Original Article

Sirolimus Is Associated With Reduced Islet Engraftment and Impaired β-Cell Function

Nan Zhang,[1] Dongming Su,[2] Shen Qu,[2] Tonia Tse,[2] Rita Bottino,[2] A.N. Balamurugan,[2] Jing Xu,[1] Jonathan S. Bromberg,[1] and H. Henry Dong[2]

Successful islet transplantation depends on the infusion of sufficiently large quantities of islets, but only a fraction of transplanted islets can survive and become engrafted, and yet the underlying mechanism remains unclear. In this study, we examined the effect of sirolimus, a key component of the immunosuppressive regimen in clinical islet transplantation, on islet engraftment and function. To distinguish the effect of sirolimus on immune rejection from its effect on islet engraftment, we used a syngeneic model. Diabetic mice were transplanted with 250 islets under the renal capsule, followed by treatment with sirolimus or vehicle for 14 days. Thirty days posttransplantation, islet grafts were retrieved for the determination of insulin content and vascular density. Compared with mock-treated controls, diabetic recipient mice receiving sirolimus exhibited impaired blood glucose profiles and reduced glucose-stimulated insulin secretion, correlating with reduced intragraft insulin content and decreased vascular density. Islets exposed to sirolimus for 24 h in culture displayed significantly diminished glucose-stimulated insulin release, coinciding with decreased pancreas duodenum homeobox-1 and GLUT2 expression in cultured islets. Furthermore, sirolimus-treated diabetic recipient mice, as opposed to mock-treated controls, were associated with dyslipidemia. These data suggest that sirolimus, administered in the early posttransplantation phase, is a confounding factor for reduced islet engraftment and impaired β-cell function in transplants. *Diabetes* 55:2429–2436, 2006

The Edmonton protocol for islet transplantation depends on the infusion of ~10,000 IE (islet equivalents)/kg body wt, requiring multiple cadaver pancreata per diabetic recipient (1–4). Despite the implantation of such a large quantity of islets, <30% of transplanted islets can survive the procedure and gain stable engraftment, and yet the mechanism underlying the loss of a vast majority of islet mass in the early posttransplantation phase remains elusive (4,5). Unlike whole-organ transplantation, by which grafts are implanted as vascularized tissue, islets are transplanted as single islets or islet clusters that are considered avascular following collagenase digestion and isolation. Although residual endothelial cells in isolated islets may contribute to islet revascularization (6,7), adequate intraislet blood flow requires the formation of a functional microvascular network that links engrafted islets to surrounding tissues. These data suggest that microvascular perfusion to newly transplanted islets does not resume immediately after transplantation and can take up to 2 weeks before the reestablishment of a functional microvasculature in islet grafts (8,9). This delay in islet revascularization can potentially deprive islets of oxygen and nutrients, resulting in islet cell death, particularly within the core of engrafted islets. There is mounting evidence that impaired islet revascularization is an independent factor that limits the success rate and compromises the clinical outcome of islet transplantation (7,9–17).

Clinical islet transplantation is accompanied by a rigorous sirolimus (rapamycin)-based steroid-free immunosuppressive regimen, which is meant to prevent allograft rejection. While rapid and sufficient islet revascularization is important for islet viability and function, whether immunosuppressants adversely affect the engraftment and function of newly transplanted islets remains undetermined. To address this fundamental question, we examined the effect of sirolimus, a key component of the immunosuppressive regimen in clinical islet transplantation, on islet engraftment and β-cell function. To separate the impact of sirolimus on islet revascularization from its effect on immune rejection, we used a syngeneic model of islet transplantation. Our data indicate that sirolimus adversely affects islet engraftment and compromises β-cell function in a dose-dependent manner, contributing to reduced islet mass and diminished glucose-stimulated insulin release in islet grafts in diabetic mice.

RESEARCH DESIGN AND METHODS

Inbred BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in a pathogen-free animal facility with a 12-h light/dark cycle. Mice were fed standard rodent chow and water ad libitum. To induce diabetes, BALB/c mice (10 weeks old, body weight 25–30 g) were injected intraperitoneally with a single dose of streptozotocin (STZ; 180 mg/kg). Animals were considered diabetic when tail-vein blood glucose levels were >300 mg/dl for 2 consecutive days, as determined by glucometer (Bayer, Mishawaka, IN).

Islet isolation and transplantation. Mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg), followed by pancreatic intraductal infusion of 3-ml cold Hank's buffer containing 1.5 mg/ml of collagenase-P (Roche Diagnostics, Indianapolis, IN). The pancreas was surgically procured and digested at 37°C for 20 min. Islets were purified on a discontinuous Ficoll

From the [1]Department of Gene and Cell Medicine, Mount Sinai School of Medicine, New York, New York; and [2]Rangos Research Center, Department of Pediatrics, Children's Hospital of Pittsburgh, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania.

Address correspondence and reprint requests to H. Henry Dong, Rangos Research Center, Children's Hospital of Pittsburgh, 3460 5th Ave., Rm. 6111, Pittsburgh, PA 15213. E-mail: dongh@pitt.edu.

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AUC, area under the curve; FFA, free fatty acid; FPLC, fast-performance liquid chromatography; PDX-1, pancreas duodenum homeobox-1; STZ, streptozotocin.

