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# Inhibition of the mTOR pathway: A new mechanism of β cell toxicity induced by Tacrolimus

**Ana Elena Rodriguez-Rodriguez[1,2] | Javier Donate-Correa[1] | Jordi Rovira[3,4] | Germán Cuesto[5] | Diego Luis-Ravelo[5] | Miguel X. Fernandes[6,7] | Abraham Acevedo-Arozarena[1,7] | Fritz Diekmann[3,4,8] | Angel Acebes[5] | Armando Torres[9,10] | Esteban Porrini[11]**

[1]Research Unit, Hospital Universitario de Canarias, Tenerife, Spain

[2]Universidad de la Laguna, Tenerife, Spain

[3]Laboratori Experimental de Nefrologia i Trasplantament (LENIT), IDIBAPS, Barcelona, Spain

[4]Red de Investigación Renal (REDinREN), Madrid, Spain

[5]Department of Basic Medical Sciences, Medical School, Institute of Biomedical Technologies (ITB), Universidad de la Laguna, Tenerife, Spain

[6]Instituto Universitario de Bio-Orgánica "Antonio González" (IUBO-AG) and Centre for Biomedical Research of the Canary Islands (CIBICAN), Universidad de la Laguna, Tenerife, Spain

[7]Fundación Canaria de Investigación Sanitaria (FUNCANIS) and Instituto de Tecnologías Biomédicas (ITB), La Laguna, Tenerife, Spain

[8]Department of Nephrology and Renal Transplantation, ICNU, Hospital Clinic, Barcelona, Spain

[9]Nephrology Department, Hospital Universitario de Canarias, Tenerife, Spain

[10]Internal Medicine Department, Instituto de Tecnologías Biomédicas (ITB), Universidad de la Laguna, Tenerife, Spain

[11]Hospital Universitario de Canarias, Instituto de Tecnologías Biomédicas (ITB), University of La Laguna, Tenerife, Spain

**Correspondence**
Esteban Porrini
Email: estebanporrini72@hotmail.com
Armando Torres
Email: atorresram@gmail.com
Angel Acebes
Email: aacebesv@ull.es

The mechanisms of tacrolimus-induced β cell toxicity are unknown. Tacrolimus (TAC) and Rapamycin (Rapa) both bind to FK506-binding protein 12 (FKBP12). Also, both molecular structures are similar. Because of this similarity, we hypothesized that TAC can also inhibit the mTOR signalling, constituting a possible mechanism of β cell toxicity. Thus, we studied the effect of TAC and Rapa over the mTOR pathway, v-maf musculoaponeurotic fibrosarcoma oncogene homolog A (MafA), and insulin secretion and content in INS-1 β cells treated with or without glucose and palmitate and in islets from lean or obese rats. TAC and Rapa inhibited the mTOR pathway as reflected by lower levels of phospho-mTOR, phospo-p70S6K, and phospo-S6. The effect of Rapa was larger than TAC. Both drugs reduced the levels of MafA, insulin secretion, and content although these effects were larger with TAC. The changes on MafA and insulin metabolism were observed in cells on glucose and palmitate, in obese animals, and were absent in cells on maintenance medium or in lean animals. In silico docking and

**Abbreviations:** C, control; CNI, calcineurin inhibitor; CsA, cyclosporin A; DAPI, 4′, 6-diamidino-2-phenylindole; FKBP12, FK506-binding protein 12; FoxO1, forkhead box protein O1; FRAP, FKBP12-rapamycin binding (FRB) domain of FKBP12-rapamycin associated protein; G, glucose; GSIS, glucose-stimulated insulin secretion; IPGTT, intraperitoneal glucose tolerance test; IR, insulin resistance; KRBB, Krebs-Ringer bicarbonate buffer; LZR, lean Zucker rat; MafAv-maf, musculoaponeurotic fibrosarcoma oncogene homolog A; mTOR, mammalian target of rapamycin; NFAT2, nuclear factor of activated T cells; OZR, obese Zucker rat; P, palmitate; p70S6K, p 70 ribosomal protein S6 kinase; PBS, phosphate-buffered saline; PTDM, posttransplant diabetes mellitus; RyR2, calcium channel ryanodine receptor 2; S6, ribosomal protein S6; SRL, rapamycin; T2DM, type 2 posttransplant diabetes mellitus; TAC, Tacrolimus-FK506; TGF-β, RI transforming growth factor β (TGF-β) type I receptor.

Acebes, Torres, and Porrini contributed equally to this article.

immunoprecipitation experiments confirmed that TAC can form a stable noncovalent interaction with FKBP12-mTOR. Thus, the mTOR inhibition may be a mechanism contributing to the diabetogenic effect of TAC.

**KEYWORDS**
animal models, basic (laboratory) research/science, cellular biology, diabetes, immunosuppressant - calcineurin inhibitor, immunosuppressant - mechanistic target of rapamycin (mTOR), kidney transplantation/nephrology, molecular biology, molecular biology, murine, new onset/posttransplant, tacrolimus

## 1 | INTRODUCTION

In renal transplantation, the use of Tacrolimus (TAC) is a major risk factor for post-transplant diabetes mellitus (PTDM).[1] However, the mechanisms involved in tacrolimus-induced diabetes are unknown. Deciphering the diabetogenic effect could have important repercussions both in the prevention and treatment of PTDM.

Our group showed in previous studies that the toxicity of TAC depends on the pre-existence of β cell dysfunction related to insulin resistance (IR).[2-4] In fact, TAC potentiated the same changes in β cells already induced by IR including alterations in transcription factors crucial for the proliferation and insulin production (such as forkhead box protein O1 [FoxO1] and v-maf musculoaponeurotic fibrosarcoma oncogene homolog A [MafA]), and reductions in insulin content and secretion.[5] Interestingly, in the absence of IR, TAC did not induce these changes. Moreover, at least in this setting, the increased diabetogenic effect of TAC seemed not to be related to calcineurin and NFAT inhibition.[5]

FK506-binding protein 12 (FKBP12), the intracellular binding immunophilin of TAC, might mediate toxic effects, including diabetogenicity. This protein, which is found in higher concentrations in the β cells than in any other cell type of the pancreatic islet,[6] exerts important functions including: regulation of the transforming growth factor β (TGF-β) type I receptor (TGF-β RI),[7] prevention of uncontrolled leakage of $Ca^{2+}$ from the endoplasmic reticulum by stabilizing calcium channel ryanodine receptor 2 (RyR2),[8] and interaction with the mammalian target of rapamycin (mTOR) pathway. mTOR is a serine/threonine kinase that acts as a master regulator of the cellular metabolic status, integrating signals from nutrients and growth factors,[9] regulating the metabolism of lipids, amino acids and glucose, and therefore controlling growth. The inhibition of mTOR has been related to the appearing of PTDM both in humans[10] and in animal models.[11] The main inhibitor of this pathway is the macrolide compound Rapamycin (Rapa), which complexes with FKBP12 to inhibit the mTOR pathway.[12] TAC and Rapa are structurally similar and both bind to FKBP12. Thus, we hypothesize that the diabetogenic effect of TAC could be explained, at least in part, by the inhibition of the mTOR pathway.

