Insulin receptors in β-cells are critical for islet compensatory growth response to insulin resistance

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Edited by Donald F. Steiner, University of Chicago, Chicago, IL, and approved March 6, 2007 (received for review October 3, 2006)

Insulin and insulin-like growth factor 1 (IGF1) are ubiquitous growth factors that regulate proliferation in most mammalian tissues including pancreatic islets. To explore the specificity of insulin receptors in compensatory β-cell growth, we examined two models of insulin resistance. In the first model, we used liver-specific insulin receptor knockout (LIRKO) mice, which exhibit hyperinsulinemia without developing diabetes due to a compensatory increase in β-cell mass. LIRKO mice, also lacking functional insulin receptors in β-cells (βIRKO/LIRKO), exhibited severe glucose intolerance but failed to develop compensatory islet hyperplasia, together leading to early death. In the second model, we examined the relative significance of insulin versus IGF1 receptors in islet growth by feeding high-fat diets to βIRKO and β-cell-specific IGF1 receptor knockout (βIGFRKO) mice. Although both groups on the high-fat diet developed insulin resistance, βIRKO, but not βIGFRKO, mice exhibited poor islet growth consistent with insulin-stimulated phosphorylation, nuclear exclusion of FoxO1, and reduced expression of Pdx-1. Together these data provide direct genetic evidence that insulin/FoxO1/Pdx-1 signaling is one pathway that is crucial for islet compensatory growth response to insulin resistance.

β-cell growth | compensatory hyperplasia | β-cell apoptosis

Insulin resistance is a common feature of type 2 diabetes, obesity, and hyperlipidemias (1). In patients with type 2 diabetes and mouse models of diabetes and obesity, the β-cells compensate for the insulin resistance by increasing their mass (2, 3). One of these models, the liver-specific insulin receptor knockout (LIRKO) mouse, manifests severe insulin resistance and glucose intolerance, but the mutants do not become overtly diabetic due, in part, to a significant increase in β-cell mass (4). Although lineage tracing experiments in normal mice and studies in cyclin D2 knockouts indicate that β-cell replication is a major mechanism for regeneration of adult β-cells (5, 42), the signals that induce β-cell replication are not fully defined.

Insulin and IGFI and the proteins in their signaling pathways regulate cell growth and function (6), but their specificity in modulating β-cell proliferation is not fully explored. Thus, β-cell-specific insulin receptor (βIRKO) (7), IGF1 receptor knockout (βIGFRKO) (8, 9), or double mutants (10) surprisingly exhibited normal growth and development of β-cells consistent with data from global knockouts of insulin receptor or IGF1 receptor genes (11). Mice with global knockout of IRS1 (12, 13) or IRS2 genes (12, 14) also do not manifest defects in the development of islet cells, whereas overexpression of Akt in β-cells promotes an increase in cell size (15). Together these studies indicate that insulin/IGF1 signaling is not critical for early development and growth of β-cells.

Our follow-up studies showed that βIRKO, but not βIGFRKO, mice developed an age-dependent decrease in β-cell mass and a susceptibility to develop overt diabetes (7, 16, 17). Further, examination of compound β-cell-specific insulin receptor and IGF1 receptor knockouts suggested that insulin plays a prominent role in the proliferation of adult β-cells (10). In the current study, we directly examined the role of insulin signaling in β-cells in the compensatory proliferative response by using two complementary models. First, we explored phenotypes of insulin-resistant LIRKO mice that lack functional insulin receptors in β-cells (βIRKO/LIRKO). Second, we compared the impact of a high-fat diet on islet compensatory growth response in βIRKO mice versus mice lacking IGF1 receptors specifically in β-cells (βIGFRKO). We also examined localization and phosphorylation of FoxO1 and expression of its downstream target, Pdx-1, in βIRKO or βIGFRKO islets, and in β-cell lines with knockdown of insulin or IGF1 receptors. Together the results indicate a prominent role for insulin, FoxO1, and Pdx-1 in the compensatory islet growth response to insulin resistance.

Results

Creation of βIRKO/LIRKO. We created βIRKO/LIRKO double mutants by crossing individual knockouts (4, 7) maintained on a mixed-genetic background (129Sv × C57BL/6 × DBA/2) from a cohort generated for other studies wherein we have described specificity of knockout for each receptor (10). Qualitatively similar data were obtained in females and males. Therefore, only data from males will be reported.

Glucose Homeostasis and Metabolic Parameters in βIRKO/LIRKO Mice. Serum insulin levels were significantly elevated, as early as 2 weeks, in both LIRKO and βIRKO/LIRKO groups and remained elevated up to 6 weeks (Fig. 1*a*). The hyperinsulinemia was adequate to maintain normoglycemia in the LIRKOs similar to controls and βIRKOs up to 3 weeks (Fig. 1 *a* and *b*). By 4 weeks, however, significant hyperglycemia was evident in LIRKO mice that persisted up to 6 weeks. In contrast, the βIRKO/LIRKO double mutants developed hyperglycemia earlier (by 2 weeks), which rapidly worsened by age 6 weeks (>600 mg/dl).

Examination of acute-phase insulin release to glucose stimulation showed virtually no response in βIRKO/LIRKOs or βIRKOs, consistent with a role for insulin signaling in β-cell glucose sensing (16, 18) (Fig. 1*c*). Assessment of glucose tolerance showed intolerance in βIRKO, LIRKO, and βIRKO/LIRKOs (Fig. 1*d*). Insulin

Author contributions: T.O. and C.W.L. contributed equally to this work; T.O., M.D.M., and R.N.K. designed research; T.O., C.W.L., J.H., C.H., M.D.M., J.K., C.Y., and R.N.K. performed research; J.K., M.H., and M.S. contributed new reagents/analytic tools; T.O., C.W.L., J.H., C.H., M.D.M., J.K., C.Y., M.S., and R.N.K. analyzed data; and T.O., M.S., and R.N.K. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0608703104/DC1.

