Original Paper

Molecular Pathways Leading to Induction of Cell Death and Anti-Proliferative Properties by Tacrolimus and mTOR Inhibitors in Liver Cancer Cells

Elena Navarro-Villarán[a,b]   Patricia de la Cruz-Ojeda[a]   Laura Contreras[a,c]
Raúl González[a,b]   María Negrete[a]   María A. Rodríguez-Hernández[a,b]
Luís M. Marín-Gómez[a,b,d]   José M. Álamo-Martínez[a,b,d]   Antonio Calvo[e]
Miguel A. Gómez-Bravo[a,b,d]   Jesús de la Cruz[a,c]   Javier Padillo[a,b,d]   Jordi Muntané[a,b,d]

[a]Institute of Biomedicine of Seville (IBiS), Hospital University "Virgen del Rocío"/CSIC/University of Seville, Seville, Spain, [b]Networked Biomedical Research Center Hepatic and Digestive Diseases (CIBEREHD o Ciberehd), Instituto de Salud Carlos III, Madrid, Spain, [c]Department of Genetics, University of Seville, Seville, Spain, [d]Department of General Surgery, Hospital University "Virgen del Rocío"/CSIC/University of Seville/IBiS, Seville, Spain, [e]Department of General Surgery, Hospital University of Puerto Real, Puerto Real, Spain

Abstract
Background/Aims: Orthotopic liver transplantation (OLT) is the recommended treatment for patients at early stages of hepatocarcinoma (HCC) with portal hypertension and/or increased bilirubinemia, but without vascular-associated diseases. Tumor recurrence, which is the main drawback for the survival of patients submitted to OLT for HCC, has been related to tumor-related variables and the immunosuppressive therapies. We have previously shown that Tacrolimus (FK506) exerts a more potent pro-apoptotic and anti-proliferative effects than the mammalian target of rapamycin (mTOR) inhibitors (Sirolimus and Everolimus) in liver cancer cells. This study identified the role of the immunosuppressant partners such as FK506-binding proteins (FKBPs) in the induction of cell death and arrest of cell proliferation by immunosuppressants in two representative liver cancer cells. ***Methods:*** The regulation of endoplasmic reticulum (ER) stress, apoptosis/autophagy, cell proliferation, and FKBPs expression was determined in Tacrolimus-, Sirolimus- and Everolimus-treated primary human hepatocytes, and hepatoma HepG2 and Huh7 cell lines. The functional repercussion of FKBPs on cell death and proliferation was also addressed using the siRNA technology. The assessed

R. González, M. Negrete and M. A. Rodríguez-Hernández contributed equally to this study.

Jordi Muntané Ph.D.   Instituto de Biomedicina de Sevilla (IBiS), Hospital Universitario "Virgen del Rocío"/CSIC/
Universidad de Sevilla, Av. Manuel Siurot s/n, 41013-Seville (Spain)
Tel. +34-955923122, Fax +34-955923002, E-Mail jmuntane-ibis@us.es

antitumoral properties of the immunosuppressants were associated to microRNAs (miRNAs) pattern. ***Results:*** The enhanced pro-apoptotic and anti-proliferative properties of Tacrolimus *versus* mTOR inhibitors were associated with increased protein kinase RNA-like endoplasmic reticulum kinase (PERK)-related ER stress, ^{Ser15}P-p53/p53 ratio and p21 protein expression that may counterbalance the risk of proliferative upregulation caused by enhanced ^{Thr172}P-Cdk4/Cdk4 activation in liver cancer cells. The inhibition of the mTOR pathway by Sirolimus and Everolimus was related to an induction of autophagy; and at a high dose, these drugs impaired translation likely at a very early step of the elongation phase. Tacrolimus and mTOR inhibitors increased the protein expression of FKBP12 and FKBP51 that appeared to play pro-survival role. Interestingly, the administration of immunosuppressants yields a specific pattern of miRNAs. Tacrolimus and mTOR inhibitors decreased miR-92a-1-5p, miR-197-3p, miR-483-3p and miR-720, and increased miR-22-3p, miR-376a-3p, miR-663b, miR-886-5p, miR-1300 and miR-1303 expressions in HepG2 cells. ***Conclusion:*** The more potent pro-apoptotic and anti-proliferative properties of Tacrolimus *versus* mTOR inhibitors were associated with an increased activation of PERK and p53 signaling, and p21 protein expression. FKBP12 and FKBP51 appeared to be the most relevant partners of Tacrolimus and mTOR inhibitors exerting a pro-survival effect in HepG2 cells. The observed effects of immunosuppressants were related to a specific miRNA signature in liver cancer cells.