To determine possible effects of TAC on the mTOR pathway we evaluated: (A) the variations in mTOR and downstream effectors of this pathway (p70 S6 kinase [p70S6K] and ribosomal protein S6), (B) the changes in nuclear levels of MafA, (C) insulin content and secretion in β cells (INS-1 cell line) treated with TAC, cyclosporin-A (CsA) and Rapa in normal and glucolipotoxic conditions, and (D) the variations in the phosphorylated form of one of effectors (S6) and nuclear levels of MafA in pancreas of insulin-resistant obese Zucker rats (OZRs) and insulin-sensitive lean Zucker rats (LZRs) on TAC, CsA, and Rapa.

## 2 | MATERIALS AND METHODS

### 2.1 | Materials

CsA and FK506 (tacrolimus-TAC) were purchased from Sigma Aldrich (St. Louis, MO) and Rapa was from LC Laboratories (Woburn, MA). Rabbit antibodies against p70S6K and S6 (phosphorylated and total) and phosphorylated mTOR and Raptor were from Cell Signalling Technology (Danvers, MA). Mouse antibody against FKBP12 was from Abcam (Cambridge, UK). The antibodies against MafA, β-actin, anti-rabbit horseradish peroxidase-linked and anti-rabbit or anti-mouse Alexa Fluor secondary antibodies were from Abcam (Cambridge, UK).

### 2.2 | Experimental design

#### 2.2.1 | β cell in culture

*Cell line maintenance*

Rat insulinoma INS-1 β cells were cultured in RPMI-1640 medium as previously described.[5]

*Glucolipotoxicity*

Cells were cultured in dishes of 60 mm for 5 days with glucose (22 mmol/L), palmitate (100 μmol/L), or both as previously described.[5]

*Dose of Immunosuppressors*

CsA (250 ng/mL), TAC (15 ng/mL) or Rapa (5 nmol/L) were added for 48 hours to cells previously incubated on standard medium for 72 hours (Figure S1A). The doses of CsA and TAC were the same

used in previous experiments.[3,5] The dose of Rapa (5 nmol/L) was selected based on previous studies observing that such levels inhibited the mTOR pathway without inducing apoptosis.[13,14] Additionally, we performed a sensitivity analysis to test the effect on the mTOR pathway on different doses of Rapa (0.1, 0.3, 0.6, 1.25, 2.5, 5, 10, 30, 50, and 100 nmol/L) alone or combined with diverse doses of TAC (2.5, 5, 10, 15, and 30 ng/mL).

*Interaction between immunosuppressors and glucolipotoxicity*

Cells were treated for 5 days with glucose (22 mmol/L) and/or palmitate (100 μmol/L), and TAC, CsA, or Rapa was added the last 2 days.

*Western blot analysis*

Both phosphorylated and total levels of p70S6K and S6 were determined on total cell extracts containing 20 μg of protein subjected to SDS-PAGE following standard procedures.

*Immunofluorescence analysis*

In dishes of 60 mm, $1 \times 10^4$ cells were seeded on sterilized 12-mm-diameter coverslips. After 5 days of treatments, cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and blocked with 3% bovine serum albumin. Then, cells were incubated at 4°C overnight with the corresponding primary antibody (phospho-S6 [1:500], MafA [1:250], or phospho-mTOR [1:500]), washed and incubated again with the secondary antibody. Proliferation experiments were done using Ki67 (1:500), whereas apoptosis was tested using a cleave caspase-3 (1:250) as primary antibodies. Images were taken with a Leica confocal microscope (Leica, Wetzlar, Germany) and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD). DNA was stained with DAPI (4′, 6-diamidino-2-phenylindole).

*Insulin secretion and content*

In brief, we seeded $5 \times 10^4$ cells per well in a 24-well culture plate in 1 mL of RPMI medium.[5] After treatments (Figure S1A), cells were washed twice with Krebs-Ringer bicarbonate buffer (KRBB) and then sensitized with 2.8 mmol/L glucose in KRBB for 30 minutes at 37°C (basal insulin). To measure glucose-stimulated insulin secretion (GSIS) and content, cells were incubated for 30 minutes at 37°C in 1 mL KRBB with 16.8 mmol/L glucose in the presence of CsA, TAC or Rapa. Insulin secretion was measured in supernatants. Cells were lysed overnight at 4°C with acidified ethanol to measure the remaining insulin content. Insulin was measured by ELISA (Mercodia, Uppsala, Sweden). All experiments were repeated three times. Total insulin was calculated as insulin secretion after GSIS plus insulin content, and the percentage of secretion was calculated as: (insulin secretion/total insulin) × 100.

*Immunoprecipitation of FKBP12*

Based on Jacinto et al[15] in brief, INS-1 cells were cultured in dishes of 100 mm (about 90% confluent) and treated with Rapa 200 nmol/L or TAC 50 ng/mL for 1 hour. Then, cells were washed twice with 1× PBS-cold and scraped in lysis buffer (40 mmol/L HEPES, 120 mmol/L NaCl and 0.3% CHAPS) supplemented with protease and phosphatase inhibitors. Crude extracts (~600 μL for each immunoprecipitation) were cleared with 5 minutes spins at 600 g at 4°C and pre-cleared with 15 μL of protein A/G plus-Agarose for 30 minutes. Pre-cleared lysates were subjected to immunoprecipitation with 1 μL of the appropriate control IgG (normal mouse, for a negative control) or 4 μL of anti-FKBP12 (Abcam) overnight at 4°C, followed by the addition of 20 μL of protein A/G plus-Agarose and incubation for 3 hours. Beads were washed three times and resuspended in 2 × Laemmli buffer for western-blot analysis following the standard procedures.

### 2.2.2 | Studies in pancreata of OZRs and LZRs

*Experimental design*

We analyzed pancreata from OZRs and LZRs treated with TAC, CsA, or Rapa obtained from previous studies.[3,4,11] Animals were treated with intraperitoneal injections of vehicle (PBS), TAC (0.3 mg/kg/day), CsA (2.5 mg/kg/day), or Rapa (SRL, 1.0 mg/kg) during 11 days (Figure S1B). All studies were approved by the animal ethics committee (Universities of La Laguna and Barcelona).

*Immunohistochemistry*

Sections of pancreata (3-mm thickness) from OZRs and LZRs (n = 9 per treatment) were mounted on separate slides. Phosphorylated S6 and MafA were evaluated with antibodies against the phosphorylated form of S6 (1:50; Abcam) or MafA (1:250; Abcam), using a secondary horseradish peroxidase–conjugated antibody with diaminobenzidine as chromogen. Images were captured with an Olympus DP72 camera fitted to an Olympus DX41 microscope (Olympus, Tokyo, Japan) and analyzed using ImageJ software and the Wright Cell Imaging Facility plug-in from the Western Research Institute (Toronto, Canada).

