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ARTICLE

Pancreatic deletion of insulin receptor substrate 2 reduces beta and alpha cell mass and impairs glucose homeostasis in mice

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Abstract
Aims/hypothesis Insulin signalling pathways regulate pancreatic beta cell function. Conditional gene targeting using the Cre/loxP system has demonstrated that mice lacking insulin receptor substrate 2 (IRS2) in the beta cell have reduced beta cell mass. However, these studies have been complicated by hypothalamic deletion when the *RIPCre* (*B6.Cg-tg(Ins2-cre) 25Mgn/J*) transgenic mouse (expressing Cre recombinase under the control of the rat insulin II promoter) is used to delete floxed alleles in insulin-expressing cells. These features have led to marked insulin resistance making the beta cell-autonomous role of IRS2 difficult to determine. To establish the effect of deleting *Irs2* only in the pancreas, we generated *PIrs2KO* mice in which Cre recombinase expression was driven by the promoter of the pancreatic and duodenal homeobox factor 1 (*Pdx1*, also known as *Ipf1*) gene.
Materials and methods In vivo glucose homeostasis was examined in *PIrs2KO* mice using glucose tolerance and glucose-stimulated insulin secretion tests. Endocrine cell mass was determined by morphometric analysis. Islet function was examined in static cultures and by performing calcium imaging in Fluo3am-loaded beta cells. Islet gene expression was determined by RT-PCR.
Results The *PIrs2KO* mice displayed glucose intolerance and impaired glucose-stimulated insulin secretion in vivo. Pancreatic insulin and glucagon content and beta and alpha cell mass were reduced. Glucose-stimulated insulin secretion and calcium mobilisation were attenuated in *PIrs2KO* islets. Expression of the *Glut2* gene (also known as *Slc2a2*) was also reduced in *PIrs2KO* mice.
Conclusions/interpretation These studies suggest that IRS2-dependent signalling in pancreatic islets is required not only for the maintenance of normal beta and alpha cell mass but is also involved in the regulation of insulin secretion.

Keywords Diabetes · Insulin receptor substrate protein · Pancreatic islet

Abbreviations

FOXO1	forkhead box O1
GCG	glucagon
GCK	glucokinase
GSIS	glucose-stimulated insulin secretion
HBSS	Hanks' balanced salt solution
HPRT1	hypoxanthine phosphoribosyltransferase 1
IGF1R	insulin-like growth factor 1 receptor
INSR	insulin receptor
IRS	insulin receptor substrate
NEUROD1	neurogenic differentiation factor 1
NKX6-1	NK6 transcription factor related, locus 1

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PDX1	pancreatic and duodenal homeobox factor 1
SST	somatostatin

Introduction

Insulin regulates peripheral energy homeostasis by acting on multiple tissues to control carbohydrate, lipid and protein metabolism [1]. It has also been demonstrated that insulin receptor and post-receptor signalling mechanisms are required for pancreatic beta cell function [2]. For example, mice with global deletion of insulin receptor substrate (IRS) 2 develop type 2 diabetes due to a combination of insulin resistance and beta cell failure [3, 4]. Furthermore, cell-specific gene targeting in mice using Cre/loxP-mediated recombination strategies has shown that beta cell deletion of the insulin receptor reduces first-phase insulin release and beta cell insulin content and causes a progressive deterioration in glucose tolerance [5]. Deletion of the insulin-like growth factor 1 receptor gene (*Igf1r*) likewise impairs insulin synthesis and secretion and combined deletion of the insulin receptor gene (*Insr*) and *Igf1r* causes marked beta cell failure [6, 7]. Using similar techniques, we and others have recently generated 'beta cell-specific' *Irs2*-null mice that have utilised a rat insulin promoter 2 Cre recombinase transgenic mouse (*RIPCre* or *B6.Cg-tg(Ins2-cre)25Mgn/J*) to delete the floxed alleles of *Irs2* [8–11]. Our studies and those of others have demonstrated that beta cell deletion of *Irs2* reduces beta cell mass and impairs glucose tolerance. However, the interpretation of all these studies with respect to the precise role of INSR and IRS2 signalling in beta cell function has been complicated by features of the *RIPCre* transgenic mouse used to delete the floxed *Insr* and *Irs2* alleles. First, significant Cre recombinase expression in the hypothalamus resulted in the development of obesity, hyperleptinaemia and insulin resistance, which together per se will alter beta cell function [8–10]. Second, *RIPCre* mice themselves have been reported to display mild glucose intolerance, although it was unclear whether this was due to the influence of genetic background or the effects of Cre recombinase expression in beta cells [12].

To attempt to circumvent these problems, we have generated mice lacking *Irs2* in the pancreas using a mouse in which Cre recombinase expression is driven by the promoter of the pancreatic and duodenal homeobox factor 1 (*Pdx1,* also known as *Ipf1*) gene, one of the earliest and most specific endoderm markers of the developing pancreas [13]. These *Pdx1-cre* mice therefore efficiently delete floxed alleles in the endocrine and exocrine pancreas. Mice lacking *Irs2* in *Pdx1*-expressing cells (*PIrs2KO* mice) display impaired glucose tolerance with reduced beta and alpha cell mass, impaired insulin secretion, alterations in islet gene expression and beta cell calcium mobilisation but no reduction in insulin sensitivity or body weight alterations. Therefore these results demonstrate that intrinsic pancreatic IRS2 signalling is critical for islet endocrine cell function identifying a novel role for IRS2 in alpha cell function. These effects are independent of the central nervous system and are not due to the use of Cre recombinase technology. Furthermore, we demonstrate that in our hands *RIPCre* mice do not display glucose intolerance on two mixed genetic backgrounds, suggesting that the *RIPCre* transgene alone does not cause glucose intolerance.