Introduction

Hepatocellular carcinoma (HCC) has been the sixth most common neoplasia in the world, and the fourth most common cause of cancer-related mortality worldwide during 2018 [1]. HCC is the main primary malignancy in the liver causing death in cirrhotic patients [2]. The performance status and hepatic function of the patient, number and size of the nodules, tumor vascular invasion, and the presence of extrahepatic metastasis, are actually used for the staging, prognosis as well as the therapeutic recommendation to the patients with HCC [3]. The curative treatments such as ablation, resection and orthotopic liver transplantation (OLT) are indicated at the very early stage (Barcelona Clinic Liver Cancer or BCLC 0) and early stage (BCLC A) of the disease characterized by the presence of 1-3 tumors less than or equal to 3 cm diameter, good liver function (Child-Pugh A-B), asymptomatic (Performance Status or PS 0), and absence of vascular invasion and extrahepatic metastases. OLT is indicated in patients with potential portal hypertension and/or bilirubinemia, but without vascular-associated diseases [3]. The patients subjected to OLT receive immunosuppressive therapy with Cyclosporin A, Tacrolimus (FK506) or mammalian target of rapamycin (mTOR) inhibitors (Rapamycin or Sirolimus, and Everolimus) to reduce graft rejection.

Immunophilins consist of a family of highly conserved proteins that binds to immunosuppressive drugs such as Cyclosporin A, Tacrolimus, and mTOR inhibitors. Cyclophilins and FK506-binding proteins (FKBPs) are the major immunophilins that binds to Cyclosporin A, or Tacrolimus and mTOR inhibitors, respectively [4]. FKBPs are involved in numerous cellular functions, such as protein folding, protein stability, kinase activity, receptor signaling, and protein trafficking, as well as play a role in cancer and chemoresistance [5]. Tacrolimus inhibits antigen receptor-dependent T cell proliferation through binding to the conserved active sites of the canonical FKBP members (FKBP12, FKBP51 and FKBP52), inhibiting its peptidylprolyl cis/trans isomerase (PPIase) activity [6], but also the phosphatase activity of Calcineurin, thereby preventing the calcium/calmodulin-dependent Calcineurin-related dephosphorylation of the nuclear factor of activated T cells (NFAT). The process downregulates the expression of IL-2, protooncogenes (H-RAS, c-MYC) and receptors for cytokines (IL-2 receptor) in T cells [7]. While binding to the same FKBP partners, Sirolimus and Everolimus exert their immunosuppressive properties by preventing IL-2-dependent T cell proliferation [8]. The immunosuppressive properties of the FKBP/Sirolimus complex are related to the interaction with the mTOR complex [9, 10].

The aim of the study was the identification of the role of FKBPs in the induction of cell death and anti-proliferative properties induced by immunosuppressants in liver cancer cells. The present study showed that the increased pro-apoptotic and anti-proliferative properties induced by Tacrolimus *versus* mTOR inhibitors were due to an enhanced protein kinase RNA-like endoplasmic reticulum kinase (PERK)-related endoplasmic reticulum (ER) stress. However, the administration of mTOR inhibitors increased autophagy markers compared to Tacrolimus treatment in HepG2 and Huh7 cell lines. We have also performed siRNA functional studies which showed that FKBP12 and FKBP51 mediate cell survival in immunosuppressive-treated HepG2 cells. We have observed a specific signature of microRNAs (miRNAs) expression upon treatment of HepG2 cells with Tacrolimus and mTOR inhibitors that was associated with the induction of apoptosis and anti-proliferative properties of the treatments.

Materials and Methods

Drugs

Tacrolimus (Ref AT23293, Carbosynth Limited, Berkshire, United Kingdom), Sirolimus (Ref 37094, Sigma-Aldrich, Sant Louis, Missouri, USA), and Everolimus (Ref FE23209, Carbosynth Limited) were dissolved in DMSO (95.8, 91.5 and 80.4 µg/µl, respectively) in order to obtain working solution useful for all the experimental work.

