Increased β-Cell Mass by Islet Transplantation and *PLAG1* Overexpression Causes Hyperinsulinemic Normoglycemia and Hepatic Insulin Resistance in Mice

Jeroen Declercq,[1,5] Anujith Kumar,[1] Janna A. Van Diepen,[2] Irene O.C.M. Vroegrijk,[2] Conny Gysemans,[3] Caterina Di Pietro,[1] Peter J. Voshol,[2] Chantal Mathieu,[3] Nadine Ectors,[4] Wim J.M. Van de Ven,[5] and Catherine M. Verfaillie[1]

OBJECTIVE—It is believed that an organism remains normoglycemic despite an increase in the β-cell mass because of decreased insulin production by β-cells on a per-cell basis. However, some transgenic mouse models with β-cell hyperplasia suggest that insulin production remains excessive and that normoglycemia is maintained by insulin resistance.

METHODS—Here, we investigated the effect of an increased β-cell mass on glycemia and insulin resistance by grafting excess normal islets in normoglycemic mice, as well as using targeted *PLAG1* expression in β-cells, which leads to β-cell expansion.

RESULTS—In both models, fasting plasma insulin levels were increased, even though animals were normoglycemic. After an intraperitoneal glucose tolerance test, plasma insulin levels increased, which was associated with improved glucose clearing. Under these conditions, normoglycemia is maintained by hepatic insulin resistance as demonstrated by hyperinsulinemic euglycemic clamp experiments.

CONCLUSIONS—In conclusion, we demonstrate that when excess β-cells are grafted, insulin production on a per β-cell basis is not sufficiently decreased, leading to hyperinsulinemia and hepatic insulin resistance. This observation might be important for the design of stem cell-based islet replacement therapies. ***Diabetes* 59:1957–1965, 2010**

Patients with type 1 diabetes produce very little or no insulin because of autoimmune destruction of the insulin-producing β-cells. Several decades ago, it was shown that transplantation of isogeneic islets to replace β-cells in diabetic rats could restore normoglycemia (1). This has led to whole-pancreas or islet transplantation to treat patients with type 1 diabetes (2). One of the major hurdles for the routine use of this therapy is the scarcity of transplantable islets. This forms the basis for many studies that evaluate different methods to generate large numbers of β-cells in vitro. In this context, stem cells of both embryonic and adult origin offer interesting perspectives (3).

It has been well established that successful islet transplantation requires that sufficient islets are grafted and survive to induce normoglycemia. However, what is not known is the effect of grafting excess β-cells on glucose homeostasis. Only a single study has tested the effect of grafting 300 C57BL/6 islets in normoglycemic mice, demonstrating no effect on glucose metabolism (4). Normoglycemia, despite an increased β-cell mass, may be achieved by a decrease in insulin secretion on a per β-cell basis (5). Alternatively, the insulin secretion may remain elevated in normoglycemic animals because of decreased insulin sensitivity, documented in some, but not all, animal models in which the β-cell mass was increased by forced expression of specific transgenes (6).

Here, we used two different mouse models to address the mechanism underlying normoglycemia in animals with a very large β-cell mass: *1*) mice in which the exogenous β-cell mass is increased by grafting 1,000 syngeneic islets under the kidney capsule; and *2*) mice in which the endogenous β-cell mass is increased by targeted overexpression of the human *Pleomorphic Adenoma Gene 1 (PLAG1)* in β-cells.

PLAG1 is a developmentally-regulated zinc-finger transcription factor (7,8). *PLAG1* overexpression has been linked to tumorigenesis in humans (9–14). Forced expression of *PLAG1* in a tissue-specific manner in mice causes formation of pleomorphic adenomas of the salivary glands (15,16), adenomyoepitheliomatous lesions of the mammary glands (17), and cavernous angiomatosis (18). Because *PLAG1* overexpression leads to increased cell proliferation, we hypothesized that targeted overexpression of *PLAG1* in β-cells might result in increased β-cell proliferation and might, as such, be a good method to generate mice with an increased endogenous β-cell mass.

Using both models, we clearly demonstrate that normoglycemia is maintained in mice that display persistent hyperinsulinemia by the development of hepatic insulin resistance.

RESEARCH DESIGN AND METHODS

FVB and C57BL/6 mice were purchased from Charles Rivers (Belgium) and Janvier (France), respectively. The generation and genotyping of the *PLAG1* transgenic mouse strain has been reported previously (15). To target *PLAG1* expression to the β-cells, *PLAG1* transgenic mice were crossed with homozygous *RIP-Cre* transgenic mice (a gift from Pedro Herrera, Switzerland), and

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From the [1]Stamcel Instituut, Katholieke Universiteit Leuven, Leuven, Belgium; the [2]Department of Endocrinology and Metabolic Diseases, Leiden University Medical Center, Leiden, The Netherlands; the [3]Laboratory for Experimental Medicine and Endocrinology, Katholieke Universiteit Leuven, Leuven, Belgium; the [4]Department of Pathology, University Hospital Gasthuisberg, Leuven, Belgium; and the [5]Laboratory for Molecular Oncology, Department of Human Genetics, Katholieke Universiteit Leuven, Leuven, Belgium.

Corresponding author: J. Declercq, jeroen.declercq@med.kuleuven.be.

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The current address for P.J.V. is Metabolic Research Laboratories, Cambridge, U.K.

