adapter protein with a PH and SH2 domain (APS) and SH2 and PH domain (GAB1), GAB2, or dreadlocks (DOCK, an SH3-SH2 adaptor protein) isoforms (5). Although the role of each of these substrates merits attention, cell-based experiments and work with transgenic mice suggest that many insulin responses are directly integrated with counterregulatory hormones and proinflammatory cytokines through tyrosine and serine phosphorylation of IRS1 and IRS2 (5). Dysregulation of these IRS proteins by proinflammatory cytokines or genetic deletion impairs glucose tolerance as a result of peripheral insulin resistance; however, systemic Irs2−/− mice also develop life-threatening diabetes at 2–3 months of age because Irs2 is required for β cell growth, function, and survival (6–9). The progression of Irs2−/− mice toward diabetes is retarded or prevented by modifying elements of the insulin/IGF-signaling cascade that promote compensatory β cell function — including downregulation of protein tyrosine phosphatase Ptp1b or the transcription factor Foxo1; or upregulation of Akt (also called protein kinase B, or PKB) or pancreatic duodenal homeobox 1 (Pdx1) (9–14). Transgenic upregulation of Irs2 in pancreatic β cells also prevents diabetes in Irs2−/− mice, obese mice, and streptozotocin-induced diabetic mice by promoting sufficient and sustained compensatory insulin secretion (9). Thus, the Irs2 branch of the insulin/IGF-signaling cascade is a common link between peripheral insulin action and secretion.

A molecular basis for the close association between obesity and peripheral insulin resistance is difficult to establish. Dysregulated signaling — rather than antiodotal consumption of high-calorie diets — might contribute to the early development of obesity that progresses to diabetes (15–17). Insulin, leptin, and adiponectin are important peripheral signals that inform the brain of short- and long-term nutrient availability (17–19). Pharmacologic inhibition of insulin signaling in the hypothalamus increases food intake, and conditional KO of the insulin receptor in the brain causes obesity in mice on high-fat diets (20–23). Leptin secreted from adipocytes promotes satiety and energy utilization, at least in part by promoting α melanocyte-stimulating hormone (αMSH) production in the hypothalamus (15). Mutations that disrupt neuronal leptin signaling or melanocortin signaling increase food intake, body weight, and peripheral insulin resistance in mice and people that progresses to diabetes if β cell function also deteriorates (24–26). Adiponectin, another adipocyte-derived hormone, enhances hepatic and muscle insulin action and promotes energy expenditure through signaling in the hypothalamus (18, 27); however, adiponectin is reduced in obese people and rodents (28, 29).

Previous work suggests that Irs2 signaling plays an important role in the CNS for brain growth, female fertility, and nutrient homeostasis (30). Since IRS2 is highly expressed in the hypothalamus, a molecular basis for the close association between obesity and peripheral insulin resistance is difficult to establish. Dysregulated signaling — rather than antiodotal consumption of high-calorie diets — might contribute to the early development of obesity that progresses to diabetes (15–17). Insulin, leptin, and adiponectin are important peripheral signals that inform the brain of short- and long-term nutrient availability (17–19). Pharmacologic inhibition of insulin signaling in the hypothalamus increases food intake, and conditional KO of the insulin receptor in the brain causes obesity in mice on high-fat diets (20–23). Leptin secreted from adipocytes promotes satiety and energy utilization, at least in part by promoting α melanocyte-stimulating hormone (αMSH) production in the hypothalamus (15). Mutations that disrupt neuronal leptin signaling or melanocortin signaling increase food intake, body weight, and peripheral insulin resistance in mice and people that progresses to diabetes if β cell function also deteriorates (24–26). Adiponectin, another adipocyte-derived hormone, enhances hepatic and muscle insulin action and promotes energy expenditure through signaling in the hypothalamus (18, 27); however, adiponectin is reduced in obese people and rodents (28, 29).

Previous work suggests that Irs2 signaling plays an important role in the CNS for brain growth, female fertility, and nutrient homeostasis (30). Since IRS2 is highly expressed in the hypothalamus, its signaling cascade may be responsible for integrating central control of nutrient homeostasis and appetite regulation with peripheral insulin action and β cell function (9). Female Irs2−/− mice — which develop diabetes more slowly than do male mice — are hyperphagic and obese until severe diabetes causes weight loss (30). To test the role of selective Irs2 dysregulation in obesity and diabetes, we flanked the Irs2 gene with loxp recombination sites (floxed Irs2, or fIrs2) and crossed these mice with transgenic mice expressing Cre recombinase under control of the rat insulin-2 promoter (TgN[Irs2Cre]25Mgn, or crIrs2). Cre recombinase is expressed strongly in β cells and weakly in certain brain regions of these transgenic cr2 mice (31, 32). Thus, our strategy strongly deleted fIrs2 alleles from β cells and weakly deleted them from brain and
certain neurons of the hypothalamus. Analysis of the mice suggests that dysregulated Irs2 signaling could be a common link between obesity and β cell failure that progresses to diabetes; and that Irs2 signaling is an important element for β cell regeneration.

