β-cell–specific deletion of the Igf1 receptor leads to hyperinsulinemia and glucose intolerance but does not alter β-cell mass

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Regulation of glucose homeostasis by insulin depends on the maintenance of normal β-cell mass and function. Insulin-like growth factor 1 (Igf1) has been implicated in islet development and differentiated function[1,2], but the factors controlling this process are poorly understood. Pancreatic islets produce Igf1 and Igf2, which bind to specific receptors on β-cells[3–6]. Igf1 has been shown to influence β-cell apoptosis[7], and both Igf1 and Igf2 increase islet growth[8,9]; Igf2 does so in a manner additive with fibroblast growth factor 2 (ref. 10). When mice deficient for the Igf1 receptor (*Igf1r*$^{+/-}$) are bred with mice lacking insulin receptor substrate 2 (*Irs2*$^{-/-}$), the resulting compound knockout mice show a reduction in mass of β-cells[11] similar to that observed in pancreas of *Igf1r*$^{-/-}$ mice (ref. 11), suggesting a role for Igf1r in growth of β-cells. It is possible, however, that the effects in these mice occur secondary to changes in vascular endothelium[12] or in the pancreatic ductal cells, or because of a decrease in the effects of other hormones implicated in islet growth. To directly define the role of Igf1, we have created a mouse with a β-cell–specific knockout of *Igf1r* (β*Igf1r*$^{-/-}$). These mice show normal growth and development of β-cells, but have reduced expression of *Slc2a2* (also known as *Glut2*) and *Gck* (encoding glucokinase) in β-cells, which results in defective glucose-stimulated insulin secretion and impaired glucose tolerance. Thus, Igf1r is not crucial for islet β-cell development, but participates in control of differentiated function.

We created mice with a β-cell specific knockout of *Igf1r* by breeding animals carrying *Igf1r* in which exon 3 was flanked with *lox*P sites (*Igf1r*$^{tm1.1Mhz}$, hereafter referred to as *Igf1r*$^{lox/lox}$)[13] with mice expressing *cre* driven by the rat insulin promoter (TgN(ins2-*cre*)25Mgn, hereafter referred to as *cre*$^{+/-}$; Fig. 1*a*)[14]. Control (*Igf1r*$^{lox/lox}$ and *cre*$^{+/-}$) and knockout mice were born normally and survived equally into adulthood. Homozygous knockout of *Igf1r* in β-cells but not other islet cells was confirmed by RT–PCR using RNA from β*Igf1r*$^{-/-}$ islet cells separated by flow cytometry[14](Fig. 1*b*), and by immunostaining of pancreas sections for Igf1 receptors (Fig. 1*c*). Pancreas sections from six-month-old control and β*Igf1r*$^{-/-}$ mice showed no significant differences in β-cell mass (β*Igf1r*$^{-/-}$, 1.1 ± 0.3 mg per