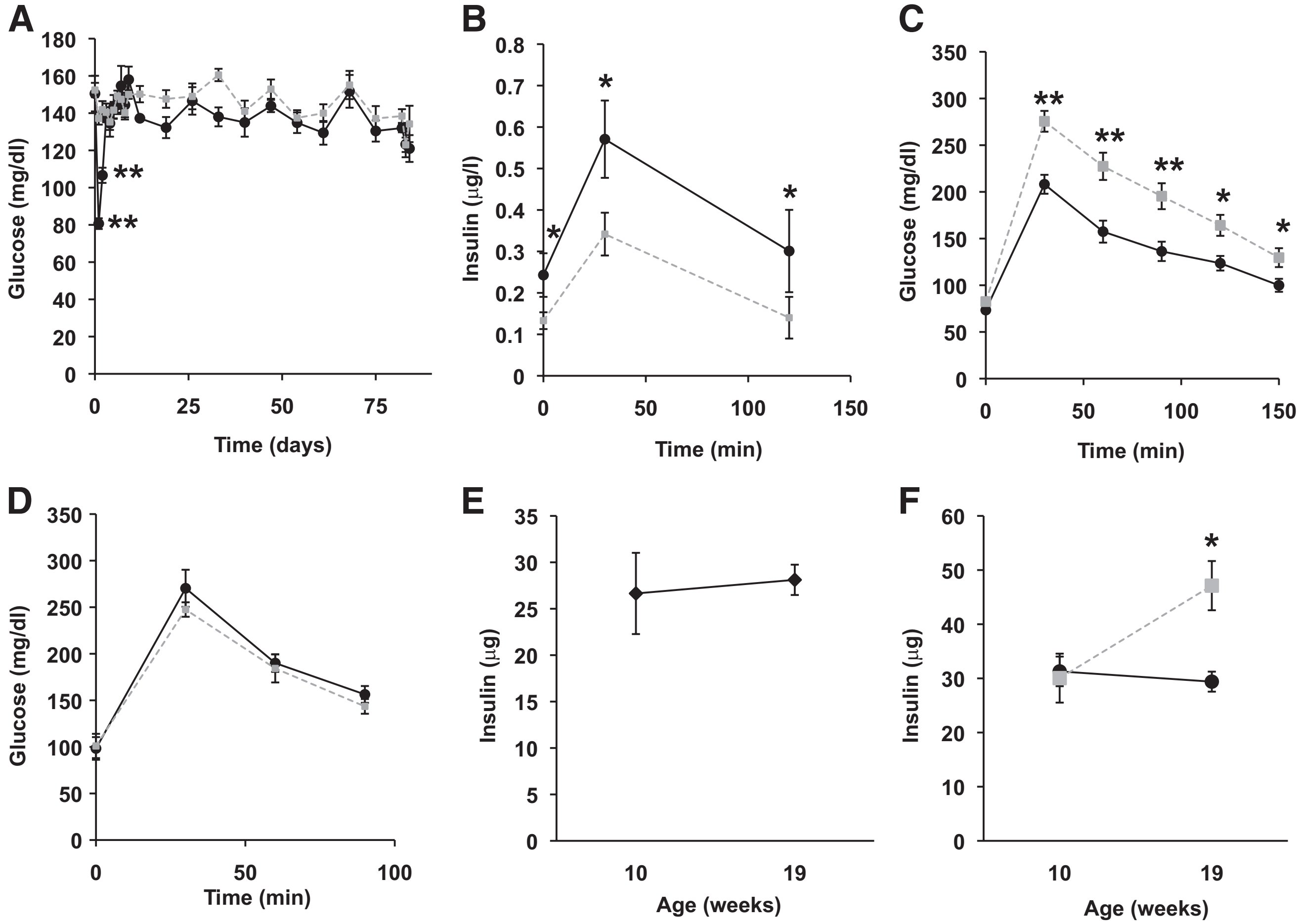

FIG. 1. Glucose homeostasis and glucose tolerance test (GTT) in mice transplanted with 1,000 islets. *A*: A quantity of 1,000 syngeneic islets were grafted under the kidney capsule of FVB mice, and blood glycemia levels were followed for 2 months (full line). The dashed line represents blood glucose levels of sham-operated littermate mice in function of time. At least 7 mice were included in both groups. Results are represented as mean ± SEM. $^*P \le 0.05$; $^{**}P \le 0.005$. *B* and *C*: GTT tests were performed on 11- to 13-week-old FVB mice transplanted with 1,000 islets 1 week before the GTT (full line) and on sham-operated littermate mice (dashed line). The mice were starved for 16 h and injected with 1 mg/g glucose. Blood samples were obtained at the indicated times, and plasma insulin (*B*) and blood glucose (*C*) levels measured. Data are shown as mean ± SEM. $^*P \le 0.05$; $^{**}P \le 0.005$. At least 15 mice were included per group. *D*: FVB mice grafted with 1,000 islets were nephrectomized 82 days after transplantation, and an intraperitoneal GTT was performed 1 week later. Blood glucose levels of nephrectomized FVB mice initially transplanted with 1,000 islets (full line) and nephrectomized sham-operated littermate mice (dashed line) are represented as mean ± SEM. Four mice were included per group. *E*: The total insulin content of the graft did not decrease between 1 day and 2 months after transplantation in C57BL/6 mice transplanted with 1,000 islets. *F*: Two months after transplantation, the total endogenous pancreatic insulin content was significantly higher in sham-operated littermate mice compared with C57BL/6 mice transplanted with 1,000 islets.

backcrossed in a FVB background (19). This resulted in double-transgenic *PLAG1*$^{+/-}$/*RIP-Cre*$^{+/-}$ offspring mice, designated as P1-RIPCre mice.

Detection of PLAG1 expression by immunofluorescence. We stained 5-μm cryostat sections using standard procedures, using a primary rat anti-HA antibody (DAKO, Glostrup, Denmark) diluted 1/100 for 1 h at room temperature and the Alexa Fluor 546 goat anti-rat secondary antibody (Invitrogen, Merelbeke, Belgium) in a dilution of 1/500 with Hoechst 33258 diluted 1/2,000 for 30 min at room temperature.

Immunohistochemical analysis of the pancreata. Pancreata were removed, fixed overnight in 4% formaldehyde, and embedded in paraffin. For each pancreas, six different sections separated by 200 μm were selected for histologic analysis. Paraffin sections (5 μm) were stained with hematoxylin and eosin. In addition, paraffin sections were stained with either a guinea pig anti-insulin antibody (DAKO) diluted 1/2, a rabbit anti-glucagon antibody (DAKO) diluted 1/1,500, or rabbit anti-somatostatin antibody (DAKO) diluted 1/750. As secondary antibodies, rabbit anti-guinea pig IgG coupled to peroxidase (DAKO) diluted 1/200 or the anti-rabbit Envision+ kit (DAKO) were used.

Morphometric analysis of β-cell mass. Pancreata obtained from 20-week-old P1-RIPCre and *RIP-Cre* mice, were stained for insulin on six sections of each pancreas separated by 200 μm. The total β-cell surface area (mm^2), that is, the surface area of insulin positive cells, was determined with Zeiss Axiovision software (Micro Imaging, Heidelberg, Germany). The percentage of β-cell surface area in the pancreas was calculated as (surface area of all insulin-positive cells in one section/the total surface area of the section) × 100. The β-cell mass was calculated by multiplying the pancreas weight by the percentage of β-cell surface area.

Determination of total pancreatic insulin content. Pancreata from P1-RIPCre and *RIP-Cre* mice at different time points after birth were homogenized in 5 ml of acidic ethanol (75% ethanol, 0.12M HCl), sonicated for 3 min, incubated overnight at −20°C, and centrifuged at 4,000 rpm. The supernatant fractions were collected for analysis of insulin content with the high range rat insulin ELISA kit (Mercodia, Uppsala, Sweden).

Quantification of β-cell proliferation. Three 5 μm cryostat sections separated by 200 μm from pancreata of five 20-week-old P1-RIPCre and *RIP-Cre* mice were stained with a mixture of a monoclonal mouse anti-insulin antibody (Sigma, Bornem, Belgium) diluted 1/1,000 and a rat anti-Ki67 antibody (DAKO) diluted 1/50 for 1 h at room temperature. Subsequently, sections were incubated with a mixture of Alexa Fluor 488 goat anti-mouse and Alexa Fluor 546 goat anti-rat antibodies (Invitrogen), each diluted 1/500, with Hoechst 33258 diluted 1/2,000 for 30 min at room temperature. β-cell proliferation was quantified as (the number of insulin$^+$/Ki67$^+$ cells of the 3 sections/total number of insulin$^+$ cells per section) × 100. At least 2,000 insulin-positive cells were counted per mouse.

Islet isolation and transplantation. Islets of 10- to 12-week-old male FVB or C57BL/6 mice were isolated using previously published isolation procedures (20).