Results

Conditional disruption of floxed Irs2 allele by cr². To determine whether partial dysregulation of Irs2 signaling links obesity and diabetes, we intercrossed mice containing homozygous firs2 alleles with cr² mice (Figure 1A). PCR analysis of islets from the compound firs2:cr² mice (4 weeks old) confirmed that firs2 alleles were almost completely deleted, which was consistent with the reported efficacy of cr² in β cells (Figure 1B). Since islets are composed mainly of β cells, Irs2 mRNA levels decreased at least 90% (Figure 1C). firs2 alleles were partially deleted in the brain, including regions of the hypothalamus (Figure 1, B and D), which is consistent with the reported expression of cr² in some neurons (31, 32). At 8 weeks, Irs2 protein levels were reduced at least 30% in firs2:cr² hypothalamic extracts (Figure 1E). By contrast, firs2 was intact and protein levels were normal in other tested tissues, including muscle, liver, and fat (Figure 1B and data not shown).

To verify that firs2 was disrupted in hypothalamic neurons, we immunostained sections of the arcuate nucleus with antibodies against Irs2 (green stain) and Cre recombinase (red stain); low background was confirmed in systemic Irs2/– brain sections that lack both proteins (Figure 1F). Irs2 was detected in the arcuate nucleus from WT or cr² mice, and Cre recombinase was detected in cr² mice, where it was expressed in some (yellow staining) but not all Irs2-positive neurons (Figure 1F). However, coexpression of Irs2 and Cre was largely abolished in firs2:cr² sections, which confirmed that firs2 was deleted wherever Cre was expressed (Figure 1F); similar results were found in paraventricular nucleus (data not shown).

Growth of the firs2:cr² mice. The firs2:cr² mice reached a normal body weight at 4 weeks of age but then grew more rapidly and were 30% heavier than controls when the experiment was terminated at 32 weeks (Figure 2A). At 8 weeks, firs2:cr² mice were 8 grams heavier than the controls (firs2, 38.2 ± 0.6 g, P < 0.01 compared with firs2, 29.8 ± 0.5 g; cr², 29.4 ± 0.8 g; and WT, 29.1 ± 0.6 g); and they consumed 60% (P < 0.05) more chow (9% fat) and 65% (P = 0.001) more water than did the control mice during a 24-hour interval (Figure 2, B and C). Unlike systemic Irs2/– mice, which have small brains (33), the firs2:cr² mice had a normal brain size (firs2:cr², 0.41 ± 0.01 g; firs2, 0.40 ± 0.01 g; cr², 0.40 ± 0.02 g; and WT, 0.40 ± 0.02 g).

The firs2:cr² mice were 10% longer — snout to anus — than the controls (firs2, 107.9 ± 0.5 mm, P < 0.01 compared with firs2, 98 ± 2 mm; cr², 96.3 ± 0.4 mm; WT, 98 ± 1 mm). On the normal 9% fat diet, 8-week-old firs2:cr² mice had 2-fold more body fat and increased lean body mass (Figure 2D). Chemical analysis confirmed that fat mass was increased disproportionately in firs2:cr² mice (firs2:cr², 18% ± 3% fat, n = 5; firs2, 11% ± 1% fat, n = 4; P = 0.005). Consistent with the increased adiposity, serum leptin and insulin levels were elevated 2-fold in the firs2:cr² mice compared with controls (Figure 2E). Because of their larger size, firs2:cr² mice produced more heat than control mice during a 24-hour period (firs2:cr², 141.1 ± 0.1 kcal; WT, 110.0 ± 1.5 kcal). Although caloric restriction achieved by feeding the mice a low-fat (5%) diet reduced the weight of all the mice, the firs2:cr² mice were consistently heavier (Figure 2A).

Glucose homeostasis in the firs2:cr² mice. Diabetes developed in the firs2:cr² mice between 8 and 10 weeks of age, as fasting glucose levels exceeded those of the control mice (firs2:cr², 110 ± 9 mg/dL, P < 0.01 compared with firs2, 63 ± 2 mg/dL; cr², 60 ± 4 mg/dL; WT, 62 ± 6 mg/dL). Moreover, random-fed glucose was consistently 2-fold higher in the firs2:cr² mice than in the controls (Figure 2A). Glucose disposal rates measured in the firs2:cr² mice — during a hyperinsulinemic/
euglycemic clamp test — were reduced 50%, which revealed peripheral insulin resistance, an important hallmark of type 2 diabetes (Figure 2B). By contrast, hepatic glucose output measured before and during experimental hyperinsulinemia was similar in all of the mice examined (Figure 3C).