Primary human hepatocytes, cell lines and culture conditions

Human hepatocytes were prepared from healthy portions of liver resections obtained from three patients (two men and one woman, aged 63±5.2 years) submitted to surgical resection for liver metastasis from colorectal cancer. Isolation of human hepatocytes was based on a two-step collagenase procedure [11]. HepG2 (HB-8065™) cells were obtained from ATCC/LGC Standards (Barcelona, Spain). Huh7 cells were obtained from Apath LLC (New York, New York, USA). Both cells lines were negative for mycoplasma contamination. Cells (100000 cells/cm^2) were cultured in MEM with Earle's salts with L-glutamine (Ref E15-825, PAA Laboratories Inc, Toronto, Ontario, Canada) with 10 % FBS (F7524, Sigma-Aldrich, Lot No: 022M3395, endotoxin <0.2 EU/ml), sodium pyruvate (1 mM) (Ref S11-003, PAA Laboratories Inc), non-essential amino acids (Ref M11-003, PAA Laboratories Inc), Penicillin-Streptomycin solution (100 U/mL-100 µg/ml) (P11-010, PAA Laboratories Inc), at 37°C in a humidified incubator with 5 % CO_2. Drugs were added at a broad range of concentrations (0, 10 nM, 10 µM and 100 µM) 24 h after plating. Cell lysates were obtained at different time points according to the assays.

Measurement of apoptosis

Caspase-3-associated activity was determined using caspase-Glo® 3 assay systems (G8091, Promega, Fitchburg, Wisconsin, USA). Cells were treated with caspase-Glo® 3 reagent in an "add-mix-measure" format resulting in cell lysis, caspase-3-dependent cleavage of the substrate and generation of a "glow-type" luminescent signal. The signal generated is proportional to the amount of caspase-3 activity. The values are extrapolated into a calibration curve included in the assay. Chemiluminiscence was measured using an Infinite 200 PRO Microplate Reader (TECAN, Männedorf, Switzerland).

Evaluation of protein expression markers related to ER stress, autophagy, cell cycle, apoptosis and FKBPs

Cells were treated with lysis solution including 50 mM HEPES pH. 7.5, 5 mM EDTA, 150 mM NaCl, 1 % NP-40, commercial proteases inhibitor cocktail (P8340-5 ml, Sigma-Aldrich), 1 mM PMSF, 1 mM NaF and 1 mM Na_3VO_4. Protein expression was determined by SDS-PAGE coupled to Western-blot analysis. Proteins (50-100 µg) were separated by Any kD™ Criterion™ TGX Stain-Free™ Protein Gel, 18 well, 30 µl (#5678124, BioRad, Hercules, California, USA) and transferred to PVDF membranes. The membranes were incubated with the corresponding commercial primary and secondary antibodies coupled to horseradish peroxidase revealing protein content by Clarity™ Western ECL substrate (Ref 170-5061, BioRad) and analyzed in a ChemiDoc™ Touch Imaging System. Antibodies for Western-blot were obtained commercially and included 4E-BP1 (#9452), $^{Thr37/Thr46}$P-4E-BP1(#9459), Cdk4 (#12790), eIF2α (#5324), ^{Ser51}P-eIF2α (#9721)

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and ^{Ser15}P-p53 (#9284) obtained from Cell Signaling Technology (Danvers, Massachusetts, USA); LC3 (PM036) purchased from MBL International (Woburn, Massachusetts, USA); GADD153 (C/EBP homologous protein or CHOP) (sc-575), Beclin (sc-48341), p21 (sc-397) and p53 (sc-6243) obtained from Santa Cruz Biotechnology (Dallas, Texas, USA); ^{Thr172}P-Cdk4 (PA5-64482) obtained from ThermoFisher (Waltham, Massachusetts, USA); FKBP12 (Ref NB300-508) and FKBP38 (Ref NBP1-77909) obtained from Novus Biologicals (Centennial, Colorado, USA); and FKBP51 (Ref MAB4094) and FKBP52 (Ref MAB4095) obtained from R&D Systems (Minneapolis, Minnesota, USA). The corresponding secondary antibodies anti-rabbit (sc-2004), anti-mouse (sc-2005) and anti-goat (sc-2768) IgG-HRP labelled were purchased from Santa Cruz. Densitometric analysis was performed using the software Image Lab 6.0 of BioRad.

