Alterations in growth and apoptosis of IRS-1 deficient beta-cells

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Abstract
Insulin and insulin-like growth factor-I (IGF-I) activate anti-apoptotic pathways via insulin receptor substrate (IRS) proteins in most mammalian cells including β-cells. IRS-1 knockout (IRS-1KO) mice show growth retardation, hyperinsulinemia and hyperplastic but dysfunctional islets without developing overt diabetes, while IRS-2KOs develop insulin resistance and islet hypoplasia leading to diabetes. Since both models display insulin resistance it is difficult to differentiate islet response to insulin resistance from islet defects due to loss of proteins in the islets itself. We used a transplantation approach, as a means of separating host insulin resistance from islet function, to examine alterations in proteins in insulin/IGF-I signaling pathways that may contribute to β-cell proliferation and/or apoptosis in IRS-1KO islets. Islets isolated from wildtype (WT) or IRS-1KO mice were transplanted into WT or insulin resistant IRS-1KO males under the kidney capsule. The β-cell mitotic rate in transplanted islets in IRS-1KO recipients was increased 1.5-fold compared to WT recipients, and was similar to that in endogenous pancreases of IRS-1KOs, while β-cell apoptosis was reduced by ~80% in IRS-1KO grafts in IRS-1KO recipients compared to WT recipients. Immunohistochemistry showed a substantial increase in IRS-2 expression in IRS-1KO islets transplanted into IRS-1KO mice as well as in endogenous islets from IRS-1KOs. Furthermore, enhanced cytosolic Forkhead transcription factor (FoxO1) staining in IRS-1KO grafts suggests intact Akt/PKB activity. Together, these data indicate that even in the absence of insulin resistance, β-cells deficient in IRS-1 exhibit a compensatory increase in IRS-2, which is associated with islet growth and is characterized by both proliferative and anti-apoptotic effects that likely occur via an insulin/IGF-1/IRS-2 pathway.

Key words: IRS proteins, islets, transplantation, insulin resistance, beta-cell growth
Running Title: beta-cell growth in IRS-1 deficient mice
Introduction

Type 2 diabetes is a metabolic disease characterized by insulin resistance in peripheral organs including liver, skeletal muscle and adipose tissue coupled with a failure of the β-cells to compensate for the increasing demand (15; 32; 33; 48). Naturally occurring rodent models of diabetes and obesity, such as the ob/ob or db/db mouse, and genetically engineered models of insulin resistance such as mice heterozygous for deletion of the insulin receptor and insulin receptor-substrate-1 (IR/IRS-1) or homozygous for deletion of IRS-1 (IRS-1KO) all show islet hyperplasia (18; 25; 34). Although the precise pathway(s) that underlie the islet hyperplasia in each of the different models are not fully defined, several factors including glucose, insulin, growth hormone, prolactin, placental lactogen, glucagon-like peptide-1, hepatocyte growth factor/scatter factor, and the insulin-like growth factors (IGFs) have all been implicated in the β-cell proliferation response (25; 46; 47)(11; 39).

In addition to the effects on classic target tissues, including the skeletal muscle, liver and adipose, it is now well established that the insulin/IGF-I signaling pathway plays an important role in non classical tissues such as pancreatic islets (21) and the central nervous system (4). For example, mice lacking functional receptors for insulin (βIRKO) or IGF-I (βIGFKO) in β-cells manifest a type 2 diabetes phenotype characterized by loss of first phase insulin response to glucose stimulation and progressive glucose intolerance (22; 23; 51). Although, neither βIRKO nor βIGFKO mice exhibit developmental defects in islets, the βIRKO mice do show failure to increase β-cell mass in an age-dependent manner suggesting a role for insulin in growth and/or survival of β-cells. Furthermore, we have previously shown that islets from normal WT mice transplanted under the renal capsule of insulin-resistant normoglycemic insulin receptor/insulin receptor substrate-1 double heterozygous (DH) or ob/ob mice, proliferate at a higher rate
compared to WT recipients (10), suggesting that either insulin itself and/or a glucose-independent factor promotes islet growth in these models.