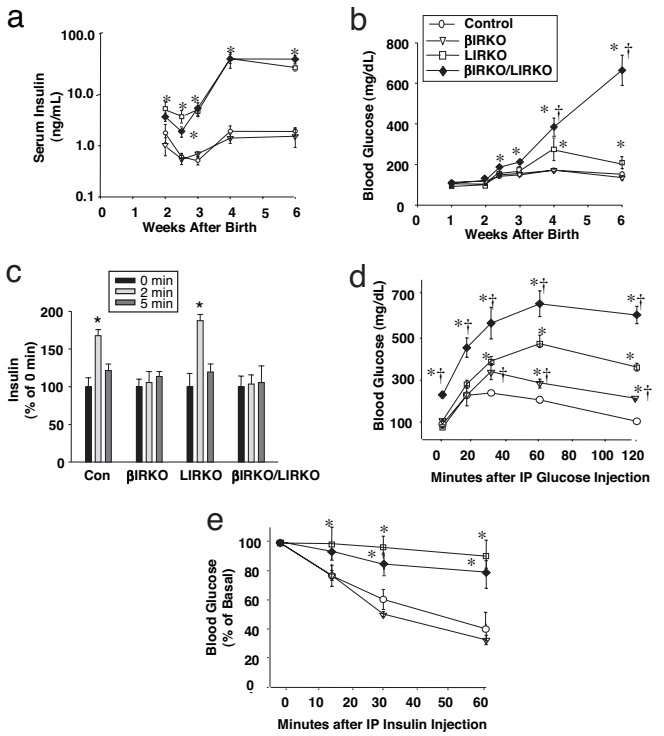

Fig. 1. βIRKO/LIRKO mice display early hyperinsulinemia, hyperglycemia, glucose intolerance, loss of acute phase insulin release, and insulin resistance. (*a–e*) Serum insulin (*a*) levels and blood glucose (*b*) were measured 7, 14, 17, 21, 28, and 42 days after birth. *, $P < 0.01$, βIRKO/LIRKO vs. control, βIRKO, or LIRKO; †, $P < 0.05$, βIRKO/LIRKO vs. LIRKO. Insulin is plotted on log scale on *y* axis. (*c*) Acute (first)-phase insulin secretion following an intraperitoneal (i.p.) injection of glucose (3 g/kg body weight). Data are expressed as percentage increase at 2 and 5 min relative to time 0. *, $P < 0.01$, vs. 0 or 5 min; $n = 8–10$. (*d*) Blood glucose after i.p. injection of glucose (2 g/kg body weight). *, $P < 0.001$ βIRKO vs. control; †, $P < 0.05$, βIRKO vs. control, or LIRKO vs. βIRKO/LIRKO; $n = 8$. (*e*) Blood glucose after i.p. injection of insulin (Humulin, 1 unit/kg body weight). *, $P < 0.01$ βIRKO/LIRKO or LIRKO vs. control and βIRKO; $n = 8$. Experiments described in *c–e* are from male mice ages 4–5 weeks..

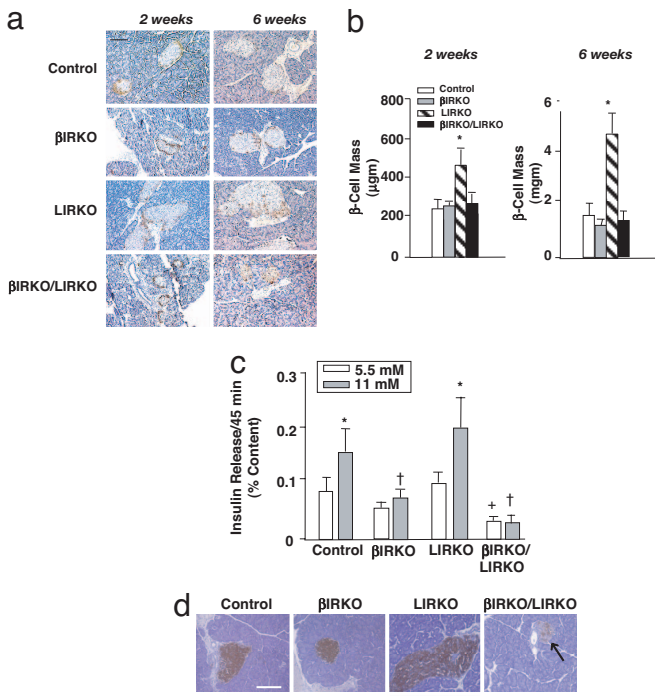

Fig. 2. Absent compensatory islet hyperplasia in βIRKO/LIRKO mice. (*a*) Immunostaining of pancreas sections for non-β-cells by using a mixture of antibodies to glucagon, somatostatin, and pancreatic polypeptide as described in *Methods*. Representative sections from 2- and 6-week-old mice. (Magnification: ×10.) (Scale bar: 50 μm.) (*b*) β-cell mass at 2 (in micrograms) and 6 (in milligrams) weeks assessed by morphometric analysis as described in *Methods*. *, $P < 0.05$ LIRKO vs. all other groups. (*c*) After overnight culture, size-matched islets were incubated in different concentrations of glucose for 45 min. Then the medium was removed and assayed for insulin. Islet insulin content was assayed by acid ethanol extraction as described in *Methods*. *, $P < 0.05$, 5.5 mM vs. 11 mM glucose; †, $P < 0.05$, βIRKO/LIRKO or βIRKO vs. control or LIRKO; +, $P < 0.05$, βIRKO/LIRKO vs. control or βIRKO or LIRKO. (*d*) Representative pancreas sections from 4-week-old male mice immunostained for insulin by using DAB as a substrate as described in *Methods*. (Magnification: ×40.) (Scale bar: 50 μm.)