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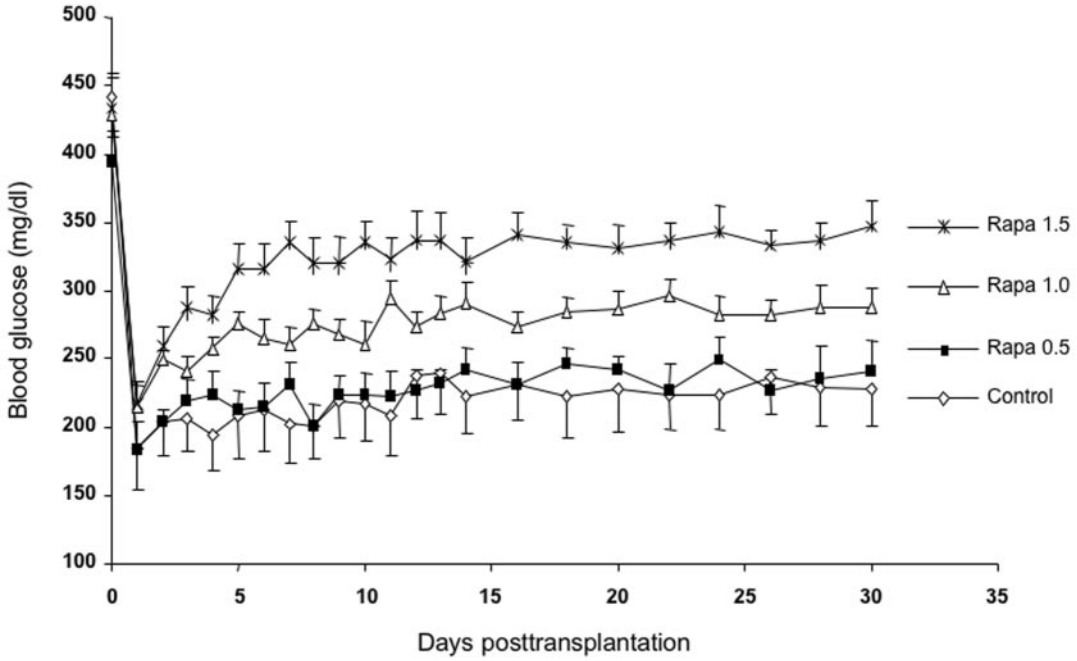

FIG. 1. Blood glucose profiles. Significant differences ($P < 0.05$) in blood glucose levels were detected between mock- and sirolimus (rapamycin [Rapa])-treated diabetic recipient mice receiving 1.0 or 1.5 mg/kg sirolimus.

gradient (Sigma, St. Louis, MO) as described (15). For islet transplantation, 250 handpicked islets were implanted beneath the renal capsule of STZ-induced diabetic mice using the established procedure as described (15). Transplanted mice were randomly divided into four groups ($n = 12$). Three groups were administered intraperitoneally with 400 μl sirolimus (Sigma) at three different doses (0.5, 1.0, and 1.5 mg · kg^{-1} · day^{-1}) per mouse daily starting from day 1 posttransplantation for 2 weeks. These doses were based on previous preclinical studies (18–20) and were within the range of pharmacological doses in human islet transplantation (1,21). As control, one group was treated with 400 μl vehicle carboxymethyl-cellulose. Islet graft function was defined as elevated blood glucose levels in diabetic recipient mice reduced to <200 mg/dl on 2 consecutive days posttransplantation. All procedures were approved by the institutional animal care and usage committee of Children's Hospital of Pittsburgh (Pittsburgh, PA).

Glucose tolerance test. Animals were fasted for 5 h and injected intraperitoneally with 50% dextrose solution (Abbott Laboratories, Chicago, IL) at 3 g/kg body wt. Blood glucose levels were determined before and after glucose infusion as described (15). Area under the curve (AUC) of blood glucose profiles during glucose tolerance was calculated using the KaleidaGraph software (Synergy Software, Reading, PA). To determine glucose-stimulated insulin release, aliquots (25 μl) of tail-vein blood were sampled before and 5 min after glucose infusion for the determination of plasma insulin levels by an ultrasensitive mouse insulin enzyme-linked immunosorbent assay kit (ALPCO, Windham, NH).

Plasma lipid determination. Blood was collected from tail vein into capillary tubes precoated with potassium-EDTA (Sarstedt, Nümbrecht, Germany) for the preparation of plasma or for the determination of blood glucose levels using Glucometer Elite (Bayer, IN). Plasma levels of triglyceride and cholesterol were determined using ThermoDMA Infinity triglycerides and cholesterol reagents (ThermoDMA, Louisville, CO). Plasma free fatty acid (FFA) levels were determined using the Wako FFA assay kit (Wako Chemical, Richmond, VA).

Fast-performance liquid chromatography fractionation of lipoproteins. Aliquots (250 μl) of plasma pooled from individual diabetic recipient mice from each group were applied to two head-to-tail linked Tricorn high-performance Superose S-6 10/300GL columns using a fast-performance liquid chromatography (FPLC) system (Amersham Biosciences), followed by elution with PBS at a constant flow rate of 0.5 ml/min. Fractions (500 μl) were eluted and assayed for triglycerides and cholesterol concentrations using the ThermoDMA Infinity triglycerides and cholesterol reagents (ThermoDMA).

Immunohistochemistry. Immunohistochemistry of islet grafts and morphometric analysis were performed as previously described (15). Mice were fasted for 16 h and killed by CO_2 inhalation 30 days posttransplantation, and islet grafts were retrieved from individual diabetic recipient mice. After fixing in 10% phosphate-buffered formalin overnight, islet grafts were embedded in paraffin. Consecutive sections (8-μm thick) of paraffin-embedded islet grafts were immunostained with guinea pig anti-insulin (1:200 dilution; DAKO, Carpentaria, CA) and rat anti-CD31 (1:50 dilution; Research Diagnostics, Flanders, NJ), as described (15). The immunoreactivity was detected using the multilink-HRP ultrasensitive system (BioGenex, San Ramon, CA). After immunostaining, slides were examined at 200× magnification in a microscope that was linked to a computerized charge-coupled device camera. Microscopic views covering engrafted islets under the kidney capsule that were immunostained by insulin and CD31 antibodies were captured as digitized micrographic pictures using Adobe Photoshop software (Adobe Systems, San Jose, CA). Using the color range section option of Adobe Photoshop, insulin- or CD31-positively immunostained color (brown in each case) was selected for quantification of the relative content per islet graft by densitometry using NIH Image 1.62 software (National Institutes of Health, Bethesda, MD). For the determination of vascular density, microvessels that were positively stained by anti-CD31 immunohistochemistry within a given islet graft as well as in graft stroma were collectively quantified. Using this procedure, the relative contents of insulin or CD31 from 10 nonoverlapping fields covering 3–5 islets per field were evaluated for the determination of mean values, which were subsequently compared among groups.