Materials and methods

Mouse breeding and genotyping strategies Mice with a floxed allele of *Irs2* (*Irs2*^{tm1With} mice) [8] were intercrossed with *Tg(Pdx1-cre)1Herr* (*Pdx1-cre*) mice [13] to generate compound heterozygote mice. Double heterozygote mice were crossed with *Irs2*^{tm1With} mice to obtain wild-type, *Irs2*$^{tm1With/tm1With}$, *Pdx1-cre* and *Irs2*$^{tm1With/tm1With}$*Pdx1-cre* mice. The last of these mice, which lack *Irs2* in *Pdx1-cre*-expressing cells, are designated *PIrs2KO* mice. Mice were maintained on a 12-h light–dark cycle with free access to water and standard mouse chow (4% fat; RM1, Special Diet Services Ltd, Essex, UK) and housed in specific pathogen-free barrier facilities. Mice were handled and all in vivo studies were performed in accordance with the 1986 Home Office Animal Procedures Act (Home Office, London, UK). *PIrs2KO* mice were studied on a mixed *129Sv/C57Bl/6* background with appropriate litter-mate controls. Wild-type, *Cre* transgenic and *Irs2*$^{tm1With/tm1With}$ mice were phenotypically indistinguishable and mice of these genotypes were used as controls. *RIPCre* (*B6.Cg-tg(Ins2-cre)25Mgn/J*) mice were maintained as previously described [8]. Genotyping of the mice was performed by PCR amplification of tail DNA as described previously [8].

Metabolic studies Blood samples were collected from mice via tail vein bleeds or from cardiac puncture on terminally anaesthetised mice. Blood glucose, plasma insulin levels, glucose and insulin tolerance tests were performed as previously described [3, 8]. Glucose-stimulated insulin secretion in vivo was performed using an i.p. dose of 3 g/kg of glucose and tail vein bleeds at the times indicated.

Tissue preparation and western blotting Western blotting was performed as previously described [8]. In brief, tissues were homogenised in lysis buffer, solubilised for 30 min on ice and clarified by centrifugation. Supernatant fractions

were snap frozen in aliquots and stored at −80°C. For analysis of IRS2 production, tissue extracts (200 μg total protein) were immunoprecipitated for 2 h with polyclonal anti-IRS2 antibody. Immune complexes were collected with 40 μl of a 50% slurry of Protein-A agarose, washed with lysis buffer and resolved on 8% SDS-PAGE and transferred to nitrocellulose. The blots were probed with a polyclonal antibody against IRS2 and enhanced chemiluminescence (Amersham/GE Healthcare UK Ltd, Little Chalfont, UK) detection performed. For other western blots the antibodies used were rabbit anti-forkhead box O1 (FOXO1) (Cell Signaling Technology, Inc., Danvers, MA, USA) and mouse anti-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Sheep anti-GCK antibody was a kind gift from M. Magnuson. Densitometer quantification was undertaken as previously described [14].

RNA isolation and real-time quantitative RT-PCR RT-PCR was performed as previously described [8] using FAM/TAMRA-labelled fluorescent probes (Applied Biosystems, Foster City, CA, USA). Proprietary sequence RT primers (Applied Biosystems) used were: *Ins2* (insulin gene) Mm00731595_gH, *Gcg* (glucagon gene) Mm00801712_m1, *Irs1* Mm00439720_s1, *Irs2* MIRS 396412, *Slc2a2* (solute carrier family 2 [facilitated glucose transporter], member 2 gene, previously known as *Glut2*) Mm00446224_m1, *Gck* (glucokinase gene) Mm00439129_m1, *Hprt1* (hypoxanthine phosphoribosyltransferase 1 gene) Mm01545399_m1, *Foxo1* Mm00490672_m1, *Pdx1* Mm00558275_m1, *Neurod1* (neurogenic differentiation factor 1 gene) Mm01280117_m1, *Nkx6-1* (NK6 transcription factor related, locus 1 gene) Mm00454962_m1 and *Sst* (somatostatin gene) Mm00436671_m1. The relative amount of mRNA was calculated from an internal standard curve following normalisation to *Hprt1* mRNA levels.

Pancreatic immunocytochemistry and measurement of endocrine cell mass Pancreases were removed, cleared of fat and lymph nodes, fixed in Bouin's solution, embedded in paraffin and cut into 5-μm sections. Insulin and GCG staining and morphometric analysis were performed as previously described [8]. Beta cell proliferation was assessed using Ki67 immunostaining [15] and apoptosis detected using activated caspase 3 immunostaining [16]. Antibodies used were mouse anti-insulin and anti-GCG (Sigma-Aldrich Company Ltd, Poole, UK), rabbit anti-Ki67 and anti-GCG and guinea pig anti-insulin (all Abcam PLC, Cambridge, UK), rabbit anti-active caspase 3 (BD PharMingen, San Diego, CA, USA), rabbit anti-PDX1 (Millipore/Upstate, Dundee, UK), rabbit anti-SST (Millipore/Chemicon International Inc., Temecula, CA, USA), chicken anti-mouse AlexaFluor 488 and 594, chicken anti-rabbit AlexaFluor 488 and 594 and goat anti-guinea-pig AlexaFluor 546 (all Molecular Probes/Invitrogen, Paisley, UK). Rabbit anti-SCL2A2 antibody was a kind gift from B. Thorens (University of Lausanne).