The insulin content of the fIrs2:cr2 pancreas was reduced more than 2-fold compared with that in the control mice (Figure 3D). This result is consistent with a positive role for IRS2 in β cell function, especially during compensation for peripheral insulin resistance (9). Intraperitoneal glucose injections following a 16-hour overnight fast confirmed glucose intolerance and diabetes in fIrs2:cr2 mice (Figure 3E). Although glucose tolerance improved when the fIrs2:cr2 mice were placed on the low-fat diet, fIrs2:cr2 mice remained hyperinsulinemic (fIrs2:cr2, 2.5 ± 0.2 ng/ml, P < 0.01 compared with fIrs2, 0.7 ± 0.1 ng/ml; cr2, 0.6 ± 0.1 ng/ml; WT, 0.5 ± 0.1 ng/ml) and relatively glucose intolerant in comparison with control mice on the low-fat diet (Figure 3F).

β cell growth and function in fIrs2:cr2 mice. The growth, function, and survival of β cells are essential for normal glucose homeostasis, especially during compensation for peripheral insulin resistance. At 4 weeks of age when the fIrs2:cr2 mice had a normal body weight, relative β cell content — estimated by the average β cell area in pancreas sections — and β cell size were indistinguishable from those of control mice (Table 1). The expression of several genes that promote β cell function and growth — hepatocyte nuclear factor 3 (Hnf3β), Hnf4α, and Pdx1 — were decreased in fIrs2:cr2 islets; however, glucose transporter 2 (Glut2) mRNA levels were nearly normal.
between 1 and 12 months of age suggests that regenerated fIrs2:cr mice (9), 68 ± 2 g; fIrs2, 51 ± 2 g; cr, 53 ± 1 g; and owing to a larger pancreas at 10 months, the total β cell mass in fIrs2:cr mice was slightly greater than that in controls (Table 1). At 10 months, pancreas sections from fIrs2:cr mice contained more islets, but a disproportionate number of the islet sections contained fewer than 10 β cells (Table 1). Thus, persistent insulin resistance was balanced by the growth of functional β cells that maintained compensatory hyperinsulinemia in mice at 10 months of age (Figure 4B); and during the next year, these mice did not develop diabetes.

What reversed the progression to diabetes? We isolated individual islets from fIrs2:cr mice at 4, 6, and 10 months of age and used PCR to identify the presence or absence of the fIrs2 allele. At 4 months, most islets lacked fIrs2, whereas a few islets displayed a mixture of deleted and intact fIrs2 (Figure 4C). At 6 months, islets contained either deleted fIrs2, intact fIrs2, or a mixture of these alleles; and by 10 months, deleted fIrs2 was never detected (Figure 4C). Thus, fIrs2−/− β cells were progressively replaced by β cells containing fIrs2. The progressive decline of Cre recombinase in islets between 1 and 12 months of age suggests that regenerated β cells arise from cells that weakly express Cre recombinase (Figure 4D); however, we cannot establish the source of these new β cells.

**The role of IRS1 in β cell regeneration.** Peripheral insulin action is mediated by both Irs1 and Irs2 (5); however, systemic Irs1−/− mice never develop diabetes, as Irs2 promotes β cell growth and function to compensate for peripheral insulin resistance (34). Previous work shows that Irs1 haploinsufficiency strongly inhibits residual growth, function, and survival of β cells in systemic Irs2−/− mice, as β cells are nearly undetected when systemic Irs2−/−:Irs1−/− mice die at 30 days of age (34). To determine whether Irs1 contributes to the regeneration of β cells in fIrs2:cr mice, we intercrossed fIrs2:cr mice with systemic Irs1−/− mice to produce compound [fIrs2:cr]:Irs1−/− mice. Unlike systemic Irs1−/− mice or fIrs2:cr mice, the [fIrs2:cr]:Irs1−/− mice developed diabetes at 4 weeks (Figure 4A). The early-onset diabetes progressed rapidly to severe hyperglycemia as serum insulin levels fell during the next several months (Figure 4B). As a result of progressive and severe hypoinsulinemia, the [fIrs2:cr]:Irs1−/− mice gained less weight than the fIrs2:cr mice during the study period (Figure 4E). When the [fIrs2:cr]:Irs1−/− mice were sacrificed at 6 months, relative β cell content was reduced 8-fold in comparison with the insulin-resistant but normal-sized Irs1−/− mice (Figure 4F and Table 1). Although β cells containing insulin were detected in the [fIrs2:cr]:Irs1−/− pancreas at 6 months, the small islets were disproportionately populated by α cells (Figure 4F). The striking discrepancy between the β cell mass of fIrs2:cr:Irs1−/− mice compared with fIrs2:cr mice was unexpected, as β cells retaining fIrs2 alleles should grow in both cases; unfortunately, it was impossible to isolate intact islets from 6 month old [fIrs2:cr]:Irs1−/− mice for genotyping.