*Docking experiments*

Interactions of FKBP12 and the FRB domain of FKBP12-Rapa associated protein (FRAP) of mTOR with the compounds were analyzed by computational docking using Maestro software (Schrödinger, LLC, New York, NY). Crystallographic structure of human FKBP12-FRAP complex was obtained from Protein Data Bank (PDB code 2FAP). Protein structure was energetically minimized using OPSL3 force field, and a maximum root mean square deviation (RMSD) of 0.3 Å from crystallographic positions was selected. Grid generation for ligand binding was established around the volume where Rapa was co-crystallized with FKBP12-FRAP; exclusion volumes were not established and rotation of hydroxyl and thiol groups of serine, threonine, tyrosine, and cysteine was blocked. Ligand structures were drawn in Maestro software, and their energy was minimized using OPSL3 force field. Interaction energy was defined by the equation $\Delta Gbind = \Delta Gelec\ trostatic + \Delta GvdW + \alpha\Delta Gelectrostatic + \beta\Delta GvdW$. β scaling factor of nonpolar interactions was set to 0.8, with a partial charge limit of 0.15. Standard precision method was used for docking calculations, allowing total flexibility of ligands. Nonplanar conformations of amides

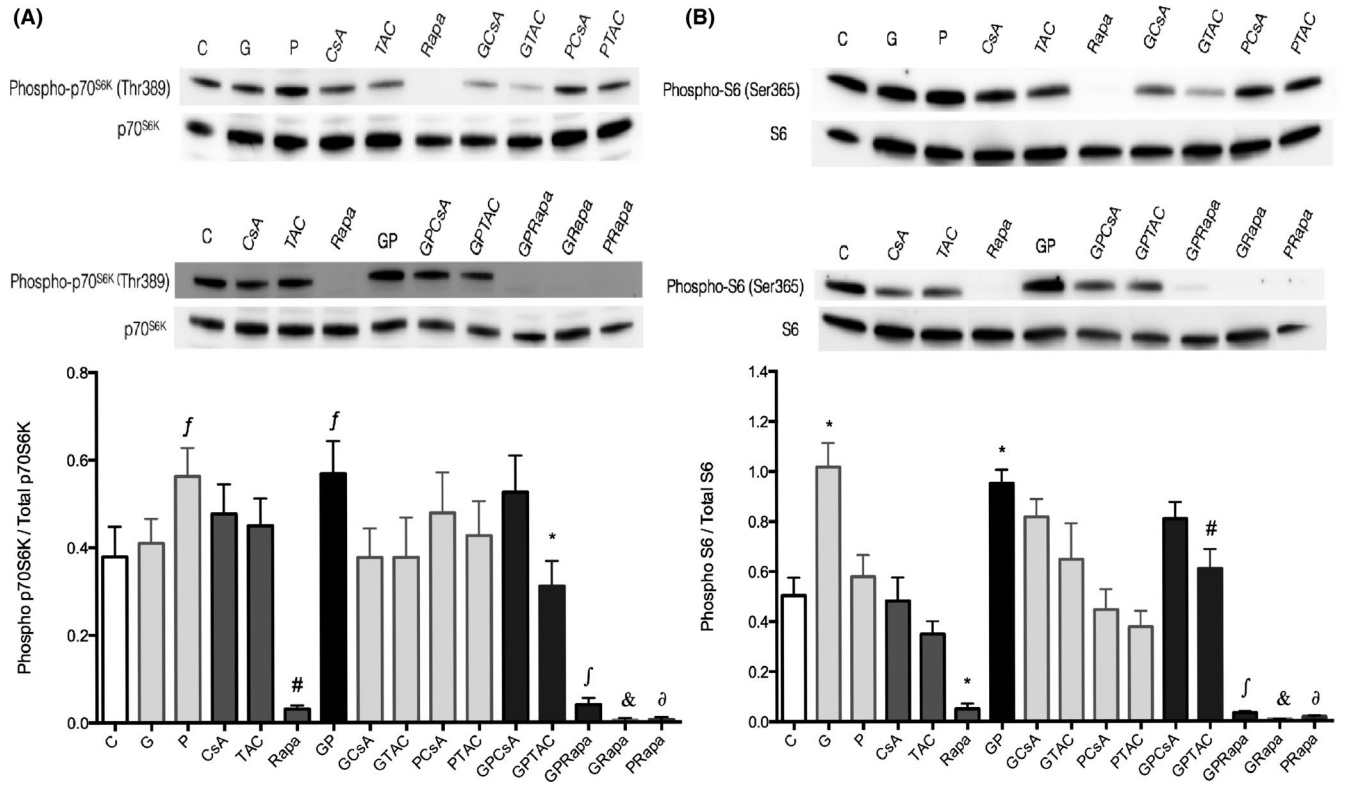


**FIGURE 1** Combined effect of metabolic treatments and Rapa, TAC, or CsA on the mTOR pathway. Inmunoblot showing (A) p70S6K and (B) S6 levels on INS-1 cells. All experiments were done seven times. Data expressed as mean ± standard deviations. Histograms showing changes in (A) p70S6K: # vs. C, $P \leq .0001$; ƒ vs. C, $P = .05$; * vs. GPCsA, $P = .047$; ∫ vs. GP, vs. GPCsA, vs. GPTAC, $P \leq .0001$; & vs. G $P \leq .012$; ∂ vs. P, $P \leq .001$, and (B) S6: * vs. C, $P \leq .0001$; # vs. GP, $P = .01$, vs. GPCsA, $P = .05$; ∫ vs. C, $P = .05$; $ vs. GP, $P \leq .001$. C, control; G, glucose; P, palmitate; CsA, cyclosporin-A; TAC, tacrolimus; Rapa, rapamycin