Islet perifusion. Glucose-stimulated insulin release was assayed by dynamic perifusion of cultured islets, as described (22). Aliquots of 100 freshly isolated murine islets were cultured in 1.5 ml RPMI-1640 medium (Cellgro Mediatech, Herndon, VA) in the absence or presence of 30 ng/ml sirolimus at 37°C for 24 h, followed by islet perifusion, as described (22). Islets were first equilibrated by perifusing with Kreb KCl s-Ringer bicarbonate buffer (pH 7.4, 2.4 mmol/l $CaCl_2$, 120 mmol/l NaCl, 1.2 mmol/l $MgSO_4$, 5.4 mmol/l KCl, 1.2 mmol/l KH_2PO_4, 20 mmol/l HEPES) supplemented with 2.8 mmol/l glucose for 30 min, followed by perifusion with Krebs-Ringer bicarbonate buffer containing 2.8 or 20 mmol/l glucose at a constant flow rate of 1 ml/min. Fractions (1 ml) were collected for the determination of insulin concentrations by ultrasensitive mouse insulin enzyme-linked immunosorbent assay (ALPCO, Windham, NH).

Effect of sirolimus on cultured islets. Aliquots of freshly isolated murine islets ($n = 500$) were cultured in RPMI-1640 medium (Cellgro Mediatech) in the absence and presence of sirolimus (0, 10, 20, and 30 ng/ml). After 24 h of incubation, islets were collected and total RNA was prepared for real-time quantitative RT-PCR analysis of pancreas duodenum homeobox (PDX)-1, GLUT2, and glucokinase expression using 18S rRNA as control, as previously described (23). The primers used were as follows: PDX-1 forward 5′-AGCAG TACTACGCGGCCACACA-3′ (corresponding to mouse PDX-1 cDNA 14-35 nucleotide [nt]) and PDX-1 reverse 5′-GCACTTCGTATGGGGAGATG-3′ (mouse PDX-1 cDNA 192-211 nt), glucokinase forward 5′-CAAGCTGCAC CCGAGCTT-3′ (mouse glucokinase cDNA 12391-256 nt) and glucokinase reverse 5′-ACTGGCCCAGCATGCAAG-3′ (mouse glucokinase cDNA 13791-396 nt), GLUT2 forward 5′-TGGCACATCCTACTTGGCCTATC-3′ (mouse

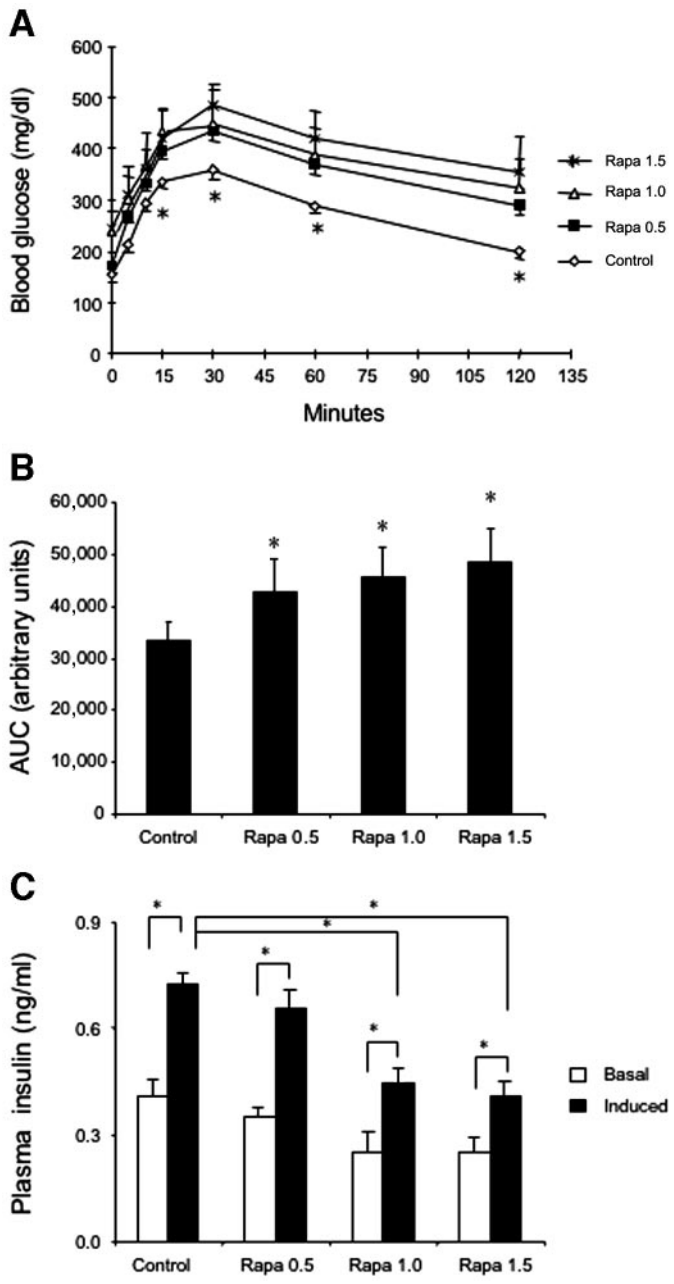

FIG. 2. Glucose tolerance test. *A*: Blood glucose profiles during glucose tolerance test 2 weeks posttransplantation. *B*: Calculated AUC of blood glucose profiles. *C*: Glucose-stimulated insulin release in islet grafts. During glucose tolerance test, aliquots (25 μl) of blood were collected from tail vein before and 5 min after glucose infusion. Plasma insulin levels were determined under basal and glucose-stimulated conditions. Similar results were reproduced at 3 weeks posttransplantation. *$P < 0.05$ vs. controls. Rapa, rapamycin.

GLUT2 cDNA 649-671 nt) and GLUT2 reverse 5′-GATCACGGAGACCTTCT GCTC-3′ (mouse GLUT2 853-873 nt), and 18S rRNA forward 5′-CGCCGCTA GAGGTGAAATTC-3′ (mouse 18S rRNA 949–968 nt) and 18S rRNA reverse 5′-TTGGCAAATGCTTTCGCTC-3′ (mouse 18S rRNA 992-1010 nt).

Statistical analysis. Statistical analysis was performed by ANOVA with Dunnett's post hoc test for the comparisons of parameters between sirolimus and control groups using StatView software (Abacus Concepts, Berkeley, CA). Data are expressed as the mean ± SE. P values <0.05 are statistically significant.