Cell proliferation assay

The measurement of bromodeoxyuridine (BrdU) incorporation was used as marker of cell proliferation (Ref 11 647 229 001, Roche Diagnostics, Mannheim, Germany). Cells were seeded at low density (12500 cells/cm^2) at 37°C in a humidified incubator with 5 % CO$_2$. After 24 h of stabilization cells were treated with drugs including corresponding controls without BrdU. Two hours before evaluation of cell proliferation (12 h) 20 μl of BrdU (10 μM) was added to culture. DNA was denaturalized with 200 μl FixDenat solution included in the commercial assay for 30 min a room temperature. After removal, cells were incubated with 100 μl monoclonal anti-BrdU antibodies conjugated with horseradish peroxidase for 90 min at room temperature. Afterwards, cells were washed with phosphate buffer saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na$_2$HPO$_4$), and incubated with 100 μl revealing solution including hydrogen peroxide, luminol and 4-iodophenol for 15 min at room temperature. Absorbance was measured at 370 nm, using as reference wavelength 492 nm, and with an Infinite 200 PRO Microplate Reader (TECAN, Männedorf, Switzerland).

Assessment of protein translation

The protocol for polysome preparation of liver cancer cells was adapted from that we routinely employ for yeast cells [12]. Cells were grown up to 80 % confluency in 150 cm^2 dishes. Before cell harvesting, 200 μg/ml cycloheximide was added and incubated for 5 min at 37°C. Each dish was then placed on ice, the medium was collected, and the cultures washed twice with PBS without Ca^{2+} and Mg^{2+} containing 200 μg/ml cycloheximide. Then, 800 μl of lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM MgCl$_2$, 200 μg/ml cycloheximide, 200 μg/ml heparin, 2 mM DTT, 0.5 % NP40) was added to one dish, cells were scrapped, and transferred to the second dish. Cell lysate was incubated at 4°C with gentle end-over-end rotation for 10 min and then centrifuged at 12000 g for 8 min at 4°C in a refrigerated microfuge. The corresponding supernatants were recovered and the A$_{260}$ measured using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific). About 10 absorption units of A$_{260}$ were layered on top of 7-50 % (w/v) sucrose gradients prepared in 50 mM Tris-acetate, pH 7.5, 50 mM NH$_4$Cl, 12 mM MgCl$_2$, 1 mM DTT. The gradients were centrifuged at 260110 g (39000 rpm) in a Beckman SW41 rotor at 4°C for 2 h 45 min. The dissociation of the vacant 80S ribosomes was achieved by addition of high-salt (0.25 M NaCl) concentration within the gradients. Gradient analysis was performed with an ISCO UA-6 system (Isco, Inc. Lincoln, NE, USA) equipped to continuously monitor the A$_{254}$.

Regulation of FKBP expression by siRNA technology

Cells were seeded the day before the experimental intervention to reach 60 % confluency at the moment of cell transfection in 24 h. siRNA (25 nM) were mixed with the transfection reagent DharmaFECT 4 Transfection Reagent (77T-2004-02; GE Healthcare Dharmacon, Horizon-Discovery, Lafayette, Colorado, USA) for 20 min at room temperature and transferred to cell culture without antibiotic/antimycotic solution for 24 h. Afterwards, cells were maintained in fully complemented culture medium, and drugs were added 48 h after cell transfection. The siRNA of FKBP12 (on-target plus smart pool Human FKBP1A 2280 siRNA, ref L-009494), FKBP38 (on-target plus smart pool Human FKBP8 23770 siRNA, ref L-009673), FKBP51 (on-target plus smart pool Human FKBP4 2288 siRNA, ref L-006410) and FKBP52 (on-target plus smart pool Human FKBP5 2289 siRNA, ref L-004224), as well as non-targeting siRNA (on-target plus non-targeting siRNA pool, ref 77D-001810-10) used as negative control were also obtained from GE Healthcare Dharmacon.

Expression of miRNAs

Total RNA was extracted using the miRNeasy kit (Ref 217004, Qiagen, Hilden, Germany). Briefly, HepG2 cells and primary hepatocytes were lysed with QIAzol Lysis Reagent (Ref 79306, Qiagen). Chloroform was added to homogenates and these were centrifuged for 15 min at 12000 g. Upper aqueous phases containing RNA were transferred to clean tubes. After the addition of ethanol, RNA was bound RNeasy Mini spin column (Ref 74104, Qiagen) and washed in subsequent centrifugation steps. Eventually, RNA was eluted in 50 µl of RNase-free water. RNA was quantified using NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific). Quality control and RNA integrity assessment were performed with 2100 Bioanalyzer (Agilent Technologies, California, USA). All samples showed RIN higher than 8.