secretion during glucose tolerance revealed an increase in serum insulin 30 min after glucose injection in controls that was absent in βIRKOs, whereas LIRKOs showed a trend toward an increase. The large variation within groups, in part due to the limited number of mice studied, indicates that these experiments require further investigation [supporting information (SI) Table 1]. In the βIRKO/LIRKO double mutants, the significantly high fasting glucose (≈250 mg/dl) that increased even further, after an i.p. bolus, indicates frank diabetes due to additive effects of defects in β-cells and hepatocytes. βIRKO/LIRKOs and LIRKOs exhibited insulin resistance, in contrast to normal sensitivity in βIRKOs and controls (Fig. 1*e*). Due to loss of insulin signaling in hepatocytes, LIRKOs and βIRKO/LIRKOs showed significant alterations in hepatic function (SI Table 2).

Islet Growth Response in βIRKO/LIRKOs. To directly evaluate the role of insulin receptors in compensatory islet hyperplasia, we examined β-cell mass in βIRKO/LIRKO mice. The normoglycemic and hyperinsulinemic LIRKO mice showed ≈2-fold elevation in β-cell mass by 2 weeks, which increased to ≈4-fold by 6 weeks (Fig. 2 *a* and *b*) (4). A similar trend was evident in total islet cell mass (SI Table 2). In striking contrast and despite a similar level of hyperinsulinemia, we did not detect an increase in β-cell mass in βIRKO/LIRKO double mutants even at 6 weeks (Fig. 2*b*). The increase in β-cell mass in LIRKO mice was, in part, due to mitosis (SI Table 2). At 6 weeks, we did not observe significant differences between groups in TUNEL+ β-cells (data not shown), α-cell mass, or circulating glucagon levels (SI Table 2).

The compensatory hyperplasia in 2-week-old LIRKO mice, in the absence of hyperglycemia, indicates that potential circulating factors (19), including insulin, could contribute to an increase in β-cell mass (17, 20). The absence of islet growth in response to hyperglycemia in βIRKO/LIRKOs further supports the concept of a glucose-independent circulating β-cell growth factor that requires functional insulin receptors on β-cells (19). It is unlikely that IGF1 contributes to the compensatory β-cell growth in the double mutants because circulating levels of IGF1 are equally low in both LIRKO and βIRKO/LIRKO mice at age 6 weeks (SI Table 2).

To gain some insight into functional defects, we generated a fresh cohort of mice and isolated islets and pancreas for secretion and immunohistochemical studies, respectively. Insulin secretion at baseline levels (5.5 mM glucose) was significantly lower in the βIRKO/LIRKO group compared with all other groups in size-matched batch-incubated islets (Fig. 2*c*). Increasing glucose concentrations to 11 mM promoted stimulation of insulin secretion in controls and LIRKOs, whereas βIRKO and βIRKO/LIRKO groups showed blunted insulin release, consistent with loss of acute-phase insulin secretion. Insulin content in islets was mildly but significantly reduced in 4-week-old βIRKO/LIRKO mice (SI Table 2), consistent with a patchy and reduced intensity of immunostaining for insulin in pancreas sections (Fig. 2*d*).

Most of the double mutants (82%) died at ≈6 weeks of age,

whereas some mutants survived a few weeks longer. βIRKO/LIRKO mice, in terminal stages, showed classic signs of severe diabetes, including an ≈30% loss of body weight (SI Table 2), glycosuria (SI Table 2), polydispsa (almost empty drinking water in bottles), and polyuria (wet bedding). It is likely that the overt hyperglycemia adversely impacts β-cells in the βIRKO/LIRKO mutants in terminal stages of the disease, consistent with a role for glucotoxicity in β-cell dysfunction (21, 22).

To examine whether the islet hyperplasia persisted in adult LIRKO mice, we studied the mutants, along with βIRKO and control groups, up to 12 months of age. Some of the βIRKO mice died due to severe hyperglycemia, whereas the surviving βIRKOs exhibited a significant decrease in β-cell mass compared with controls (Fig. 3*a*) (7, 16, 17). Interestingly, the blood glucose levels were significantly lower in 10- to 12-month-old LIRKOs compared with controls (control, 128 ± 22; LIRKO, 74 ± 21; βIRKO, 188 ± 41 mg/dl; $P < 0.05$ LIRKO vs. control or βIRKO; $P < 0.05$, βIRKO vs. control; $n = 5–9$). Analyses of pancreas sections in LIRKO mice revealed a massive 27-fold increase in β-cell mass that was characterized by a significant ≈8-fold increase in BrdU+ β-cells, indicating enhanced replication of β-cells (Fig. 3 *a–c*). Using TUNEL staining, we also observed a slight but significant increase in the number of apoptotic β-cells, suggesting an increased β-cell death rate in the hyperplastic LIRKO islets at this older age (control, 0.4 ± 0.05; LIRKO, 0.7 ± 0.06% of total β-cells; $P < 0.05$; $n = 4$). It is likely that the significant increase in replication outweighed the rate of β-cell death, leading to enhanced β-cell mass in the LIRKO group. Longitudinal assessment of blood glucose, serum insulin, and BurdU+ β-cells in control and LIRKO mice, from ages 6 weeks to 20 months, revealed a positive correlation between circulating insulin levels and BrdU+ β-cells (Fig. 3*d*). The persistence of robust islet hyperplasia in 10- and 20-month-old normoglycemic LIRKOs further supports the concept of a glucose-independent β-cell proliferation factor (19).

