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# mTOR Inhibition: Reduced Insulin Secretion and Sensitivity in a Rat Model of Metabolic Syndrome

Jordi Rovira, PhD,<sup>1</sup> María Jose Ramírez-Bajo, PhD,<sup>1</sup> Elisenda Banon-Maneus, PhD,<sup>1</sup> Daniel Moya-Rull,<sup>1</sup> Pedro Ventura-Aguiar, MD,<sup>1,2</sup> Natalia Hierro-Garcia,<sup>1</sup> Marta Lazo-Rodriguez, BVSc,<sup>1</sup> Ignacio Revuelta, MD, PhD,<sup>1,2</sup> Armando Torres, MD, PhD,<sup>3</sup> Federico Oppenheimer, MD, PhD,<sup>1,2</sup> Josep M. Campistol, MD, PhD,<sup>1,2</sup> and Fritz Diekmann, MD, PhD<sup>1,2</sup>

**Background.** Sirolimus (SRL) has been associated with new-onset diabetes mellitus after transplantation. The aim was to determine the effect of SRL on development of insulin resistance and  $\beta$ -cell toxicity. **Methods.** Lean Zucker rat (LZR) and obese Zucker rat (OZR) were distributed into groups: vehicle and SRL (0.25, 0.5, or 1.0 mg/kg) during 12 or 28 days. Intraperitoneal glucose tolerance test (IPGTT) was evaluated at days 0, 12, 28, and 45. Islet morphometry,  $\beta$ -cell proliferation, and apoptosis were analyzed at 12 days. Islets were isolated to analyze insulin content, insulin secretion, and gene expression. **Results.** After 12 days, SRL treatment only impaired IPGTT in a dose-dependent manner in OZR. Treatment prolongation induced increase of area under the curve of IPGTT in LZR and OZR; however, in contrast to OZR, LZR normalized glucose levels after 2 hours. The SRL reduced pancreas weight and islet proliferation in LZR and OZR as well as insulin content. Insulin secretion was only affected in OZR. Islets from OZR + SRL rats presented a downregulation of *Neurod1*, *Pax4*, and *Ins2* gene. Genes related with insulin secretion remained unchanged or upregulated. **Conclusions.** In conditions that require adaptive  $\beta$ -cell proliferation, SRL might reveal harmful effects by blocking  $\beta$ -cell proliferation, insulin production and secretion. These effects disappeared when removing the therapy.

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Posttransplantation diabetes mellitus (PTDM) is a serious problem and affects up to 20% of kidney transplant patients. It is associated with worse cardiovascular outcome and worse graft and patient survival.<sup>1-4</sup> Therefore, it is of utmost importance to identify and modify risk factors associated with PTDM. Immunosuppressive medication including calcineurin inhibitors and steroids has been identified as one of the most important risk factors. Furthermore, examining the pathogenesis of PTDM may offer new preventive strategies.

In terms of timing, risk factors for PTDM can be classified as preexisting factors at the time of transplantation and as additional posttransplant risk factors. The most important pretransplant risk factors are: age, obesity, hypertriglyceridemia, and metabolic syndrome. The pretransplant risk factors can hardly be modified; however, the posttransplant risk factors, such as immunosuppression, can be adjusted.

Pretransplant risk factors, such as metabolic syndrome, obesity, and high triglyceride levels, are markers of insulin

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<sup>1</sup> Laboratori Experimental de Nefrologia i Trasplantament (LENIT), CRB CELLEX, Fundació Clínic, IDIBAPS, Barcelona, Spain.

<sup>2</sup> Department of Nephrology and Renal Transplantation, Hospital Clínic, Barcelona, Spain.

<sup>3</sup> Department of Nephrology, Hospital Universitario de Canarias, Centro de Investigaciones Biomédicas de Canarias (CIBICAN), Universidad de La Laguna, Santa Cruz de Tenerife, Spain.

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Correspondence: Fritz Diekmann, MD, Hospital Clínic, Department of Nephrology and Renal Transplantation, Villarroel 170 (Escala 12-Planta 5), E-08036 Barcelona, Spain. (fdiekman@clinic.ub.es).

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resistance (IR).<sup>5</sup> The IR and  $\beta$ -cell dysfunction are the main features of type 2 diabetes. Often, these 2 features are associated.<sup>6-8</sup> In this sense, pretransplant IR may indicate a serious metabolic condition in combination with additional impairment caused by posttransplant immunosuppression favoring PTDM. Calcineurin inhibitors have been demonstrated to be associated with a diabetogenic potential, the effect of tacrolimus being more potent compared with that caused by cyclosporine A.<sup>9-11</sup>

However, inhibitors of the mammalian target of rapamycin (mTOR-I) as well have shown to be diabetogenic.<sup>12,13</sup> Calcineurin and mTOR-I were used at low doses in the Symphony study to reduce side effects; unfortunately, all of them retain their toxicity profiles which include PTDM.<sup>14</sup> So far, it remained unclear, by which mechanism and to which extent mTOR-I could be associated with PTDM.

Therefore, the aim of this study was to evaluate the role of mTOR inhibition in the pathogenesis of PTDM, especially to differentiate whether mTOR inhibition provokes  $\beta$ -cell toxicity, peripheral IR, or both.

## MATERIALS AND METHODS

### Experimental Animals

Six-week-old male lean Zucker rat (LZR) and obese Zucker rats (OZR) were purchased from Charles River Laboratories España (Barcelona, Spain). Male OZR are homozygotic for a mutation in the leptin receptor (*fa/fa*) resulting in a phenotype of hyperphagia, obesity, hyperlipidemia, and severe IR. We included LZR as a control group, which have a normal leptin receptor and therefore none of the above-mentioned characteristics of the IR phenotype. The animals were kept at constant temperature, humidity, and at a 12-hour light/dark cycle. The animals had free access to water and standard rat chow (Harlan Interfauna Ibérica, S.L., Barcelona, Spain). All animal studies were approved by and conducted according to the guidelines of the local animal ethics committee (Comitè ètic d'experimentació animal CEEA, Decret 214/97).