RESULTS

Effect of sirolimus on islet function in diabetic recipient mice. To address the effect of sirolimus on islet engraftment and function in vivo, we chose to use a syngeneic islet transplantation model. BALB/c mice were rendered diabetic by intraperitoneal injection of STZ. One week post-STZ administration, diabetic mice were stratified by degree of hyperglycemia into different groups to ensure a similar mean blood glucose level per group (n = 12), followed by the implantation of 250 handpicked islets under the renal capsule. Such a syngeneic model allows the examination of the net effect of sirolimus on islet engraftment in the absence of immune response. After islet transplantation, groups of diabetic recipient mice were treated with once-daily intraperitoneal administration of sirolimus at 0.5, 1.0, and 1.5 mg/kg, respectively. As shown in Fig. 1, transplantation of 250 islets resulted in near-normal glycemia in diabetic recipient mice that were mock treated. In contrast, diabetic recipient mice treated with sirolimus at 1.0 mg/kg or higher doses maintained hyperglycemia during the course of study.

To study the effect of sirolimus on glucose-stimulated insulin secretion from transplanted islets, we performed a glucose tolerance test on diabetic recipient mice 2 and 3

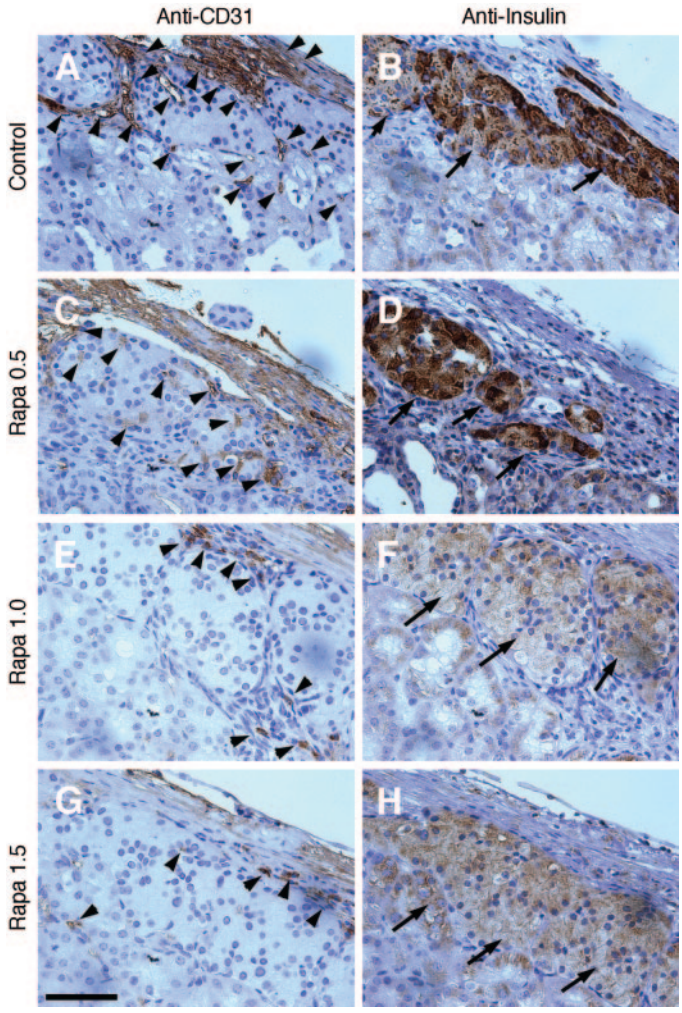

FIG. 3. Immunohistochemistry of islet grafts. Islet grafts were retrieved from recipient diabetic mice transplanted with mock-treated (*A* and *B*) and sirolimus-treated diabetic recipient mice at concentrations of 0.5 mg/kg (*C* and *D*), 1.0 mg/kg (*E* and *F*), and 1.5 mg/kg (*G* and *H*). Islet grafts were embedded in paraffin, and consecutive sections of paraffin-embedded islet grafts were cut and immunostained with anti-CD31 (*A*, *C*, *E*, and *G*) and anti-insulin (*B*, *D*, *F*, and *H*). Arrowheads in *A*, *C*, *E*, and *G* indicate microcapillaries that were positively stained by anti-CD31. Arrows in *B*, *D*, *F*, and *H* indicate islet grafts that were positively stained by anti-insulin. Bar, 100 μm. Rapa, rapamycin.

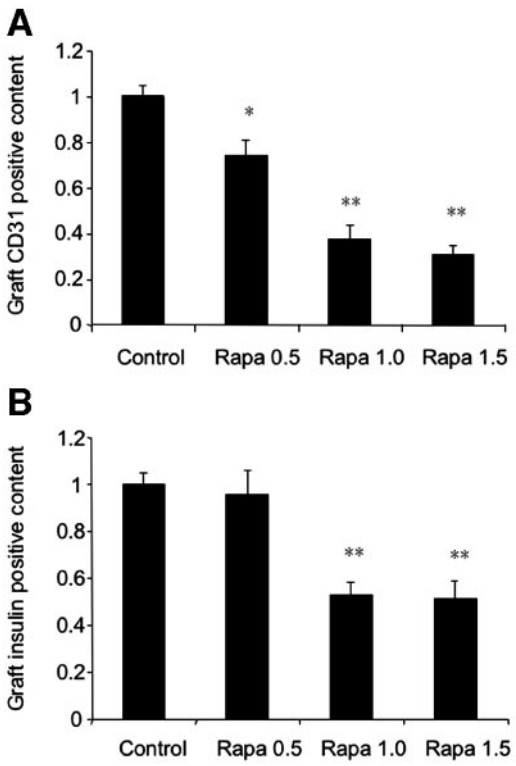

FIG. 4. Islet graft CD31 and insulin content. The relative contents of anti-CD31 (*A*) and anti-insulin (*B*) immunostaining were compared between different groups, as determined by morphometric analysis of immunohistochemistry. *$P < 0.05$, **$P < 0.01$ vs. control. Rapa, rapamycin.

weeks posttransplantation. In response to glucose challenge, blood glucose levels were elevated and returned to normal levels within 2 h in mock-treated diabetic recipient mice (Fig. 2*A*). In contrast, significantly impaired blood glucose tolerance was detected in diabetic recipient mice that were treated with sirolimus at all three different doses (Fig. 2*A*). These results were corroborated by the quantification of the AUC of blood glucose profiles during the glucose tolerance test. In keeping with their impaired blood glucose profiles, diabetic recipient mice in sirolimus treatment groups were associated with significantly higher AUC values, a reflection of relatively poor glycemic control (Fig. 2*B*). Similar results were produced at 2 and 3 weeks posttransplantation. Notably, diabetic recipient mice receiving the lowest dose of 0.5 mg $\cdot$ kg^{-1} $\cdot$ day^{-1} sirolimus also exhibited significantly impaired glucose tolerance (Fig. 2*A*), although their daily blood glucose profiles were not significantly different from those of mock-treated controls (Fig. 1).