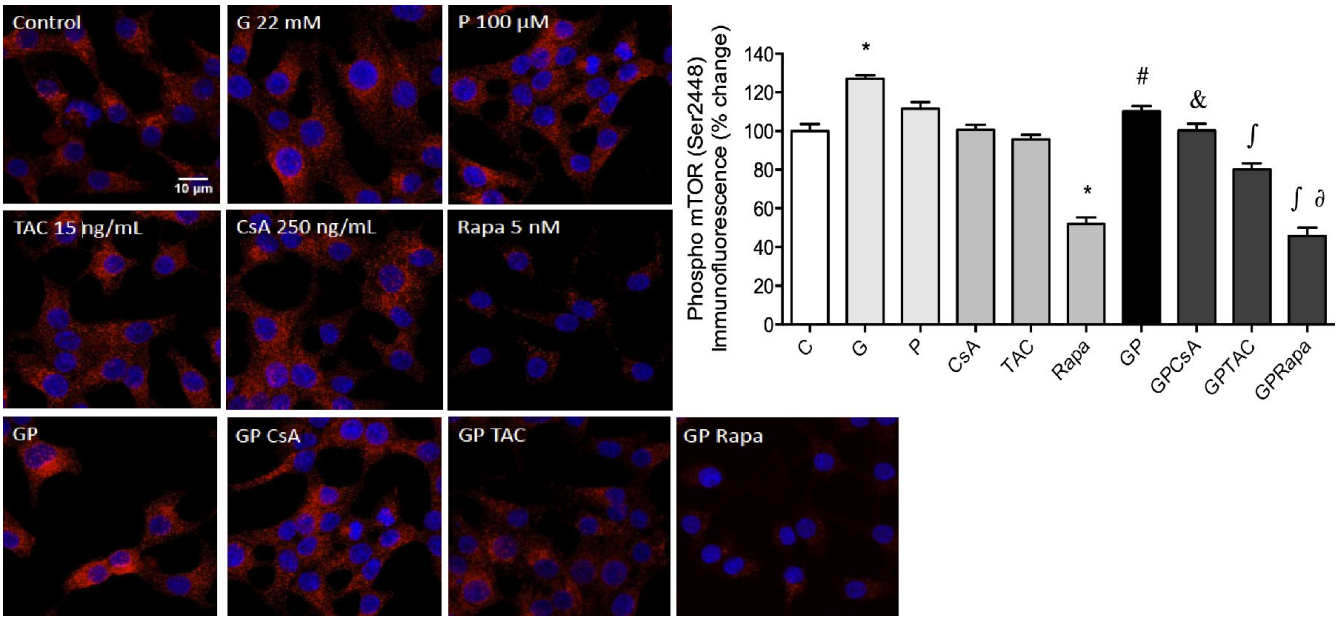


**FIGURE 2** Combined effect of metabolic treatments and Rapa, TAC, or CsA in the expression of mTOR. All experiments were done thrice. Data are expressed as mean ± standard deviation. * vs. C, $P \leq .0001$; # vs. C, $P = .05$; & vs. GP, $P = .039$; ∫ vs. GP, vs. GPCsA, $P = .001$; ∂ vs. GPTAC, $P \leq .0001$. C, control; G, glucose; P, palmitate; CsA, cyclosporin-A; TAC, tacrolimus; Rapa, rapamycin

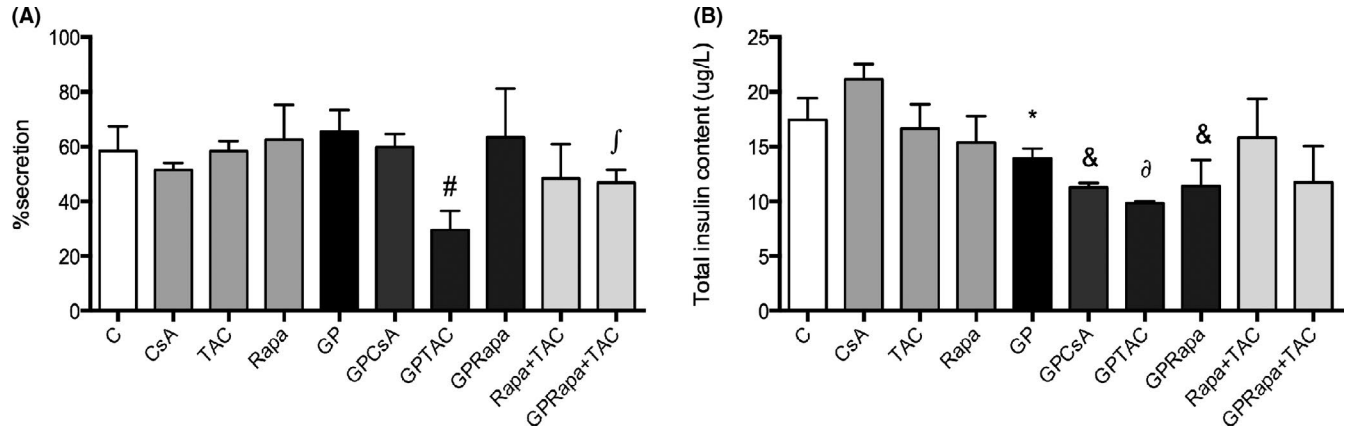


**FIGURE 3** Insulin secretion (A) and content (B). All experiments were done thrice. Data are expressed as mean ± standard deviation. * vs. C, $P = .003$; # vs. GP, vs. GPRapa, $P \leq .0001$; ∫ vs. GP, vs. GPCsA, $P = .003$; & vs. GP, $P \leq .01$; ∂ vs. GP, $P \leq .0001$. C, control; G, glucose; P, palmitate; CsA, cyclosporin-A; TAC, tacrolimus; Rapa, rapamycin

were penalized as well as low probability tautomers. Their docking score values were penalized using Epik algorithm and post-docking energy minimization was performed. Two-dimensional diagrams were obtained from Maestro software and three-dimensional illustrations of docked ligands were obtained using Pymol software.

### 2.3 | Statistical analyses

Data are expressed as mean ± SD. Statistical analyses were performed by ANOVA. Post hoc pairwise comparisons were performed with the Tukey test using statistical program SPSS 20.0 (IBM, Armonk, New York).

## 3 | RESULTS

### 3.1 | β cells in culture

#### 3.1.1 | mTOR pathway

The combination of glucose (22 mmol/L) and palmitate (100 μmol/L) increased the levels of phospho-S6, phospho-p70S6K, and phospho-mTOR (Figures 1,2, and S2) compared to controls. In cells on maintenance medium, neither CsA (250 ng/mL) nor TAC (15 ng/mL) induced changes in these factors (Figures 1,2, and S2). Interestingly, in β cells on glucose plus palmitate, TAC, but not CsA, reduced the levels of phospho-S6, phospho-p70S6K and phospho-mTOR (Figures 1,2, and S2). Rapa (5 nM), both on maintenance medium or with glucose and palmitate, induced a total inhibition of phospho-S6 and phospho-p70S6K and a mild decrease in phospho-mTOR levels (Figures 1,2, and S2). The combination of Rapa and TAC with or without glucose + palmitate inhibited phospho-S6 and phospho-p70S6K similarly (Figure S3). In dose-response experiments, the inhibition of phospho-S6 started with very low doses of Rapa (0.1-0.3 nmol/L) (Figure S4). Interesting, the addition of TAC on top of such doses of Rapa induced an even larger inhibitory effect of the pathway (Figure S4).

#### 3.1.2 | Insulin secretion and content

In the absence of metabolic stress, the immunosuppresses did not influence insulin secretion and content (Figure 3). Glucose plus palmitate alone reduced insulin content but did not affect insulin secretion (Figure 3). On top of glucose plus palmitate, TAC, CsA and Rapa reduced insulin content ($P \leq .0001$, $P = .008$, and $P = .03$, respectively) but only TAC reduced insulin secretion ($P \leq .0001$) (Figure 3), and the combination of Rapa and TAC improved insulin secretion (Figure 3).