Hepatic Gene Expression Patterns in Diabetic βIRKO/LIRKO Mice. To assess whether hepatic gene expression is altered in βIRKO/LIRKOs, we harvested livers and subjected them to RT-PCR (23). As expected, no significant differences were observed in the expression of any of the hepatic genes examined between controls and βIRKOs. In contrast and consistent with severe hepatic insulin resistance in LIRKOs (4) and βIRKO/LIRKOs, expression of several genes involved in gluconeogenesis and glycolysis was altered favoring increased hepatic glucose output (SI Fig. 5). We only measured mRNA levels due to limited samples, and it is possible that protein expression levels differ from mRNA expression patterns due to posttranslational regulation. Although the overall hepatic gene expression patterns were strikingly similar between LIRKOs and βIRKO/LIRKO groups, the latter group developed early hyperglycemia, leading to premature death that was likely due to inappropriate compensation by β-cells.

βIRKO, but Not βIGFRKO, Mice Fail to Manifest Compensatory Islet Hyperplasia in Response to High-Fat Diet-Induced Insulin Resistance. Compensatory islet hyperplasia is a consistent feature of obesity-induced insulin resistance (17). To examine the specificity of insulin versus IGF1 receptors in mediating the β-cell growth response, we subjected 8-week-old βIRKO and βIGFRKO mice to a high-fat diet for 20 weeks and examined the consequences on compensatory islet hyperplasia. Controls included mice on a mixed-genetic background expressing RIP-Cre, IR Lox, or wild-type mice. Consistent with other reports, no significant phenotypic differences were observed among controls in regard to glucose tolerance tests, circulating insulin, or blood glucose levels (SI Fig. 6). Therefore, we used RIP-Cre mice as controls for all subsequent analyses.

As expected, all mice on the high-fat diet had significantly increased body weights compared with their respective chow-fed controls, and all groups on the high-fat diet manifested hyperinsulinemia and reduced insulin sensitivity consistent with development of obesity-induced insulin resistance (Fig. 4*a*; SI Table 3). Although all groups developed elevated blood glucose levels as a consequence of the high-fat diet, the βIRKO group displayed the most severe hyperglycemia, and 30% died after ≈16 weeks on the high-fat diet (Fig. 4*b*; SI Table 3). In contrast, fat-fed βIGFRKO mice showed mild hyperglycemia similar to control mice on the high-fat diet, and both βIGFRKO and controls survived to the end of the study. The surviving βIRKO and βIGFRKO mice exhibited intolerance compared with controls on normal chow (Fig. 4*b*) (7, 8). All groups on the high-fat diet showed a higher excursion of glucose compared with the respective chow-fed controls. Again, βIRKO mice exhibited severe fasting hyperglycemia and the most severe intolerance