### Experimental Design

The LZR were randomly assigned to 2 groups: vehicle (VEH), mTOR-I (sirolimus, SRL, 1.0 mg/kg injected 3 times a week), whereas OZR were randomly assigned to 4 groups: VEH, SRL (0.25, 0.5, and 1.0 mg/kg 3 times a week). The VEH consisted of polysorbate 80 (Sigma-Aldrich, Madrid, Spain), dimethylacetate, and polyethylene glycol 400 (Merck S.A., Madrid, Spain). The experiment lasted 12 days. All animals received a morning intraperitoneal injection of VEH or SRL for 11 days. At baseline (day 0) and days 6, 9, and 12, fasting tail-blood glucose was measured using an automatic glucose monitoring device (Accu-Chek Sensor, Roche). At baseline and at day 12, after a 12-hour fast, animals underwent an intraperitoneal glucose tolerance test (IPGTT) (2 g/kg glucose, and insulin levels were measured at 0, 15, 30, 60, 90, and 120 minutes after glucose injection. Body weight (BW) was recorded daily. To analyze food and water intake, as well as urinary parameters, some animals were housed in metabolic cages separately for 24 hours at days 0 and 11. In another set of rats, we extended SRL exposure until day 28, and then SRL was withdrawn and rats were followed up until day 45. In these rats, IPGTT was performed at 28

and 45 days to study the reversibility of the effects produced by mTOR-I.

Animals were anesthetized at days 12, 28, or 45, serum samples collected, and their pancreas removed for histological, islet isolation, or gene expression analyses. Blood urea nitrogen, creatinine, triglycerides, and cholesterol determinations were performed in the Central Laboratory of the Hospital Clínic de Barcelona.

### Insulin

Serum samples were collected before and 30 minutes after initiation of IPGTT. Total insulin content and insulin secretion were analyzed from pancreas and isolated islets, respectively. Insulin detection was determined with an ultrasensitive rat insulin ELISA kit from Mercodia (Uppsala, Sweden).

### IR and Sensitivity

Insulin resistance was assessed using homeostasis model assessment-IR (HOMA-IR):  $HOMA-IR = \{[fasting\ insulin\ (\mu U/mL) \times fasting\ glucose\ (mmol/L)]/22.5$ . Insulin sensitivity was assessed by the quantitative insulin sensitivity check index:  $quantitative\ insulin\ sensitivity\ check\ index\ (QUICKI) = 1 / \{log[fasting\ insulin\ (\mu g/mL)] + log[fasting\ glucose\ (mg/dL)]\}$ .

### Isolation of Langerhans Islets

Pancreas from 3 rats per group (LZR + VEH, LZR + SRL, OZR + VEH and OZR + SRL 1 mg/kg) were harvested at the end of the protocol and used to perform islet isolation. Rat islets were isolated using digestion with collagenase P (Roche, Basel, Switzerland). Islets were handpicked under a stereomicroscope after separation with a Ficoll density gradient.<sup>15,16</sup> The handpicked islets were seeded in culture wells for insulin secretion experiments, and the rest islets were frozen for gene expression. Each experiment was repeated 4 times.

### Insulin Secretion Analysis

Rat isolated islets were stabilized in Krebs Ringer Buffer with 2.8 mM glucose for 2 hours at 37°C. Groups of 6 islets were then incubated in Krebs Ringer buffer with 2.8 mM or 16.7 mM glucose for 2 hours at 37°C. Each condition was assessed in 5 wells, and the experiment was repeated in 4 independent islet batches. Supernatant fractions were sampled, and insulin release was quantified.

### Immunohistochemistry and Immunofluorescence

At the end of the study, 6 rats per group were sacrificed, and pancreas samples were harvested for inclusion in paraffin. For the morphometric analysis, pancreases were immunostained with a polyclonal guinea pig anti-insulin antibody (Dako, Glostrup, Denmark) previously diluted with antibody diluent and background-reducing components (Dako). Antiguinea pig IgG conjugated with peroxidase (Sigma) was used as the secondary antibody. Detection was performed using DAB system (Dako) according to the manufacturer's instructions. Langerhans islets size was analyzed in all islets from 2 nonconsecutive sections of each pancreas. For each section, both the whole pancreas and the islet area were traced manually.

Proliferation and apoptosis rates were evaluated in 3 nonconsecutive section of each pancreas. Proliferation was assessed by the estimated number of Ki67-positive cells adjusted for the total number of 4',6-diamidino-2-phenylindole (DAPI)-positive cells into Langerhans islet. Antigen retrieval

using citrate buffer (pH = 6.0) for 15 minutes was required before the immunostaining. Monoclonal mouse antirat Ki67 (Dako, Glostrup, Denmark) and polyclonal guinea pig anti-insulin antibody was used as the primary antibody for proliferation and islet localization, respectively. Secondary antibodies used were conjugated with fluorochromes, antiguinea pig with Cyanine2 and antimouse with Alexa-Fluor 555. DAPI was used to visualize nuclei.

$\beta$ -cell apoptosis analysis was performed on pancreas paraffin sections by terminal deoxynucleotidyl transferase-mediated 2'-Deoxyuridine, 5'-Triphosphate nick-end labeling combined with insulin and DAPI stains. All images were acquired using an Olympus BX51 clinical microscope and DP70 digital camera and software (Olympus, Tokyo, Japan).

### RNA Extraction and Quantitative Real-Time PCR

The handpicked islets were kept in batch of 250 to 300 islets to perform gene expression analysis. Isolated islets were rapidly frozen in buffer D containing guanidium thiocyanate. Total RNA was extracted using Trizol from Invitrogen following the manufacturer's instructions. The purity and concentration of RNA was determined by Nano-drop 2000 (Thermo-Fisher, Boston, MA). First-strand complementary DNAs were synthesized from total RNA using High Capacity complementary DNA Reverse Transcription Kit (Applied Biosystems). Quantitative real-time PCR was performed with 7900HT Fast Real-Time PCR System (Applied Biosystems). Insulin genes 1 and 2 were analyzed using our primers and probes (Table S1, SDC, <http://links.lww.com/TXD/A22>), whereas all genes were analyzed using TaqMan Gene Expression Assays (Table S2, SDC, <http://links.lww.com/TXD/A22>). Relative changes in gene expression were calculated with the following formula: fold change =  $2^{-(\Delta\Delta C_t)}$ , where  $\Delta\Delta C_t = \Delta C_t$  treated samples -  $\Delta C_t$  control samples, and  $\Delta C_t$  values =  $C_t$  target gene -  $C_t$  reference gene.