**Hypothalamic function in fIrs2:cr mice.** The fIrs2:cr mice closely resemble agouti mice or mice deficient in pro-opiomelanocortin (Pomc−/−) or melanocortin 4 receptor (Mc4r−/−) (15), which suggests that Irs2 mediates some effects of insulin upon the melanocortin pathway in the hypothalamus. To evaluate insulin signaling, we prepared hypothalamic extracts from WT or fIrs2:cr mice 15

<table>
<thead>
<tr>
<th>4 weeks</th>
<th>fIrs2:cr</th>
<th>fIrs2:cr:Irs1+/−</th>
<th>fIrs2</th>
<th>fIrs2:Irs1+/−</th>
<th>cr</th>
<th>WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>β Cell area</td>
<td>0.5 ± 0.1 (4)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.5 ± 0.1 (5)</td>
</tr>
<tr>
<td>β Cell size</td>
<td>75 ± 6 (6)</td>
<td>–</td>
<td>75 ± 6 (6)</td>
<td>–</td>
<td>86 ± 10 (3)</td>
<td>78 ± 7 (5)</td>
</tr>
<tr>
<td>BrdU (%)</td>
<td>1.5 ± 0.2 (6)</td>
<td>–</td>
<td>1.5 ± 0.2 (6)</td>
<td>–</td>
<td>1.1 ± 0.2 (3)</td>
<td>1.6 ± 0.3 (5)</td>
</tr>
<tr>
<td>Real-time PCR</td>
<td>Hnf3β (%)</td>
<td>41 ± 7 (4)</td>
<td>–</td>
<td>98 ± 11 (5)</td>
<td>–</td>
<td>94 ± 15 (4)</td>
</tr>
<tr>
<td></td>
<td>Pdx1 (%)</td>
<td>46 ± 4 (4)</td>
<td>–</td>
<td>103 ± 9 (5)</td>
<td>–</td>
<td>107 ± 2 (4)</td>
</tr>
<tr>
<td></td>
<td>Hnf4a (%)</td>
<td>30 ± 1 (4)</td>
<td>–</td>
<td>99 ± 12 (5)</td>
<td>–</td>
<td>89 ± 23 (4)</td>
</tr>
<tr>
<td></td>
<td>Glut2 (%)</td>
<td>69 ± 12 (4)</td>
<td>–</td>
<td>93 ± 10 (5)</td>
<td>–</td>
<td>97 ± 15 (4)</td>
</tr>
<tr>
<td>8 weeks</td>
<td>β Cell area</td>
<td>0.45 ± 0.06 (5)</td>
<td>–</td>
<td>0.94 ± 0.08 (6)</td>
<td>–</td>
<td>0.9 ± 0.1 (4)</td>
</tr>
<tr>
<td>6 months</td>
<td>β Cell area</td>
<td>1.1 ± 0.1 (5)</td>
<td>0.20 ± 0.05 (6)</td>
<td>–</td>
<td>1.6 ± 0.2 (6)</td>
<td>–</td>
</tr>
<tr>
<td>10 months</td>
<td>β Cell area (% of total)</td>
<td>3.4 ± 0.1 (5)</td>
<td>–</td>
<td>3.5 ± 0.6 (4)</td>
<td>–</td>
<td>3.7 ± 0.1 (4)</td>
</tr>
<tr>
<td></td>
<td>Cell mass (µg/pancreas)</td>
<td>1,698 ± 79 (5)</td>
<td>–</td>
<td>1,414 ± 246 (4)</td>
<td>–</td>
<td>1,341 ± 91 (4)</td>
</tr>
<tr>
<td></td>
<td>Islets/pancreas (&lt;10 β cells)</td>
<td>52 ± 9 (4)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Islets/pancreas (&lt;10 β cells)</td>
<td>25 ± 6 (4)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>22 ± 3 (3)</td>
</tr>
<tr>
<td></td>
<td>Total islets/pancreas</td>
<td>77 ± 11</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Table 1

Islet characteristics were calculated as previously described (9) and summarized briefly in Methods. The number of animals used for each genotype and the average ± SE is reported. *P < 0.05; **P < 0.01, –, not determined.
minutes after intravenous insulin or saline injections. Irs2 protein levels and tyrosine phosphorylation were reduced 30% in fIrs2:cr2 extracts in comparison with WT, whereas levels of signal transducer and activator of transcription 3 (Stat3) protein — measured as a control — were equal in both mice (Figure 5A). Moreover, less p85α/β was detected in Irs2 immunoprecipitates, and insulin-stimulated Akt phosphorylation was decreased in fIrs2:cr2 hypothalamic extracts (Figure 5A). These results confirmed that insulin signaling was reduced in fIrs2:cr2 hypothalamus. By comparison, leptin-stimulated Stat3 or Akt phosphorylation were not significantly changed in fIrs2:cr2 mice (data not shown).