Islet isolation Mice were killed by cervical dislocation and the common bile duct was cannulated and its duodenal end occluded by clamping. Two millilitres of Liberase (Roche, Basel, Switzerland) solution (0.25 mg/ml in Hanks' balanced salt solution [HBSS]) were injected into the duct to distend the pancreas. The pancreas was excised, incubated at 37°C for 15 min, and mechanically disrupted in 10 ml HBSS. Cellular components were collected by centrifugation (201 g for 1 min) and re-suspended in 10 ml HBSS. Islets were hand-picked under a microscope and washed once in HBSS. Prior to DNA or protein extraction, islets were centrifuged at 5,724 *g* for 5 min and stored at −80°C. RNA was extracted from islets using an RNAeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For insulin secretion studies, isolated islets were cultured overnight in DMEM with 11 mmol/l glucose supplemented with 10% fetal bovine serum. After a 1-h pre-incubation in HBSS (supplemented with 0.5% BSA and 20 mmol/l HEPES) containing 2 mmol/l glucose, insulin release was assessed by static incubation of groups of eight islets size-matched by hand-picking in HBSS containing 2 mmol/l glucose for 30 min, then 11 mmol/l glucose for 30 min, at 37°C with 5% CO_2. Secreted insulin was assayed using an insulin ELISA kit (Millipore/Linco Research Inc., St Charles, MI, USA). Insulin and GCG were measured in pancreatic extracts by RIA as previously described [17].

Analysis of calcium fluxes in beta cells by confocal microscopy Islets isolated as above were dissociated into single cells by incubation in trypsin at 21°C for 2.5 min with gentle agitation. Beta cells were washed once before being allowed to attach to poly-L-lysine-treated glass cover-slips. Beta cells were incubated for 48 h in DMEM culture medium with 2 mmol/l L-glutamine supplemented with 10% heat-inactivated fetal bovine serum and 11 mmol/l glucose. The medium was changed to HBSS supplemented with 20 mmol/l HEPES and 2 mmol/l glucose 1 h prior to dye loading. Cells were loaded with 10 μg/ml Fluo3am (Molecular Probes) and 0.025% Pluronic detergent for 30 min and were then imaged on a 37°C heated stage on a Biorad TE300 confocal microscope as glucose concentration was increased from 2 to 11 mmol/l. The response to 200 μmol/l tolbutamide was also determined. Beta cells were identified after imaging by staining with dithizone [18].

Statistical methods All statistics were performed using GraphPad Prism4 software (Graphpad Software Inc., San

Diego, CA, USA) and paired and unpaired *t* tests and two-way ANOVA with Bonferroni post-tests performed as appropriate. A p value of <0.05 was regarded as significant.

Results

Efficient deletion of Irs2 in the pancreas but not hypothalamus of PIrs2KO mice To assess the sites and extent of the recombination of floxed alleles by the *Pdx1-cre* mice used in these studies, we analysed DNA extracted from tissues of control and *PIrs2KO* mice. In *PIrs2KO* mice, recombination and deletion of the *Irs2* allele was detected in DNA from both whole pancreases and isolated islets but not in hypothalamic samples or other tissues (Fig. 1a). No recombination was seen in tissues from control mice (Fig. 1a). IRS2 protein was not detected in the pancreas or isolated islets from *PIrs2KO* mice (Fig. 1b) and *Irs2* mRNA was normal in the hypothalamus of these animals (*Irs2* mRNA