In many different types of cells, insulin exerts anti-apoptotic and proliferative effects by activating IRS proteins (35; 44). All four IRS proteins, including IRS-1, -2, -3 and –4, are expressed in mouse islets (27). Each IRS-protein contains a highly conserved N-terminal pleckstrin homology (PH) domain followed by a phosphotyrosine-binding domain (PTB), which together couple IRS proteins to the activated insulin or IGF-1 receptors. Upon phosphorylation, IRS proteins bind to SH2 domains of effector proteins, including the regulatory subunit of the lipid kinase phosphatidylinositol 3-kinase (PI 3-kinase) (37). Products of PI 3-kinase activate a network of serine/threonine kinases implicated in the pleiotropic effects of insulin including glucose transport, glycogen and protein synthesis, hepatic gluconeogenesis, anti-apoptosis and proliferation (6). Although the precise pathways that mediate growth and anti-apoptotic effects of insulin in pancreatic β-cells have not been fully explored, the forkhead transcription factor, FKHR, has been recently implicated in β-cell growth (17), while other studies suggest a role for signals from endothelial cells including vascular endothelial growth factor A (VEGFA) (29; 31).

IRS-1 is an important mediator of insulin/IGF-1 receptor signaling in most tissues, including islets. IRS-1KO mice develop hyperplastic islets that is likely due to the insulin resistance and/or ambient hyperinsulinemia (1; 27; 41). In contrast, IRS-2KOs manifest hepatic insulin resistance and islet hypoplasia of varying severity depending on the genetic background, leading to a phenotype ranging from mild to severe diabetes, and suggesting that IRS-2 is involved in β-cell growth and/or survival (20; 49; 50). The potential role of IRS-2 in β-cell growth and/or anti-apoptosis prompted us to examine whether this substrate protein is also involved in the islet hyperplastic response in mice lacking IRS-1. Using a transplantation
approach, as a means of separating host insulin resistance from islet function, we show that enhanced β-cell proliferation and survival in islets deficient in IRS-1 is associated with a compensatory increase in expression of IRS-2.

**Materials and Methods:**

**Animals**

IRS-1 knockout mice (IRS-1KO) were backcrossed onto the C57Bl/6 background at Taconic Farms and were essentially syngeneic. All mice were maintained on a 12-hour light/dark cycle and housed at the Joslin Diabetes Center in accordance with IACUC protocols. Mice were fed standard mouse chow *ad libitum* and were housed individually after transplantation.

**Islet Isolation and Transplantation**

Islets were isolated from 8-week-old male mice as described previously (27) using the intraductal liberase technique and hand-picked under a stereomicroscope (Stereozoom GZ7, Leica, Deerfield, IL). Following isolation, 150 islets were hand-picked and kept on ice until transplantation. We chose 150 size-matched islets based on pilot studies wherein 150 islets did not alter metabolic variables including serum insulin and blood glucose levels in the recipients. In the event some of the isolated islets were larger we transplanted similar islet equivalents (IEs) so that total amount of islet tissue was comparable between all groups. Surgery was performed under anesthesia that was induced by an intra-peritoneal injection of a 1:1 mixture of 2,2,2-tribromoethanol and tert-amyl alcohol and diluted 1:50 in PBS (pH 7.4) at a dose of 15 µl/g body weight. Using a retroperitoneal approach, the capsule of one of the kidneys was incised, and islets were implanted near the upper pole in 8-week-old male recipient mice. The capsule was cauterized, and mice were allowed to recover on a heating pad.
Metabolic analysis.

Body weight, blood glucose and serum insulin levels were followed weekly after transplantation. Blood was obtained from the tail vein in the fed state and glucose levels were measured using Glucometer Elite (Bayer). For insulin measurement, blood was collected in chilled heparinized capillaries and immediately centrifuged to obtain plasma that was stored at –20 C for subsequent insulin ELISA (Crystal Chem, Chicago, IL) or for serum IGF-I by RIA (ALPCO).