Next, 10- to 12-week-old male isogeneic recipient mice were anesthetized by intraperitoneal injection of 100 μg ketamine and 10 μg xylazine/gr body

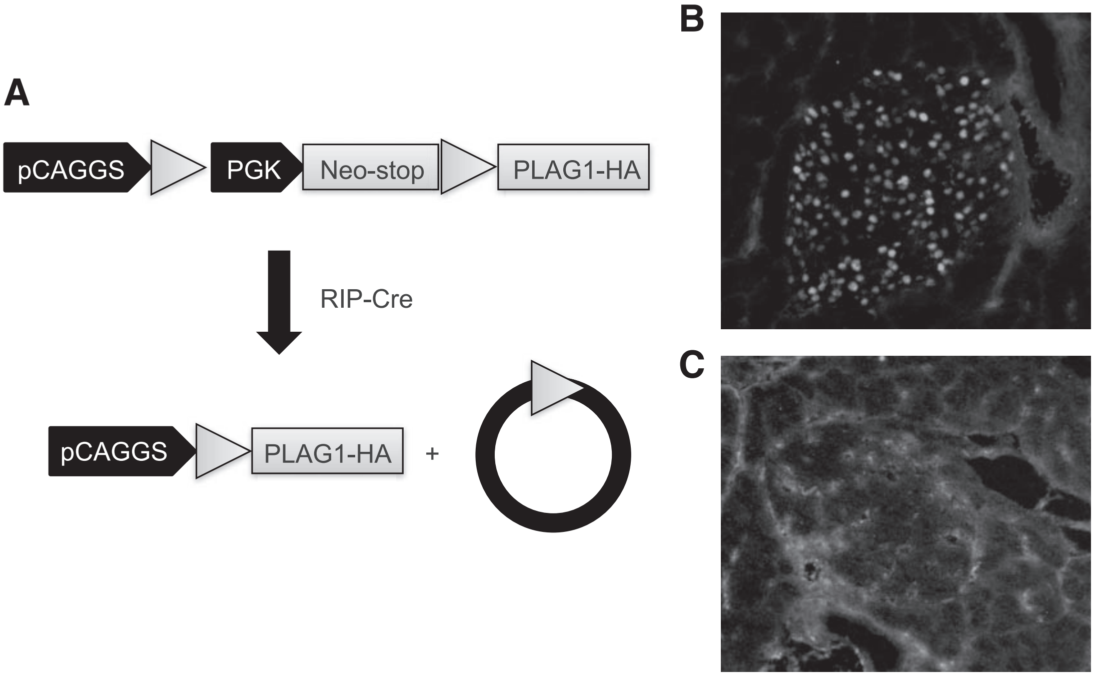

FIG. 2. Generation of *PLAG1* transgenic mice. *A*: Schematic overview of the construct used to generate the *PLAG1* transgenic founder line. After crossing the *PLAG1* transgenic founder line with *RIP-Cre* mice, the neomycin stop cassette becomes excised in β-cells. This leads to expression of *PLAG1* in β-cells. *B* and *C*: Immunofluorescent staining using an antibody against hemaglutinin clearly demonstrates the expression of the *PLAG1* transgene in β-cells of 20-week-old P1-RIPCre mice (*B*), but not in littermate *RIP-Cre* mice (*C*).

weight. We transplanted 1,000 fresh islets under the left kidney capsule. Sham-operated littermate mice were used as controls.

Nonfasting blood glucose levels and body weight of recipient and sham-operated mice were measured daily during the first week and weekly afterwards using a Contour glucose meter (Bayer, Diegem, Belgium).

Glucose tolerance tests and measurement of plasma insulin and glucagon levels. Glucose (1 mg/g body weight) was administered in mice fasted for 16 h via intraperitoneal injection. Blood was drawn from the tail vein at 0, 30, 60, 90, 120, and 150 min after injection, and blood glucose levels measured with a Contour glucometer.

Blood samples were collected in heparin-coated capillaries (Analis, Suarlééé, Belgium) before and 30 and 120 min after glucose injection, and plasma was prepared by centrifugation for 10 min at 1,000g.

Plasma insulin and glucagon levels were measured in 30 and 100 μl of plasma with the Mercodia ultra sensitive mouse insulin ELISA and the Gentaur glucagon EIA (Kampenhout, Belgium).

Western blotting. After 16 h of starvation, P1-RIPCre and *RIP-Cre* mice were injected intraperitoneally with 1U/kg of insulin and killed 10 min later. The liver lysates were analyzed by Western blot analysis using Akt (#4,691), phosphoAkt(Ser473) (#4,060), and phosphoAkt(Thr308) (#2,965) antibodies (Cell Signaling Technologies, Leiden, the Netherlands).

Homeostasis model assessment of insulin resistance test. A measure of insulin resistance, the homeostasis model assessment of insulin resistance, was calculated as fasting insulin (μU/ml) × fasting glucose (mmol/l)/22.5 (21).

Hyperinsulinemic euglycemic clamp study. The hyperinsulinemic euglycemic clamp study was performed as published previously (22–24). In short, a continuous infusion of D-^{3}H glucose, 0.3 μCi/kg/min, (Perkin Elmer) was started, and blood samples (60 μl) were taken after 50 and 60 min of tracer infusion to determine basal glucose, insulin, and free fatty acids (FFA). The hyperinsulinemic study started with a bolus (100 mU/kg or 150 mU/kg for P1-RIPCre and *RIP-Cre* mice, Actrapid, Novo Nordisk, Bagsvaerd, Denmark) followed by continuous infusion of insulin (5 mU/h or 7.5 mU/h for *RIP-Cre* and P1-RIPCre mice) and D-^{3}H glucose (0.3 μCi/kg/min). A variable infusion of 12.5% D-glucose (in PBS) solution was adjusted to maintain euglycemia 70, 80, and 90 min (steady state) after the start of the hyperinsulinemic period. Blood samples (60 μl) were taken for determination of plasma D-^{3}H glucose, insulin, and FFA concentrations.

For determination of plasma D-^{3}H glucose, plasma was deproteinized with 20% trichloroacetic acid, dried to remove $^{3}H_2O$, resuspended in demiwater, and counted with scintillation fluid (Ultima Gold, Packard, Meridien, CT).

Plasma glucose, insulin, and FFA levels were determined using commercially available kits (Instruchemie, Crystal Chemical and Wako Pure Chemical Industries, respectively).

Under steady state conditions for plasma glucose concentrations, the rate of glucose disposal equals the rate of glucose appearance. The glucose disposal rate was calculated as the ratio of the infusion rate of D-^{3}H glucose (dpm/min) and the steady state plasma D-^{3}H glucose specific activity (dpm/mg/kg glucose). The hepatic glucose production was calculated as the difference between the rate of glucose disposal and the infusion rate of exogenous D-glucose.

Statistical analysis. Where appropriate, results are expressed as means ± SEM. Statistical analysis was performed by unpaired Student *t* test, where $P < 0.05$ was considered significant.

RESULTS

Transplantation of exogenous islets results in hyperinsulinemia. To formally test whether an organism remains normoglycemic despite an increase in the β-cell mass, we transplanted 1,000 syngeneic islets under the kidney capsule of normoglycemic FVB mice and evaluated glycemia and insulin levels for 2 months afterward. The glucose levels of mice transplanted with 1,000 islets decreased significantly for 2 days after transplantation compared with sham-operated mice, but all animals were normoglycemic from day 3 onwards (Fig. 1*A*). However, plasma insulin levels in animals grafted with 1,000 islets were 1.8-fold increased compared with sham-operated mice, 1 week and 2 months after transplantation (ad libitum fed: 1.93 ± 0.27 μg/l in sham-operated mice; 3.38 ± 0.64 μg/l in mice transplanted with 1,000 islets; starved mice: 0.16 ± 0.02 μg/l in sham-operated mice; and 0.30 ± 0.05 μg/l in mice transplanted with 1,000 islets). The basal plasma glucagon level in starved FVB mice transplanted with 1,000 islets (520 ± 45 pg/ml) was also significantly increased compared with sham-operated mice (300 ± 7 pg/ml). After intraperitoneal glucose tolerance test (GTT), insulin levels in mice transplanted with 1,000 islets rose to significantly higher levels compared with sham-operated mice (Fig. 1*B*), which was associated with a significantly greater ability to dispose of glucose in mice grafted with