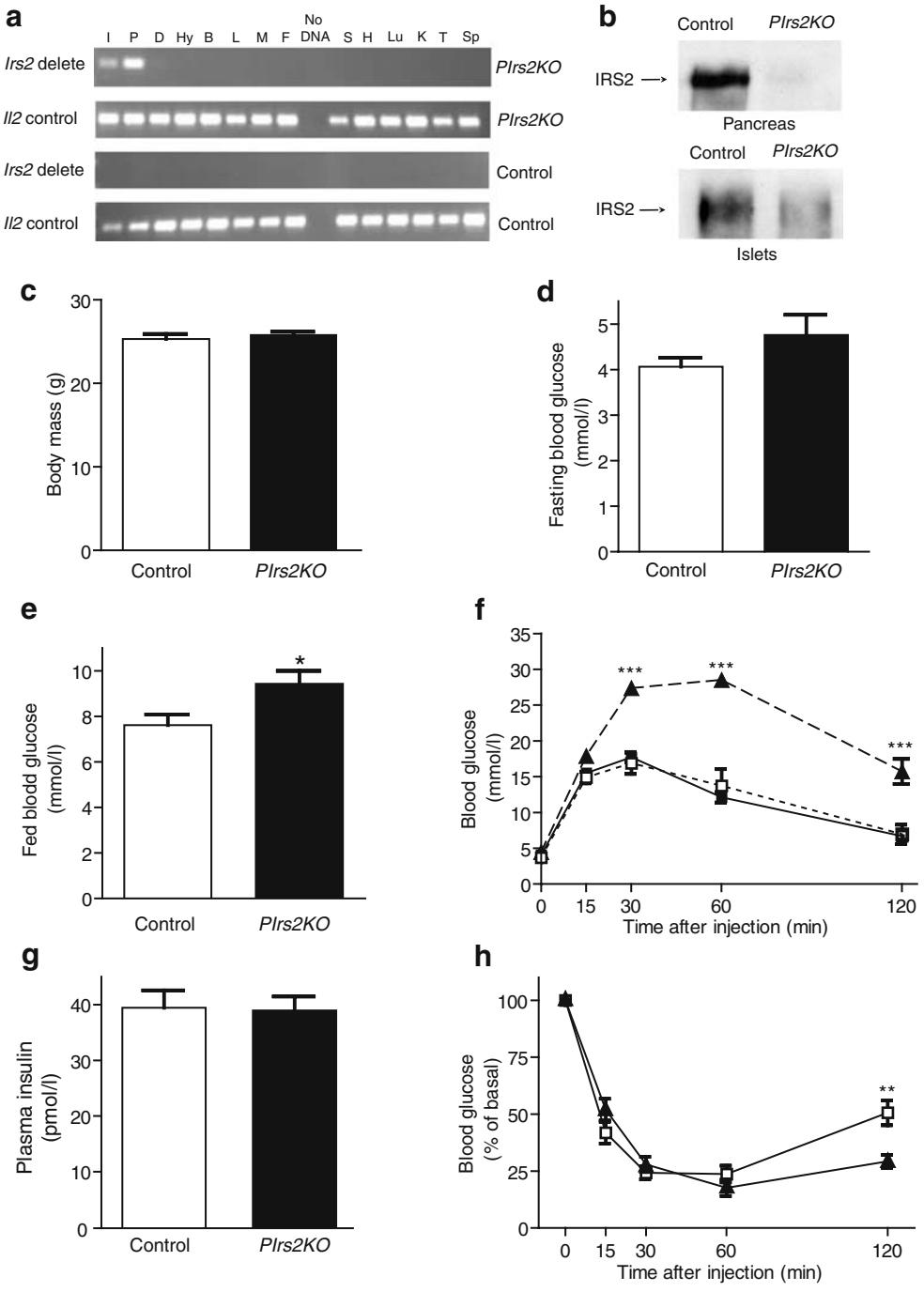

Fig. 1 Deletion of *Irs2* in the pancreas impairs glucose tolerance in mice. **a** Detection of deletion of *Irs2* allele in DNA extracted from tissues (*I*, islets; *P*, pancreas; *D*, duodenum; *Hy*, hypothalamus; *B*, brain; *L*, liver; *M*, skeletal muscle; *F*, fat; *S*, stomach; *H*, heart; *Lu*, lung; *K*, kidney; *T*, testis; *Sp*, spleen) from *PIrs2KO* and control mice. An interleukin 2 (*Il2*) control PCR reaction is also shown. **b** Western blot of IRS2 protein production in pancreas and islets from control and *PIrs2KO* mice. Results are representative of three independent experiments. **c** Body mass in 12-week-old male control and *PIrs2KO* mice, n=13. **d** Fasting blood glucose levels in 12-week-old male control and *PIrs2KO* mice, n=20. **e** Random fed blood glucose levels in 12-week-old male control and *PIrs2KO* mice, n=8. **f** Glucose tolerance in 12-week-old *Pdx1-cre* (*open squares*), $Irs2^{tm1With/tm1With}$ (*closed squares*) and *PIrs2KO* mice (*closed triangles*), n=7–18. **g** Fasting blood insulin levels in 12-week-old male control and *PIrs2KO* mice, n=10. **h** Insulin tolerance in 12-week-old male control (*open squares*) and *PIrs2KO* mice (*closed triangles*), n=8. Values are means±SEM, *p<0.05, **p<0.01 and ***p<0.001

expression relative to *Hprt*: *PIrs2KO* mice 0.0012±0.0005 vs control 0.0014±0.0005, n=3, p=NS). Therefore *PIrs2KO* mice represent a good model for determining the effects of complete deletion of *Irs2* in the pancreas without concomitant deletion in the hypothalamus.

PIrs2KO mice develop impairment of glucose homeostasis but display normal bodyweight The *PIrs2KO* mice were born with the expected Mendelian frequency and developed and grew normally. At 12 weeks of age, a time when mice lacking *Irs2* in *RIPCre*-expressing cells (*RIPCreIrs2KO* mice) have a marked obesity phenotype [8–10], male and female *PIrs2KO* mice and their littermate controls had equivalent body mass (Fig. 1c and Electronic Supplementary Material [ESM] Fig. 1a) and there was no difference in food intake between the genotypes, consistent with the lack of hypothalamic deletion (food intake per mouse [g/24 h]: *PIrs2KO* mice, 2.9±0.2 vs control, 2.9±0.1, n=6, p=NS). Examination of fasting blood glucose levels at 12 weeks of age showed no significant difference between *PIrs2KO* mice and control animals in both sexes (Fig. 1d and ESM Fig. 1b). In contrast, random fed blood glucose levels were significantly elevated and glucose tolerance was markedly impaired in 12-week-old male animals (Fig. 1e,f). Of particular note was that glucose handling in both *Pdx1-cre* and $Irs2^{tm1With/tm1With}$ mice was normal, demonstrating no adverse effects of the *Pdx1-cre* transgene or the loxP-flanked *Irs2* allele upon glucose homeostasis (Fig. 1f). Consistent with our previous reports [3, 19], female mice had a milder glucose homeostasis phenotype than male mice (ESM Fig. 1c). Fasting insulin levels in 12-week-old *PIrs2KO* mice were equivalent to control mice, suggesting that *PIrs2KO* mice are not insulin resistant (Fig. 1g). Indeed, insulin tolerance tests in *PIrs2KO* mice revealed no evidence of reduced insulin sensitivity, but at the 60-min time-point a reduced recovery in glucose levels after insulin treatment was seen (Fig. 1h).