### Statistical Analysis

Statistical analysis was performed using the SPSS 14.0 statistics package. Values are given as mean  $\pm$  standard deviation. The Kruskal-Wallis or Mann-Whitney *U* tests were used where applicable.

## RESULTS

### Effect of mTOR-I on Glucose Metabolism

#### Baseline Characteristics

The OZR had higher BW, triglycerides, and cholesterol levels and lower BUN levels than LZR (Table 1). Insulin levels were higher, whereas fasting glucose levels were similar (Table 1). In the IPGTT assay, OZR showed a lower area under the curve (AUC) than LZR. Insulin sensitivity (QUICKI) was lower and IR (HOMA-IR) higher in OZR than LZR (Table 1).

#### General Characteristics After 12 Days

The LZR and OZR enrolled in VEH groups increased BW after 12 days. The SRL treatment reduced the increase of BW in both LZR and OZR in a dose-dependent manner (Table 2). Triglyceride levels were increased after SRL treatment, but only in the OZR group (Table 2). The SRL treatment did not change hepatic function analyzed by ALT and AST levels (data not shown). At the same sirolimus dose,

**TABLE 1.**

### Comparison of lean and obese Zucker rats at baseline

	Lean Zucker Rats	Obese Zucker Rats	P
N	40	52	
Weight (g)	146.8 $\pm$ 16.3	181.7 $\pm$ 10.9	<sup>a</sup>
Biochemical			
BUN, mg/dL	15.42 $\pm$ 1.56	26.2 $\pm$ 3.43	<sup>a</sup>
Creatinine, mg/dL	0.27 $\pm$ 0.03	0.26 $\pm$ 0.05	ns
Triglycerides, mg/dL	46.8 $\pm$ 11.1	205.7 $\pm$ 47.3	<sup>a</sup>
Cholesterol, mg/dL	103.2 $\pm$ 7.8	139.1 $\pm$ 29.6	<sup>a</sup>
IPGTT			
Glycemia, mg/dL	N = 14	N = 28	
Baseline	84.3 $\pm$ 12.1	87.7 $\pm$ 19.0	ns
30 min	279.1 $\pm$ 48.7	204.0 $\pm$ 62.4	<sup>b</sup>
60 min	241.4 $\pm$ 51.0	138.0 $\pm$ 57.2	<sup>c</sup>
120 min	109.7 $\pm$ 21.3	74.25 $\pm$ 15.0	<sup>a</sup>
Insulin, $\mu$ U/mL			
Baseline	0.08 $\pm$ 0.05	3.21 $\pm$ 1.55	<sup>a</sup>
30 min	0.47 $\pm$ 0.44 <sup>d</sup>	9.62 $\pm$ 2.64 <sup>d</sup>	<sup>a</sup>
AUC	1749 $\pm$ 608	1511 $\pm$ 256	ns
Indexes			
HOMA-IR	0.01 $\pm$ 0.01	0.63 $\pm$ 0.29	<sup>a</sup>
QUICKI	1.86 $\pm$ 0.81	0.42 $\pm$ 0.03	<sup>a</sup>

<sup>a</sup> *P* < 0.001 (significantly different when compared to Lean Zucker rat group).

<sup>b</sup> *P* < 0.05 (significantly different when compared with Lean Zucker rat group).

<sup>c</sup> *P* < 0.01 (significantly different when compared to Lean Zucker rat group).

<sup>d</sup> *P* < 0.05 (significantly different when compared to baseline).

obese rats had higher sirolimus blood levels than lean rats (20.3  $\pm$  3.1 and 12.1  $\pm$  1.8 ng/mL, respectively) (Figure S1, SDC, <http://links.lww.com/TXD/A22>).

#### IPGTT After 11 Days of Treatment (Day 12)

Fasting glucose levels were increased only in OZR under high-dose SRL (1.0 mg/kg) treatment. The OZR (54.5%) after SRL (1.0) treatment were considered diabetic rats, because 120-minute glucose in the IPGTT was higher than 200 mg/dL (Table S3, SDC, <http://links.lww.com/TXD/A22>). Fasting insulin levels were 10 times higher in obese rats compared with lean rats. The SRL treatment did not modify fasting insulin levels in lean rats. However, obese rats treated with SRL at any dose showed higher insulin levels than the rats in the VEH group. Intraperitoneal glucose injection induced an increase of insulin levels after 30 minutes in baseline and VEH-treated groups from lean and obese Zucker rats and SRL-treated lean rats. Insulin levels of obese rats treated with SRL—already at a high baseline level—did not further increase after 30 minutes of glucose injection (Table 2).

The OZR treated with SRL had higher IR and lower insulin sensitivity than VEH-treated obese rats, whereas SRL treatment did not affect IR and insulin sensitivity on LZR (Table 2).

#### Effects of mTOR-I on Islet Histomorphometry, Proliferation, and Apoptosis

Macroscopically, SRL treatment significantly reduced the pancreas weight in both LZR and OZR (Figure 1A). Obese Zucker rats had larger islets of Langerhans than lean rats. The SRL treatment reduced islet size in both lean and obese rats (Figure 1B).