Impact of sirolimus on functional islet mass in diabetic recipient mice. To estimate the functional islet mass, we determined the amplitude of glucose-stimulated insulin secretion in diabetic recipient mice at 2 and 3 weeks posttransplantation. Aliquots (25 μl) of tail vein blood were sampled from diabetic recipient mice before and 5 min after intraperitoneal glucose infusion for the determination of plasma insulin levels. As shown in Fig. 2*C*, significantly lower levels of plasma insulin were detected in diabetic recipient mice treated with sirolimus, which correlated with their impaired abilities to tolerate intraperitoneally infused glucose (Fig. 2*A*). It has been suggested that the amplitude of glucose-stimulated insulin release represents the insulin secretory reservoir that best predicts functional islet mass (4,24). These results indicate that diabetic recipient mice treated with sirolimus even at the lowest dose were associated with significantly reduced functional islet mass.

Effect of sirolimus on islet microvascularity in diabetic recipient mice. To determine the effect of sirolimus on islet revascularization, diabetic recipient mice were killed under fasting conditions 30 days posttransplantation and islet grafts retrieved for immunohistochemistry using antibodies against the endothelial marker CD31. This approach allows the visualization of endothelium in a cross-section of islet grafts (15). We noted that most neovessels developed in the connective tissue surrounding engrafted islets in both mock- and sirolimus-treated diabetic recipient mice. Similar results have been reported in subcapsular islet grafts in diabetic recipient mice (9,12,13). In light of these observations, we determined total vascular density collectively from microvessels within islet grafts as well as in connective tissue by morphometric analysis following anti-CD31 immunohistochemistry (Fig. 3). When compared with mock-treated controls, islet grafts retrieved from sirolimus-treated diabetic recipient mice were associated with relatively lower total microcapillary densities, as evidenced by anti-CD31 immunohistochemistry (Fig. 3*A*, *C*, *E*, and *G*) and quantification of anti-CD31 immunostaining content by morphometric analysis (Fig. 4*A*).

To correlate the degree of islet revascularization with insulin content under the kidney capsule, islet grafts retrieved from killed animals were immunostained for insulin (Fig. 3*B*, *D*, *F*, and *H*). Consistent with the data of anti-CD31 immunohistochemistry, islet grafts in diabetic recipient mice treated with sirolimus at 1.0 mg/kg or higher doses displayed significantly lower levels of insulin content than control islet grafts, as quantified by morphometric analysis (Fig. 4*B*). This difference in insulin content in engrafted islets between mock- and sirolimus-treated diabetic recipient mice correlated closely with their blood glucose profiles on glucose tolerance tests and glucose-stimulated insulin secretion (Fig. 2).

Impact of sirolimus on plasma lipid metabolism in diabetic recipient mice. Sirolimus-based immunotherapy has been linked to the pathogenesis of diabetic dyslipidemia in transplants (25–27). To study the effect of sirolimus on plasma lipid metabolism, plasma levels of triglyceride, cholesterol, and FFAs were determined in diabetic recipient mice 30 days posttransplantation. When compared with mock-treated controls, diabetic recipient mice that were pretreated with sirolimus exhibited significantly elevated plasma triglyceride, cholesterol, and FFA levels, which were indicative of impaired lipid metabolism (Fig. 5*A*–*C*). The severity of impairment in plasma lipid metabolism was further pronounced with increasing doses of sirolimus administered to diabetic recipient mice. To confirm these results, aliquots (250 μl) of plasma pooled from individual groups of diabetic recipient mice were subjected to FPLC fractionation of lipoproteins. As shown in Fig. 5*D*, plasma VLDL-triglyceride levels were markedly elevated in response to sirolimus treatment. Furthermore, in keeping with significantly elevated total plasma cholesterol levels, sirolimus-treated diabetic recipient mice were associated with increased plasma HDL cholesterol levels when compared with mock-treated controls (Fig. 5*E*).

Effect of sirolimus on glucose-stimulated insulin release in cultured islets. To study the effect of sirolimus on β-cell function, we treated islets in vitro with sirolimus