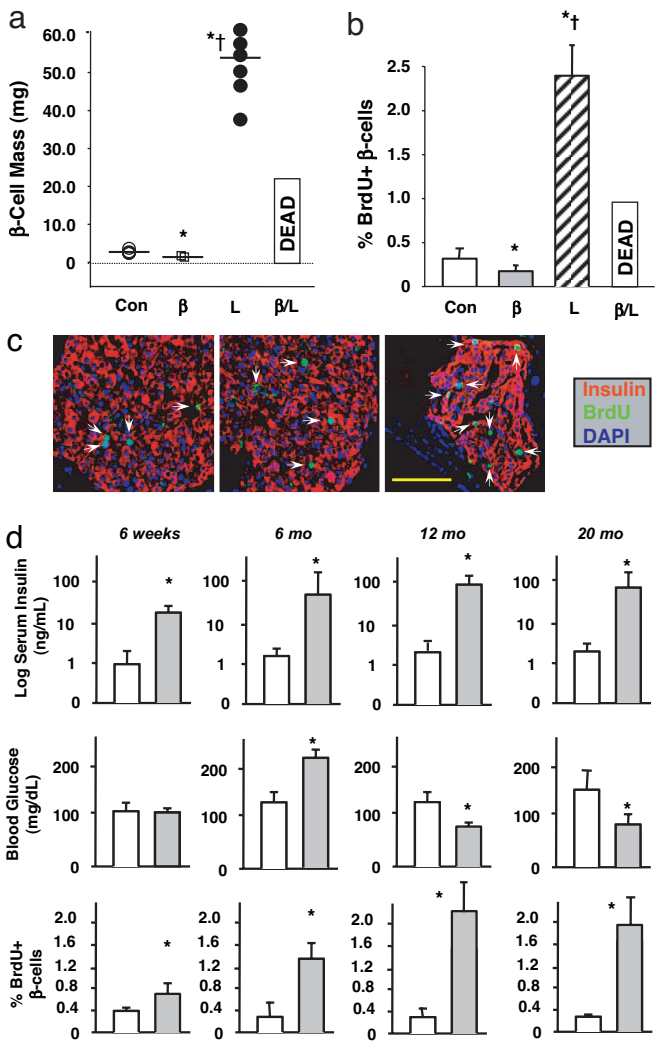

Fig. 3. LIRKO mice show massive islet hyperplasia secondary to enhanced β-cell replication. (*a*) Morphometric analyses of β-cell mass in 10- to 12-month-old control, βIRKO, and LIRKO mice as described in *Methods*. *, $P < 0.05$ control vs. βIRKO or LIRKO; †, $P < 0.01$, βIRKO vs. LIRKO ($n = 4–6$). βIRKO/LIRKO mice were dead by ≈8 weeks. Analyzed by single observer blinded to genotypes. (*b*) BrdU+ β-cells in 10-month-old control, βIRKO, and LIRKO mice. *, $P < 0.05$ control vs. βIRKO or LIRKO; †, $P < 0.01$, βIRKO vs. LIRKO ($n = 4–6$). (*c*) Representative islets from three male LIRKOs immunostained for insulin (red), BrdU (green), and nuclear stain DAPI (blue). Arrowheads point to BrdU+ cells. (Magnification: ×63.) (Scale bar: 100 μM.) (*d*) Alterations in blood glucose, serum insulin, and BrdU+ β-cells in 6-week- and 6-, 10-, and 20-month-old control and LIRKO male mice. *, $P < 0.05$ LIRKO vs. control; $n = 4–7$. Serum insulin is plotted on log scale. BrdU immunostaining was performed as described in *Methods*.

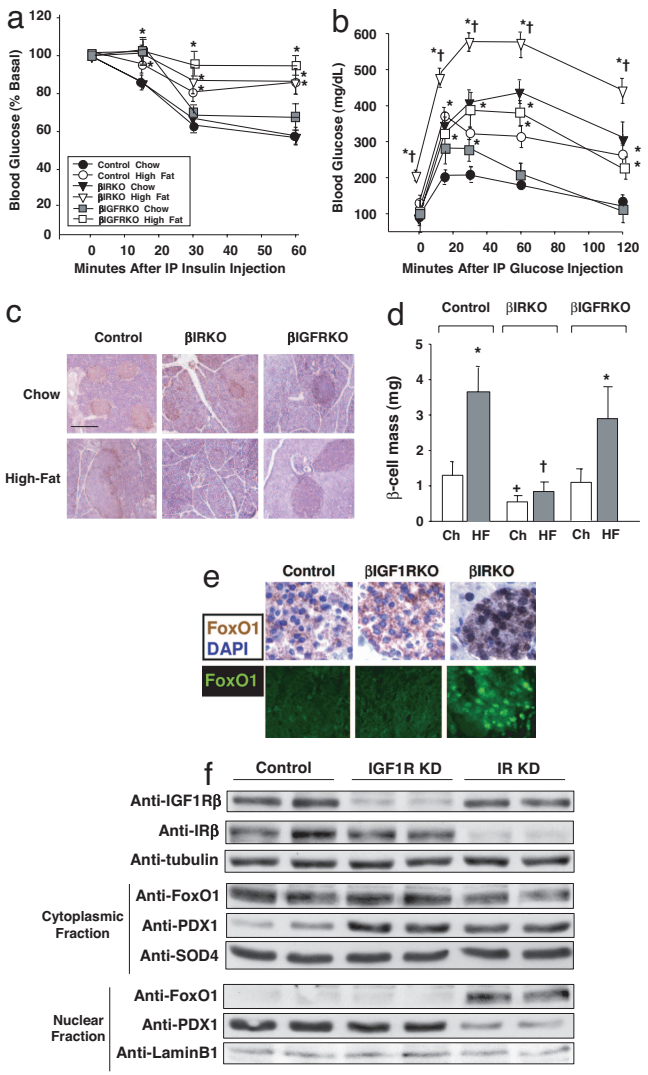

Fig. 4. βIRKO, but not βIGFRKO, mice fail to manifest compensatory islet hyperplasia in response to high-fat diet-induced insulin resistance due to nuclear restriction of FoxO1. (*a*) Insulin tolerance tests in control, βIRKO, and βIGFRKO mice on chow or high-fat diets at the end of 20 weeks. *, $P < 0.05$ high-fat diet vs. chow ($n = 9–16$). (*b*) Glucose tolerance tests in control, βIRKO, and βIGFRKO mice on chow and high-fat diets at the end of 20 weeks. *, $P <$ 0.05 high-fat diet vs. chow; †, $P < 0.01$, βIRKO (high-fat) vs. control (high-fat) or βIGFRKO (high-fat). βIRKO mice on chow diet are significantly different from control group on chow diet at 15, 30, 60, and 120 min. βIGFRKO mice on chow diet are significantly different from control mice on chow diet at 15 and 30 min. βIRKO mice on chow diet are significantly different from βIGFRKO mice on chow diet at 30, 60, and 120 min ($n = 9–16$). (*c*) Representative pancreas sections from control, βIRKO, and βIGFRKO mice at the end of 20 weeks on chow or high-fat diets immunostained with mixture of antibodies against non-β-cell hormones as described in *Methods*. (Magnification: ×20.) (Scale bar: 100 μM.) (*d*) Morphometric analyses of β-cell mass in control, βIRKO, and βIGFRKO mice on chow or high-fat diets as described in *Methods*. *, $P < 0.05$ chow vs. high-fat diet in each group; +, $P < 0.05$ βIRKO (chow) vs. control (chow); †, $P < 0.01$, βIRKO (high-fat) vs. control (high-fat) or βIGFRKO (high-fat) ($n = 5–7$). Analyzed by a single observer blinded to genotypes. (*e*) Representative pancreas sections of control, βIGFRKO, and βIRKO mice on high-fat diet immunostained with anti-FoxO1 antibody (dark brown) and nuclear stain DAPI (blue) (*Upper*) or for FoxO1 only (*Lower*) by using immunofluorescent antibody as described in *Methods* ($n = 3–4$ per group). (Magnification: ×63.) (*f*) Representative Western blot of control β-cell lines, β-cells with a 95% knockdown of insulin receptors, or β-cells with a ≈90% knockdown of IGF1 receptors. Expression of insulin receptors (IR) and IGF1 receptors (IGF1R) was examined in whole-cell lysates, and expression of FoxO1 and PDX-1 protein was examined in cytosolic and nuclear fractions as described in *Methods*.

compared with βIGFRKOs and controls, indicating a differential response to high-fat feeding.