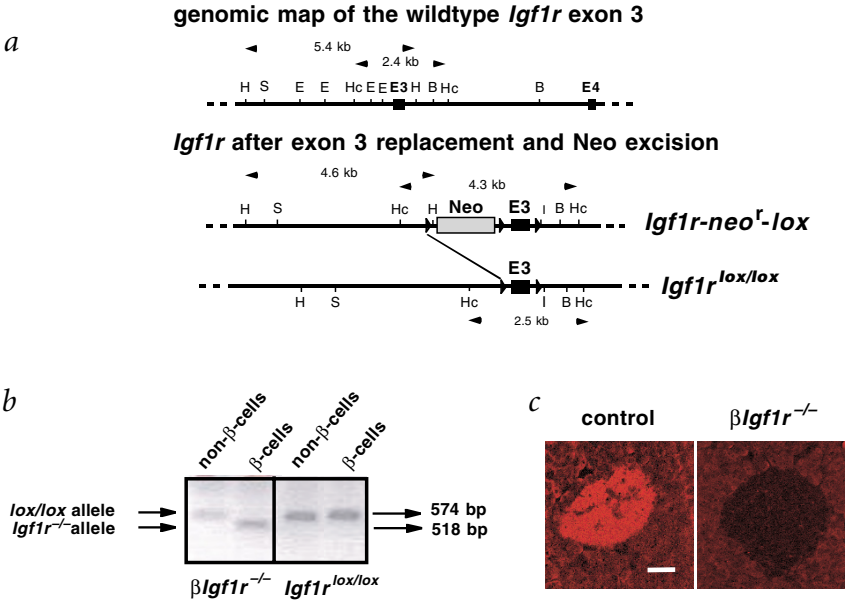

Fig. 1 Conditional *Igf1r* targeting allows β-cell–specific *Igf1r* knockout. ***a,*** The upper panel shows a partial genomic map of wildtype *Igf1r*. Individual letters represent restriction sites. The lower panel shows the targeted locus before and after excision of the neomycin selection cassette (Neo) that is flanked by one *lox*P site upstream and two *lox*P sites downstream of exon (E) 3. ***b***, RT–PCR analysis of RNA obtained from islet cells sorted by flow cytometry into β- and non–β-cells. Amplification from the knockout transcript (518 bp) is evident in the lane corresponding to the β*Igf1r*$^{-/-}$ β-cells, indicating absence of exon 3. All other lanes show the intact *lox/lox* allele (574 bp). ***c,*** Representative islets from pancreas sections of a *Igf1r*$^{lox/lox}$ control and a β*Igf1r*$^{-/-}$ mouse stained for Igf1 receptors. Scale bar, 25 μm.

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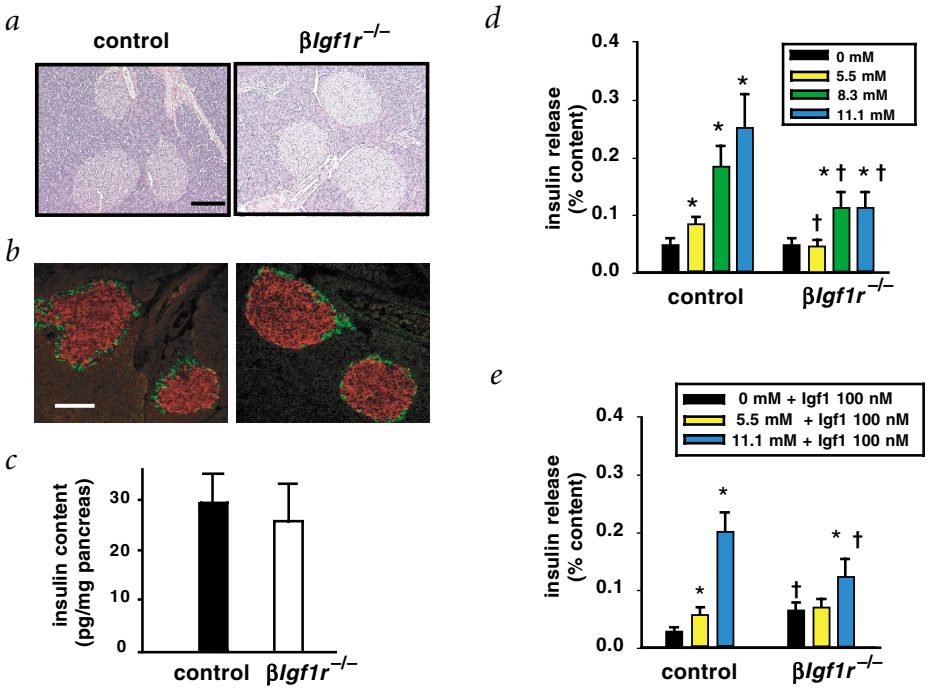