Reduced beta and alpha cell mass in PIrs2KO mice To investigate further the defect in glucose tolerance we measured pancreatic hormone content, determined endocrine cell mass and analysed islet anatomy in *PIrs2KO* mice. Pancreatic insulin and GCG peptide and islet insulin, *Gcg* and *Sst* mRNAs from *PIrs2KO* mice were reduced (Fig. 2a–c). Morphometric analysis in 12-week-old *PIrs2KO* mice revealed a reduced total beta cell and alpha cell mass in these animals but preservation of pancreatic mass (Fig. 2d–f). However, analysis of beta cell mass in 2-week-old *PIrs2KO* mice revealed a normal beta cell mass, suggesting that IRS2 was required for the maintenance of a normal adult beta cell mass and not islet development (Fig. 2d). To understand the basis for the reduced beta cell mass we examined proliferation and apoptosis in these cells. Reduced Ki67 staining was detected in beta cells in *PIrs2KO* mice, demonstrating reduced proliferation of this cell type (Fig. 2g), but we observed no increase in apoptosis as determined by staining for activated caspase 3 (Fig. 2g). Furthermore, islet architecture was preserved with no obvious disruption of the normal organisation of alpha, beta cells or delta cells (Fig. 2h,i).

Impaired in vivo and in vitro glucose-stimulated insulin secretion in PIrs2KO mice To assess the impact of these abnormalities upon islet function, we analysed glucose-stimulated insulin secretion (GSIS) both in vivo and in isolated islets. GSIS in vivo was severely perturbed with loss of first-phase insulin secretion in *PIrs2KO* mice but was normal in both *Pdx1-cre* and $Irs2^{tm1With/tm1With}$ mice (Fig. 3a). Similarly, in static incubation studies, GSIS was attenuated in islets isolated from *PIrs2KO* mice (Fig. 3b).

Gene expression and calcium mobilisation defects in PIrs2KO mice To investigate the mechanisms underlying the secretory defects in *PIrs2KO* islets, we studied glucose-stimulated calcium fluxes in beta cells and analysed the expression of key genes in isolated islets. Examination of calcium fluxes revealed that, in response to an increased glucose concentration, there was a robust increase in intracellular calcium in control beta cells but this was attenuated in *PIrs2KO* beta cells, consistent with the defects seen in GSIS (Fig. 3c). Tolbutamide-stimulated calcium release was largely preserved in *PIrs2KO* beta cells (Fig. 3c). *Foxo1* and *Nkx6-1* mRNA levels were significantly reduced but *Pdx1* and *Neurod1* expression was unchanged (Fig. 3d). *Slc2a2* expression was reduced but *Gck* expression was unaltered in *PIrs2KO* islets (Fig. 3d). *Irs1* expression was also unchanged (Fig. 3d). To assess the impact of these alterations of mRNA levels we examined protein production of SLC2A2, PDX1, GCK, FOXO1 and NKX6-1 in *PIrs2KO* mice. Immunostaining of islets revealed reduced membrane staining for SLC2A2 in *PIrs2KO* islets (Fig. 3e) but normal PDX1 staining (ESM Fig. 1d). Western blotting for FOXO1 demonstrated a trend to reduced protein production (p= 0.07). Likewise there was a non-significant reduction in NKX6-1, but GCK protein was normal (Fig. 3f).

Normal glucose tolerance in RIPCre mice maintained on two genetic backgrounds As discussed above, it has been suggested that *RIPCre* (*B6.Cg-tg(Ins2-cre)25Mgn/J*) transgenic mice develop glucose intolerance in the absence of any gene deletion [12]. This was particularly apparent in mice obtained from the Jackson Laboratory maintained on a C57Bl/6 background. In light of these observations and our findings of normal glucose homeostasis in mice with Cre recombinase expression in islets driven by the *Pdx1* promoter, we studied our own colonies of *RIPCre* mice,