Food intake and insulin secretion increases αMSH secretion from POMC neurons in the hypothalamus, which stimulate MC4R signaling that promotes satiety (35). Our results were consistent with characteristics of reduced MC4R signaling, including excess appetite: Pomc1 mRNA levels — measured on Affymetrix GeneChip Mouse Expression Array 430A — were reduced almost 2-fold (90% confidence) in the hypothalamus of 16-hour–starved fIrs2:cr2 mice (Figure 5B). Other neuropeptide mRNAs — neuropeptide Y (Npy), agouti-related protein (Agrp), and cocaine and amphetamine regulated transcript (Cart) — were not changed; and the mRNA levels encoding Stat3, suppressor of cytokine signaling 3 (Socs3), and pro-protein convertase-2 — which processes αMSH — were normal (Figure 5B).

Since αMSH is a product of the POMC transcript in the arcuate nucleus, we immunostained hypothalamic sections from 8-week-old WT or fIrs2:cr2 mice with antibodies against insulin (green) or glucagon (red).

stimulated Akt phosphorylation was decreased in fIrs2:cr2 hypothalamic extracts (Figure 5A). These results confirmed that insulin signaling was reduced in fIrs2:cr2 hypothalamus. By comparison, leptin-stimulated Stat3 or Akt phosphorylation were not significantly changed in fIrs2:cr2 mice (data not shown).

Food intake and insulin secretion increases αMSH secretion from POMC neurons in the hypothalamus, which stimulate MC4R signaling that promotes satiety (35). Our results were consistent with characteristics of reduced MC4R signaling, including excess appetite: Pomc1 mRNA levels — measured on Affymetrix GeneChip Mouse Expression Array 430A — were reduced almost 2-fold (90% confidence) in the hypothalamus of 16-hour–starved fIrs2:cr2 mice (Figure 5B). Other neuropeptide mRNAs — neuropeptide Y (Npy), agouti-related protein (Agrp), and cocaine and amphetamine regulated transcript (Cart) — were not changed; and the mRNA levels encoding Stat3, suppressor of cytokine signaling 3 (Socs3), and pro-protein convertase-2 — which processes αMSH — were normal (Figure 5B).

Since αMSH is a product of the POMC transcript in the arcuate nucleus, we immunostained hypothalamic sections from 8-week-old WT or fIrs2:cr2 mice with antibodies against Ins2 or αMSH (Figure 5C). The size of the hypothalamus appeared normal. In WT mice, Irs2 and αMSH were coexpressed — as indicated by the yellow staining — in many but not all of the neurons near the 3rd ventricle; however, yellow staining was absent in the fIrs2:cr2 sections, which suggested that many αMSH neurons no longer expressed Irs2. These results show that some POMC neurons in the arcuate nucleus of fIrs2:cr2 mice were uncoupled from Irs2 signaling.
To confirm that weight gained by the \textit{firs2:cr} mice during the first 8 weeks of life was due to dysregulated \textit{Irs2} signaling in the CNS and not \(\beta\) cell failure, we restored \(\beta\) cell function by introducing transgenic \textit{Irs2} into the \(\beta\) cells of \textit{firs2:cr} mice. This was accomplished through an intercross with \textit{rip13ln2} mice, which express recombinant \textit{Irs2} in the \(\beta\) cells but not in the hypothalamus (9). Hyperglycemia did not occur in the \textit{firs2:cr} mice; however, the mice continued to gain excess weight (Figure 5D). Thus, partially dysregulated \textit{Irs2} signaling in the brain, rather than in \(\beta\) cells, appears to be responsible for hyperphagia and obesity in the \textit{firs2:cr} mice.

**Discussion**

Our results show that partial dysregulation of \textit{Irs2} signaling in \(\beta\) cells and brain — including the hypothalamus — may explain the close association between obesity, peripheral insulin resistance, and \(\beta\) cell failure that characterizes type 2 diabetes. Whether dysregulation of \textit{Irs2} signaling contributes to type 2 diabetes and obesity in humans is unknown. However, allelic variations at the human \textit{Irs2} locus appear to be linked to obesity, glucose intolerance, and polycystic ovarian syndrome (36–38). Moreover, \textit{Irs1} and \textit{Irs2} functions are strongly inhibited by many heterologous signals, especially proinflammatory cytokines produced during acute trauma or chronic metabolic stress (5). Moreover, \textit{Irs2} expression is strongly induced by the \(\text{cAMP} \rightarrow \text{CREB}\), which is activated by \(\text{cAMP} \rightarrow \text{AGP}\) agonists, including glucagon-like peptide 1 (GLP1) in \(\beta\) cells (10).

Disregulation of GLP1 signaling has been reported in type 2 diabetes, which might impair IRS2 expression in \(\beta\) cells and the brain (39, 40).