Fig. 2 β*Igf1r*$^{-/-}$ islets show normal morphology and insulin content but have blunted glucose-stimulated insulin secretion and loss of Igf1-mediated suppression of insulin secretion. ***a***, Hematoxylin and eosin staining of representative sections of pancreas isolated from control and β*Igf1r*$^{-/-}$ mice. Scale bar, 25 μm. ***b***, Representative pancreas sections visualized by immunofluorescence after staining for insulin (red) using guinea pig anti-human insulin antibody and glucagon (green) using rabbit anti-glucagon antibody. Scale bar, 25 μm. ***c***, Insulin content in islets extracted with acid ethanol. ***d***, After overnight culture, islets isolated from the two groups were incubated in fresh medium for 30 min with increasing concentrations of glucose. The medium was then removed and assayed for insulin using an ELISA kit. The asterisk indicates $P<0.01$ versus basal (0 mM), and the cross indicates $P<0.05$, β*Igf1r*$^{-/-}$ versus controls (*Igf1r*$^{lox/lox}$ and *cre*$^{+/-}$); $n=5$. ***e***, Cultured islets were placed in fresh medium containing Igf1 and different concentrations of glucose for 30 min. The medium was then removed and assayed for insulin. The asterisk indicates $P<0.01$ versus basal (0 mM), and the cross indicates $P<0.01$, β*Igf1r*$^{-/-}$ versus controls; $n=5$.

pancreas versus control, 1.4 ± 0.2 mg per pancreas, $n=4$, P=NS) or non–β-cell mass (β*Igf1r*$^{-/-}$, 0.22 ± 0.08 mg per pancreas versus control, 0.28 ± 0.06 mg per pancreas, $n=4$, P=NS; Fig. 2*a*). Similarly, insulin and glucagon content, as determined by immunostaining (Fig. 2*b*) or islet hormone gene expression assessed by semi-quantitative RT–PCR (Fig. 4), and acid ethanol extraction of pancreas for insulin (Fig. 2*c*), were not significantly different between the two groups. The lack of an alteration in islet size in the β*Igf1r*$^{-/-}$ mice, as compared with the decrease in islet mass in *Igf1r*$^{-/-}$ mice[11], indicates that the islet growth defect in the *Igf1r*$^{-/-}$ mice probably results from loss of Igf1 action in cells other than the β-cell itself.

In addition to its potential role in islet growth, Igf1 has been shown to suppress insulin release[15]. To determine the presence of potential secretory defects, islets isolated from β*Igf1r*$^{-/-}$ and control mice were cultured overnight and then stimulated with glucose. Control islets showed a concentration-dependent stimulation in insulin secretion with an increase of roughly threefold at 11.1 mM glucose (Fig. 2*d*). In the β*Igf1r*$^{-/-}$ islets, we detected no stimulation at 5.5 mM glucose and only small increases at higher glucose concentrations (Fig. 2*d*). To determine the effect of Igf1 on insulin secretion *in vitro*, we carried out similar experiments on islets cultured in the presence of 100 nM Igf1. In the basal state and in the absence of glucose, β*Igf1r*$^{-/-}$ islets secreted higher amounts of insulin than controls, suggesting the normal suppressive effect of Igf1 is reduced in β*Igf1r*$^{-/-}$ islets (Fig. 2*e*). Despite the higher basal secretion, upon glucose challenge, the β*Igf1r*$^{-/-}$ islets had significantly lower insulin secretion than the controls (Fig. 2*e*), indicating a glucose-stimulatory defect similar to that observed *in vivo*.

We detected a mild increase in *in vivo* fasting and fed circulating insulin levels in β*Igf1r*$^{-/-}$ mice, whereas we did not find significant differences in circulating glucagon and glucose levels between the groups (Table 1). These data suggest that *in vivo*, similar to the results obtained *in vitro*, inhibition of insulin secretion by Igf1 is lost in the β*Igf1r*$^{-/-}$ mice, leading to an increase in circulating insulin. We also found significantly higher C-peptide levels, a surrogate marker for insulin secretion, in the β*Igf1r*$^{-/-}$ mice in the fasting and fed states (Table 1). The normal sensitivity of these knockout mice to injected insulin rules out the possibility of insulin resistance (Fig. 3*d*). In addition, to evaluate whether peripheral insulin sensitivity is altered in β*Igf1r*$^{-/-}$ mice secondary to changes in hormones and metabolites, we measured Igf1, leptin, corticosterone, free fatty acids, triglycerides and cholesterol. No significant differences were detected between knockout mice and controls at approximately two months of age (Table 1). Taken together, these results indicate that the mild elevation in circulating insulin *in vivo* may compensate to maintain blood glucose in the normal range in β*Igf1r*$^{-/-}$ mice.