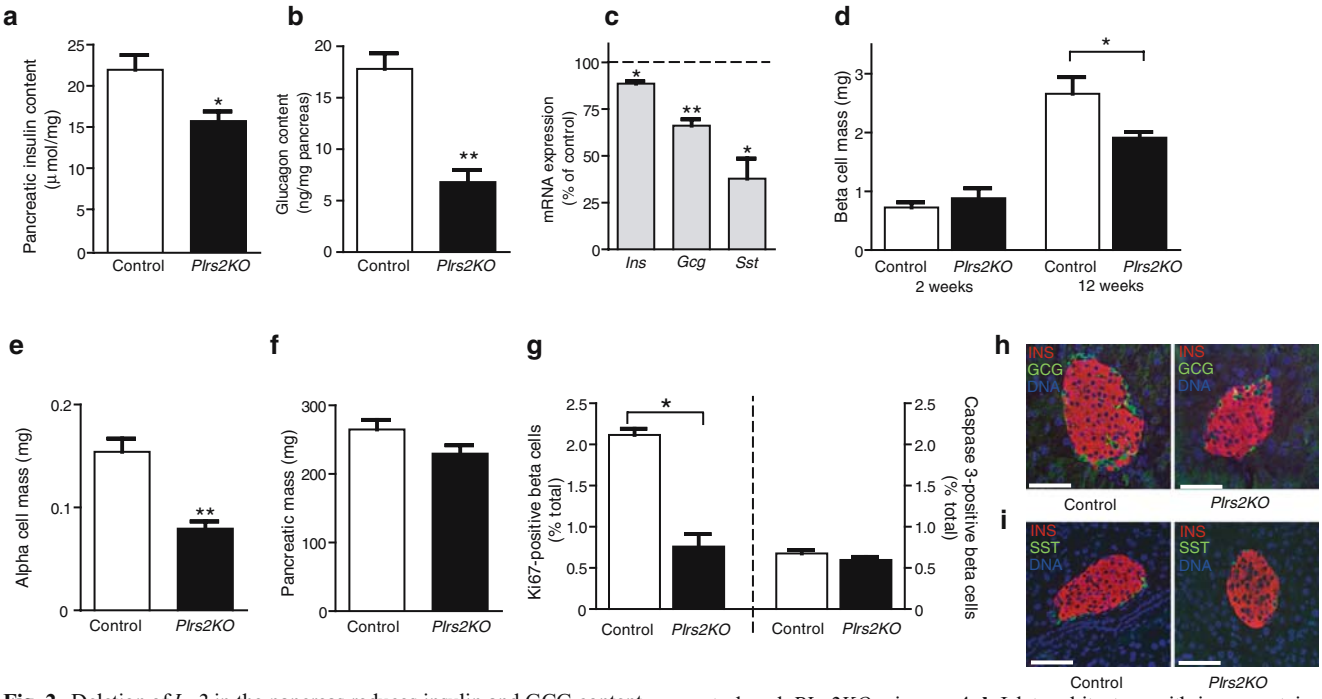

Fig. 2 Deletion of *Irs2* in the pancreas reduces insulin and GCG content and expression, and reduces alpha and beta cell mass and beta cell proliferation. **a**, **b** Pancreatic insulin and GCG peptide content in 12-week-old male control and *PIrs2KO* mice, $n=5$. **c** *Ins*, *Gcg* and *Sst* mRNA expression in islets of *PIrs2KO* mice expressed relative to control islets, $n=5$ mice. **d** Beta cell mass in 2- and 12-week-old male control and *PIrs2KO* mice, $n=3$–6. **e** Alpha cell mass in 12-week-old male control and *PIrs2KO* mice, $n=3$. **f** Pancreatic mass in 12-week-old male control and *PIrs2KO* mice, $n=6$. **g** Beta cell proliferation determined by Ki67 and insulin co-staining and apoptosis determined by activated caspase 3 and insulin co-staining in islets of 12-week-old control and *PIrs2KO* mice, $n=4$. **h** Islet architecture with immunostaining for INS (*red*), GCG (*green*) and 4,6-diamidino-2-phenylindole (DAPI) staining for nuclei (*DNA*: *blue*) in 12-week-old control and *PIrs2KO* mice. Images are typical of islets sampled from multiple pancreatic levels from four mice of each genotype. **i** Islet architecture with immunostaining for insulin (*INS*: *red*), SST (*green*) and DAPI staining for nuclei (*DNA*: *blue*) in 12-week-old control and *PIrs2KO* mice. Images are typical of islets sampled from multiple pancreatic levels from four mice of each genotype. Values are means±SEM, $*p<0.05$, $**p<0.01$. Scale bars are 50 μm

which were originally obtained from the Jackson Laboratory but intercrossed with floxed alleles generated with C57Bl/6 mice obtained from other sources as well as other strains. We therefore performed these studies in two mixed genetic backgrounds: 129Sv/C57Bl/6/BALB/c and 129Sv/C57Bl/6. At 12 weeks of age, wild-type and *RIPCre* littermates of both sexes from the 129Sv/C57Bl/6/BALB/c intercross had identical glucose tolerance (ESM Fig. 1e). At 24 weeks of age, wild-type and *RIPCre* littermates of both sexes from the 129Sv/C57Bl/6 intercross had identical glucose tolerance (ESM Fig. 1f).

Discussion

Through the use of both global and conditional mouse gene targeting strategies we and others have demonstrated that IRS2 plays a key role in the maintenance of pancreatic beta cell mass [3, 4, 8–10]. Furthermore both INSR and IGF1R have also been demonstrated to act as key regulators of beta cell function using Cre/loxP techniques [5–7]. However, many of these studies have been compromised by two key features of the *RIPCre* (*B6.Cg-tg(Ins2-cre)25Mgn/J*) transgenic mice used to delete the floxed alleles. First, in this animal there was extensive expression in hypothalamic neurons, which resulted in obesity, hyperleptinaemia and hyperinsulinaemia when either *Irs2* or indeed *Insr* was deleted in these cells [8–10, 20]. Second, it has been suggested that the *RIPCre* mouse is intrinsically glucose intolerant [12]. Our current studies have utilised a *Pdx1-cre* mouse to delete *Irs2* only in the pancreas, thus generating *PIrs2KO* mice. These animals do not display obesity, insulin resistance or hyperinsulinaemia and therefore represent a good model for determining the role of IRS2 in the pancreas in the absence of these phenotypes. Furthermore, the *Pdx1-cre* allele alone does not impair glucose homeostasis. Our analysis shows that *PIrs2KO* mice have a reduced beta cell mass with reduced beta cell proliferation, confirming the key role of IRS2 in this process. No alteration in beta cell mass was seen before weaning, consistent with previous observations [8–10] demonstrating that IRS2 is not required for beta cell development. Our findings also suggest that IRS2-dependent mechanisms are required not only for beta cell compensation under conditions of increased insulin