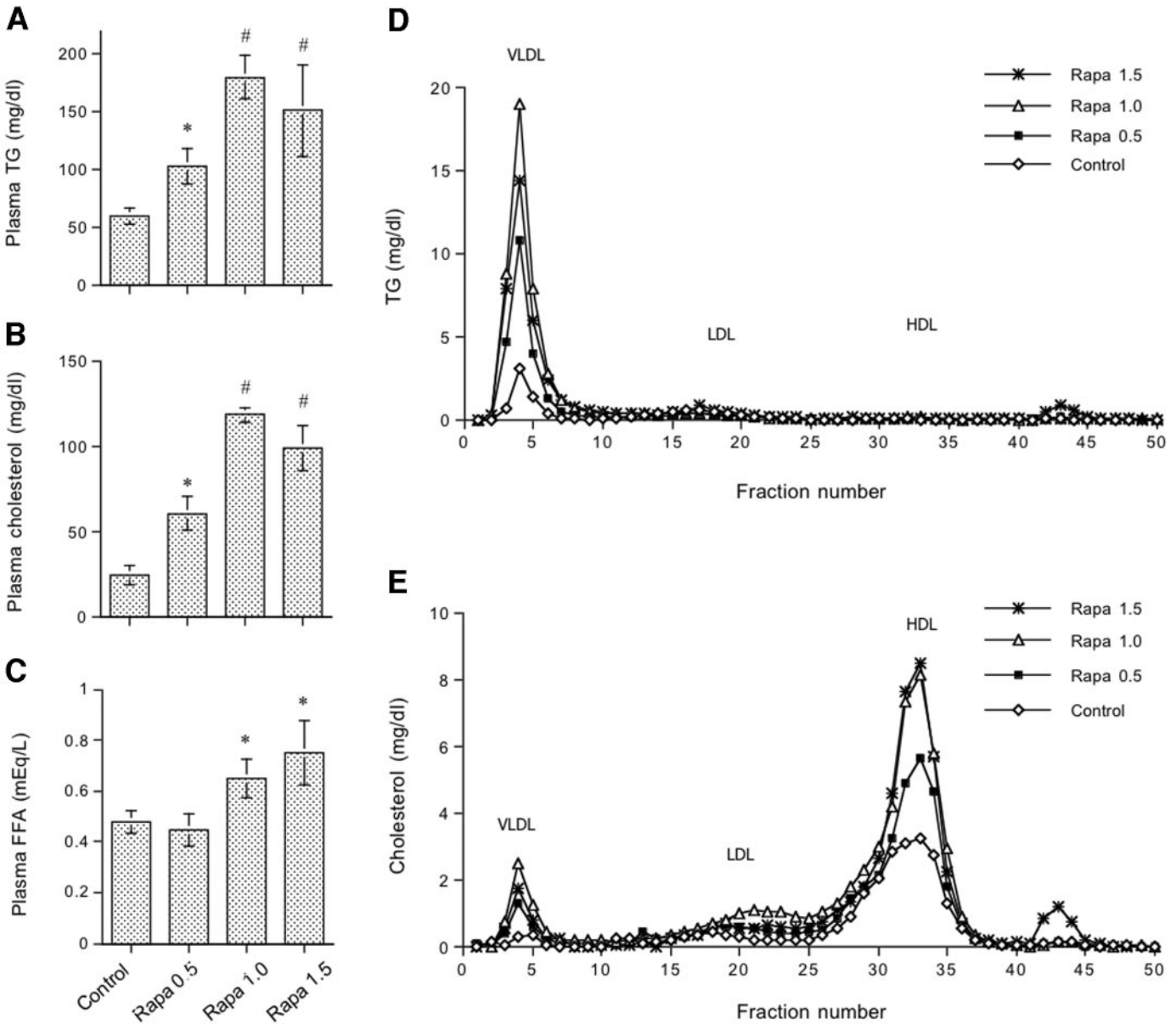

FIG. 5. Plasma lipid and lipoprotein profiles. Plasma triglyceride (TG) (*A*), plasma total cholesterol (*B*), and plasma FFA (*C*) levels. *$P < 0.05$. #$P < 0.005$ vs. controls. *D*: Triglyceride distributions in VLDL, LDL, and HDL fractions. *E*: Cholesterol distributions in VLDL, LDL, and HDL fractions. Aliquots (250 μl) of plasma pooled from killed mice in each group were subjected to gel filtration column chromatography through two consecutive Tricorn high-performance Superose S-6 10/300GL columns in an FPLC system. A total of 50 fractions (500 μl) were collected for the determination of triglyceride and cholesterol concentrations. Data were obtained on day 30 posttransplantation. Rapa, rapamycin.

and assayed the amplitude of insulin release in response to a glucose challenge. Aliquots of 100 freshly isolated islets from BALB/c mice were cultured in the absence and presence of sirolimus at final concentrations ranging from 10 to 30 ng/ml in 2 ml culture media. After a 24-h incubation, islets were challenged with glucose by shifting the culture medium from low (5 mmol/l) to higher (20 mmol/l) glucose concentrations. Aliquots (50 μl) of conditioned media were collected before and after glucose concentration shift for the determination of insulin concentrations. As shown in Fig. 6*A*, about a sixfold induction of insulin release was observed in cultured islets in response to a glucose challenge in the absence of sirolimus. In contrast, prior culturing of islets in the presence of sirolimus for 24 h at 20 ng/ml or higher concentrations significantly impaired glucose-stimulated insulin release.

To corroborate these findings, islet perifusion experiments were performed to study the effect of sirolimus on the kinetics of insulin release in cultured islets in response to a glucose challenge. Aliquots of 100 handpicked islets were cultured in the absence and presence of 30 ng/ml sirolimus for 24 h, followed by perifusion. As shown in Fig. 6*B*, control islets displayed markedly elevated insulin secretion with the typical first and second phases of glucose-stimulated insulin secretion when the perifusion media was shifted from low (2.8 mmol/l) to higher (20 mmol/l) glucose concentrations. Under the same perifusion condition, islets pre-exposed to sirolimus were associated with impaired β-cell function, as both the first and second phases of glucose-stimulated insulin release were significantly diminished (Fig. 6*B*).

Mechanism of sirolimus-mediated impairment in glucose-stimulated insulin release in islets. To gain insight into the mechanism underlying sirolimus-mediated