To assess the impact of localized Igf1 resistance on regulated insulin secretion *in vivo*, we examined insulin secretion in

Table 1 • Metabolic parameters in control and β*Igf1r*$^{-/-}$ mice

Metabolic parameters		Controls	β*Igf1r*$^{-/-}$
glucose (mg/dl)	fast	94 ± 6	82 ± 9
	fed	164 ± 8	172 ± 18
insulin (ng/ml)	fast	0.4 ± 0.1	0.9 ± 0.2*
	fed	1.3 ± 0.4	2.4 ± 0.3*
C-peptide (pmol/l)	fast	71 ± 12.4	119 ± 6.8*
	fed	188 ± 10.2	294 ± 14.6*
glucagon (pg/ml)	fast	69 ± 11	65 ±13
	fed	58 ± 8	54 ± 9
leptin (ng/ml)	fed	2.3 ± 0.6	1.8 ± 0.7
Igf-I (ng/ml)	fed	216 ± 24	197 ± 28
corticosterone (ng/ml)	fed	176 ± 13	184 ± 10
free fatty acids (mEq/l)	fast	742 ± 58	795 ± 64
triglycerides (mg/dl)	fast	88.7 ± 7.0	82.8 ± 5.5
cholesterol (mg/dl)	fast	78.0 ± 5.5	81.4 ± 4.1

*$P<0.05$ β*Igf1r*$^{-/-}$ versus control (*Igf1r*$^{lox/lox}$ and *cre*$^{+/-}$) mice, $n=12$–14.

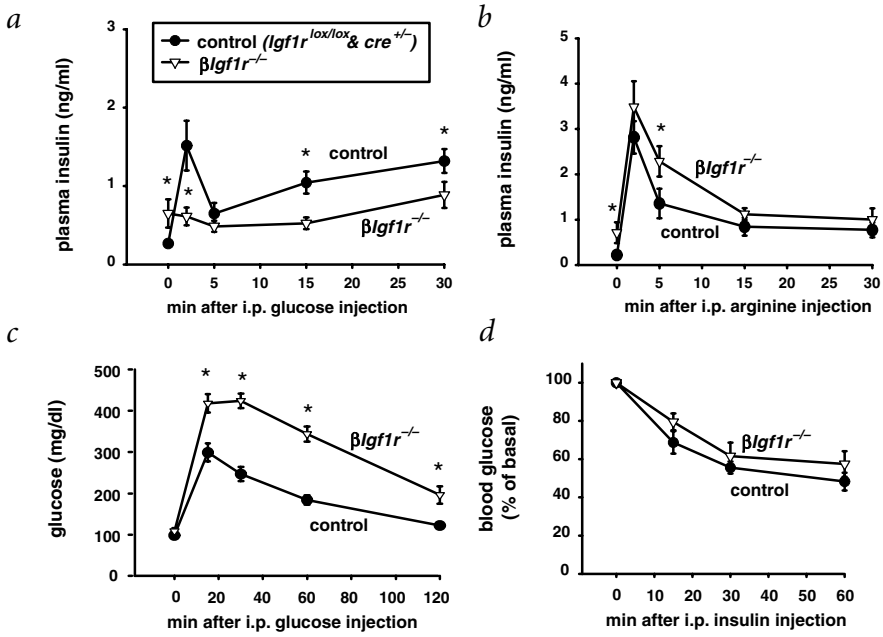

Fig. 3 β*Igf1r*$^{-/-}$ mice lack glucose-stimulated, acute-phase insulin secretion and have glucose intolerance. ***a***,***b***, Acute (first) phase and second-phase insulin secretion after an i.p. injection of glucose (*a*) or L-arginine (*b*), in male β*Igf1r*$^{-/-}$ and control mice. The asterisk indicates $P<0.01$, β*Igf1r*$^{-/-}$ mice versus controls; $n=8$–12. ***c***, Blood glucose levels following an i.p. injection of glucose. The asterisk indicates $P<0.001$, β*Igf1r*$^{-/-}$ mice versus controls; $n=8$–10. ***d***, Insulin tolerance tests were carried out on random-fed β*Igf1r*$^{-/-}$ and control male mice at 10 wk. Blood glucose levels before and after an i.p. injection of human regular insulin are shown; $n=8$–12.