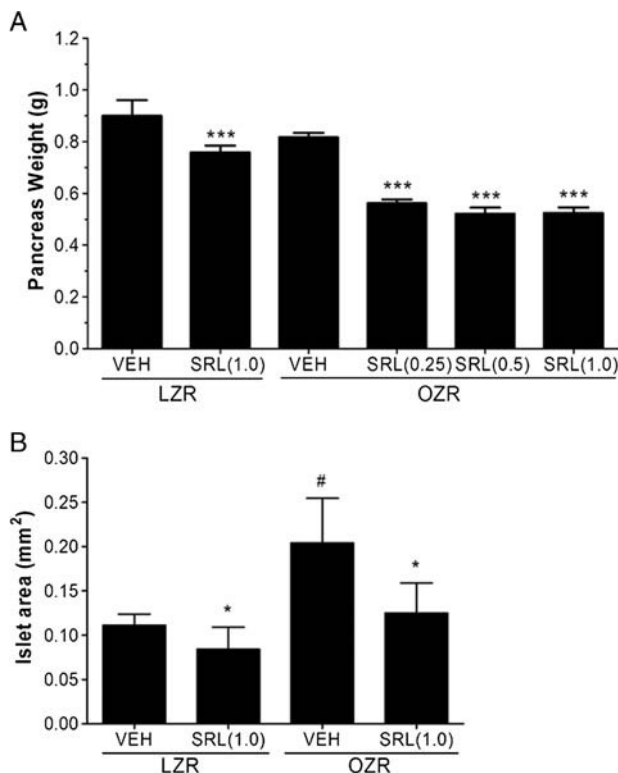
**TABLE 2.****Biochemical data and IPGTT results at day 12 in the lean and obese Zucker rats**

	Lean Zucker Rats		Obese Zucker Rats			
	VEH	SRL 1.0	VEH	SRL 0.25	SRL 0.5	SRL 1.0
N	17	19	14	10	10	16
Weight (g)	200.3 ± 22.4	188.6 ± 25.1 <sup>a</sup>	270 ± 41.3	219.4 ± 9.9 <sup>b</sup>	199.2 ± 23.1 <sup>b</sup>	187.5 ± 16.7 <sup>b</sup>
Biochemical						
BUN, mg/dL	16.00 ± 4.06	17.3 ± 2.79	29.87 ± 5.46	29.1 ± 5.95	33.1 ± 8.03	31.2 ± 8.72
Creatinine, mg/dL	0.35 ± 0.07	0.33 ± 0.07	0.53 ± 0.19	0.52 ± 0.07	0.61 ± 0.08	0.51 ± 0.15
Triglycerides, mg/dL	60.2 ± 16.2	48.6 ± 5.0	185.9 ± 79.4	281.6 ± 96.4 <sup>b</sup>	276.8 ± 122.1 <sup>b</sup>	260.1 ± 103.8 <sup>b</sup>
Cholesterol, mg/dL	93.0 ± 12.2	110.0 ± 16.3	120.7 ± 21.4	123.5 ± 18.1	122.5 ± 17.7	114.0 ± 19.5
IPGTT	N = 9	N = 10	N = 9	N = 8	N = 7	N = 10
Glycemia—baseline, mg/dL	93.0 ± 17.7	96.2 ± 18.5	88 ± 3.4	73 ± 16.3	93 ± 23.0	131 ± 23.6 <sup>b</sup>
30 min	280.7 ± 95.4 <sup>c</sup>	270.3 ± 40.5 <sup>c</sup>	226 ± 40.4	150 ± 70.9	135 ± 45.9	214 ± 68.2
60 min	197.9 ± 88.6 <sup>c</sup>	231.0 ± 80.7 <sup>b, c</sup>	143 ± 32.6	123 ± 57.1	136 ± 31.7	218 ± 69.4 <sup>b</sup>
120 min	102.8 ± 16.5	154.2 ± 31.1 <sup>a</sup>	79 ± 14	123 ± 68.3 <sup>a</sup>	121 ± 44.5 <sup>a</sup>	191 ± 76.9 <sup>b</sup>
Insulin (μU/mL) - Baseline	0.25 ± 0.20	0.26 ± 0.21	2.83 ± 1.53	8.68 ± 2.24 <sup>b</sup>	9.45 ± 3.62 <sup>b</sup>	7.05 ± 3.61 <sup>b</sup>
30 min	1.20 ± 0.89 <sup>c</sup>	1.34 ± 0.98 <sup>c</sup>	9.84 ± 3.02 <sup>c</sup>	4.45 ± 4.58	1.36 ± 1.92 <sup>c</sup>	4.03 ± 5.05
AUC	1950 ± 509	2165 ± 341	1552 ± 239	1841 ± 429	1961 ± 1281	2057 ± 485 <sup>b</sup>
Indexes						
HOMA-IR	0.06 ± 0.05	0.06 ± 0.07	0.63 ± 0.36	1.58 ± 0.50 <sup>b</sup>	2.17 ± 0.99 <sup>b</sup>	1.86 ± 1.00 <sup>b</sup>
QUICKI	1.22 ± 0.81	0.95 ± 0.41	0.43 ± 0.04	0.36 ± 0.02 <sup>b</sup>	0.35 ± 0.04 <sup>b</sup>	0.36 ± 0.04 <sup>b</sup>

<sup>a</sup>  $P < 0.001$  (significantly different when compared with VEH group).<sup>b</sup>  $P < 0.05$  (significantly different when compared with VEH group).<sup>c</sup>  $P < 0.05$  (significantly different when compared with baseline).

Proliferation analysis was performed by Ki67 stain; SRL treatment reduced proliferation in the islets of Langerhans in OZR (Figure 2A). There were no differences in the

apoptosis analysis, which was performed by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling stain (Figure 2B).



**FIGURE 1.** Effect of mTOR-I on pancreas and islet size. A, Pancreas weight (n = 15 per group). B, Quantification of islet area (n = 6 rats per group). \*Significantly different when compared with VEH treated group ( $^*P < 0.05$ ;  $^{***}P < 0.001$ ). #Significantly different when compared with LZR group ( $^{\#}P < 0.05$ ).

### Effects of mTOR-I on Insulin Content and Secretion

Insulin content was analyzed from a complete pancreas; SRL-treated rats showed lower numerical levels of insulin than VEH-treated rats in both LZR and OZR; however, this reached statistical significance only in OZR on SRL treatment (Figure 3A).