#### 3.1.3 | Expression of nuclear MafA

In cells on maintenance medium, the immunosuppresses did not affect nuclear MafA (Figure 4). Glucose plus palmitate alone reduced nuclear MafA (Figure 4). Whereas, in cells on glucose and palmitate: (1) both TAC and Rapa reduced nuclear MafA, (2) this reduction was greater with TAC than with Rapa, and (3) comparable between TAC + Rapa and TAC alone (Figure 4). CsA alone or in combination with glucose and palmitate did not modify the levels of nuclear MafA (Figure 4).

#### 3.1.4 | INS-1 cells proliferation and apoptosis

In cells on maintenance medium, TAC or CsA did not induce cellular proliferation (Ki67) (Figure S5). Glucose plus palmitate alone induced cellular proliferation (Figure S5). However, in cells on glucose and palmitate both TAC and CsA significantly reduced cell proliferation which was larger with TAC. Rapa reduced cells proliferation alone or in combination with glucose and palmitate (Figure S5).

No changes in nuclear localization of active caspase 3 (apoptosis) were observed in the studied groups (Figure S6).

### 3.2 | Pancreata from obese and lean Zucker rats

#### 3.2.1 | mTOR pathway

The levels of phospho-S6 in pancreatic islets were higher in obese than in lean animals ($P \leq .0001$) (Figure 5A,B). TAC reduced phospho-S6 both

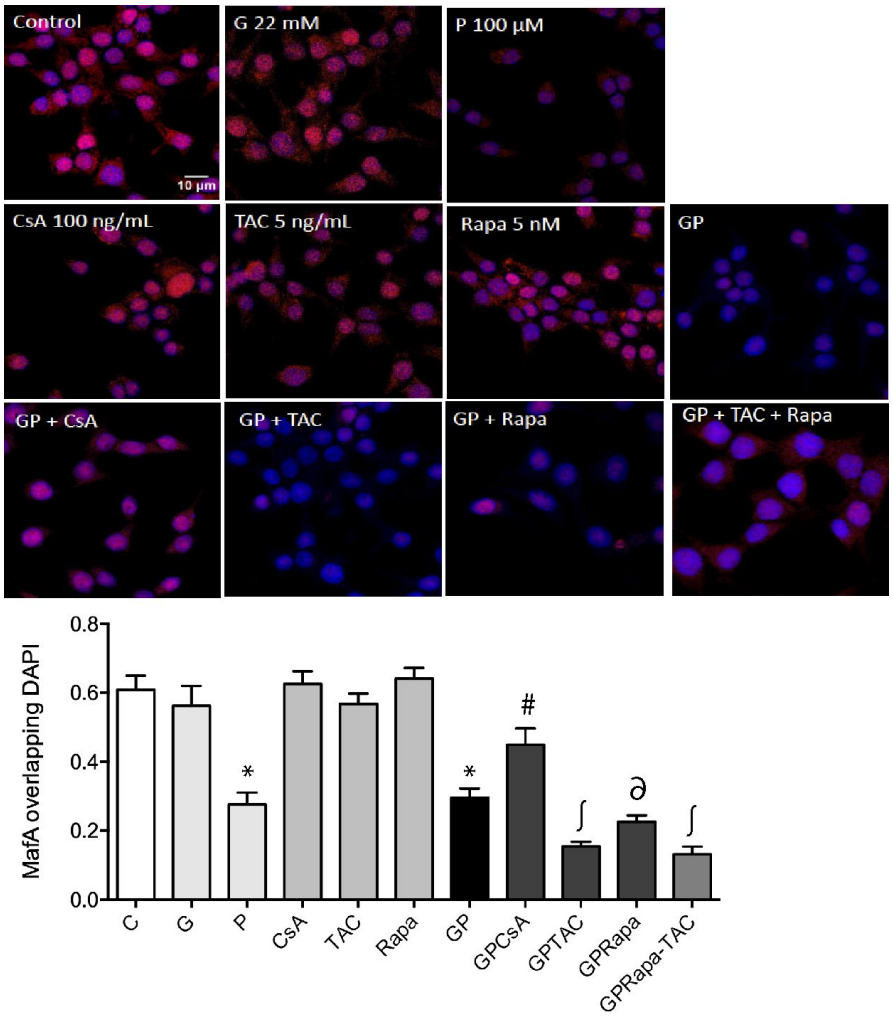


**FIGURE 4** Combined effect of metabolic treatments and Rapa, TAC, or CsA in nuclear factor MafA. All experiments were done thrice. Data are expressed as mean ± standard deviation. * vs. Control, $P \leq .0001$; # vs. GP, $P = .006$; ∫ vs. GP, $P = .01$, vs. GPCsA, $P \leq .0001$; ∂ vs. GP, vs. GPTAC, vs. GPRapa-TAC, $P = .05$. C, control; G, glucose; P, palmitate; CsA, cyclosporin-A; TAC, tacrolimus; Rapa, rapamycin

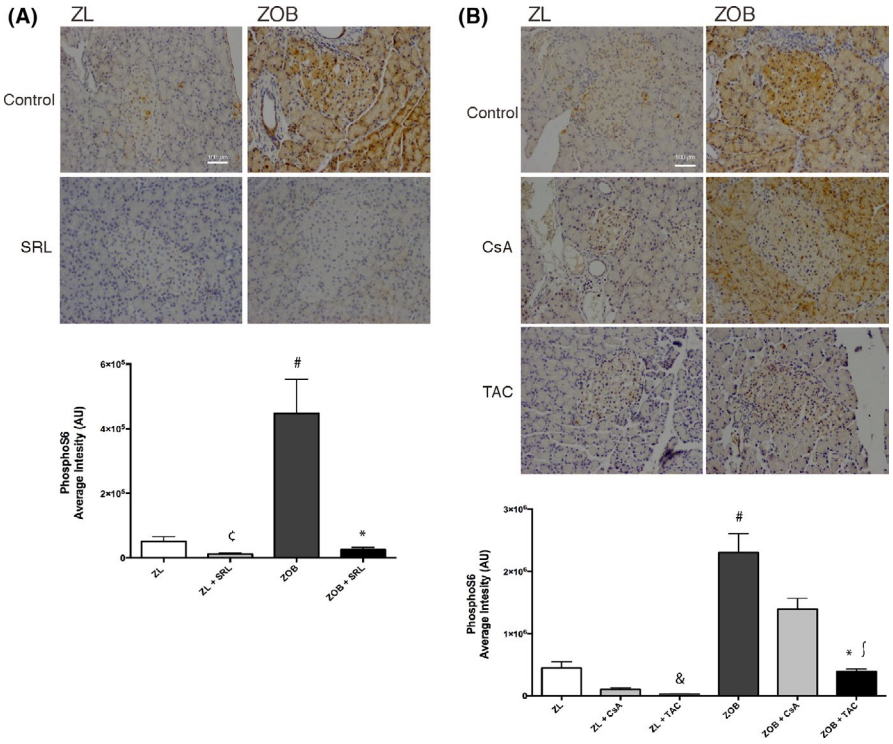


**FIGURE 5** Effect of Rapa, TAC, or CsA in phospho-S6 on pancreatic islets in lean (LZRs) and obese Zucker rats (OZRs). Images and histograms showing phospho-S6 levels in pancreata from lean or obese Zucker rats treated with Rapa (SRL) (A), TAC or CsA (B). Data are expressed as mean ± standard deviation. * vs. ZOB, $P \leq .0001$. # vs. ZL, $P \leq .0001$. ¢ vs. ZL, $P = .008$. ∫ vs. ZOB+CsA, $P = .001$. & vs. ZL, vs. ZL+TAC, $P \leq .001$

in lean ($P = .001$) and obese rats ($P \leq .0001$) compared with controls (Figure 5B). CsA did not induce changes in phospho-S6 (Figure 5B). Rapa reduced phospho-S6 both in lean ($P = .008$) and obese animals ($P \leq .0001$) (Figure 5A).