response to intraperitoneal injection of glucose or L-arginine. In agreement with the findings *in vitro*, we observed an absent acute (first phase) insulin response after glucose stimulation and a blunted second-phase insulin secretion pattern in the β*Igf1r*$^{-/-}$ mice, compared with robust responses in both the *Igf1r*$^{lox/lox}$ and *cre*$^{+/-}$ controls (Fig. 3*a*). Consequently, during the glucose tolerance test, glucose levels remained high in the β*Igf1r*$^{-/-}$ mice for up to two hours, indicating severe glucose intolerance (Fig. 3*c*). By contrast, we observed similar insulin secretory responses between the two groups after L-arginine injection, confirming a glucose-specific secretory defect in the knockouts (Fig. 3*b*).

To identify the mechanism underlying the loss of glucose-stimulated insulin secretion, we evaluated steady-state mRNA levels of glucose-sensing proteins and the hepatocyte nuclear factor (*Hnf*) family of transcription factors[16] in islet RNA from the two groups. mRNA from β*Igf1r*$^{-/-}$ islets showed a nearly complete loss of *Igf1r* expression, whereas levels of insulin receptor mRNA were similar between the groups (Fig. 4). Notably, we detected decreases of 35–50% in *Slc2a2* and *Gck* expression levels in the β*Igf1r*$^{-/-}$ mice, indicating that loss of the β-cell *Igf1r* negatively influences the expression of the glucose-sensing proteins (Fig. 4). Moreover, we observed significantly reduced *Foxa2* (also known as *Hnf3b*) and *Tcf2* (also known as *HNF-1β*) mRNA levels in the β*Igf1r*$^{-/-}$ islets, suggesting a role for Igf1r in modulating the expression of early transcription factors.

These results indicate that Igf1r in β-cells is not crucial for islet growth, but is important in differentiated function of the β-cell. These results seem to conflict with studies showing that *Igf1r*$^{-/-}$ or *Irs2*$^{-/-}$ mice, or mutants with a combination of the two genotypes, have decreased β-cell mass[11]. In these earlier models, however, it is not clear whether the alterations are due to a defect in Igf1 signaling in the β-cells themselves or due to defects in Igf1 signaling at all. Thus, in global knockouts (*Igf1r*$^{-/-}$), Igf1 might affect either primordial stem cells or ductal cells that may give rise to β-cells, or endothelial cells or other cells that support islet growth[12], or might provide important paracrine or endocrine factors for islet growth and differentiation. Similarly, the defects in islet development in *Irs2*$^{-/-}$ mice might reflect defects in Igf1-independent signaling pathways, as Irs2 acts in growth hormone, prolactin and cytokine signaling[17,18], all of which may influence islet growth, survival and function. In addition, genetic background may contribute to the differences in phenotypes observed between these mouse models[19].

In the β*Igf1r*$^{-/-}$ model, the results are more clear-cut, as the defect is in a single signaling pathway in a single cell type. However, because the β*Igf1r*$^{-/-}$ mice were created using a *cre* transgene controlled by the rat insulin promoter, we cannot rule out the possibility that Igf1r is important before insulin expression at