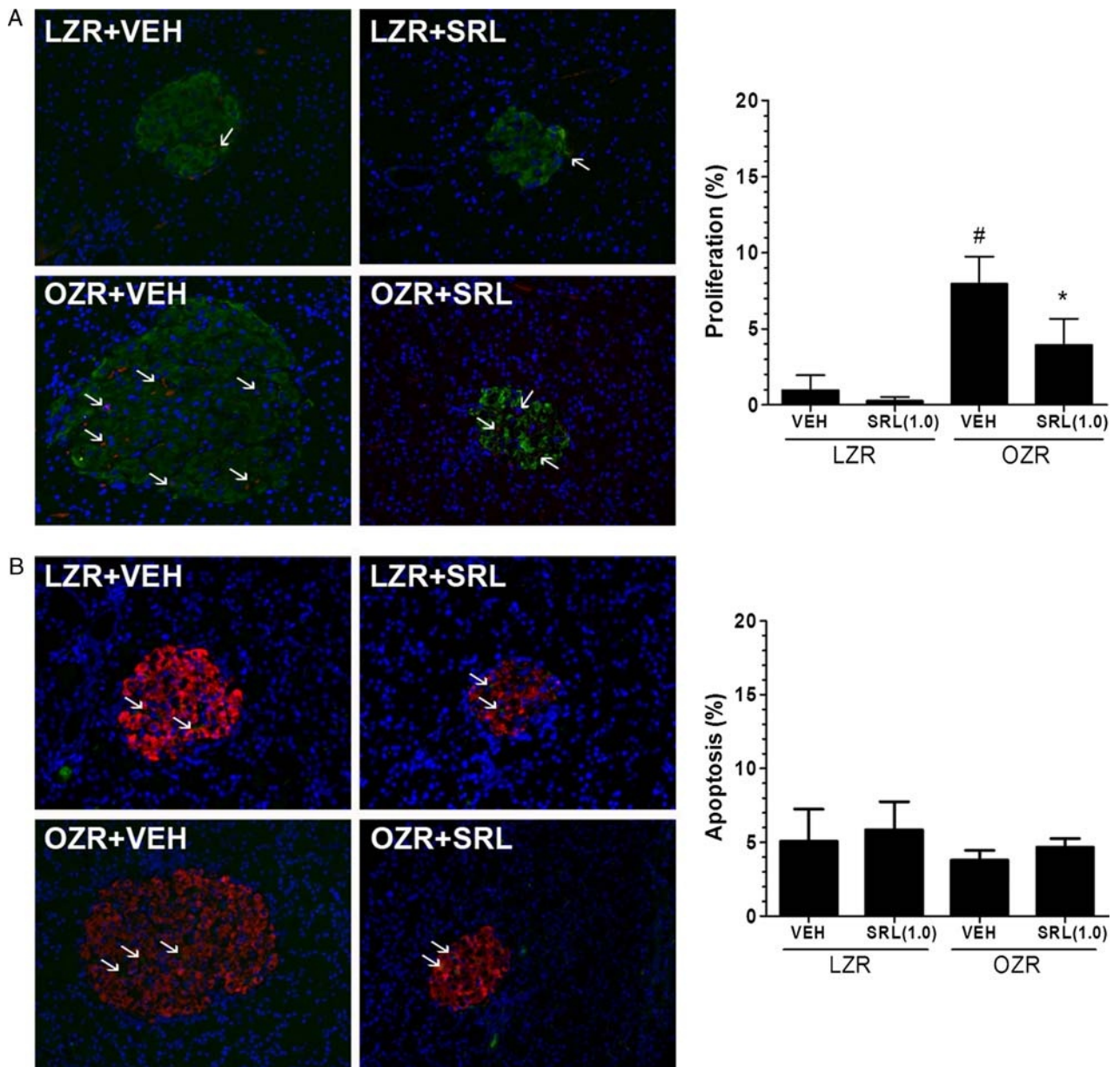
Insulin secretion was performed ex vivo using isolated islets. All islets, even islets from SRL treated rats, released insulin after the incubation time (2 hours) with high concentration of glucose (16.7 mmol/L). The insulin release was statistically lower in islets from OZR treated with SRL compared with VEH group ( $0.29 \pm 0.1$  vs  $1.24 \pm 0.3$  ng/mL), whereas islets from LZR did not show changes in insulin release depending on treatment (VEH vs SRL;  $0.67 \pm 0.2$  vs  $0.73 \pm 0.2$  ng/ml) (Figure 3B). However, insulin content per islet revealed huge differences between both VEH- and SRL-treated groups (Figure 3C).

### Effects of mTOR-I on Islet Gene Expression

In isolated purified islets, we analyzed the expression of several transcription factor genes (Figure 4A: *Pdx1*, *Neurod1*, and *Pax4*), genes related with insulin processing (Figure 4B: *Cpe*, *Pcsk1*, and *Pcsk2*), genes related with insulin signalling (Figure 4C: *Insr*, *Irs1*, and *Irs2*), genes related with insulin secretion (Figure 4D: *Abcc8*, *Kcnj11*, and *Cacna1c*), genes related with glucose signalling (Figure 4E: *Glut2* and *Gck*), islet hormone genes (Figure 4E: *Ins1*, *Ins2*, *Gcg*, and *Smst*).

The first approach was to compare the gene expression of LZR + VEH and OZR + VEH groups. The OZR + VEH group showed an upregulation of *Pcsk1* and *Ins1* (Figures 4B,





**FIGURE 2.** Effect of mTOR-I on proliferation and apoptosis. A, Proliferation by Ki67 immunohistochemistry. A representative islet from each group has been shown; Insulin (green), Ki67 (red) and DAPI-nuclei (blue). White arrows indicate cells in proliferation. Quantification of percentage of Ki67 insulin-positive nuclei within insulin-positive cells (n = 6 rats per group). B, Apoptosis by in situ TUNEL assay. A representative islet from each group has been shown: insulin (red), TUNEL (green), and DAPI-nuclei (blue). White arrows indicate apoptotic cells. Quantification of percentage of TUNEL insulin-positive nuclei within insulin-positive cells (n = 6 rats per group). \*Significantly different when compared with VEH-treated group ( $*P < 0.05$ ). #Significantly different when compared with LZR group ( $\#P < 0.05$ ). TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