Male \textit{firs2:cr} mice develop diabetes between 2 and 3 months of age, at least in part because \(\beta\) cells fail to compensate for peripheral insulin resistance. Unlike systemic \textit{Irs2} mice that develop life-threatening diabetes earlier in life owing to complete loss of \(\beta\) cells, the \textit{firs2:cr} mice survive with diabetes until \(\beta\) cell regeneration repopulates the islets with functional \(\beta\) cells that cure diabetes. In \textit{firs2:cr} mice, the new \(\beta\) cells must arise from cells that never expressed Cre. The progressive decline of Cre recombinase in islets is consistent with this mechanism. Inactivation of \(\text{c}^2\) by hyperglycemia — as shown for the insulin-2 gene itself—might explain the progressive loss of Cre that prevented deletion of \textit{firs2} alleles in the new \(\beta\) cells (41); however, more work is needed to understand the exact mechanism involved.

New \(\beta\) cells are thought to arise by division of \(\beta\) cells themselves; by differentiation of islet precursors residing in pancreatic duct epithelium; or from bone marrow (42). Direct lineage tracing in adult mice suggests that terminally differentiated \(\beta\) cells retain a significant proliferative capacity in vivo and are an important — and possibly the only — source of new \(\beta\) cells in adult mice (42). If \(\beta\) cells that never express Cre recombinase are the sole source of \(\beta\) cell regeneration in the \textit{firs2:cr} mouse, the regenerative capacity must be significant. Intact \textit{firs2} is barely detected in pooled islets at 2 months of age; however, previous reports showing that \(\text{c}^2\) mediates nearly complete deletion of floxed genes in \(\beta\) cells (32, 43, 44). At least a portion of the weak PCR signal revealing...
intact fIrs2 in these islets arises from non-β cells. Thus, β cells in fIrs2:cr mice that retain Irs2 expression represent a distinct minority. Regardless of the source of regenerated β cells, IRS2 signaling must play an important role in the process.

By contrast, systemic deletion of one Irs1 allele from fIrs2:cr mice caused early-onset diabetes and impaired β cell regeneration. This result is unexpected, because Irs1 plays a limited role in islet growth (34). The absence of 1 Irs1 allele should not inhibit regeneration if the new β cells arise by replication of a few β cells that contain intact fIrs2. It remains possible that Irs1-dependent precursors contributed to the expansion of β cell mass in the fIrs2:cr mice. Alternatively, Irs1 signaling might sustain the function of Irs2-deficient β cells long enough to permit the few Irs2-containing β cells an opportunity to undergo division and repopulate the islets.

Diabetes is averted by the expression of transgenic Irs2 in the fIrs2:cr β cells, which suggests that strategies to promote Irs2 expression may prevent or cure type 2 diabetes. Irs2 mRNA and Irs2 protein is strongly upregulated in β cells by cAMP → CREB signaling (10). A relevant example of this mechanism is the increased expression of IRS2 during activation of the GLP1 receptor by GLP1 itself or the long-acting homolog exendin-4 (9, 10). Improved insulin secretion, increased insulin sensitivity, and better appetite regulation reported in diabetic rodents and people treated with GLP1 or exendin-4 can be explained by increased expression of IRS2 (5, 45). Since Irs2 function is inhibited by serine phosphorylation and ubiquitin-mediated degradation that is mediated by proinflammatory cytokines (46), increased IRS2 expression together with effective management of chronic inflammation might prevent the progression of insulin resistance to diabetes.

The mild disruption of fIrs2 by the cr in the hypothalamus displays the features of dysregulated melanocortin signaling — increased appetite, lean and fat body mass, linear growth, and peripheral insulin resistance (15). Although obesity is usually thought to contribute directly to peripheral insulin resistance, lean fIrs2:cr mice were also relatively insulin resistant, hyperinsulinemic, and glucose intolerant compared with the control mice. The results from the hyperinsulinemic clamp suggest that the insulin resistance in the fIrs2:cr mice resides in extra hepatic tissues — most likely the skeletal muscle — as hepatic glucose output is regulated normally by insulin, whereas glucose disposal is impaired. Previous work shows that dysregulated melanocortin signaling causes peripheral insulin resistance even before obesity develops, possibly because activation of MC4Rs in the paraventricular nucleus promotes peripheral insulin action (47, 48). The reduced levels of Pomc1 mRNA in fIrs2:cr hypothalamus and the loss of Irs2 from neurons that express αMSH reveal a potential mechanism for these effects. Thus, dysregulated Irs2 signaling in the brain, especially the hypothalamus, could contribute to central and peripheral insulin resistance that predisposes individuals to obesity and diabetes (25).