The miRNA expression profiling was obtained by qRT-PCR using the using the TaqMan® OpenArray® Human miRNA Panel (Ref 4470187, Life Technologies, Carlsbad, California, USA) and accessory kits (Applied Biosystems, Foster City, California, United States). Briefly, isolated miRNAs were reversed transcribed into cDNA using the TaqMan MicroRNA Reverse Transcription Kit (Ref 4366596, Life Technologies) and Megaplex RT Primers Human in a set of predefined pools A and B (Ref 4444750, Life Technologies). Prior to PCR, resulting cDNA was pre-amplified with Megaplex PreAmp Primers of gene-specific Human Pools A and B and the TaqMan PreAmp Master Mix (Ref 4391128, Life Technologies). The pre-amplified product was diluted 40 folds and mixed 1:1 v/v with the TaqMan OpenArray Real-Time PCR Master Mix (Ref 4462159, Life Technologies). Aliquots of the mixture were dispensed on a microfluidic OpenArray 384-well sample plate (Ref 4406947, Life Technologies). Next, TaqMan Open-Array Human MicroRNA Panels were loaded with the OpenArray AccuFill System and the PCR reactions were carried out with QuantStudio™ 12 K Flex OpenArray® Platform (QS12KFlex, Thermo Fisher, Waltham, Massachusetts, USA) following the manufacturer's instructions.

Thermo Fisher Cloud Relative Quantification software was used to obtain qPCR data. First, expression levels were calculated using the relative threshold cycle (Crt) method. TaqMan® OpenArray® Human miRNA Panel aims to obtain a total of 758 Crt values for each sample, which include 754 unique miRNAs, one negative control (ath-miR159a) and three endogenous positive controls (RNU48, RNU44 and U6). Crt values were normalized using the global mean strategy. Therefore, ΔCrt values were calculated as Crt miRNA-Crt global mean. Samples were clustered according to ΔCrt values of miRNAs expressed in all samples using the Pearson Correlation (uncentered) and average linkage methods with the Cluster (v 3.0) software. Dendogram illustration was created with Java TreeView software. Relative miRNA expression levels between control and experimental groups were calculated by using the ΔΔCrt method and exported for further analysis. Fold change values were calculated as 2-ΔΔCrt.

The bioinformatic analysis was also carried out based on significant differentially expressed miRNAs showing ≥2.0-fold or <0.5 fold changes, and with a *p*-value <0.05 that were used for in silico predictions. Target prediction was done using the TargetScan Database. Only highly conserved predicted targets with a cumulative weighted context score <-0.4 were selected. A gene regulatory network of differentially expressed miRNAs and their targets was constructed using Cytoscape (v3.6.0). In order to investigate the biological functions of miRNA targets, we carried Functional Annotation and Enrichment analysis of Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways with the Database for Annotation, Visualization, and Integrated Discovery (DAVID) tool. Results were considered statistically significant when *p*-value <0.05 and False Discovery Rate (FDR) <0.05.

Statistical analysis

All results are expressed as mean ± SEM of independent experiments (n=3-8). Data were compared using the analysis of variance (ANOVA) with the Least Significant Difference's test as post-hoc multiple comparison analysis (homogeneity of variances) or Games-Howell (non-homogeneity of variances). If Shapiro-Wilks's test showed non-normal distribution of data non-parametric Kruskal-Wallis coupled to U-Man-Whitney post-hoc analysis with Finner's correction was done. The level of significance was set at *p≤0.05, **p≤0.01, ***p≤0.001. The groups significantly different (p≤0.05) were indicated with different letters.