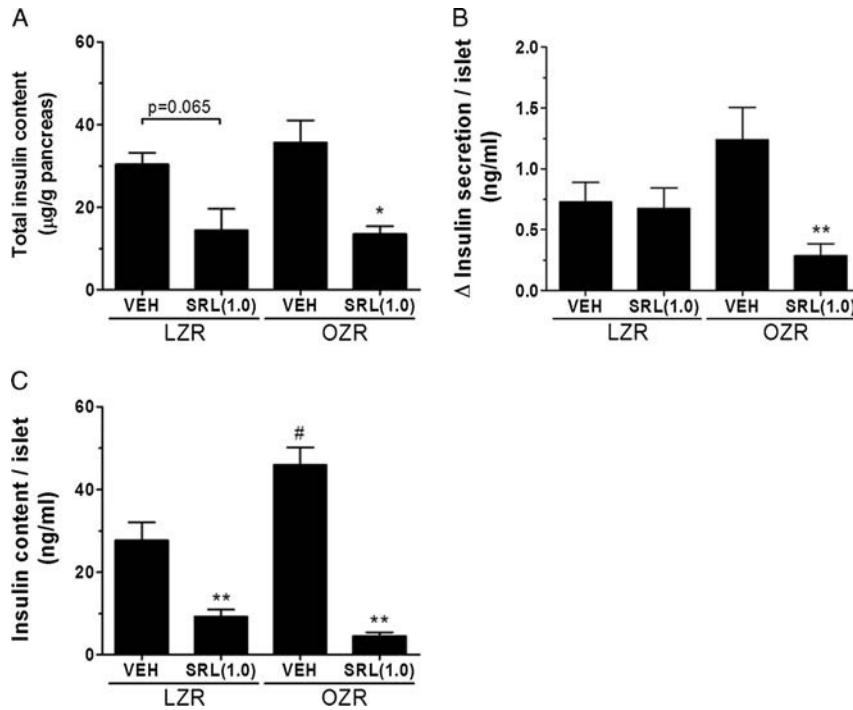
left and 4F, left), whereas *Gcg* and *Smst* were downregulated (Figure 4F, left). Second, the impact of SRL was investigated. The SRL treatment upregulated *Pdx1* expression, these results were observed only in the OZR group (Figure 4A, right). None of the genes related with insulin processing showed differences between VEH and SRL treatments (Figure 4B, right).

The OZR treated with SRL showed increased *Insr* expression, whereas SRL treatment increased *Irs2* expression in both LZR and OZR rats (Figure 4C, right). The SRL treatment upregulated *Abcc8* expression in OZR rats (Figure 4D, right). *Glut2* was overexpressed in OZR rats after SRL treatment (Figure 4E, right).

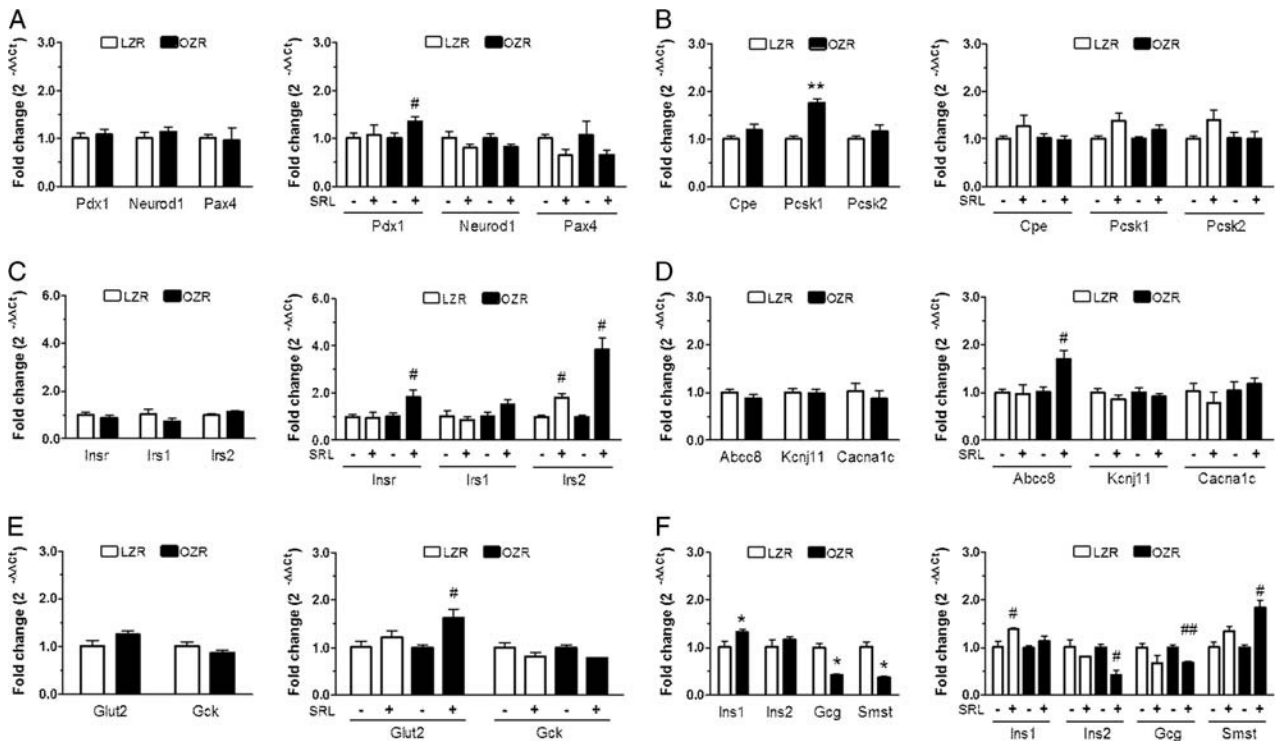
The analysis of islet hormone genes showed that SRL increased *Ins1* expression in LZR, whereas *Ins2* and *Gcg* expressions were downregulated, and *Smst* was upregulated in OZR under SRL treatment (Figure 4F, right).