DNA Laddering

To examine whether islet cells lacking IRS-1 are more resistant to cell death we treated WT and IRS-1KO islets with TNFα and subjected the extracted DNA to laddering using standard protocols (Biosource Kit) (12). Briefly, islets are plated on 6 cm dishes and cultured overnight. Following treatment with TNFα (50 ng/mL), the supernatant is discarded and attached cells are removed. Lysis buffer and enzyme are added following manufacturers instructions; the solution is vortexed and placed at 37 C for 10 min. Ammonium acetate and cold methanol (-20 C) are added and tubes placed in -20C for 15 min. The tubes are centrifuged at 12,000 rpm for 5 min and pellet is washed with cold 70% ethanol. The supernatant is discarded and pellet is allowed to dry at RT for 5 min. The DNA pellet is resuspended in 30 µl of DNase-free water and allowed to dissolve for 24 h. DNA content is measured using a spectrophotometer and 1µg is run on a 1.5% agarose gel. Three mice from each group were evaluated.

Sectioning of Endogenous Pancreas and Islet Grafts.
Five weeks after transplantation, the random fed recipient mice were injected with 5-bromo-2-deoxyuridine (BrdU, 100 µg/g b.wt; Roche, IN) and 6 hours later were anesthetized and sacrificed by perfusion using 4% PFA solution. The endogenous pancreas and kidney tissue including islet graft was rapidly harvested from recipient mice and fixed in 4% PFA for 16 hours. Pancreas were embedded in paraffin and sectioned (7-10 µm thickness). Further, kidney tissue was embedded in Araldite 502 resin and cured at 60º C for 48 hours. One micron sections were cut, adhered to glass slides with heat and stained with 1% methylene blue containing 1% sodium borate. Capillaries were counted as mean ± SEM of capillaries over number of β-cell nuclei per section, 3 sections per animal and at least 3 mice per group (at least 900 capillaries per animal).

**Semi-quantitative fluorescence immunocytochemistry.**

Following re-hydration and permeabilization (1 % Triton X-100), sections were incubated with guinea pig anti-insulin (Zymed Laboratories, Inc.), rabbit anti-IRS-2, or rabbit anti-FKHR (Santa Cruz) antibodies overnight at 4°C. To minimize variability between sections, the staining procedures were performed in parallel with the same batch of antisera and same incubation times for fixation, permeabilization, blocking, and exposure to antisera were employed for all sections. Islet proliferation was estimated by following double-label insulin and BrdU immunohistochemistry. Detection was performed with rhodamine and fluorescein-conjugated secondary antibodies (Jackson Immunoresearch). We identified apoptotic cells in de-paraffinized sections using a Rhodamine DNA fragmentation detection assay (TUNEL, Intergen Company). BrdU and TUNEL positive cells were calculated as the mean ± SEM of at least 500 insulin positive β-cells over total β-cells per section, 3 sections per animal and at least 3 mice per group.
To determine the cell distribution of IRS-2 in islets we immunostained pancreas sections from WT mice with anti-IRS-2 antibody (Santa Cruz) using standard protocols (22).

Western blotting: Islets were lysed with buffer A containing 25 mM Tris HCl (pH 7.4), 2 mM Na3VO4, 10 mM NaF, 10 mM Na4P2O7, 1 mM EGTA, 1 mM EDTA, 5 µg/ml of leupeptin, 5 µg/ml of aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 1 % Nonidet P-40. The lysates were subjected to immunoblotting and visualized by an enhanced chemiluminescence system (Roche Applied Science, Indianapolis, IN). Antibodies recognizing Akt and phospho-Akt (Ser 473) were purchased from Cell Signaling Technology (Beverly, MA) and used at dilution 1:1000 for immunoblotting (45).

Statistics.
Results are expressed as mean ± SEM. For comparison between groups the unpaired Student’s t-test (two tailed) was used. p-values less than 0.05 were considered significant.

Results:
Four groups of 8-week-old WT or IRS-1KO male mice on a pure C57Bl/6 background were transplanted with either 150 WT or IRS-1KO islets under the kidney capsule and followed weekly with measurements of body weight, serum glucose and insulin levels. Before transplantation, IRS-1KO mice were ~35% smaller than WT mice as previously described (20; 27) (Fig. 1a). The transplanted islet mass did not alter blood glucose or body weight over the 5-week transplantation period (Fig. 1a,b). Eight-week-old IRS-1KO mice were hyperinsulinemic with ~3-fold elevated serum insulin levels compared to WT mice as previously observed (1; 41), and these did not alter significantly over the transplantation period (Fig. 1c). Serum IGF-I
Concentrations were comparable in IRS-1KO and WT mice (284 ± 29 vs 310 ± 37 ng/mL; n=6; p = NS).