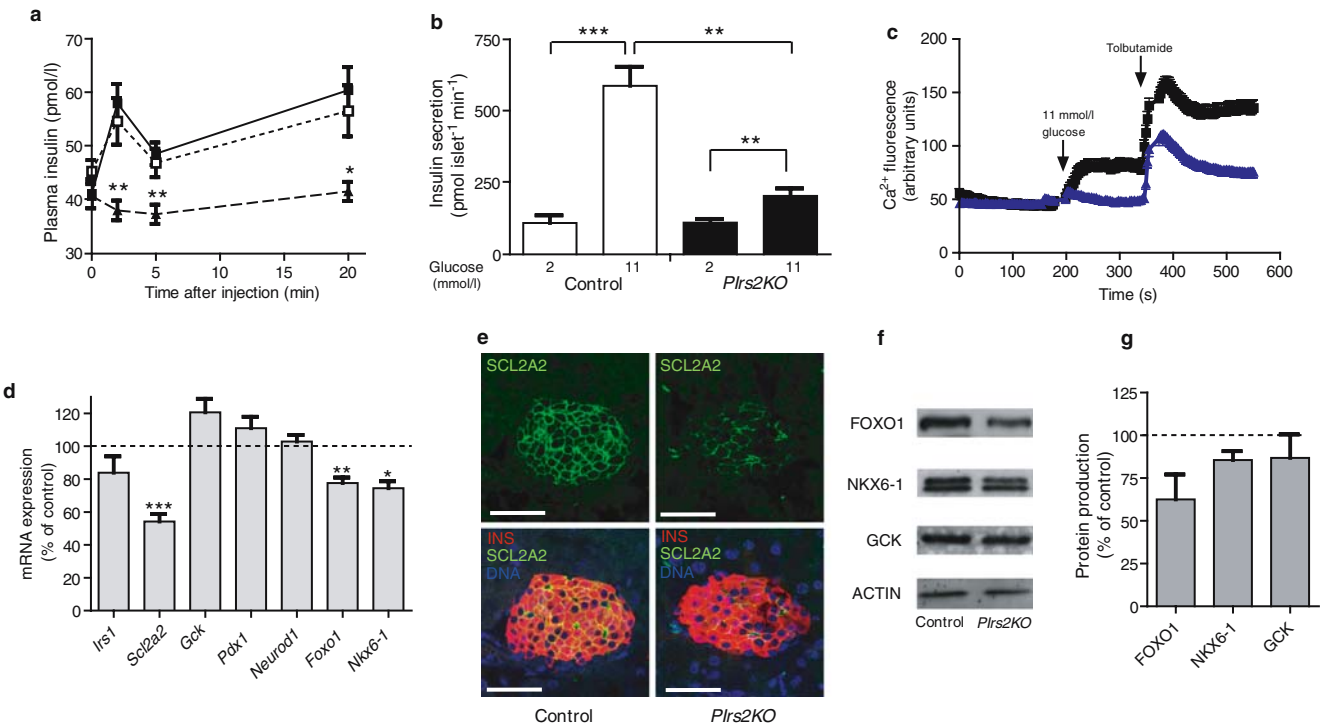

Fig. 3 Impaired in vivo and in vitro GSIS and calcium fluxes in *PIrs2KO* islets. **a** Plasma insulin levels before and after an i.p. glucose load in 12-week-old *Pdx1-cre* (*open squares*) *Irs2*$^{tm1With/tm1With}$ (*closed squares*) and *PIrs2KO* (*closed triangles*) mice, n=9–11. **b** Glucose-stimulated insulin secretion in response to increased glucose (from 2 to 11 mmol/l) during static incubation studies in *PIrs2KO* and control islets, n=16. **c** Calcium-bound Fluo3am fluorescence in response to increased glucose (from 2 to 11 mmol/l) and tolbutamide (200 μmol/l) in *PIrs2KO* (*blue triangles*) and control (*black squares*) islets. Recordings were made from 50 cells of each genotype from two animals of each genotype. **d** mRNA expression of indicated genes in islets of *PIrs2KO* mice expressed relative to control islets, n=5 mice. **e** Immunostaining for INS (*red*), SCL2A2 (*green*) and 4,6-diamidino-2-phenylindole staining for nuclei (*DNA*: *blue*) in 12-week-old control and *PIrs2KO* mice. Images are typical of islets sampled from multiple pancreatic levels from four mice of each genotype. **f** Western blotting for FOXO1, NKX6-1 and GCK in control and *PIrs2KO* islets. Blots are representative of those obtained from three mice of each genotype. An actin loading control is also shown. **g** Western blot scanning densitometry quantification of protein production for FOXO1, NKX6-1 and GCK in isolated control and *PIrs2KO* islets. Results are expressed as percentage of control islet values, n=3 and for FOXO1 p=0.07. Values are means±SEM, *p<0.05, **p<0.01 and ***p<0.001

requirement but also for maintenance of a normal postnatal beta cell mass.