### 3.2.2 | Functionality

No LZR developed diabetes after 11 days of treatment with TAC, CsA, or Rapa. On the other hand, 100% and 40% of OZR with TAC and CsA, respectively, developed diabetes.[3,4] Fifty percent OZR on Rapa developed diabetes.[11]

### 3.2.3 | Expression of nuclear MafA

In Zucker rats, nuclear MafA levels were significantly increased in obese compared with lean animals ($P \leq .0001$) (Figure 6). In obese animals, TAC (but not CsA) reduced nuclear MafA ($P \leq .0001$) (Figure 6B and C). Rapa reduced nuclear MafA in obese rats ($P \leq .0001$) but not in lean animals (Figure 6A,C). The reduction of MafA in obese rats was larger with TAC than with Rapa ($P = .013$) (Figure 6C).

### 3.3 | Docking experiments

Both TAC and Rapa form a stable noncovalent interaction with FKBP12-FRAP complex with a docking score of −11.2 and −16.3 kcal/mol, respectively (Figure 7). The binding model of both drugs was very similar (Figure 7).

### 3.4 | Immunoprecipitation of FKBP12

FKBP12 co-precipitated mTOR and Raptor from extracts of cells of treated with Rapa or TAC (Figure S7).

## 4 | DISCUSSION

### 4.1 | Main findings

Our major finding was that TAC inhibited the mTOR pathway in β cells. This was only observed on top of a previous overactivation of this route induced by glucolipotoxicity, obesity, and insulin resistance. In INS-1 β cells and in pancreatic islets of obese animals, TAC reduced the levels of phospho-mTOR, -p70S6K and -S6, nuclear MafA, insulin content, and secretion. Our data indicate that the inhibition of the mTOR pathway may explain at least in part the diabetogenic effect of TAC.

The effects of TAC on the mTOR pathways were evaluated both in INS-1 β cells cultured with glucose and palmitate (to mimic glucolipotoxicity) and obese insulin-resistant and lean insulin-sensitive rats. These models were previously used in studies showing that preexisting metabolic stress is necessary to induce β cell toxicity by TAC and Rapa.[3-5,11]

### 4.2 | TAC: A new mTOR inhibitor?

A novel finding of our study was that TAC inhibited the mTOR pathway in β cells as reflected by the lower levels of phospho-mTOR, -p70S6K, and -S6. TAC and Rapa are structurally similar and share the

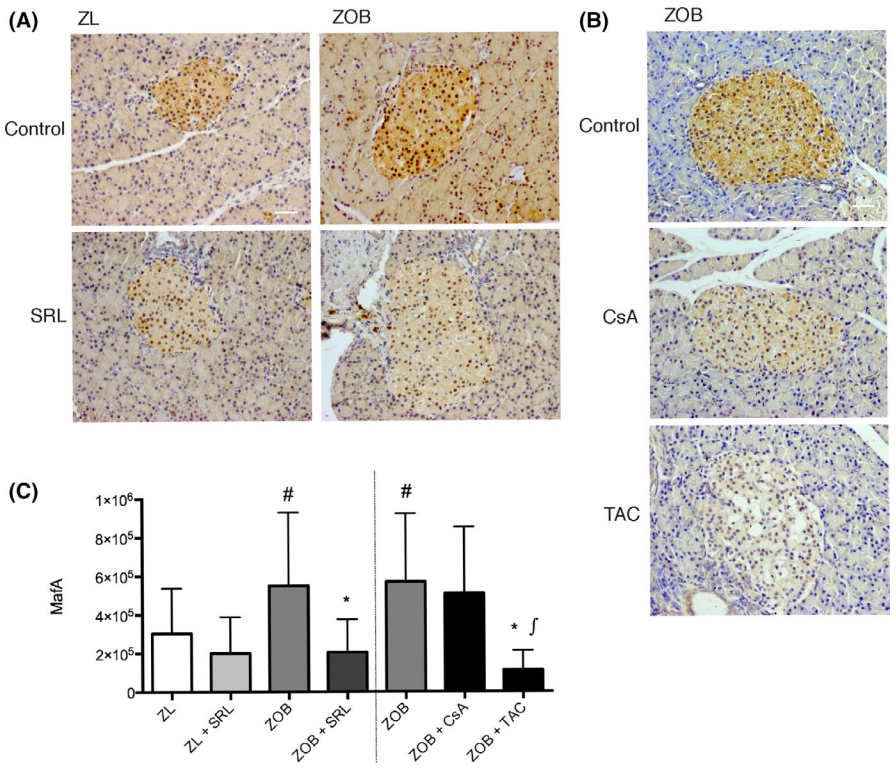


**FIGURE 6** Effect of Rapa, TAC, or CsA in MafA on pancreatic islets in lean Zucker rats (LZRs) and obese Zucker rats (OZRs). Images and histograms quantification (C) showing nuclear MafA levels in pancreata from lean or obese Zucker rats treated with Rapa (SRL) (A), TAC or CsA (B). Data are expressed as mean ± standard deviation. # vs. ZL, $P \leq .0001$. * vs. ZOB; vs. ZOB+CsA, $P \leq .0001$. ∫ vs. ZOB+SRL1, $P = .013$