#### Effect of mTOR-I Withdrawal on Glucose Metabolism

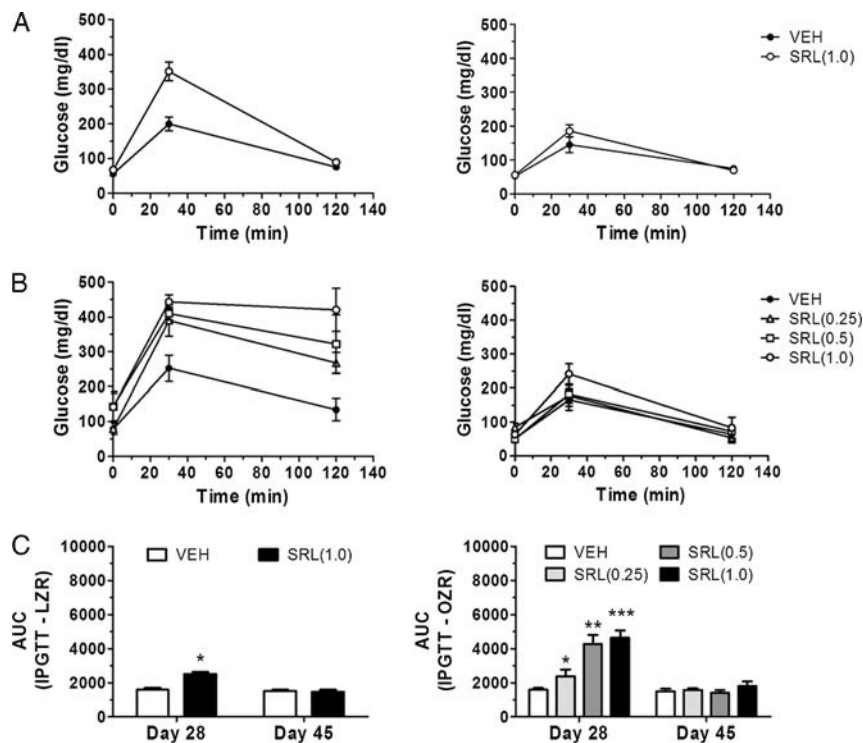
Only OZR rats treated with SRL presented a worse IPGTT, because these animals had problems to reduce the glucose injected at day 12, showing a higher AUC (Table 2). The prolongation of the treatment until day 28 induced an increase of the AUC of IPGTT in both LZR and OZR (Figure 5); however, all LZR normalized glucose levels after 2 hours, whereas OZR treated with SRL did not normalize these



**FIGURE 3.** Effect of mTOR-I on insulin content and insulin secretion. A, Total insulin content from complete pancreas samples (n = 9 rats per group). B, Insulin secretion performed ex vivo using isolated islets. C, Insulin content from isolated islets. Four independent experiments using pooled handpicked islets from 3 rats per group have been performed. \*Significantly different when compared with VEH-treated group (\**P* < 0.05; \*\**P* < 0.01). #Significantly different when compared with LZR group.



**FIGURE 4.** Effect of mTOR-I on gene expression. A, Transcription factors genes: *Pdx1*, *Neurod1*, and *Pax4*. B, Insulin processing genes: *Cpe*, *Pcsk1*, and *Pcsk2*. C, Insulin signalling genes: *Insr*, *Irs1*, and *Irs2*. D, Insulin secretion genes: *Abcc8*, *Kcnj11*, and *Cacna1c*. E, Glucose signalling genes: *Glut2* and *Gck*. F, Hormones genes: *Ins1*, *Ins2*, *Gcg*, and *Smst*. All left plots show gene expression of LZR + VEH and OZR + VEH at day 12, whereas right plots show the impact of mTOR-I (SRL at 1 mg/kg) versus VEH treatment on lean and obese rats. Four independent islet pellets (from 3 rats each one) have been used to perform gene expression analysis. TATA box binding protein (*Tbp*) was used as housekeeping gene in plots A to D, whereas the reference gene in plots E and F was Eukaryotic 18S rRNA (*18S*). \*Significantly different when compared to LZR group. #Significantly different when compared to VEH-treated group.



**FIGURE 5.** Effect of mTOR-I withdrawal on glucose metabolism. A, IPGTT performed on LZR treated with VEH or sirolimus (SRL, 1.0 mg/kg) at day 28 (left) and 17 days after SRL withdrawal, day 45 (right) ( $n = 5$  rats per group). B, IPGTT performed on OZR treated with VEH or SRL (0.25, 0.5, or 1.0 mg/kg) at day 28 (left) and 17 days after SRL withdrawal at day 45 (right) ( $n = 5$  rats per group). C, AUC of IPGTT assay performed on LZR (left) and OZR (right). \*Significantly different when compared to VEH treated group ( $*P < 0.05$ ;  $**P < 0.01$ ;  $***P < 0.001$ ).

levels (Figure 5). Obese rats treated with SRL, at any dose, revealed higher insulin blood levels at baseline. Additionally, these rats did not respond to IPGTT, having equal insulin blood levels after 30 minutes of glucose administration (Figure S2A, SDC, <http://links.lww.com/TXD/A22>).

The LZR and OZR recovered a normal IPGTT after the withdrawal of SRL (Figure 5). At day 45, all lean and obese rats responded to glucose administration increasing insulin blood levels after 30 minutes (Figure S2B, SDC, <http://links.lww.com/TXD/A22>). The SRL treatment induced diabetes in a dose-dependent manner only in obese Zucker rats after 28 days, whereas the SRL withdrawal reverses all diabetic conditions (Table S4, SDC, <http://links.lww.com/TXD/A22>).

## DISCUSSION

The  $\beta$ -cell compensation characterized by  $\beta$ -cell mass expansion in response to peripheral IR is a well-known mechanism during nutrition overload or type 2 diabetes. During  $\beta$ -cell compensation Akt and mTOR/S6K1 signalling present a persistent activation.<sup>17,18</sup> Therefore, theoretically, mTOR inhibition should minimize these effects of the metabolic syndrome.<sup>18,19</sup> Chronic mTOR inhibition seems to decrease adiposity and to protect against diet-induced obesity,<sup>20,21</sup> while promoting hyperglycemia and hyperinsulinemia, together with a downregulation of muscle glucose transporters (GluT1 and GluT4) that produce a strong muscle IR.<sup>21</sup> Considering  $\beta$ -cell compensation, mTOR-Is exacerbate IR and reduce  $\beta$ -cell function.<sup>22</sup> Recently, Barlow et al<sup>23</sup> concluded that rapamycin had profound effects on glucose homeostasis.