Examination of pancreata from surviving animals at the end of 20 weeks on chow and high-fat diets revealed a robust islet hyperplastic response in controls, indicating compensation to overcome the hyperinsulinemia (Fig. 4 *c* and *d*). Surprisingly, βIGFRKO mice also showed a similar compensatory response, with a 3-fold increase in β-cell mass, suggesting that lack of functional IGF1 receptors is unlikely to be critical for β-cell growth even in the postdevelopmental period. By contrast, high-fat-fed βIRKO mice showed significantly reduced β-cell mass compared with controls and βIGFRKOs, which contributed, in part, to the severe intolerance in the βIRKO group. Consistent with a reduced β-cell mass, serum C-peptide levels were also significantly low in βIRKO mice on the high-fat diet (SI Table 3).

Disruption of Insulin Signaling Prevents the Nuclear Exclusion of FoxO1. We next immunostained pancreas sections from βIRKO and βIGFRKO mice for proteins in the insulin/IGF1 signaling pathway, including IRS-2, *p*-Akt, *p*-4EBP-1, and FoxO1 (SI Fig. 7). Although subtle differences for several of these proteins were evident between βIRKOs and controls or βIGFRKOs, the data were difficult to quantify. In contrast, we observed a striking effect on FoxO1 localization. Thus, β-cells lacking functional insulin receptors showed clear nuclear restriction of the transcription factor in contrast to control and βIGFRKO mice, wherein β-cells showed only cytosolic immunostaining for FoxO1 (Fig. 4*e*). We observed a significantly high percentage of β-cells exhibiting FoxO1 internalization in βIRKOs compared with control or βIGFRKOs on a high-fat diet (control, 12 ± 7; βIRKO, 58 ± 10; βIGFRKO, 14 ± 8; percentage of 2,000 β-cells counted; $P < 0.05$ βIRKO vs. control or βIGFRKO; $n = 3$), whereas very few β-cells showed FoxO1 nuclear localization in chow-fed controls (4 ± 2%). To examine whether a similar response could be elicited *in vitro*, and to better quantify the data, we examined β-cell lines with a knockdown of insulin or IGF1 receptors (13). Again, β-cells with a 95% knockdown of insulin receptors showed nuclear restriction of FoxO1, in contrast to β-cells with ≈90% knockdown of IGF1 receptors that exhibited immunostaining for FoxO1 in the cytosol, but a virtual absence of the transcription factor in the nucleus (Fig. 4*f*). Analyses of PDX-1, a downstream target of FoxO1, showed a reduced nuclear expression in βIRKO compared with βIGFRKO or control cell lines. A similar pattern was evident in pancreas sections from high-fat-fed mice (SI Fig. 7*d*). Further, evaluation of phosphohistone H3 (pHH3), a marker of proliferation, revealed significantly reduced pHH3+ β-cells in βIRKO compared with controls or βIGFRKO (control, chow, 0.4 ± 0.06; high fat, 0.8 ± 0.05; βIGFRKO, chow, 0.3 ± 0.04; high fat, 0.7 ± 0.06; βIRKO, chow, 0.2 ± 0.03; high fat, 0.3 ± 0.03% +β-cells, 500 cells counted; $P <$ 0.05 βIRKO high fat vs. control high fat or βIGFRKO high fat; $P <$ 0.05 control chow vs. control high fat or βIGFRKO chow vs. βIGFRKO high fat; $P =$ NS, βIRKO chow vs. βIRKO high fat; $n =$ 3 mice from each group). Evaluation of apoptosis by using anti-cleaved caspase 3 antibody did not reveal significant differences between groups (data not shown). These data indicate that insulin- but not IGF1-regulated nuclear restriction of FoxO1 is one mechanism that prevents the ability of β-cells to proliferate.

Discussion

Islet compensatory response to insulin resistance is a recognized feature in obesity and type 2 diabetes. However, the signals and proteins that mediate this important adaptive response are poorly understood (17). To dissect the signaling pathways regulating β-cell growth, we and others have focused on insulin and IGF1, which modulate proliferation and metabolism of most tissues in mammals, including the endocrine pancreas. Indeed, receptors for insulin, IGF1, and growth hormone are expressed in β-cells (reviewed in ref. 24), and IGF1 has been suggested to

prevent apoptosis and act as a mitogen in β-cells (reviewed in ref. 25). Contrary to traditional thought, however, we and others, by using genetically engineered mice, have directly demonstrated that the insulin/IGF1 signaling pathway is not critical for early development of β-cells (7–11).