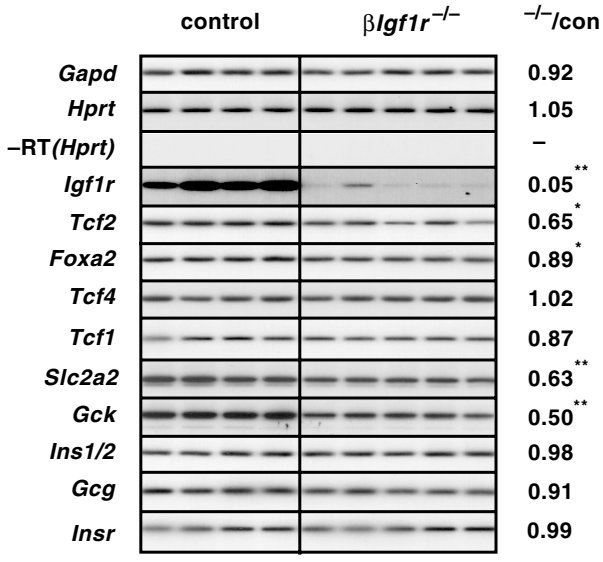

Fig. 4 Reduced steady-state mRNA levels of islet-enriched transcription factors and glucose-sensing proteins in β*Igf1r*$^{-/-}$ islets. RT–PCR analysis of islet-enriched transcription factors, glucose-sensing proteins, islet hormones and insulin receptors in islets isolated from β*Igf1r*$^{-/-}$ and control male mice. Samples were normalized using *Gapd* and the mitochondrial marker hypoxanthine guanosine phosphoribosyl transferase (*Hprt*). Quantitative measurements were obtained by densitometry and expressed as a ratio of the mean values of β*Igf1r*$^{-/-}$ mice ($^{-/-}$) to controls (con). The single asterisk indicates $P<0.05$, and the double asterisk indicates $P<0.001$, β*Igf1r*$^{-/-}$ versus controls; $n=4$–5. *Igf1r*, Igf1 receptor; *Tcf2*, transcription factor 1; *Foxa2*, forkhead Box A2; *Tcf4*, transcription factor 4; *Tcf1*, transcription factor 1; *Slc2a2*, solute carrier family 2 member 2; *Gck*, glucokinase; *Ins1/2*, insulin I/II; *Insr*, insulin receptor.

day 9 of embryogenesis[20], similar to the role of Fgf-7 (refs 10,21). Another caution is that we have examined the role of Igf1 receptors in islet growth only under normal circumstances. Further study will be required to determine whether Igf1 receptors are important in the islet compensatory response observed in many diabetic states[22], or in response to injury[23].

Finally, it is worth comparing the effects of loss of *Igf1r* with loss of the insulin receptor gene (*Insr*) in β-cells. Although neither receptor is essential for prenatal islet growth, the role of Insr may be more important than Igf1r in postnatal growth. Mice lacking Insr in β-cells (β*Insr*$^{-/-}$ mice) show a decrease in the age-dependent growth of islets[14], whereas postnatal growth is normal in β*Igf1r*$^{-/-}$ mice. By contrast, both β*Insr*$^{-/-}$ and β*Igf1r*$^{-/-}$ mice have glucose-sensing defects. It has been shown that insulin stimulation can increase intracellular calcium concentrations in β-cells[24] and increase the levels of glucokinase mRNA (refs 25,26), and that insulin signaling is required for normal glucose sensing by pancreatic β-cells[14,25,26]. These effects depend on signaling through the Irs proteins[24–28], involving both the PI 3-kinase and p70 S6 kinase pathways[24–26,28]. The similarity of the defects in the β*Igf1r*$^{-/-}$ and β*Insr*$^{-/-}$ mice might reflect the use of insulin/Igf1 hybrid receptors for these overlapping signaling pathways[3,4]. Preliminary findings in mice with β-cell–specific knockout of *Insr* and *Igf1r* indicate that the defects due to functional loss of both receptors are additive and lead to early-onset severe diabetes (R.N.K. and C.R.K., unpublished data). Thus, defects in insulin and Igf1 signaling in the β-cell indicate an important link between Igf1 and insulin action, Igf1 and insulin resistance and alterations in the function of the pancreatic β-cell.

Methods

Animals. *Igf1r* floxed mice were created as described previously[13] and maintained on a mixed (C57BL/6 × 129/Sv) genetic background. Mice expressing *cre* recombinase under a rat insulin promoter[14] were maintained on a mixed (C57BL/6 × 129Sv × DBA/2) genetic background and bred with the floxed mice in the Joslin Animal Facility. All mice were housed in pathogen-free facilities on a 12-h light/dark cycle and had free access to water and food. We used male mice at 2–6 mo for all experiments. All protocols for animal use and euthanasia were reviewed and approved by the Animal Care Committee of the Joslin Diabetes Center and were in accordance with the guidelines of the National Institutes of Health. We carried out genotyping by PCR using genomic DNA isolated from the tail tip.