The mTOR inhibition has been associated with a higher incidence of PTDM in kidney allograft recipients.<sup>12,13</sup> There

are some hints that the pathophysiology might be different from that evidenced in tacrolimus-associated PTDM.<sup>24</sup> In the present study, we analyzed whether a pre-established IR potentiates the diabetogenic effect of mTOR-Is. The main findings of our study are that mTOR inhibition has a double effect in an environment of IR: it not only further increases IR, but additionally leads to an inhibition of an adaptive  $\beta$ -cell response. Interestingly, OZR treated with SRL at 0.25 mg/kg reached the same SRL trough levels than LZR treated at 1 mg/kg. However, equal SRL trough levels generate a complete different response between OZR and LZR; OZR showed a pathological profile, whereas LZR group does not modify glucose metabolism. It is remarkable that mTOR inhibition in an environment of normal insulin sensitivity did not cause diabetes in rats. However, the picture changed in an environment of preestablished IR in a dose-dependent manner. About 54.5% of obese rats with high-dose SRL treatment had to be classified as diabetic according to the IPGTT.

To maintain normal glucose levels in conditions of IR,  $\beta$  cells have to increase their mass and capacity of insulin production and secretion leading to a  $\beta$ -cell hyperplasia as can be seen in VEH-treated OZR, also described in previous studies.<sup>25</sup> Apparently, mTOR inhibition attenuates this compensatory response as well. We observed that mTOR inhibition increased insulin blood levels, which are already high in these rats, at the expenses of total insulin content into the Langerhans islets. The mTOR inhibition induced an anti-proliferative effect on the growth of the pancreas in OZR as evidenced by a smaller islet size and pancreas mass. Our results are in line with Zahr et al,<sup>26</sup> which have demonstrated that in a pregnancy mice model, rapamycin significantly



reduces  $\beta$ -cell proliferation. Moreover, at a histological level, we could observe that mTOR inhibition blocked the hyperplastic response to IR in OZR without changing the number of islets and without an increase of apoptosis.

Additionally, the peak insulin secretion 30 minutes after a glucose challenge seems to be inhibited. Apparently, the already high level of insulin secretion evidenced in the OZR under baseline conditions cannot be increased further, not even with a glucose challenge, such as an IPGTT. A similar phenomenon has been evidenced by Rodríguez-Rodríguez and colleagues<sup>11</sup> who named it “ $\beta$ -cell exhaustion” in a study of tacrolimus in LZR and OZR.

In gene expression analysis of isolated islets, it could be shown that SRL treatment downregulated insulin production by lower *Ins2* gene expression. Moreover, islets should be more sensitive to glucose and insulin because *Glut2*, *Insr*, and *Irs2* were upregulated. In the pancreatic  $\beta$  cell, several components of mTOR signalling are crucial. The mTOR/S6K1 signalling in  $\beta$  cell is involved in regulating  $\beta$ -cell size and inhibits insulin/insulin-like growth factor-1.

Insulin receptor substrate 2 (IRS2) is the most abundant and functionally important IRS family member, which promotes  $\beta$ -cell growth, proliferation, and survival.<sup>27,28</sup> Previous observations demonstrated that mTOR activation promotes proteasomal degradation of IRS2-inducing  $\beta$ -cell apoptosis. The mTOR blockade with rapamycin treatment prevents apoptosis.<sup>28</sup> Additionally, we observed a higher expression of *Pdx1* on islets from rats treated with SRL, upregulation of this transcription factor has been related with a reduction of  $\beta$ -cell apoptosis.<sup>29</sup>

Interestingly, in our ex vivo experiments, isolated islets from rats treated with mTOR-Is could respond to glucose at the same level as islets from nontreated rats, indicating that the effects produced by mTOR inhibition are transient. Importantly, the withdrawal of SRL of 14 days led to a complete recovery of these alterations even in rats treated with higher doses.

Insulin resistance is the main feature of the metabolic syndrome.<sup>30</sup> Most probably, many patients on the waiting list for a kidney transplant have IR.<sup>31</sup> Moreover, IR is considered to be one of the risk factors for developing new-onset diabetes after transplantation, but insulin indices calculated pretransplantation do not predict PTDM.<sup>32</sup> Insulin resistance is a condition in which the metabolism fails to respond adequately to a given amount of insulin. The causes of IR are multifactorial. Obesity, diet, sedentary life style, genetic predisposition, certain medications, such as steroids, protease inhibitors, and diseases, such as hepatitis C, have been identified as causes. A higher than normal insulin secretion can be considered as a normal physiological response to IR. This can be observed in many patients with metabolic syndrome. Only when finally despite of an initially increased insulin response this secretion gets insufficient to maintain normal glucose blood concentrations hyperglycemia in insulin-resistant patients becomes manifest as diabetes mellitus type 2. In our study, mTOR inhibition contributes as an additional factor to IR. Indicators of  $\beta$ -cell dysfunction, rather than IR, may be predictors for the development of PTDM.<sup>33</sup>

The possible limitation of this study is that data obtained from animal models quite often cannot be translated into human pathophysiology. Moreover, in kidney transplant patients, the situation is much more complex than the

metabolic conditions in our animal model. Quite often, transplant patients already have a certain age, which already is a risk factor by itself for developing diabetes or IR. Moreover, patients are submitted to multiple simultaneous pharmacological treatments, and several of these treatments might alter the glucose metabolism.

Nevertheless, our observations are reflected in observations from clinical trials. Recently, we have presented data from a randomized clinical trial of early transition from tacrolimus-based immunosuppression to sirolimus-based immunosuppression 3 months after kidney transplantation. Surprisingly, we could observe that despite withdrawal of tacrolimus, more patients in the sirolimus group developed abnormalities of the glucose metabolism, indicating that sirolimus must have an additional effect on glucose metabolism beyond the known effect on  $\beta$ -cell proliferation associated with tacrolimus. This could serve as a clinical correlate to our findings in rats.<sup>34</sup>

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