The deletion of floxed insulin or Igf1 receptors by cr expression does not alter food intake or body weight (43, 49). If more careful analysis reveals the deletion of these receptor genes in the hypothalamus — as reported here for fIrs2 and elsewhere for floxed Stat3 (32) — then loss of insulin or Igf1 receptor signaling might not promote obesity. This potential result would be similar to that obtained using the phenotype of neuron-specific insulin receptor KO (NIRKO) mice; only when placed on a diet containing 60% fat do NIRKO mice develop obesity.

Our work shows that Irs2 signaling is important for β cell growth, function, and survival. Moreover, our experiments show that β cell regeneration in middle age can cure diabetes in mice and that Irs2 signaling provides a significant advantage for this regeneration. Now we must determine whether the new β cells arise from the division of a few preexisting β cells that escaped fIrs2 deletion or from non-β cell precursors that never express Cre recombinase when they differentiate at middle age. Independent of the mechanism, strategies to enhance the Irs2 branch of the insulin/IGF-signaling pathway in β cells and hypothalamus could be a rational approach to prevent obesity and diabetes.

**Methods**

**Targeting vector and generation of mice.** All methods discussed in this section involving animals have been approved by the Institutional Animal Care and Use Committee (IACUC) of Children’s Hospital Boston, and by the Harvard School of Public Health review board. Irs2 genomic sequence was isolated from a 129 mouse genomic library as previously described (6). Two fragments of Irs2 gene were cloned into a pPNT backbone vector that contained a neomycin selection cassette flanked by LoxP sites (Figure 1A). A 2.3-kb 5′ Nhel/MscI fragment was inserted in front of the neo cassette, and an 8-kb Msel/Sall fragment — including a 200-bp 5′-untranslated region, 4-kb full-length coding sequence and the 3.8-kb 3′ region — was cloned from the neo cassette. The latter sequence contains the third LoxP site located 60-bp downstream from the stop codon. The linearized targeting vector was transfected into male R1 embryonic stem cells derived from 129 mouse strain (51). Double selection was performed with G418 and ganciclovir, and drug-resistant clones were screened for homologous recombination by Southern blotting. Recombinants were subject to transfection by a Cre-containing plasmid, and clones maintaining fIrs2 but losing the neo cassette were selected by Southern analysis and injected into blastocysts from C57BL/6 mice. Chimeric male pups were bred with C57BL/6 females to confirm germ-line transmission.

fIrs2 mice were purchased from The Jackson Laboratory and maintained in a heterozygous state. Generation of Irs1 KO mice and rip13Irs2 transgenic mice was as previously described (6, 9). To generate fIrs2:cr mice (mice homozygous for the fIrs2 allele and carrying cr transgene), we bred heterozygous fIrs2 with cr first, and then mice double heterozygous for fIrs2 and cr were mated with fIrs2 heterozygous mice to obtain fIrs2:cr and control mice (mice homozygous for the fIrs2 allele, cr2 transgenetic, and WT). To generate fIrs2:cr:ir1crβ and fIrs2:cr:ir1β mice, fIrs2:cr females were bred with fIrs2:cr:ir1crβ and fIrs2:cr:ir1β mice, respectively. All mice were maintained on a mixed C57BL/6 x 129Sv genetic background, housed in a pathogen-free environment in a 12-hour light/dark cycle facility, and fed a normal (9%) or low-fat (5%) diet (Research Diet). Routine genotyping was performed on tail DNA by PCR. Primers for fIrs2 allele were 5′-ACTTGAGGAAGCCACAGTCG and 3′-GTC-CACCTTCTGACAGGC. They flanked the last LoxP site and amplified a 200-bp product in WT allele and a 240-bp product in fIrs2 allele. Genotyping for ir1, np13β, and cr2 mice has been described (6, 9, 43).

**Assessment of recombination.** Mice were anesthetized with sodium amobarbital (100 mg kg–1). Islets were isolated by intraductal collagenase digestion (13). Various tissues were collected for genomic DNA extraction. Recombination was assessed by PCR analysis using two 5′ and one 3′ primers. The first 5′ primer, TCCGATCATATTCAATAACCCTTA, which included the first of the last LoxP sequence, paired with the 3′ primer, TACATCTGAGAAGCCACAGTCG and 3′-GTC-CACCTTCTGACAGGC, which was located downstream of the last LoxP site, to give rise to a 250-bp recombinated product. The other 5′ primer, AGCTC-GTCGCCACAGTCCAGG, in the coding region of ir2, paired with the same 3′ primer to amplify a 750-bp un-recombined fIrs2 product.

**Metabolic analysis.** Body weight was measured weekly beginning on postnatal day 21. Food intake during 24 hours was determined using the Comprehensive Lab Animal Monitoring System (Columbus Instru-
ments). Lean and fat body mass were determined by dual-energy x-ray absorptiometry (52). Carcasses were digested by alcoholic potassium hydroxide hydrolysis at 60°C overnight, and body lipid was determined as described previously (53).