Compensation for insulin resistance can occur either by enhanced insulin secretion, an increase in β-cell mass or both (9; 16; 24). An enhanced β-cell mass can be secondary to replication of preexisting β-cells or an increase in β-cell survival (2; 3). To measure β-cell proliferation and apoptosis, we performed in situ assays using 5-bromodeoxyuridine (BrdU) and terminal deoxyribotransferase (TdT)-UTP nick end labeling (TUNEL) staining on endogenous pancreas and islet graft sections harvested 5 weeks after transplantation. As expected, the percentage of BrdU positive β-cells was increased in pancreas sections of IRS-1KO mice, likely due to peripheral insulin resistance (Fig 2a). In grafts of IRS-1KO islets into IRS-1KO hosts (IRS-1-IRS-1), a profound increase in BrdU incorporation was observed while replication in all other groups was unchanged (Fig. 2b). The increase in BrdU positive cells in IRS-1KO transplanted islets into IRS-1KO mice was similar to that present in endogenous islets of the IRS-1KO host animal and represented a ~41% increase compared to WT. Interestingly, IRS-1KO islets transplanted into WT animals (IRS-1-wt) exhibited a slight, but non-significant, decrease in BrdU incorporation compared to WT islets transplanted into WT mice (wt-wt). Conversely, BrdU incorporation was increased in WT islets transplanted into IRS-1KO mice (wt-IRS-1), suggesting that insulin resistance in IRS-1KO mice is one potential factor promoting β-cell mitosis in WT grafts. In fact, examination of serum insulin levels of host animals in the WT to IRS-1KO group revealed a positive and linear correlation with BrdU incorporation (Fig. 2c), suggesting that insulin itself is a potential islet growth factor (25; 26).

To assess apoptosis, we stained pancreas and graft sections using the terminal deoxyribotransferase (TdT)-UTP nick end labeling (TUNEL) assay. By contrast to proliferation,
TUNEL staining showed a 50% decrease in apoptosis in endogenous IRS-1KO islets as compared to WT islets (Fig. 3a). Next, TUNEL staining in graft tissue showed a ~3-fold increase in apoptosis in the IRS-1KO into WT group, while the number of apoptotic cells in the IRS-1KO into IRS-1KO group was lower but did not reach significant levels (Fig. 3b, c). To confirm the alterations in cell death in IRS-1 knockout islet cells we used a different approach by treating freshly isolated islets with TNF-α for 24 h and subjecting the isolated DNA to laddering. As expected, significant laddering was evident in WT islets and significantly minimal effects were observed in the IRS-1KO group (Fig 3d). These data clearly indicate that cell death is lower in the absence of IRS-1 and it is likely that there are compensatory effects that contribute to protection against cell death in the mutants. Maximal cell death in a transplant setting has been reported to occur two weeks after surgery (8). Therefore, to assess whether a significant reduction in cell death in IRS-1KO beta cells in the presence of hyperinsulinemia can be unmasked at an earlier time after transplantation, we examined apoptosis in the grafts 2 to 3 weeks after transplanting IRS-1KO islets in IRS-1KO recipients. Again, we observed a trend towards a decrease in apoptosis, but no significant differences were evident (data not shown). It is possible that several factors including a greater level of circulating insulin is necessary to significantly impact on apoptosis in β-cells (25).

To determine potential alterations in proteins in the insulin/IGF-I signaling pathway that promote β-cell proliferation and anti-apoptosis we performed immunohistochemistry on endogenous pancreas and islet graft sections. IRS-2 is one of the intermediates suggested to promote β-cell development, proliferation and survival (50). Although we could barely detect IRS-2 expression in WT islets of 13-week old mice, we were able to easily detect IRS-2 in islets from IRS-1KO mice by immunohistochemistry (Fig. 4a). Furthermore, immunostaining for IRS-2
in islet grafts also showed an increase in IRS-2 expression in the IRS-1KO into IRS-1KO group (Fig. 4b), suggesting that the compensatory increase in IRS-2 promotes both proliferation and anti-apoptotic signals in these islets. IRS-2 staining in IRS-1KO islets transplanted into WT mice, on the other hand, was comparable to WT islets, suggesting that elevated serum insulin levels in IRS-1KO host animals likely promote expression of IRS-2 in the absence of IRS-1 (Fig. 4b). Interestingly, we observed expression of IRS-2 protein in \( \alpha \)-cells in WT islets using immunohistochemistry (Fig 4c). Further work is necessary to evaluate whether IRS-2 indeed has a potential role in regulation of islet \( \alpha \)-cell growth and/or function.