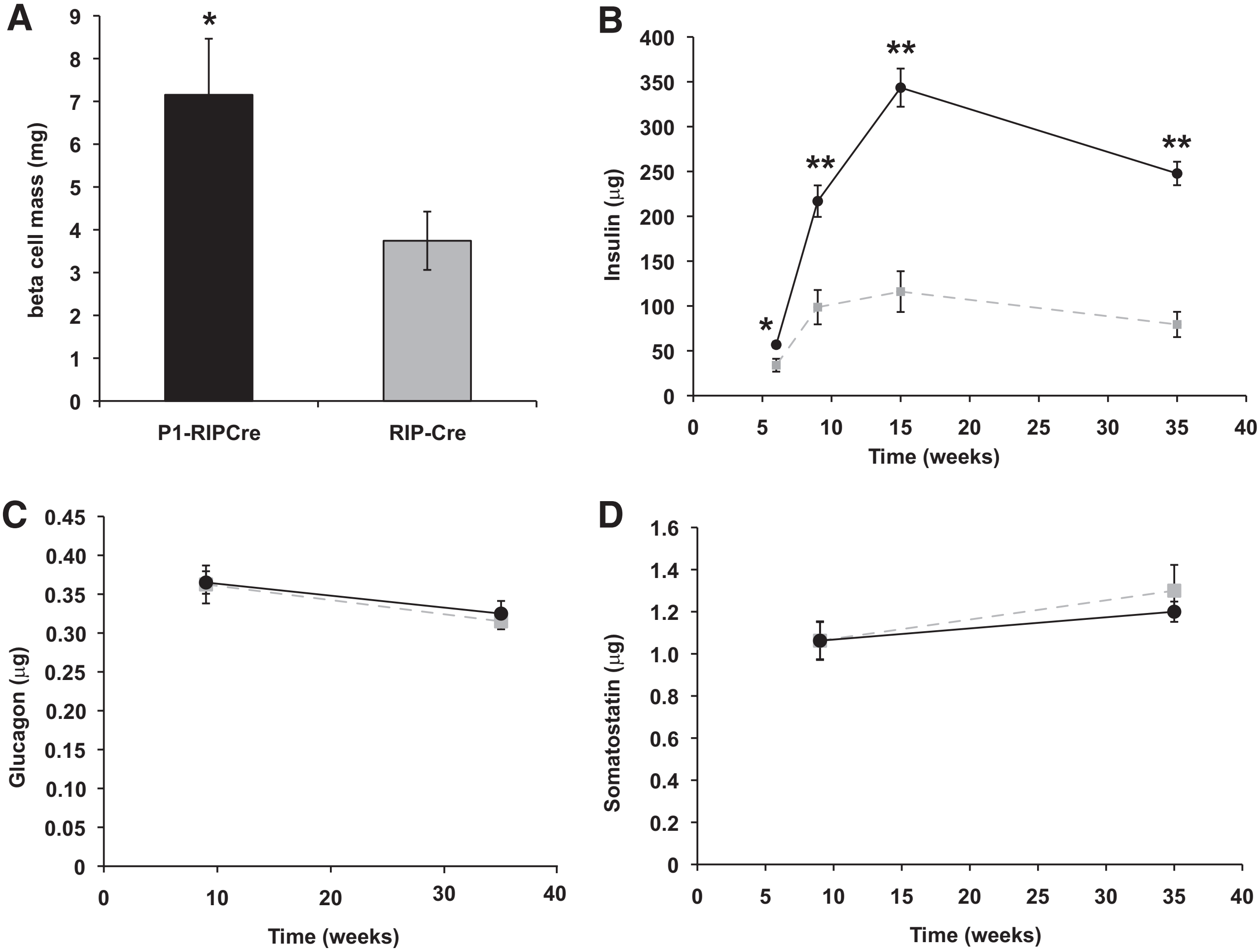

FIG. 3. Quantification of β-cell hyperplasia. *A*: The β-cell mass of 20-week-old P1-RIPCre mice (black bar) is significantly increased compared with those of littermate *RIP-Cre* mice (gray bar). Results are represented as mean ± SEM. Five mice were analyzed per group. *B*: The total pancreatic insulin content of P1-RIPCre mice (full line) was significantly increased compared with those of littermate *RIP-Cre* mice (dashed line) at different time points after birth. *C* and *D*: The total pancreatic glucagon (*C*) and somatostatin (*D*) content of P1-RIPCre mice was similar to those of littermate *RIP-Cre* mice at different time points after birth. Results are represented as mean ± SEM. *$P \leq 0.05$; **$P \leq 0.005$. At least 10 mice were included for each group at each time point.

islets compared with sham-treated mice (Fig. 1*C*). Mice were nephrectomized 82 days after transplantation, and an intraperitoneal GTT was performed 1 week later. There were no differences in the glucose levels of nephrectomized mice initially transplanted with 1,000 islets and sham-operated mice (Fig. 1*D*), indicating that differences in mice harboring excess islets were caused by the transplanted islets.

These observations are independent of the genetic background of the mice, as C57BL/6 mice responded in a similar way to the increased islet cell mass as FVB mice (these data can be found in supplementary Fig. 1, available in an online appendix at http://diabetes.diabetesjournals.org/cgi/content/full/db09-1446/DC1). The total insulin content of the graft remained unaltered 1 day and 2 months after grafting 1,000 islets in C57BL/6 mice. (Fig. 1*E*) and was ~85% of the total pancreatic insulin content. In contrast to sham-operated C57BL/6 mice, in which the endogenous pancreatic insulin content increased with age, the pancreatic insulin content of C57BL/6 mice transplanted with 1,000 islets did not increase with time (Fig. 1*F*).

RIP-Cre-mediated overexpression of PLAG1 in β-cells results in an endogenous increase of the β-cell mass. To confirm that mice with an increased β-cell mass remain normoglycemic, a *PLAG1* transgenic mouse strain with β-cell hyperplasia was developed (15) (schematically represented in Fig. 2*A*). *PLAG1* transgenic mice were crossed with homozygous *RIP-Cre* transgenic mice (19) to target stop-cassette excision and subsequent *PLAG1* expression activation to the β-cells. Such crossing resulted in *RIP-Cre*$^{+/-}$/*PLAG1*$^{+/-}$, *RIP-Cre*$^{+/-}$/*PLAG1*$^{-/-}$ offspring mice, designated P1-RIPCre and *RIP-Cre* mice, respectively. Pancreata of P1-RIPCre mice expressed the transgene as shown by immunofluorescence using an anti-HA tag antibody in the nuclei of most β-cells (Fig. 2*B*), whereas no expression of the transgene could be detected in the pancreata of littermate *RIP-Cre* mice (Fig. 2*C*).

Histologic analysis of 12-week- to 1-year-old male and female mice revealed that targeted expression of *PLAG1* in the β-cells resulted in islet hyperplasia in P1-RIPCre mice compared with littermate *RIP-Cre* mice. The β-cell mass was 1.9 times increased in 20-week-old male P1-RIPCre compared with *RIP-Cre* mice (Fig. 3*A*). The pancreatic weight of 9-, 15- and 35-week-old P1-RIPCre mice was also significantly increased compared with those of littermate *RIP-Cre* mice (supplementary Fig. 2*A*). In contrast, the total body weight was similar (supplementary Fig. 2*B*).