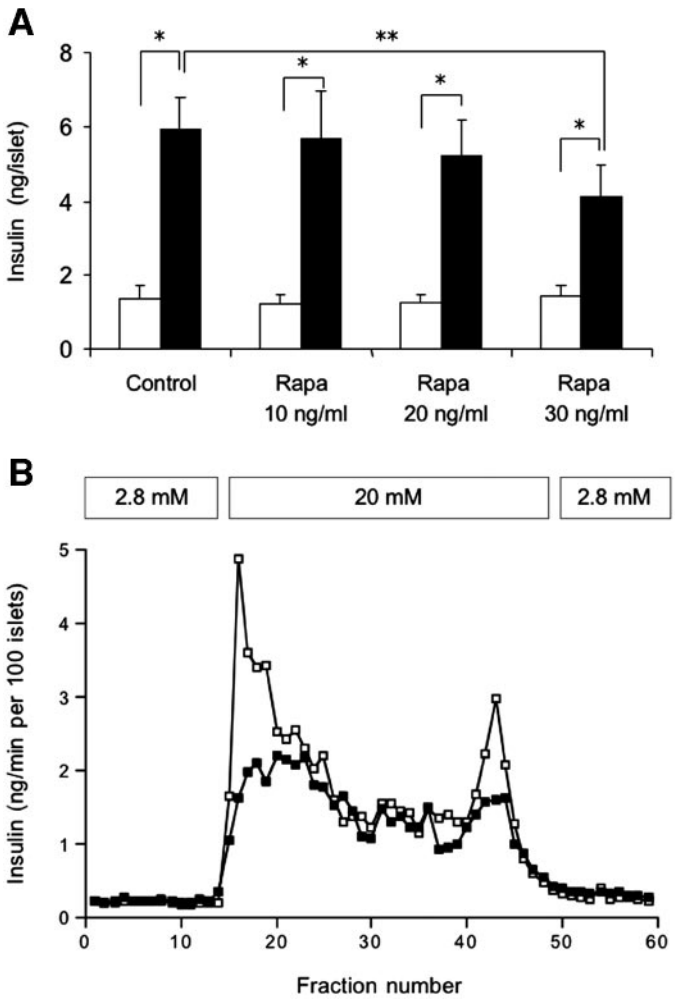

FIG. 6. Effect of sirolimus on islet function. *A*: The effect of sirolimus on islet function was studied by determining glucose-stimulated insulin release from cultured islets. Aliquots of 100 hand-picked islets were cultured in RPMI-1640 medium in the absence and presence of sirolimus at different concentrations for 24 h, followed by shifting glucose concentration of culture media from 5 to 20 mmol/l. Fifteen minutes later, aliquots (50 μl) of media were sampled for the determination of insulin concentrations. *$P < 0.001$. **$P < 0.05$. □, basal; ■, induced. *B*: The kinetics of glucose-stimulated insulin release was assayed by dynamic perifusion of cultured islets. Aliquots of 100 handpicked islets were cultured in the absence or presence of 30 ng/ml sirolimus for 24 h, followed by glucose perifusion. Fractions (1 ml) were collected for the determination of insulin concentrations. Rapa, rapamycin. □, control; ■, rapamycin.

impairment in glucose-stimulated insulin release in islets, we determined the expression levels of key functions in β-cell functional integrity, namely PDX-1, glucokinase, and GLUT2 in cultured islets following a 24-h treatment with sirolimus. PDX-1 acts in β-cells as a house-keeping transcription factor for insulin gene expression (28). Glucokinase and GLUT2 are two key components of the glucose-sensing machinery responsible for glucose-inducible insulin release (29–31). When compared with mock-treated islets, islets cultured in the presence of sirolimus at 20 ng/ml or higher concentrations were associated with significantly reduced PDX-1 and GLUT2 mRNA levels, as determined by real-time quantitative RT-PCR assay (Fig. 7). Relatively lower glucokinase mRNA levels were detected in cultured islets following sirolimus treatment, but the difference between sirolimus- and mock-treated islets did not reach a significant level (Fig. 7). These data indicate that prolonged exposure of islets to sirolimus is deleterious to β-cell function, accounting for impaired glucose-stimulated insulin release in islets.

DISCUSSION

The development of a sirolimus-based steroid-free immunosuppressive regimen represents a breakthrough that significantly improves the success rate of 1-year insulin independence in islet recipient subjects by more than 80% (4). However, there is emerging evidence that such sirolimus-based immunotherapy may not be optimal for the survival and revascularization of newly transplanted islets. Our present studies indicate that sirolimus administration resulted in decreased functional islet mass, as reflected in significantly diminished glucose-stimulated insulin release in response to a glucose challenge. This effect, which was more pronounced with increasing doses of sirolimus, cannot be ascribed to immune rejection because of the syngeneic model used in this study. Furthermore, we demonstrate that sirolimus suppressed the expression of PDX-1 and GLUT2, contributing to the abolition of first-phase glucose-stimulated insulin secretion in cultured islets. Bell et al. (18) show that sirolimus affects the viability of islets by inducing islet cell apoptosis in vitro. Together these data suggest that sirolimus administered at relatively higher doses, especially during the early post-transplantation phase, can compromise islet viability and deteriorate islet function, contributing to reduced islet engraftment in diabetic recipient subjects.

We must acknowledge that the immunohistochemistry used for determining intragraft insulin content is limited, as persistent hyperglycemia can potentially exhaust β-cells for insulin release in islet grafts, leading to underestimation of graft insulin content. Likewise, this effect may have contributed to relatively lower levels of insulin secretion in response to a glucose challenge in sirolimus-treated diabetic mice. Although significantly lower amplitudes of glucose-stimulated insulin release and reduced intragraft insulin content were detected in sirolimus-treated diabetic mice, caution needs to be exercised when these data are used for inferring functional islet mass in transplants because of the potential exhaustive effect of persistent hyperglycemia on β-cell insulin secretion. Indeed, we show that sirolimus-treated diabetic recipient mice maintained hyperglycemia along with impaired glucose tolerance posttransplantation during the course of the study when compared with controls.

It is noteworthy that we limited the administration of sirolimus to the first 14 days posttransplantation, based on previous findings that islet engraftment initiates as early as on day 2 posttransplantation and concludes with the reestablishment of blood flow to islets grafts within 2–3 weeks in rodent models of diabetes (9,32). Strikingly, we found that sirolimus-mediated impairment in islet graft function was irreversible, as hyperglycemia, glucose intolerance, and hyperlipidemia persisted after discontinuation of sirolimus treatment at day 15 posttransplantation. These results underscore the critical importance of rapid islet revascularization in graft survival and function, corroborating previous observations that once implanted islets become stably engrafted, islet revascularization does not seem to improve over time (9,13).

One potential mechanism accounting for impaired islet engraftment in sirolimus-treated diabetic recipient mice is due to the intrinsic antiproliferating effect associated with