The IRS-2-PI 3-kinase cascade controls many downstream elements, including Akt/PKB, BAD/Bcl2, and the FoxO family of transcription factors (AFX, FKHR, and FKHRL1) (17; 36). FKHR is located in the nucleus under basal conditions where it is transcriptionally active. Insulin and IGF-I, acting via Akt, stimulate phosphorylation of FKHR, which causes it to bind to 14-3-3 proteins and accumulates in the cytosol where it is unable to regulate gene expression. Compared to WT islets, IRS-1KO islets transplanted into IRS-1KO mice exhibited increased phosphorylation of FKHR as determined by nuclear exclusion using immunohistochemistry (Fig. 5). Moreover, IRS-1KO islets in WT mice show lower level of cytoplasmic FKHR, suggesting that these cells are undergoing apoptosis. This is consistent with nuclear restriction of FoxO1 (FKHR) in \( \beta \)-cells lacking insulin receptors (T. Okada and R. N. Kulkarni, unpublished observations). Together, these findings are in agreement with an increased number of apoptotic cells in IRS-1KO into WT group compared to transplanted IRS-1KO islets into IRS-1KO mice. We did not detect significant differences in the expression of total or phospho-Akt protein levels between WT and IRS-1 KO groups by Western blotting of freshly isolated islets in the basal state (data not shown). It is possible that exposing the isolated islets to the ambient environment of
hyperinsulinemia found in vivo in the IRS-1KO mice is necessary to unmask differences in Akt levels between groups. Thus, increased expression of IRS-2 is associated with alterations in gene transcription and anti-apoptosis that likely occurs via a PKB/forkhead-transcription-factor-mediated pathway.

PDX-1 is critical for the development of the pancreas in mice and humans and complete disruption of PDX-1 results in pancreatic agenesis (28; 40). Expression of PDX-1 has been shown to be diminished or unchanged in islets from IRS-2KO mice depending on the genetic background of the mutants (28; 42). Immunostaining for insulin and PDX-1 revealed no significant changes among the groups in our study suggesting that proliferation in cells lacking IRS-1 is independent of changes in PDX-1 in this model (Fig. 6).

Microvascularization of islets is essential for successful engraftment and long-term function of islet grafts. To investigate whether islets from IRS-1KO mice show a revascularization pattern similar to those from WT donors, we measured capillary density in plastic sections of islet grafts (Fig. 7a). A significant increase in capillary density was detected in IRS-1KO islets transplanted into IRS-1KO mice compared with other groups (Fig. 7b). Preliminary data suggest that expression of VEGF is up-regulated in grafts that exhibit more capillaries (data not shown) and other reports indicate a role for morphogens derived from endothelial cells as potential signals for islet cell growth (19; 30; 31). Whether the increase in capillary density is a consequence of increased β-cell proliferation in IRS-1KO islets transplanted into IRS-1KO mice or is secondary to a specific process involving IRS-signaling in endothelial cells (19; 30; 31) requires further investigation.
Discussion

Islet hyperplasia is a feature of many metabolic disorders including obesity, type 2 diabetes, glucocorticoid excess and elevated levels of growth hormone. Although insulin and IGF-I promote growth and anti-apoptosis in most mammalian tissues, a role for these hormones in mediating the islet proliferative response in hyperinsulinemic states has not been fully explored. In the present study we used a transplantation approach to demonstrate that enhanced IRS-2 expression is associated with enhanced growth and anti-apoptosis of β-cells in IRS-1KO islet grafts.