The islet cell distribution of P1-RIPCre mice was mildly

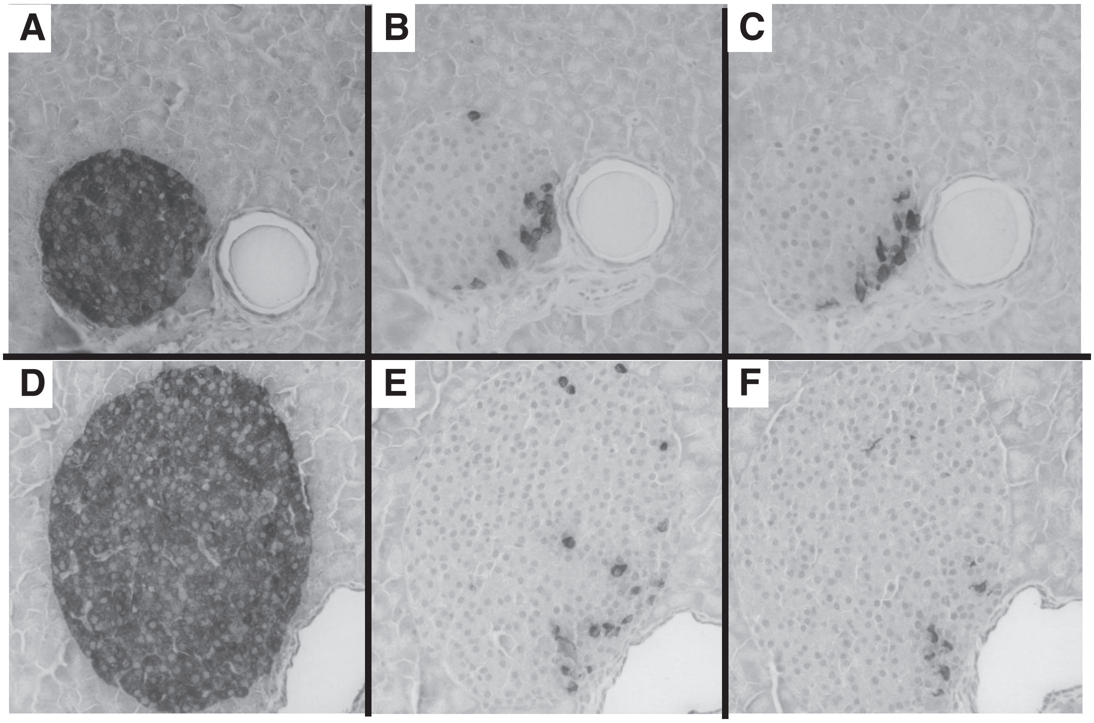

FIG. 4. Islet cell distribution in P1-RIPCre mice. Stainings for insulin (*A* and *D*), glucagon (*B* and *E*), and somatostatin (*C* and *F*) on representative sections of the pancreas of a 2-month-old *RIP-Cre* (*A–C*) and P1-RIPCre (*D–F*) mouse show that the islet cell distribution of P1-RIPCre mice is slightly disturbed, with non-β-cells in the center of the islets.

disturbed as demonstrated by immunostaining for insulin, glucagon, and somatostatin. In contrast to islets of control *RIP-Cre* mice in which the non-β-cells are located at the periphery of the islets (Fig. 4*A–C*), some islets of P1-RIPCre mice had non-β-cells randomly distributed throughout the islet (Fig. 4 *D–F*).

In P1-RIPCre mice, the total pancreatic insulin content was significantly increased in 6-, 9-, 15-, and 35-week-old mice compared with littermate *RIP-Cre* mice (Fig. 3*B*), indicating that the islet hyperplasia is present early after birth. The total pancreatic somatostatin and glucagon content was not increased in P1-RIPCre mice compared with littermate *RIP-Cre* mice, consistent with the specific expression of *PLAG1* in β-cells, and not in δ-cells and α-cells of P1-RIPCre mice (Fig. 3*C* and *D*).

Increased proliferation contributes to the islet hyperplasia in P1-RIPCre mice. The islet/β-cell hyperplasia observed in P1-RIPCre mice could be caused by increased proliferation and/or decreased apoptosis of *PLAG1*-overexpressing β-cells. To determine β-cell proliferation in vivo, pancreata from 20-week-old male P1-RIPCre and *RIP-Cre* mice were stained with antibodies against insulin and Ki67, and the percentage of proliferating β-cells was determined. In islets of *RIP-Cre* mice, almost no proliferating β-cells were detected (Fig. 5*A*), whereas in islets of P1-RIPCre mice, proliferating β-cells were easily detected (Fig. 5*B*). The percentage of Ki67$^+$/insulin$^+$ cells in P1-RIPCre mice was 3.2-fold increased compared with littermate *RIP-Cre* mice (Fig. 5*C*). Hence, increased proliferation is at least one of the mechanisms causing islet β-cell hyperplasia in P1-RIPCre mice.

P1-RIPCre mice exhibit increased β-cell mass, hyperinsulinemia, and normoglycemia. Like FVB and C57/BL/6 mice transplanted with 1,000 islets, P1-RIPCre mice remained normoglycemic (Fig. 6*A*). Nevertheless, the basal plasma insulin levels of 12-week-old and 35-week-old starved P1-RIPCre mice were significantly increased (data not shown). Similarly, the basal plasma C-peptide levels of 35-week-old P1-RIPCre mice were also significantly increased compared with littermate *RIP-Cre* mice, pointing toward an increased insulin secretion in these mice (Fig. 6*B*). In contrast, the basal plasma glucagon and somatostatin levels were not altered in P1-RIPCre mice compared with *RIP-Cre* mice (Fig. 6*B*). Like FVB mice transplanted with 1,000 islets, P1-RIPCre mice showed an increased ability to dispose injected glucose after GTT (Fig. 6*C*), and accordingly, the plasma insulin levels were significantly increased during GTT (Fig. 6*D*). Together, these data suggest that P1-RIPCre mice that have an endogenous increase in β-cell mass respond in a similar way to the increased β-cell mass as mice in which the β-cell mass has been increased by islet transplantation.

Mice with an increased β-cell mass become insulin resistant. Because mice in which the β-cell mass is expanded by transplantation of excess islets or by forced expression of *PLAG1* in β-cells have persistently elevated insulin levels under starved or ad libitum fed conditions, we hypothesized that normoglycemia may be maintained because of insulin resistance. To evaluate this hypothesis, we examined mice using the homeostasis model assessment of insulin resistance (HOMA-IR). HOMA-IR was 80, 130, and 60% increased in FVB/C57BL/6 mice transplanted with 1,000 islets and in P1-RIPCre compared with control littermates, respectively. To confirm that P1-RIPCre mice develop insulin resistance, insulin-activated Akt phosphorylation was measured in liver lysates of P1-RIPCre and *RIP-Cre* mice. As expected, acute insulin treatment (1 unit/kg) induced Akt phosphorylation in both groups. However, insulin-activated Akt phosphorylation was reduced in P1-RIPCre mice compared with littermate *RIP-Cre* mice, indicating that P1-RIPCre mice develop insulin resistance (Fig. 6*E*).