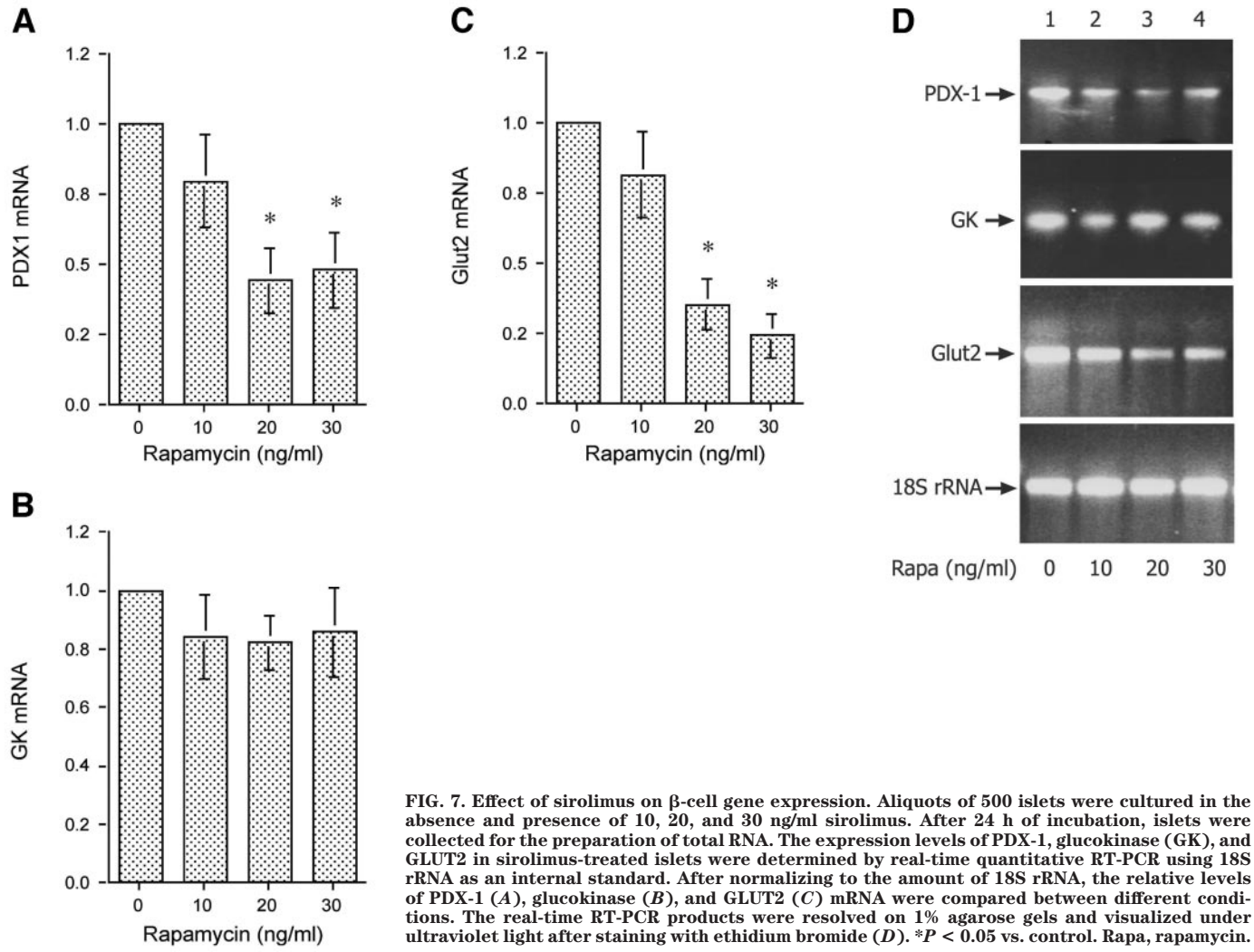

FIG. 7. Effect of sirolimus on β-cell gene expression. Aliquots of 500 islets were cultured in the absence and presence of 10, 20, and 30 ng/ml sirolimus. After 24 h of incubation, islets were collected for the preparation of total RNA. The expression levels of PDX-1, glucokinase (GK), and GLUT2 in sirolimus-treated islets were determined by real-time quantitative RT-PCR using 18S rRNA as an internal standard. After normalizing to the amount of 18S rRNA, the relative levels of PDX-1 (*A*), glucokinase (*B*), and GLUT2 (*C*) mRNA were compared between different conditions. The real-time RT-PCR products were resolved on 1% agarose gels and visualized under ultraviolet light after staining with ethidium bromide (*D*). *$P < 0.05$ vs. control. Rapa, rapamycin.

sirolimus. Sirolimus is known to bind and inhibit mTOR (mammalian target of rapamycin protein) kinase-mediated signaling in cell cycle progression, resulting in the arrest of cell cycle in various cell types including T- and B-cells and contributing to prolonged allograft survival (33,34). Because of its antiangiogenic activity, sirolimus has been used in sirolimus-eluting stents to suppress neointimal hyperplasia in patients with coronary artery disease (35). Furthermore, several recent studies indicate that sirolimus antagonizes angiogenesis by inhibiting the proliferation and differentiation of endothelial cells (36–38). This suggests that sirolimus counteracts the effect of angiogenesis, resulting in reduced islet engraftment and decreased islet mass in diabetic recipient mice.

Sirolimus, considered an effective immunosuppressant devoid of chronic toxicity, is shown to exhibit deleterious effects on the viability and function of cells in a number of cell types including β-cells (18,39–41). Our present studies indicate that sirolimus-treated diabetic recipient mice were associated with dyslipidemia, as reflected in markedly elevated plasma VLDL-triglyceride levels. These results were consistent with previous observation that chronic use of sirolimus is closely associated with the pathogenesis of hyperlipidemia (25–27). More recently, clinical application of sirolimus monotherapy in kidney transplants has been linked to the development of post-transplantation insulin resistance and impaired glucose metabolism (42). Together these data raise a serious concern that sirolimus-based immunotherapy in islet transplantation, intended primarily for mitigating islet allograft rejection, may play a causative role in impairing β-cell function and retarding islet revascularization in the immediate posttransplantation phase. In support of this notion, two recent studies (43,44) report that the blood levels of immunosuppressants in the portal vein are about twofold higher than in the peripheral circulation following intraportal delivery of islets in diabetic recipients. This elevated local level of immunosuppressants in the portal vein, interpreted as "portal storm of immunosuppression," is considered harmful to islets, particularly during the immediate posttransplantation period when the survival and function of newly transplanted islets depends on rapid revascularization for deriving blood from the host for oxygen and nutrient supplies (44).

In addition to sirolimus, other immunosuppressants and steroids, such as cyclosporine and prednisolone, have been shown to impact islet revascularization in rodent models of type 1 diabetes (45–47). Although the underlying mechanisms remain to be characterized, these studies together with our present data support the conclusion that immunosuppressants administered in the immediate post-transplantation phase constitute an independent factor that adversely affects islet function and hampers islet

engraftment, contributing to impaired glucose-stimulated insulin secretion and reduced islet mass in transplants.

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