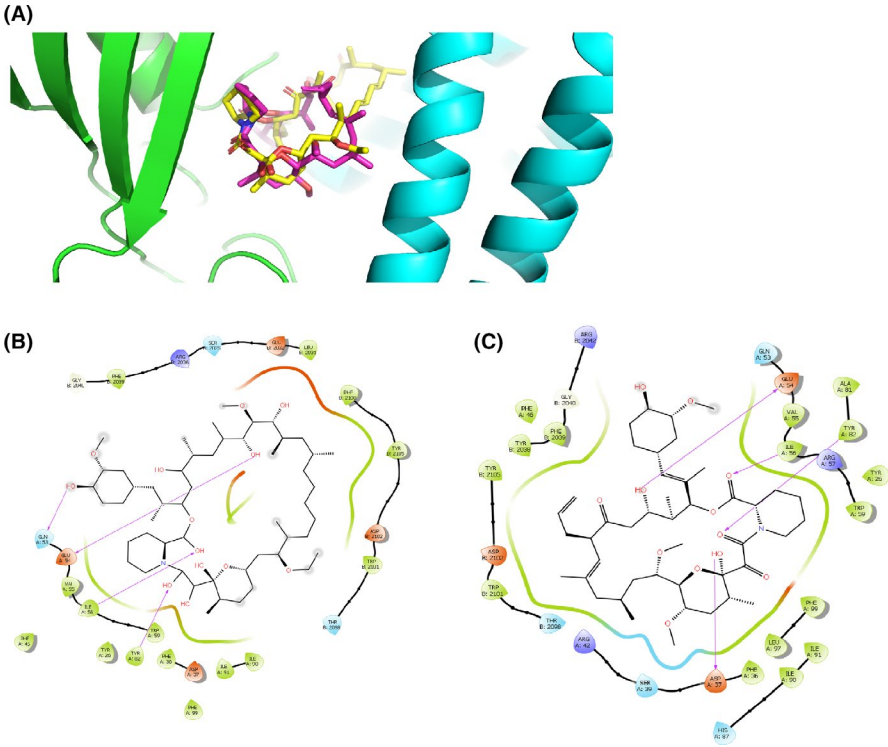


**FIGURE 7** Docking experiment. Superimposed representation of TAC (magenta) and Rapa (yellow) in the binding site of FKBP12-FRAP complex (A). Two-dimensional representation of Rapa interaction pattern at binding site of FKBP12-FRAP complex (B). Two-dimensional representation of tacrolimus interaction pattern at binding site of FKBP12-FRAP complex (C)

same immunophilin: FKBP12, which is crucial for both drugs to exert their functions.[12] How TAC can inhibit the mTOR pathway is unclear. We found evidence both in the docking and immunoprecipitation experiments that the complex TAC + FKBP12 + mTOR is feasible. Thus, it is plausible that TAC inhibits the mTOR pathway like Rapa by binding to FKBP12. In fact, CsA, which has a different immunophilin (cyclophilin-A), did not influence this pathway.

The inhibition was "almost total" with Rapa and moderate with TAC. This was not unexpected since Rapa showed in the docking experiment stronger affinity with the complex mTOR+FKBP12 than TAC. In fact, the inhibition of mTOR with Rapa was observed with doses lower than those used in our experiment (Figure S4), a fact that may limit the evaluation of the combined effect of Rapa and any other drug on this pathway. Only on top of very reduced doses of Rapa (0.1-0.3 nmol/L) TAC added an inhibitory effect of this pathway. Thus, according with our results, TAC seems to inhibit the mTOR pathway showing similarities with the action of Rapa. This finding and its consequences needs further study.

## 4.3 | mTOR inhibition and PTDM

The mTOR pathway integrates signals from growth factors and nutrients to regulate cell growth and proliferation and is implicated in several conditions like cancer, obesity, neurodegeneration, and diabetes mellitus.[16] In β cells, the pathway is important under nutrient excess, increased insulin demand, obesity, and insulin resistance. In this environment, mTOR is robustly activated in β cells of rodents and humans.[17-19] In humans, the inhibition of mTOR pathway proved to be diabetogenic in kidney transplant recipients.[11,20-22] Several mechanisms may explain this diabetogenic effect like impaired insulin-mediated suppression of hepatic glucose production,[18] insulin resistance from ectopic triglyceride deposition,[23,24] direct β cell toxicity,[25,26] and alterations in insulin secretion.[25] In lean or obese rats, Rapa was only diabetogenic in obese animals.[11] In our study, the over-activation of the mTOR pathway was confirmed both in INS-1 β cells treated with glucose and palmitate and in pancreatic islets of obese animals. Moreover, the inhibition of the mTOR by Rapa or TAC reduced insulin content and the levels of MafA, a crucial transcription factor in beta cells[5,27] (Figures 3 and 4). On the other hand, in cells without glucose and palmitate or in islets of lean animals, Rapa inhibited the mTOR pathway without inducing these diabetogenic changes. This may indicate that the inhibition of this route is diabetogenic only if previously overactivated. Finally, the disruption of Raptor, which is an essential component of mTOR complex 1, reduced the expression of MafA.[28] Therefore, our results seem to indicate that the diabetogenic effect of mTOR inhibition may be explained by reducing nuclear levels of MafA.

## 4.4 | Beta-cell toxicity and TAC-induced mTOR inhibition

In the context of glucolipotoxicity, TAC inhibited the mTOR pathway, decreased the proliferation, and induced damage in β cells by reducing the levels of nuclear MafA, insulin secretion, and content, without changes in apoptosis. Rapa also decreased proliferation and reduced the levels of MafA and insulin content but did not modify insulin secretion. A paradoxical finding was that despite a greater inhibition of mTOR by Rapa compared to TAC, the reductions of nuclear MafA or insulin secretion were lower with Rapa than with TAC. Clearly, if the harmful effect on β cells would depend only in the

inhibition of the mTOR pathway, Rapa should be more diabetogenic than TAC, which seems to be the opposite. This fact does not have a simple explanation. On the one hand, diverse studies in humans and animal models observed that Rapa presents a lower diabetogenic effect than TAC.[5,11,29-31] Thus, the inhibition of mTOR, although harmful to β cells and diabetogenic, does not induce a larger toxic effect compared with TAC. On the other hand, it may be plausible that TAC-induced toxicity could be attributed in part to mTOR inhibition as well as to other factors not yet elucidated. Another interesting finding was that on top of glucose and palmitate, the combination of Rapa and TAC increased insulin secretion despite maintaining low insulin content compared with the effect of TAC that reduced both insulin secretion and content. This finding might indicate a compensatory effect of Rapa on insulin secretion when combined with TAC. However, we acknowledge that our study was not designed to analyze the specific effect of the interaction between TAC and Rapa on insulin secretion, which is worth investigating.

This study has limitations. First, we want to acknowledge those derived from the use of cellular and animal models of disease and the reliability of extrapolating its results to humans. Second, we only measured S6 as mTOR downstream effector in the pancreata of the animal study. There are not reliable antibodies for P70S6K designed for immunohistochemical techniques.

## 5 | CONCLUSIONS

TAC inhibits the mTOR pathway in β cells both in vitro and in animal models under an environment of glucolipotoxicity and obesity. This may explain at least in part the diabetogenic effects of TAC.