In this study, we asked whether insulin or IGF1 is critical in mediating the compensatory islet hyperplastic response to insulin resistance in adult mice. Our findings, using two different models of insulin resistance, clearly indicate that in postdevelopmental stages, insulin signaling but not the IGF1 pathway plays an important role in modulating the islet proliferation response to insulin resistance. The presence of islet hyperplasia in LIRKO mice, which show significantly low circulating levels of IGF1, also suggests that this growth factor is unlikely to be a major mediator of β-cell proliferation in this model. The critical significance of compensatory islet hyperplasia to prevent and/or delay development of overt diabetes is highlighted by striking differences in phenotypes between the LIRKO and βIRKO/LIRKO groups, both of whom showed hepatic gene expression patterns favoring increased hepatic glucose output. The mild hyperglycemia in the LIRKOs, compared with the early onset and progressive hyperglycemia in βIRKO/LIRKO, suggests that the β-cell proliferation response in LIRKOs is able to limit the hyperglycemia in LIRKOs, whereas the virtual lack of this proliferative response triggers diabetes in the βIRKO/LIRKO double mutants. In a second series of experiments, we used the high-fat diet-induced insulin resistance model and again observed a poor islet compensatory growth response in βIRKOs compared with βIGFRKO and control mice. Although the absence of a β-cell proliferation defect in adult mice lacking IGF1 receptors in β-cells is intriguing, a recent report that homozygous deletion of the IGF1 gene in islets leads to hyperplastic islets (26) questions the dogma that IGF1 is indeed a β-cell growth factor in normal islets.

Because insulin resistance affects all tissues in the body, it has been a challenge over the years to design experiments to isolate the signal that promotes islet growth in insulin-resistant states. The availability of tissue-specific models of insulin resistance such as the LIRKO provides us with unique tools to begin to identify the potential source of the putative islet cell growth factor in insulin-resistant states. Based on the absence of detectable islet hyperplasia in other tissue-specific models of insulin resistance, including muscle-specific insulin receptor knockout (27) and fat-specific insulin receptor knockout mice (28), it is tempting to speculate that the liver is the source of a putative β-cell growth factor in insulin-resistant states. However, in the context of the singular difference in genotypes between LIRKOs and βIRKO/LIRKOs, i.e., absence of functional insulin receptors in β-cells in the latter, our experiments provide strong evidence that insulin is an important β-cell proliferation factor (20, 29). This is supported by studies in βIRKO mice, which show an age-dependent decrease in β-cell mass (7, 16), and experiments in β-cell lines treated with insulin receptor siRNA, which show defects in proliferation and cell cycle progression (30). Furthermore, as reported in transplantation models of fetal rat pancreas, however, insulin may also act permissively by modifying the regenerative ability of β-cells (31, 32). On the contrary, mice lacking the insulin gene have been reported to exhibit islet cell proliferation and increased vascularity, suggesting a negative role for insulin in islet growth (33). It is worth noting that a complete lack of insulin in these animals, however, may allow IGF1 and IGF2 to act freely via insulin receptors (6) and promote proliferation of islet cells. Indeed, the local levels of IGF2 have been reported to be high in the neonatal pancreas (34). Alternatively, enhanced vascularity in the islet, in the absence of insulin, may increase local concentrations of morphogens to influence cell growth (35). Finally, it is logical that nutrients including glucose necessarily promote growth of virtually every cell that is dependent on the sugar for survival. However, identifying proteins that mediate purely nutritional effects of glucose from proteins that transmit true mitogenic effects will allow integration of a unified pathway in the overall regulation of β-cell proliferation and survival.

Our recent findings that haploinsufficiency of PDX-1 limits the ability of islets to proliferate in response to ambient insulin resistance point to the homeodomain protein as another important player in the proliferation response of adult β-cells (36). The transcription factor FoxO1, which links insulin signaling to PDX-1 modulation of β-cell growth (37), has been directly implicated in cell cycle control in several cell types (38). The nuclear restriction of FoxO1 and the reduced expression of its target, PDX-1, in β-cells from βIRKO, but not control or βIGFRKO mice, indicates that insulin- but not IGF1-stimulated exclusion of FoxO1 from the nucleus is one essential signal for the β-cell proliferative response (39). The *in vitro* model we have used may not precisely reflect the effects of "insulin resistance" growth signals that are occurring *in vivo*, and it is possible that other cell cycle proteins that are regulated by Akt and/or FoxO1 can also contribute to β-cell growth in insulin-resistant states.

In summary, we provide direct genetic evidence that insulin signaling is necessary and specific for the proliferation response in adult β-cells. Defining the link between insulin/FoxO1 signaling, PDX-1 expression, and cell cycle regulation in β-cells will provide crucial clues to understanding this important biological feedback mechanism that underlies compensatory islet hyperplasia with the aim of planning therapeutic strategies to counter loss of β-cell mass in both type 1 and type 2 diabetes.

Methods

Animals. Insulin receptor or IGF1 receptor-floxed mice were maintained on the original mixed-genetic background (C57BL/6 × DBA/2 × 129/Sv) and crossed with mice expressing *Cre* recombinase under a rat insulin promoter (7) or albumin promoter (4) in the Joslin Animal Facility to generate βIRKO, βIGFRKO, or βIRKO/LIRKO mutants and littermate controls. We used RIP-Cre mice, used for deriving both βIRKO or βIGFRKO mice, as controls. All mice were housed in pathogen-free conditions on a 12-h light/dark cycle and had free access to water and food (Mouse Diet 9F; Taconic Farms, Germantown, NY). Genotyping was performed by PCR by using genomic DNA from tail biopsies. All protocols were approved by the Animal Care Committee of the Joslin Diabetes Center and were in accordance with National Institutes of Health guidelines.