Glucose and insulin levels and the various tolerance tests were performed upon fasted mice (16-hour overnight fast) or random-fed mice (test conducted in the morning between 9:00 and 11:00 without an overnight fast). Glucose levels were measured in mouse-tail blood using a Glucometer Elite (Bayer), and glucose tolerance tests were performed on mice after a 16-hour overnight fast (6). Blood insulin and leptin levels were determined from tail bleeds using a Rat Insulin ELISA kit and Mouse Leptin ELISA kit (Crystal Chem Inc.) (9).

Euglycemic hyperinsulinemic clamp. Euglycemic hyperinsulinemic clamp was performed on fasted conscious mice as previously described (54). [3-3H] glucose (NEN) was continuously infused during a 4-hour period at the rate of 0.05 μCi/min. Basal hepatic glucose output was measured in blood collected at 100 and 120 minutes after initiation of the [3-3H] glucose infusion. Then a primed continuous infusion of human regular insulin (Humulin; Eli Lilly and Co.) was initiated at a rate of 20 pmol/μg of supernatants was separated by SDS-PAGE and transferred to nitrocellulose. Membranes were probed with phospho-specific anti-Akt antibody (Cell Signaling Technology). The same membranes were stripped and re-probed with anti-Akt antibody (Cell Signaling Technology) to reveal Akt expression levels. Membranes were probed with anti-Stat3 antibody (Cell Signaling Technology) to serve as a protein loading control. The antibodies were detected by enhanced chemiluminescence (Calbiochem) and quantified using ImageQuant software (Amersham Biosciences).

Immunohistochemistry and morphometric analysis. Pancreata were removed from mice, cleared of fat and lymph nodes, fixed overnight in 4% paraformaldehyde, and embedded in paraffin. Sections (5 μm in thickness) were stained with anti-insulin antibody (Jackson ImmunoResearch Laboratories Inc.), β Cell area was presented as percentage of the total surveyed pancreatic area occupied by β cells and determined by point-counting morphometry as previously described (6). Briefly, sections were viewed using a Zeiss Axiosvert 100TV (Carl Zeiss) microscope at a magnification of ×10. The islet cross-sectional area and total pancreatic area were measured using Openlab Image analysis software (version 3.14; Improvision). β Cell mass was calculated by multiplying β cell area by pancreatic weight. β Cell size was determined by dividing β cell area by β cell number. β Cell proliferation was determined by BrdU incorporation (100 μg/g body weight; Roche Molecular Biochemicals) was injected intraperitoneally 6 hours before pancreas was collected. Double immunostaining for insulin and BrdU (Roche Molecular Biochemicals) was performed on pancreas sections. To distinguish between large (>10 β cells) and small (<10 β cells) islets, 1 entire section of pancreas immunostained by insulin—from 3 or 4 mice as indicated—was examined. For immunostaining of brain sections, mice were perfused with 10% formalin, and then whole brain was sliced with Microtome (American Optical Company). Thirty-μm-thick sections were stained with antibodies against Irs2 (UBI), Cpe (Covance), or αMSH (Chemicon). Grayscale images were normalized using ImageJ software, aligned using the Turbo Registration plug-in to ImageJ, then recolored as shown in the figures (55).

Microarray analysis. After an overnight fast, the hypothalamus was collected from 2 WT, 2 firs2, 2 cr2 mice, and 3 firs2 cr2 8-week-old mice. Total RNA was extracted and puriﬁed from each sample using RNAqueous-4PCR kit (Ambion Inc.). cDNA synthesis and in vitro transcription was carried out using SuperScript Choice System (Invitrogen) and MEGAscript T7 Kit (Ambion Inc.) according to the manufacturers’ instructions. Labeled probes were prepared by in vitro transcription using second-cycle cDNA synthesis products, and the samples were hybridized to Affymetrix GeneChip Mouse Expression Array 430A in a GeneChip Fluidics Station 400 and scanned with a Hewlett-Packard GeneArray Scanner (56). The data files (dat format) were normalized to the median array using dCHIP software (version 1.31), and the expression levels were calculated using the perfect match–only model (57). All of the reported genes were flagged as present by both Affymetrix GeneChip Mouse Expression Array 430A and dCHIP. The fold change and the 90% conﬁdence interval were calculated for average firs2 cr2 samples (n = 3) against the average control samples (n = 6).

Acknowledgments
This work was supported by NIH grant DK55236. X. Lin was a Juvenile Diabetes Foundation (JDF) postdoctoral fellow while this work was conducted.

Received for publication May 20, 2004, and accepted in revised form July 20, 2004.

Address correspondence to: Morris F. White, Howard Hughes Medical Institute Children’s Hospital, Division of Endocrinology, Harvard Medical School, Karp Research Building, Room 04210, 300 Longwood Avenue, Boston, Massachusetts 02115, USA. Phone (617) 919-2846; E-mail: morris.white@childrens.harvard.edu.