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### DISCLOSURE

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

### DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author, EP, upon reasonable request.


### ORCID

*Ana Elena Rodriguez-Rodriguez* https://orcid.org/0000-0002-0803-864X

*Jordi Rovira* https://orcid.org/0000-0001-8737-5005


### REFERENCES


1. Vincenti F, Friman S, Scheuermann E, et al.; DIRECT (Diabetes Incidence after Renal Transplantation: Neoral C Monitoring Versus Tacrolimus) Investigators. Results of an international randomized trial comparing glucose metabolism disorders and outcome with cyclosporine versus tacrolimus. *Am J Transplant*. 2007;7(6):1506-1514.
2. Porrini E, Delgado P, Alvarez A, et al. The combined effect of pretransplant triglyceride levels and the type of calcineurin inhibitor in predicting the risk of new onset diabetes after renal transplantation. *Nephrol Dial Transplant*. 2008;23(4):1436-1441.
3. Rodriguez-Rodriguez AE, Triñanes J, Velazquez-Garcia S, et al. The higher diabetogenic risk of tacrolimus depends on pre-existing insulin resistance. A study in obese and lean Zucker rats. *Am J Transplant*. 2013;13(7):1665-1675.
4. Rodríguez-Rodríguez AE, Triñanes J, Porrini E, et al. Glucose homeostasis changes and pancreatic β-cell proliferation after switching to cyclosporin in tacrolimus-induced diabetes mellitus. *Nefrologia*. 2015;35(3):264-272.
5. Triñanes J, Rodriguez-Rodriguez AE, Brito-Casillas Y, et al. Deciphering tacrolimus-induced toxicity in pancreatic β cells. *Am J Transplant*. 2017;17(11):2829-2840.
6. Tamura K, Fujimura T, Tsutsumi T, et al. Transcriptional inhibition of insulin by FK506 and possible involvement of FK506 binding protein-12 in pancreatic beta-cell. *Transplantation*. 1995;59(11):1606-1613.
7. Aghdasi B, Ye K, Resnick A, et al. FKBP12, the 12-kDa FK506-binding protein, is a physiologic regulator of the cell cycle. *PNAS*. 2001;98(5):2425-2430.
8. Santulli G, Pagano G, Sardu C, et al. Calcium release channel RyR2 regulates insulin release and glucose homeostasis. *J Clin Invest*. 2015;125(5):1968-1978.
9. Hay N, Sonenberg N. Upstream and downstream of mTOR. *Genes Dev*. 2004;18(16):1926-1945.
10. Johnston O, Rose CL, Webster AC, et al. Sirolimus is associated with new-onset diabetes in kidney transplant recipients. *J Am Soc Nephrol*. 2008;19(7):1411-1418.
11. Rovira J, Ramírez-Bajo MJ, Banon-Maneus E, et al. mTOR inhibition: reduced insulin secretion and sensitivity in a rat model of metabolic syndrome. *Transplant Direct*. 2016;2(2):e65.
12. Banaszynski LA, Liu CW, Wandless TJ. Characterization of the FKBP.rapamycin.FRB ternary complex. *J Am Chem Soc* 2005;127(13):4715-4721.
13. Bell E, Cao X, Moibi JA, et al. Rapamycin has a deleterious effect on MIN-6 cells and rat and human islets. *Diabetes*. 2003;52(11):2731-2739.
14. Chatterjee A, Mukhopadhyay S, Tung K, et al. Rapamycin-induced G1 cell cycle arrest employs both TGF-β and Rb pathways. *Cancer Lett*. 2015;360(2):134-140.
15. Jacinto E, Loewith R, Schmidt A, et al. Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. *Nat Cell Biol*. 2004;6(11):1122-1128. Epub 2004 Oct 3 PubMed PMID: 15467718.
16. Laplante M, Sabatini DM. mTOR signaling in growth control and disease. *Cell*. 2012;149:274-293.
17. Gleason CE, Lu D, Witters LA, et al. The role of AMPK and mTOR in nutrient sensing in pancreatic beta-cells. *J Biol Chem*. 2007;282(14):10341-10351.

18. Syed NA, Khandelwal RL. Reciprocal regulation of glycogen phosphorylase and glycogen synthase by insulin involving phosphatidylinositol-3 kinase and protein phosphatase-1 in HepG2 cells. *Mol Cell Biochem*. 2000;211:123-136.
19. McDaniel ML, Marshall CA, Pappan KL, et al. Metabolic and autocrine regulation of the mammalian target of rapamycin by pancreatic beta-cells. *Diabetes*. 2002;51(10):2877-2885.
20. Romagnoli J, Citterio F, Nanni G, et al. Incidence of posttransplant diabetes mellitus in kidney transplant recipients immunosuppressed with sirolimus in combination with cyclosporine. *Transplant Proc*. 2006;38(4):1034-1036.
21. Teutonico A, Schena PF, Di Paolo S. Glucose metabolism in renal transplant recipients: effect of calcineurin inhibitor withdrawal and conversion to sirolimus. *J Am Soc Nephrol*. 2005;16(10):3128-3135.
22. Murakami N, Riella LV, Funakoshi T. Risk of metabolic complications in kidney transplantation after conversion to mTOR inhibitor: a systematic review and meta-analysis. *Am J Transplant*. 2014;14(10):2317-2327.
23. Lewis GF, Carpentier A, Adeli K, et al. Disordered fat storage and mobilization in the pathogenesis of insulin resistance and type 2 diabetes. *Endocr Rev*. 2002;23:201-229.
24. Mittelman SD, Bergman RN. Inhibition of lipolysis causes suppression of endogenous glucose production independent of changes in insulin. *Am J Physiol Endocrinol Metab*. 2000;279:E630-E637.
25. Bussiere CT, Lakey JR, Shapiro AM, et al. The impact of the mTOR inhibitor sirolimus on the proliferation and function of pancreatic islets and ductal cells. *Diabetologia*. 2006;49:2341-2349.
26. Kwon G, Marshall CA, Liu H, et al. Glucose-stimulated DNA synthesis through mammalian target of rapamycin (mTOR) is regulated by KATP channels: effects on cell cycle progression in rodent islets. *J Biol Chem*. 2006;281:3261-3267.
27. Nishimura W, Takahashi S, Yasuda K. MafA is critical for maintenance of the mature beta cell phenotype in mice. *Diabetologia*. 2015;58(3):566-574. https://doi.org/10.1007/s00125-014-3464-9.
28. Ni Q, Gu Y, Xie Y, et al. Raptor regulates functional maturation of murine beta cells. *Nat Commun*. 2017;8:15755.
29. Ekberg H, Bernasconi C, Nöldeke J, et al. Cyclosporine, tacrolimus and sirolimus retain their distinct toxicity profiles despite low doses in the Symphony study. *Nephrol Dial Transplant*. 2010;25(6):2004-2010.
30. Claes K, Meier-Kriesche HU, Schold JD, et al. Effect of different immunosuppressive regimens on the evolution of distinct metabolic parameters: evidence from the Symphony study. *Nephrol Dial Transplant*. 2012;27(2):850-857.
31. Flechner SM, Glyda M, Cockfield S, et al. The ORION study: comparison of two sirolimus-based regimens versus tacrolimus and mycophenolate mofetil in renal allograft recipients. *Am J Transplant*. 2011;11(8):1633-1644.

## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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