For high-fat feeding studies, a separate cohort of 8-week-old RIP-Cre control, βIRKO, and βIGFRKO male mice were fed a diet containing either normal chow (fat content by calories 16.7%; Formulab Diet, 5008) or a high-fat diet (fat content by calories 55%; Harlan Teklad, Madison, WI; TD 93075) for 20 weeks. Body weights were followed weekly, and glucose and insulin tolerance tests were performed during the last week of the feeding period as described below. Blood was collected for measurement of glucose (Glucometer Elite), serum insulin (ELISA), and serum C-peptide (RIA) levels at the end of the study. Pancreata were harvested for immunohistochemical analyses and islet morphometry as described below.

Islet Morphology and Immunohistochemistry. Tissues were fixed in Bouin's solution and 10% buffered formalin and imbedded in paraffin. Sections of pancreas were stained for non-β-cell hormones by using a mixture of antibodies to glucagon, somatostatin, and pancreatic polypeptide (7, 13). β-cell mass was evaluated by point-counting morphometry on immunoperoxidase-stained sections of pancreas (13). For estimation of β-cell proliferation, mice were injected with BrdU (100 mg per kg of body weight; Sigma–Aldrich, St. Louis, MO) 5 h before being killed, and pancreata were harvested and fixed in 4% paraformaldehyde as described earlier (19). Sections were immunostained with anti-BrdU and anti-insulin antibodies or anti-phosphohistone H3 antibody (Upstate Biotechnology, Lake Placid, NY) or FoxO1

(Cell Signaling Technology, Danvers, MA) and appropriate secondary antibodies (The Jackson Laboratory, Bar Harbor, ME; Amersham Biosciences, Piscataway, NJ) (19). At least 500–1,000 β-cell nuclei were counted per pancreas, and data were expressed as the percentage of BrdU+ or pHH3+ β-cells. Cell death was detected by anti-cleaved caspase-3 antibody (Research and Development, Minneapolis, MN) or enzymatic *in situ* labeling for DNA strand breaks by using TUNEL method by using an *in situ* cell death detection kit (Roche Diagnostics, Indianapolis, IN). Staining was performed by using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Vector Laboratories, Burlingame, CA) as the substrate. Subsequently, sections were stained with anti-insulin antibody as described earlier and visualized by peroxidase and diaminobenzidine tetrahydrochloride substrates (DAKO Cytomation, Carpinteria, CA) (40). At least 500–1,000 β-cell nuclei were counted per pancreas, and data are expressed as a percentage of apoptotic β-cells.

Blood Glucose, Serum Insulin, and Metabolites. Blood glucose (Glucometer Elite) and serum insulin levels (ELISA) were measured in the Joslin DERC Specialized Assay Core (7). Triglyceride levels in serum from fasted animals were measured by GPO-Trinder Assay (Sigma–Aldrich) (4). Free fatty acid levels were analyzed in serum from fasted animals by using the NEFA-kit-U (Amano Enzyme). Liver function tests were measured by using a Beckman CX7 analyzer (Beckman Coulter, Fullerton, CA). IGF1 and C-peptide levels were measured by RIA (Alpco, Salem, NH). Urinary glucose was measured by using reagent strips (Uristix; Bayer Corp., Emeryville, CA).

Glucose and Insulin Tolerance Tests, *in Vivo* Insulin Secretion, *in Vitro* Insulin Secretion, and Insulin Content. Glucose and insulin tolerance tests and acute insulin secretion tests and insulin secretion during GTT were performed as described previously (7). For *in vitro* insulin secretion tests, islets were cultured overnight, and size-matched islets were subjected to glucose stimulation for 45 min. Media were collected for insulin ELISA, and islet insulin content was measured by acid ethanol extraction followed by ELISA (7, 16).

Semiquantitative and Real-Time RT-PCR. For measurement of steady-state mRNA levels, semiquantitative RT-PCR was performed with liver or islet RNA (TRIzol; Invitrogen, Carlsbad, CA) as described earlier (8). For quantitative real-time PCR, total RNA samples were isolated by using RNeasy Mini Kit (Qiagen, Valencia, CA) and reverse-transcribed by using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). cDNA samples were amplified by using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and analyzed on an ABI PRISM 7000 sequence detection system (Applied Biosystems).

Knockdown of Insulin and IGF1 Receptors and Western Blot Analysis. SMARTpool small interfering RNAs (siRNAs) against an IGF1 receptor (Dharmacon RNA Technologies, Lafayette, CO) were transfected by using Lipofectamine 2000 (Invitrogen) according to the respective manufacturers' protocols to achieve final siRNA concentrations of 100 nM. Nontargeting siRNA was used as a control. Cells were harvested 48 h after transfection for protein extraction (41). Western blotting for insulin receptor, IGF1 receptor, FoxO1, and cytosolic and nuclear markers was performed by using commercially available antibodies (Abcam, Cambridge, MA) and PDX-1 (Chemicon International, Temecula, CA) by using standard protocols (27).

Statistical Analyses. Comparisons between groups were made by Student's *t* test ANOVA with Boneferroni correction or appropriate post hoc tests. The null hypothesis was rejected at the 0.05 level. All data are expressed as means ± SEM unless indicated otherwise. A minimum of $n = 3$ was used for all studies unless indicated otherwise.

The authors thank C. Ronald Kahn (Joslin Diabetes Center, Boston, MA) for critical comments and LIRKO mice. This study was supported by National Institutes of Health Grants R01 DK67536, R01 DK68721, and U10DK42502 (to R.N.K.) and R01 DK55033 (to M.S.); Harvard Stem Cell Institute (R.N.K.); the Adler and Iacocca Foundations (T.O.); and Joslin Diabetes and Endocrinology Research Center Grant P30 DK36836 (Specialized Assay and Advanced Microscopy Cores; to the Joslin Diabetes Center).

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