We have previously shown that islets deficient in IRS-1 show defects in nutrient-stimulated insulin secretion and reduced insulin content (22). However, IRS-1KO and obese IRS-1 heterozygotes are able to compensate and maintain glucose homeostasis and do not become overtly diabetic even as they age (38). The factors that promote the islet compensatory response in the absence of one or both alleles for IRS-1 are not fully understood, but show a strong correlation with insulin resistance. The basal proliferation of β-cells in the endogenous pancreas of hyperinsulinemic IRS-1KO recipients is significantly increased while basal apoptosis levels are low. When WT islets were transplanted into hyperinsulinemic but normoglycemic IRS-1KO recipients, we detected an increase in β-cell proliferation and this showed a significant correlation with circulating insulin levels. Interestingly, the IRS-1 islets transplanted into IRS-1KO recipients responded with the highest BrdU incorporation and lowest levels of apoptosis while IRS-1KO islets transplanted into WT recipients revealed enhanced apoptosis of β-cells and reduced proliferation. Together these data indicate that after a period of adaptation to hyperinsulinemnic conditions in the donor animal when β-cell proliferation is high, exposure of the islets to a relatively hypoinsulinemnic environment in the recipient leads to β-cell apoptosis.
suggesting that insulin either plays an important anti-apoptotic role or promotes β-cell growth in the hyperinsulinemic state.

In the present study we observed that IRS-2 is up-regulated in IRS-1KO islets and protein levels of IRS-2 were also increased in WT grafts exposed to the ambient hyperinsulinemia in IRS-1KO recipients. Although IGF-I has been suggested to be an important ligand in the IGF-1/IRS-2 axis (49), our data indicate that insulin likely recruits IRS-2 via insulin and/or an insulin/IGF-1 hybrid receptor since serum IGF-I levels in IRS-1KO mice are normal. Whether local IGF-I is altered in the mutant islets and potentially plays a role in this scenario is not clear (7).

Growth factors including insulin and IGF-I signal via the IRS-2, PI3-K/Akt pathway to promote cell survival (5; 14). Activation of Akt has been demonstrated to inhibit apoptosis by inhibiting the release of cytochrome c from mitochondria (13) and to phosphorylate the forkhead transcription factor, FKHR, thereby blocking its pro-apoptotic effects. In the absence of IRS-2, IGF-I-stimulated Akt phosphorylation has been shown to be reduced with consequent high levels of cleaved/activated caspase-3. Furthermore, FKHR is also phosphorylated to a lesser degree in IRS-2KO islets suggesting a partial disruption in the Akt pathway. Thus, it is possible that upregulation of IRS-2 levels in IRS-1 mutant islets activates the major survival pathway mediated by Akt activation and FKHR phosphorylation in response to the elevated circulating insulin levels. Another factor that may participate in the islet growth response is the enhanced capillary density in the grafts in IRS-1KO recipients. Indeed, continuous infusion of VEGF has been shown to improve vascularization in rats (43) and VEGF-A has been recently shown to play a role in vascularization of islets (31). Whether increased formation of capillaries in IRS-1KO islets is the consequence of increased β-cell proliferation or a specific process involving VEGF (31)
due to the deletion of IRS-1 needs further investigation. In summary, we provide evidence that IRS-2 plays a role in the islet compensatory response in IRS-1 null mice likely via an IRS-2/Akt/FoxO1 (FKHR) pathway in IRS-1 deficient islets.

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**Figure Legends:**

**Fig 1 Metabolic effects of islet transplantation**

A) Body weight of male islet recipients followed up for 5 weeks after transplantation. B) Blood glucose levels were measured in samples obtained through tail bleeds of fed male recipients at the indicated ages (weeks after transplantation). Values are means ± SEM (n=5 mice per genotype). C) Serum insulin levels were measured using ELISA in fed recipient mice. Data are means ± SEM (n=5 mice per genotype). In all labels, the donor is represented followed by the recipient.
Fig 2 β-cell proliferation.

A) BrdU incorporation analysis was performed in anti-insulin and anti-BrdU double-labeled pancreas sections of 13-week old recipient mice and expressed as % of insulin-positive β-cells. Results are means ± SEM of five mice per genotype, *p< 0.05. B) BrdU incorporation analysis in anti-insulin and anti-BrdU double-labeled islet-graft sections. Results are means ± SEM (n=5 mice per genotype); *p< 0.05. C) BrdU incorporation analysis in WT into IRS-1KO islet graft sections expressed as % BrdU-positive cells 5 weeks after transplantation. Each data point represents one animal. A significant correlation was observed with an ‘r’ value of 0.99 (P<0.002). In all labels, the donor is represented followed by the recipient.