The absence of insulin resistance in our model also permitted analysis of the effects of *Irs2* deletion upon insulin secretory function. *Insr*-, *Igf1r*- and *Irs1*-null islets display defective glucose-stimulated insulin release in vitro [6, 21, 22]. In contrast, in both global *Irs2*-null mice and *RIPCreIrs2KO* mice, insulin secretion from isolated islets has been reported to be enhanced [4, 9]. While the basis for the discordance in these observations is unclear, they potentially suggest that IRS2 signalling has a negative effect upon insulin synthesis and secretion or that development of islets under conditions of insulin resistance caused by *Irs2* deficiency in other tissues programmes the islets to hypersecrete insulin. However, we found that GSIS in *PIrs2KO* mice is reduced both in vivo and in vitro, suggesting that IRS2 pathways are required for either insulin synthesis or secretion. These findings are consistent with the reported role of INSR, IGF1R and IRS1 in these processes and with a study demonstrating that IRS2/phosphatidylinositol 3-kinase/p70S6 kinase signalling regulates insulin gene transcription [23]. The combined effect of reduced beta cell mass and impaired insulin secretion resulted in impaired glucose tolerance and fed hyperglycaemia in *PIrs2KO* mice. However, these animals displayed normal fasting blood glucose levels and never developed the progressive diabetes seen in *Irs2* global-null mice. These findings demonstrate that the interplay between beta cell dysfunction and peripheral insulin resistance is required for the development of diabetes in the *Irs2*-null mouse model.

Insulin signalling has been implicated in pancreatic alpha cell function but the role of this pathway has not been directly addressed genetically in mice. For example, insulin inhibits GCG secretion from mouse clonal alpha cell lines and primary islets in vitro [24]. RNA interference-mediated knockdown of *Insr* expression in the same cell types impairs GCG secretion in response to reduced glucose levels [25]. Therefore disruption of insulin signalling in alpha cells might be expected to lead to loss of the repression of GCG secretion by insulin or a failure to increase GCG release under conditions of hypoglycaemia.

Here we demonstrate a novel key role for IRS2 in the maintenance of alpha cell mass, which in turn is reflected in the reduced pancreatic GCG peptide and mRNA content seen in *PIrs2KO* mice. The reduced GCG levels in our model may also underlie the mild impairment in the recovery in glucose levels during insulin tolerance testing in *PIrs2KO* mice and the absence of hyperglucagonaemia may also contribute to the lack of progressive diabetes in these animals. We also demonstrate lower SST levels in the islets of *PIrs2KO* mice suggesting reduced numbers of delta cells in these animals. Our findings that both beta cell and alpha cell mass are reduced indicate that IRS2 signalling may be important in a common endocrine precursor cell type or for maintaining proliferation in mature islet endocrine cells.

Beta cell deletion of *Insr*, *Igf1r* and *Irs1* impairs glucose-stimulated insulin secretion. Defects in glucose-stimulated calcium mobilisation are thought to be a component of this abnormality. For example, *Insr*-, *Igf1r*- and *Irs1*-deficient islets display reduced increases in intracellular calcium flux, which, in the case of *Irs1*-null islets, are thought to be in part due to decreased expression of sarco(endo)plasmic reticulum Ca^{2+}-ATPase expression [7, 26, 27]. *PIrs2KO* beta cells also showed impaired calcium mobilisation in response to glucose, suggesting that IRS2 signalling is also a component of this mechanism.

Analysis of gene expression patterns in *PIrs2KO* islets revealed that *Irs1* mRNA levels were unaltered, demonstrating there was no compensatory upregulation of this gene, which contrasts with the upregulation of *Irs2* reported in *Irs1*-null islets [28]. Reduced expression of *Pdx1* has been reported in *Irs2* global-null and *RIPCreIrs2KO* mice and upregulation of *Pdx1* in the beta cell rescues the beta cell failure in *Irs2* global-null mice [10, 29]. These findings, together with those demonstrating that *Pdx1* haploinsufficiency blunts beta cell compensation in other models of insulin resistance [30], have led to the suggestion that INSR and IRS2 signalling is required for the normal expression and function of PDX1. However, we found normal expression of *Pdx1* in islets and our findings do not support this conclusion. Indeed others have also reported that *Pdx1* expression is normal in *Irs2*-null beta cells and that differences in expression may be mouse strain dependent [31]. In contrast, we found reduced *Slc2a2* expression with concomitant reduction in protein production in *PIrs2KO* islets and this may further impair beta cell function. While the mRNA expression of two other transcription factors which are critical for beta cell function, *Foxo1* and *Nkx6-1*, was attenuated, the reductions in the production of these proteins was modest. However, it is possible that such small reductions in a number of key beta cell genes also contribute to the beta cell phenotype in *PIrs2KO* mice.

Studies with our colony of *RIPCre* (*B6.Cg-tg(Ins2-cre) 25Mgn/J*) transgenic mice failed to detect glucose intolerance in this strain in either sex up to 24 weeks of age, in contrast to a recent report [12]. While we originally obtained *RIPCre* mice from the Jackson Laboratory, these animals were intercrossed for several generations with either 129Sv/C57Bl/6 or 129Sv/C57Bl/6/BALB/c hybrids used to generate floxed alleles and these latter strains were not obtained from the same source. These findings taken together with the normal glucose homeostasis observed in the *Pdx1-cre* mice in our studies suggest that Cre recombinase expression in beta cells does not impair their function. Recently it has been demonstrated that C57Bl/6J mice descended from the colony established at the Jackson Laboratory have a spontaneous in-frame five-exon deletion in the nicotinamide nucleotide transhydrogenase gene [32]. This is associated with glucose intolerance and reduced insulin secretion, which may contribute to the phenotypes reported in some studies [32]. These findings further emphasise the influence of genetic background on metabolic phenotypes when complex gene targeting strategies are employed.

In summary, through the generation of *PIrs2KO* mice, we have demonstrated a key role for IRS2 signalling events in the regulation of beta cell function without the confounding factors of hypothalamic dysfunction and insulin resistance. We also reveal a more general role for IRS2 signalling in the regulation of other pancreatic cells types such as alpha cells.

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