Islet morphology and immunohistochemistry. Tissues were fixed in Bouin solution and 10% buffered formalin and embedded in paraffin. Sections of pancreas were stained for non–β-cell hormones using a mixture of antibodies to glucagon, somatostatin and pancreatic polypeptide[14]. We evaluated β-cell mass by point-counting morphometry on immunoperoxidase-stained sections of pancreas[14,27]. We carried out immunofluorescent staining for insulin and glucagon using guinea pig anti-human insulin antibody (Linco) and rabbit anti-glucagon antibody (Zymed). Secondary antibodies included Texas Red dye–conjugated donkey anti–guinea pig IgG (Jackson Immunoresearch) for insulin and FITC-conjugated goat anti-rabbit IgG (Zymed) for glucagon. We carried out immunofluorescent staining for Igf1r using a rabbit polyclonal antibody against the Igf1r α-subunit (Santa Cruz Biotechnology) and detected with a TRITC-conjugated goat anti-rabbit IgG (Zymed). Images were captured using a confocal microscope (Zeiss).

Analysis of metabolic parameters. We determined blood glucose values from whole venous blood using an automated glucose monitor (Glucometer Elite, Bayer). We measured serum insulin and leptin levels by ELISA, using mouse insulin and leptin standards (Crystal Chem.). For glucagon, blood samples were collected in tubes containing aprotinin (1 μg ml^{-1}, Bayer), and the serum was stored at –80 °C before radio-immunoassay (Linco). We measured serum levels of Igf1 using an RIA kit (ALPCO) and determined serum levels of corticosterone by RIA (ICN Biomedicals). We measured triglyceride levels in serum from fasted animals by a colorimetric enzyme method using the GPO-Trinder Assay (Sigma). Free fatty acid levels were analyzed in serum from fasted animals using the NEFA-C-Kit (WAKO). We analyzed total cholesterol levels colorimetrically, using a kit (Sigma).

Glucose and insulin tolerance tests and *in vivo* and *in vitro* insulin secretion. We carried out glucose tolerance tests and acute insulin secretion tests on animals that had been fasted overnight for 16 h (ref. 14). Animals were injected i.p. with 2 g kg^{-1} body weight of glucose (glucose tolerance tests) or 3 g kg^{-1} body weight of glucose (acute insulin secretion) or L-arginine (10 mM in 0.2 M PBS, acute insulin secretion). For glucose tolerance tests, glucose levels were measured from blood collected from the tail immediately before, and 15, 30, 60 and 120 min after, the injection. For acute insulin secretion, blood samples were collected before and 2, 5, 15 and 30 min after injection of the stimulus, and serum insulin was measured as described above. We carried out insulin tolerance tests on animals in the random-fed state. Animals were injected i.p. with 0.75 U kg^{-1} body weight of human regular insulin (Eli Lilly). We collected blood samples before and 15, 30 and 60 min after injection. Islets were isolated using the intra-ductal collagenase method[27]. We measured *in vitro* insulin release from isolated islets by static incubation of islets cultured overnight. We measured insulin content in whole pancreas and islets in acid-ethanol extracts using an ELISA kit (Crystal Chem.).

Flow cytometry and RT–PCR. Isolated islets were dispersed into β- and non–β-cell fractions by flow cytometry, and RT–PCR analysis was carried out as previously described[14]. We prepared RNA from the cell fractions using an RNeasy Mini Kit (Qiagen). We used three oligonucleotides to detect *Igf1r* mRNA from exon 3– and exon 3+ alleles with comparable efficiency in single triplex RT–PCR (Pharmacia Biotech). To measure steady-state mRNA levels, we carried out semi-quantitative RT–PCR with islet RNA (Trizol, Gibco BRL), using a previously described method[29]. Contaminating DNA was removed using 1 μL of RNase-free DNase-I (Boehringer) per 5 μg of RNA. cDNAs were synthesized and used as templates for PCRs using specific primers at annealing temperatures of 60–65 °C in the presence of dNTPs and *Taq* polymerase. We generally used 20–25 cycles for amplification in the linear range. We quantified bands using densitometry (Biorad) and calculated ratios of expression levels of knockout and control mice. Primer sequences are available on request.

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Competing interests statement

The authors declare that they have no competing financial interests.

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