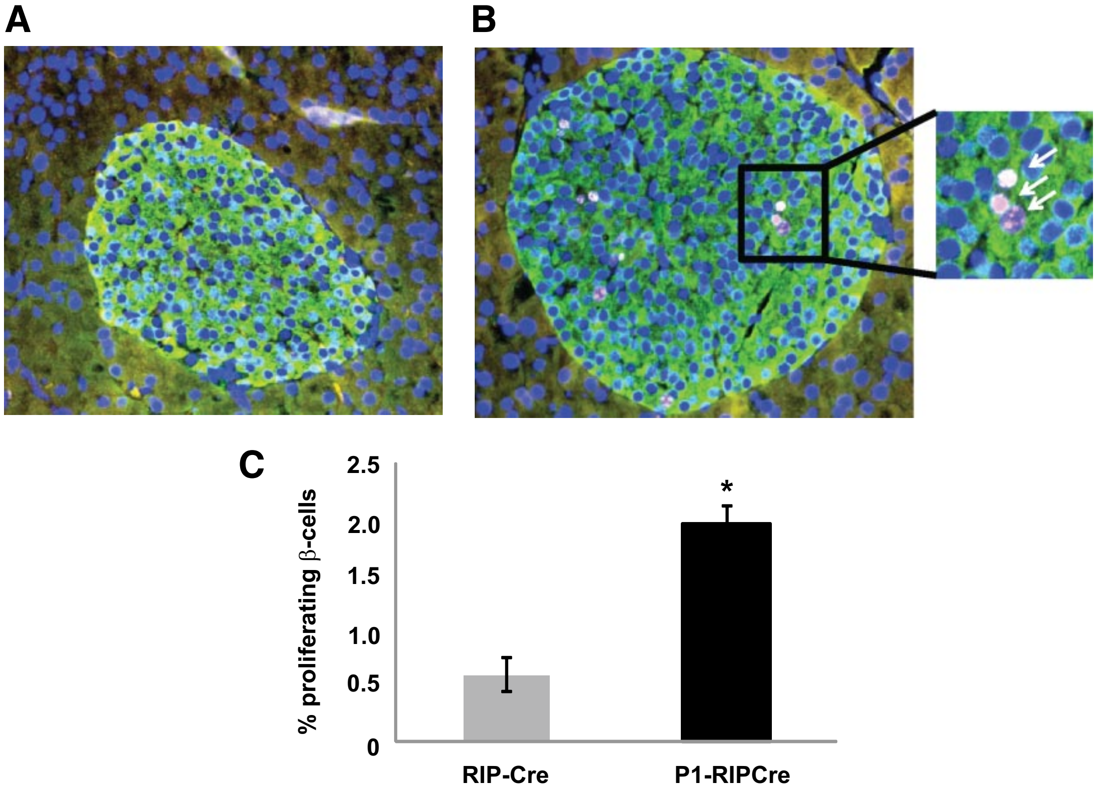

FIG. 5. Quantification of β-cell proliferation. *A* and *B*: Double immunofluorescence staining for Ki67 (orange) and insulin (green) on the pancreas of a 20-week-old *RIP-Cre* (*A*) and P1-RIPCre (*B*) mouse demonstrates increased β-cell proliferation in P1-RIPCre mice. Proliferating cells are indicated with *arrows* in the higher magnification of panel *B*. *C*: β-cell proliferation of 20-week-old P1-RIPCre mice (black bar) was 3.2 times higher compared with littermate *RIP-Cre* mice (gray bar). The results are represented as mean ± SEM. Four mice were analyzed for each group. (A high-quality digital representation of this figure is available in the online issue.)

To conclusively demonstrate that mice with an increased β-cell mass in the two models become insulin resistant, hyperinsulinemic euglycemic clamp experiments were performed. For FVB mice, hyperinsulinemia (3.50 ± 0.46 μg/l in mice transplanted with 1,000 islets vs. 3.57 ± 0.38 μg/l in wild-type mice) and euglycemia (Fig. 7*C*) were maintained during 5-mU/h insulin infusions, whereas for P1-RIPCre and *RIP-Cre* mice, hyperinsulinemia (7.90 ± 3.4 μg/l vs. 3.86 ± 0.21 μg/l, respectively) and euglycemia (Fig. 7*D*) were maintained during 7.5-mU/h insulin infusions. A higher insulin infusion concentration was used in this group because *RIP-Cre* mice responded to a lesser extent to the insulin infusion compared with wild-type FVB mice, and they are, as such, slightly insulin resistant. In FVB mice, glucose infusion rates were 55% lower in mice transplanted with 1,000 islets than controls (Fig. 7*A*), and, similarly, glucose infusion rates were 85% lower in P1-RIPCre mice compared with controls (Fig. 7*B*). The glucose disposal rates were similar for FVB mice transplanted with 1,000 islets compared with sham-operated littermates as well as for P1-RIPCre mice compared with *RIP-Cre* mice under basal (Fig. 7*G*) and hyperinsulinemic (Fig. 7*H*) conditions. This indicates that the peripheral tissues remain insulin sensitive. The basal hepatic glucose production was similar for FVB mice transplanted with 1,000 islets compared with control littermates and for P1-RIPCre mice compared with *RIP-Cre* mice (Fig. 7*I*). Nevertheless, mice with an increased β-cell mass develop hepatic insulin resistance, as indicated by increased hepatic glucose production during hyperinsulinemia in FVB mice transplanted with 1,000 islets compared with control FVB mice, as well as in P1-RIPCre mice compared with *RIP-Cre* mice (Fig. 7*J*). The fat tissue of the mice did not develop insulin resistance as no differences in insulin-mediated inhibition of FFA release were observed during the clamp in both groups (Fig. 7*K* and *L*). In conclusion, the above data indicate that mice with an increased β-cell mass in the two models remained normoglycemic, despite being hyperinsulinemic, because of hepatic insulin resistance.

DISCUSSION

Islet transplantation has been one of the reliable sources of β-cells to treat diabetic patients (2). However, several obstacles still remain for a widespread application of this method, such as the scarcity of islets for transplantation. When more abundant sources of β-cell–like cells become available—for instance, if they are derived from stem cells—it might be important to evaluate what happens if too many β-cells are transplanted. To investigate this further, we generated a mouse model with an endogenous increase of the β-cell mass via *PLAG1* overexpression in the β-cells and, more importantly, we transplanted normoglycemic mice with excess islets.

Several mouse models with islet hyperplasia have been generated. Depending on the transgene used, the impact of the β-cell hyperplasia on glucose homeostasis appears to differ. For instance, *cyclin D1* (5) and *Cdk4*- (25) mediated β-cell hyperplasia results in normoglycemia and normal insulin levels. Although this was not formally addressed, these data would be consistent with the notion that normoglycemia is preserved despite massive expansion of