Fig 3 β-cell apoptosis.

A) Apoptotic cells were detected in de-paraffinized pancreas sections using a Flourescein DNA fragmentation detection assay (TUNEL). Number of apoptotic nuclei is shown for 13-week-old mice. Values are expressed as means ± SEM of four mice per genotype. B) Number of apoptotic nuclei per β-cells is shown in grafts, 5 weeks after transplantation. Values are expressed as means ± SEM of five mice per genotype, *p< 0.05. In all labels, the donor is represented followed by the recipient. C) A representative magnified image showing coimmunostaining of insulin and TUNEL in pancreas sections from a wild type mouse. Arrows indicate cells positive for both TUNEL and insulin. D) DNA laddering in islets isolated from WT and IRS-1KO mice. Three individual mice from Controls (C1, C2, C3) and IRS-1 knockouts (IRS-1KO1, IRS-1KO2, IRS-1KO3) are shown.
Fig 4 Immunostaining for IRS-2

A) Immunohistochemistry was performed on pancreas sections of WT and IRS-1KO mice fixed in 4% PFA and embedded in paraffin. Following re-hydratation sections were incubated with anti-insulin, and anti-IRS-2 antibodies. Sections were processed identically and simultaneously as described. Representative sections of 3 mice per genotype 5 weeks after transplantation are shown. B) Anti-insulin and anti-IRS-2 co-stained sections of transplanted islets under the kidney capsule. Representative sections of 13-week-old mice are shown. In all labels, the donor is represented followed by the recipient. C) Co-immunostaining for IRS-2 and glucagon and DAPI in pancreas section from a 10-week-old WT adult male mouse. Red, IRS-2; Green, glucagon; Blue, DAPI. Magnification 40X, bar = 50 micron.

Fig 5 Immunostaining for FKHR

Immunostaining was performed on transplanted islets under the kidney capsule fixed in 4% PFA and embedded in paraffin. Following re-hydratation sections were incubated with anti-insulin and anti-FKHR antibodies. Sections were processed identically and simultaneously as described in Methods. Representative sections of 3 mice per genotype 5 weeks after transplantation are shown. In all labels, the donor is represented followed by the recipient.

Fig 6 Immunostaining for PDX-1

A) Immunohistochemistry was performed on transplanted islets under the kidney capsule fixed in 4% PFA and embedded in paraffin. Following re-hydratation sections were incubated with anti-PDX-1 antibody. Sections were processed identically and simultaneously as described in methods. Representative sections of 2 mice per genotype 5 weeks after transplantation are shown.
Fig 7 Capillary density

A) Methylene blue stained plastic sections (1µm) are shown at a magnification of 63x. In stained graft-sections, islet tissue was easily identified and capillaries counted. B) Values are expressed as number of capillaries per β-cell nucleus ± SEM of at least three mice per genotype, *p< 0.05. In all labels, the donor is represented followed by the recipient.

Reference List


Fig 3

(a) Bar graph showing the percentage of apoptotic nuclei/beta cell. The bars indicate that the percentage is higher in the IRS1KO3 (C3) group compared to IRS1KO1 (C1), IRS1KO2 (C2), and C1 groups.

(b) Bar graph showing the percentage of apoptotic nuclei/beta cell with a statistically significant difference (*). The bars indicate that the percentage is higher in the IRS1KO3 (C3) group compared to IRS1KO1 (C1), IRS1KO2 (C2), and C1 groups.

(c) Micrographs showing Insulin, TUNEL, and Merge. The arrows indicate areas of interest in the TUNEL staining.

(d) Gel electrophoresis showing bands from different conditions: C1, IRS1KO1 (C1), IRS1KO2 (C2), C3, and IRS1KO3 (C3).
Fig 4

a) wt

Irs1<sup>−/−</sup>

b) wt-wt

Irs1<sup>−/−</sup>-wt

wt-Irs1<sup>−/−</sup>

Irs1<sup>−/−</sup>-Irs1<sup>−/−</sup>
Fig 4c)