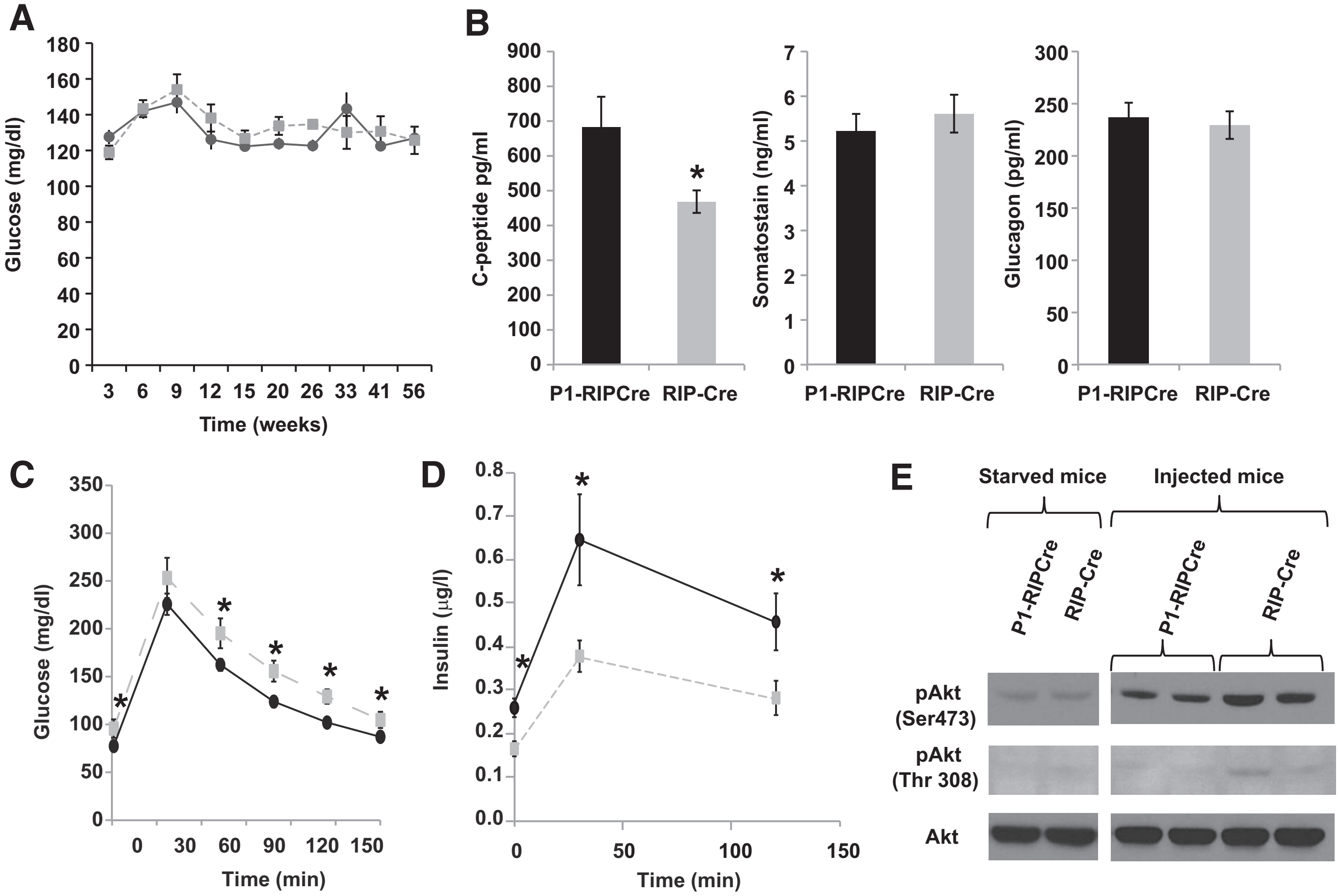

FIG. 6. Glucose homeostasis and glucose tolerance test in P1-RIPCre mice. *A*: Blood glucose levels of P1-RIPCre mice (full line) and *RIP-Cre* mice (dashed line) are shown in function of time. *B*: Plasma C-peptide levels of 35-week-old P1-RIPCre mice were significantly increased compared with littermate *RIP-Cre* mice. In contrast, the plasma somatostatin and glucagon levels were similar in 35-week-old P1-RIPCre and *RIP-Cre* mice. *C* and *D*: Intraperitoneal GTT on 2-month-old P1-RIPCre (full line) and on *RIP-Cre* mice (dashed line). The mice were starved for 16 h and injected with 1 mg/g glucose. Blood glucose (*C*) and plasma insulin (*D*) levels were measured at the indicated times, and results are represented as mean ± SEM. $^*P \leq 0.05$; $^{**}P \leq 0.005$. Thirteen P1-RIPCre and 15 *RIP-Cre* mice were measured. *E*: Starved P1-RIPCre and *RIP-Cre* mice were injected with 1 unit/kg insulin intraperitoneally. Control mice did not receive insulin. Ten minutes later, the liver was isolated, tissue extracts were prepared, and 50 μg of protein were loaded for Western blot analysis. Immunoblotting with specific phosphoserine 473, phosphothreonin 308, and total Akt antibodies are shown.

the β-cell mass because production of insulin on a per cell basis is reduced. By contrast, *Igf2*-mediated β-cell hyperplasia results in elevated plasma insulin levels associated with insulin resistance and ultimately development of type 2 diabetes (26). Finally, *Akt1* (6,27) or *Wnt*- (28) mediated β-cell hyperplasia leads to hyperinsulinemia and normoglycemia, but without development of type 2 diabetes. We also found that *PLAG1*-mediated excess of β-cells leads to hyperinsulinemic normoglycemia. Hence, under certain circumstances, excess β-cells lead to insulin resistance, thereby maintaining normoglycemia.

As all these studies, including the *PLAG1*-mediated hyperplasia of β-cells described here, are performed in mice overexpressing different transgenes in the β-cell compartment, it is possible that the unopposed increase in β-cells, but not α- and δ-cells, is responsible for the phenotype and might contribute to the impaired ability to reduce insulin secretion on a per-cell basis. Furthermore, it is not known whether mice that overexpress transgenes in the β-cell compartment are excessively producing "normal" β-cells in terms of glucose sensing, fuel metabolism, and insulin secretion dynamics.

Whether excess "normal" β-cells, such as after grafting stem cell-derived islets, will have a similar effect on glucose metabolism is not known. In fact, once it becomes possible to generate endocrine pancreatic cells from stem cells suitable for transplantation, such cells would not be genetically manipulated, and it is likely that mixtures of β-, α-, and δ-cells, similar to those found in normal islets, would be generated and would be grafted. For these reasons, it was important to mimic transplantation of excess and genetically unmanipulated islets containing normal numbers of β-, α-, and δ-cells, and to evaluate insulin production in these animals. We therefore transplanted a large excess of normal islets in normoglycemic mice. This excludes the possibility that effects seen on glucose and insulin homeostasis are caused by the presence of a transgene in endocrine pancreatic cells, which may influence the function of the β-cells. In mice transplanted with intact islets, there is an increase in all endocrine cells, which may mimic the transplantation of β-cell containing stem cell progeny. Indeed, we found that plasma glucagon levels were elevated in animals grafted with excess islets, but not in animals with *PLAG1*-induced islet hyperplasia. Despite concomitant elevation in plasma glucagon level, grafting 1,000 islets in syngeneic normoglycemic mice led to similar changes in glucose and insulin homeostasis, as was observed in mice with *PLAG1*-mediated islet hyperplasia.

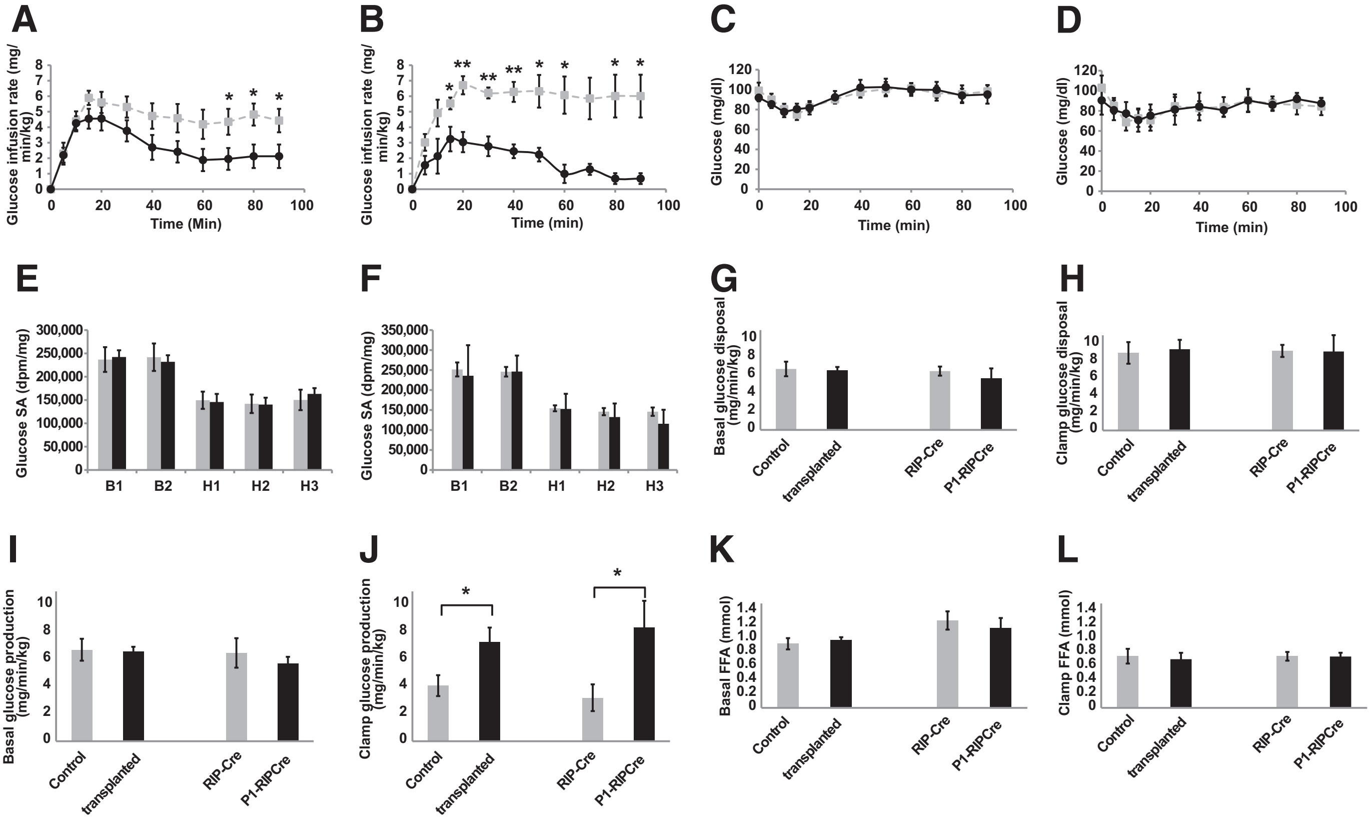

FIG. 7. Hyperinsulinemic euglycemic clamp experiment. Hyperinsulinemic euglycemic clamp experiments were performed in FVB mice transplanted with 1,000 syngeneic islets ($n = 8$) and sham-operated FVB littermate mice ($n = 7$), 2 months after transplantation and in 30- to 35-week-old P1-RIPCre ($n = 4$) and littermate *RIP-Cre* ($n = 7$) mice. *A*: Dynamics of the glucose infusion rates during hyperinsulinemic clamps of FVB mice transplanted with 1,000 islets (full line) and sham-operated littermate mice (dashed line). At time 0 min, an insulin infusion rate of 5 mU/h was started. *B*: Dynamics of the glucose infusion rates during hyperinsulinemic clamps of P1-RIPCre (full line) and *RIP-Cre* littermate mice (dashed line). At time 0 min, an insulin infusion rate of 7.5 mU/h was started. *C*: Sequential blood glucose levels during hyperinsulinemic clamps of FVB mice transplanted with 1,000 islets (full line) and sham-operated littermate mice (dashed line). An insulin infusion rate of 5 mU/h was started at time point 10 min. *D*: Sequential blood during hyperinsulinemic clamps of P1-RIPCre (full line) and *RIP-Cre* littermate mice (dashed line). At time 0 min, an insulin infusion rate of 7.5 mU/h was started. *E*: Basal glucose specific activity values, 50 (B1) and 60 (B2) min after tracer infusion and the hyperinsulinemic glucose specific activity values 70 (H1), 80 (H2), and 90 (H3) min after insulin infusion of FVB mice transplanted with 1,000 islets (black bars) and sham-operated littermate mice (gray bars) are given. *F*: Basal glucose specific activity values, 50 (B1) and 60 (B2) min after tracer infusion and the hyperinsulinemic glucose specific activity values, 70 (H1), 80 (H2) and 90 (H3) min after insulin infusion of P1-RIPCre mice (black bars) and *RIP-Cre* mice (gray bars) are given. *G*: Basal whole-body glucose disposal. *H*: Insulin-stimulated whole-body glucose disposal. *I*: Basal hepatic glucose production. *J*: Insulin-stimulated rates of hepatic glucose production during clamp. *K*: Basal plasma FFA. *L*: Plasma FFA during clamp. Data are represented as mean ± SEM. $^{*}P \leq 0.05$; $^{**}P \leq 0.005$.

After grafting 1,000 islets in normoglycemic mice, recipient mice were hypoglycemic for the initial 2 to 3 days after surgery. It is possible that islet cell death immediately after transplantation is responsible for increased insulin levels and hence hypoglycemia. It is also possible that it requires several days before insulin production and sensitivity homeostasis is established in the setting of excess islets.

Previous studies demonstrated that after transplantation of exogenous islets in normoglycemic mice, the transplanted β-cell mass decreased without significantly affecting the endogenous β-cell mass. These authors hypothesized that recipient mice remained normoglycemic because of the decrease in the transplanted β-cell mass (4). In our study, a similar plasticity of the β-cell mass was observed. In contrast to the previous study, in mice transplanted with 1,000 islets, the endogenous β-cell mass decreased, whereas the insulin content of the graft did not decrease for up to 2 months after transplantation. The decrease of the endogenous β-cell mass could not prevent the development of hyperinsulinemia in transplanted mice. We clearly demonstrate that insulin secretion from the combination of endogenous pancreas and grafted islets remains elevated for at least 2 months after transplantation. Despite the elevated basal plasma insulin levels in mice transplanted with 1,000 islets, mice did not display hypoglycemia because of the development of hepatic insulin resistance, as demonstrated by hyperinsulinemic euglycemic clamp experiments. This is consistent with what has been shown for several published models of transgenic islet hyperplasia (6) and for mice described here, in which the endogenous β-cell mass is increased by forced expression of *PLAG1* in the β-cells. After grafting cells under the kidney capsule, insulin is released into the systemic circulation which has already been described to lead to hepatic insulin resistance (29). In contrast, islets transplanted in humans are infused into the portal vein and release insulin directly into the portal vein. We demonstrate that similar insulin resistance occurs in the *PLAG1* transgenic mouse model where excess insulin and C-peptide are released in the portal tract, as when islets are grafted under the kidney capsule. When more abundant sources of islet cells become available—for instance after differentiation of stem cells into β-cell-like cells—they will likely not be grafted in the liver, but in a more accessible location, such as under the skin. Here they will also release insulin in the systemic circulation and might, as such, lead to hepatic insulin resistance as well. Although our studies were only performed in mice and still need to be further validated in humans, the results suggest that physicians will need to avoid grafting an excess numbers of β-cell–like cells, as this might lead to hyperinsulinemic normoglycemia with concomitant insulin resistance.

It has been well established that insulin resistance in the setting of obesity leads to increased β-cell mass in mice and humans (30). To our knowledge, this is the first report that demonstrates the reverse may hold true; in the face of an increased β-cell mass, hepatic insulin resistance occurs. These results should be kept in mind when designing stem cell-based islet replacement therapies.

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