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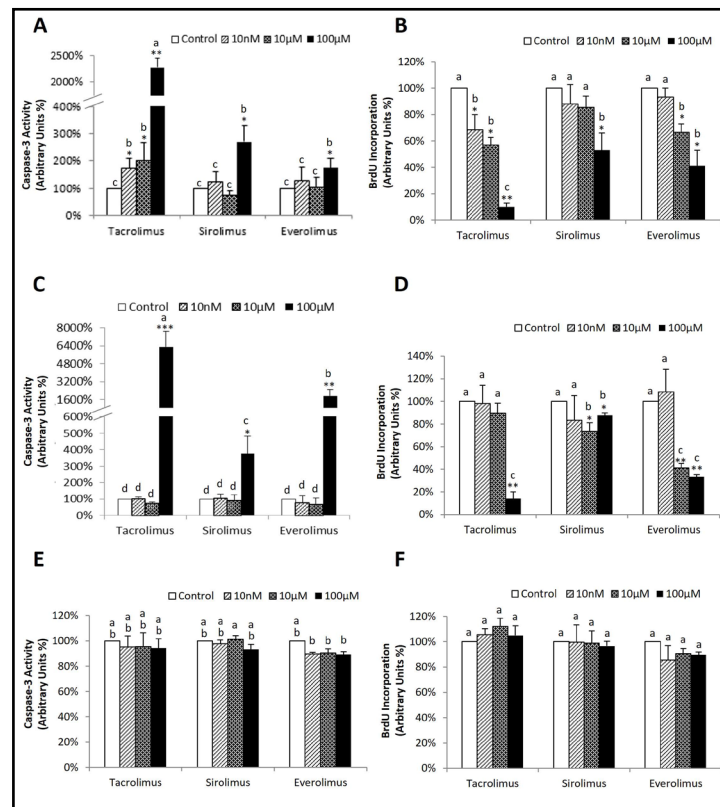Navarro-Villarán et al.: Role of Immunosuppressant and FK506-Binding Protein Complex in Liver Cancer

Results

The differential pro-apoptotic and anti-proliferative properties of Tacrolimus and mTOR inhibitors were associated with PERK-dependent activation of ER stress in liver cancer cells

Our present data confirm previous study showing that Tacrolimus exerted a more potent pro-apoptotic and anti-proliferative properties than mTOR inhibitors [13]. The induction of apoptosis was inversely correlated to the reduction of cell proliferation by Tacrolimus and mTOR inhibitors in HepG2 (Fig. 1A and 1B, respectively) and Huh7 (Fig. 1C and 1D, respectively). Primary human hepatocytes were resistant to the pro-apoptotic and anti-proliferative effects of the treatments (Fig. 1E and 1F, respectively). The ER is responsible for protein folding and regulation of intracellular calcium concentrations through the tightly regulation of three ER transmembrane

Fig. 1. Regulation of caspase-3 activity and BrdU incorporation in Tacrolimus-, Sirolimus- and Everolimus-treated HepG2 (A and B, respectively), Huh7 (C and D, respectively) and primary human hepatocytes (E and F, respectively). Apoptosis and cell proliferation were determined using commercial caspase-3 activity and BrdU incorporation assays respectively as described in Material and Methods. The variables were evaluated at 24 h after treatment administration (0, 10 nM, 10 μM, 100 μM). Results are expressed as mean ± SEM of five independent experiments. *p ≤ 0.05, **p ≤ 0.01 and ***p ≤ 0.001 between control and immunosuppressant-treated cells. The groups with different letters (a, b, c or d) were significantly different (p ≤ 0.05).

proteins: PERK, ATF6 and inositol-requiring enzyme 1 α (IRE1α) [14, 15]. Our data showed that Tacrolimus (100 μM), and to a lesser extent mTOR inhibitors (100 μM) strongly activated PERK as shown by the ^{Ser51}P-eIF2α/eIF2α ratio (Fig. 2A) and GADD153 (CHOP) protein expressions (Fig. 2B) in HepG2 cells. The impact of Tacrolimus and mTOR inhibitors in Huh7 cells followed the same pattern in ^{Ser51}P-eIF2α/eIF2α ratio and GADD153 protein expressions as that observed in HepG2 although at lower extend (Fig. 2C and 2D, respectively). The administration of the drugs does not alter ATF6- and IRE1α-dependent pathways in liver cancer cells (data not shown).

mTOR signaling appeared to be moderately downregulated in Huh7 than HepG2 cells

mTOR kinase activity induces 4E-BP1 phosphorylation, thus promoting CAP-dependent translation [16]. In agreement, the administration of high concentration of Tacrolimus (100 μM), and all tested concentrations of Sirolimus and Everolimus (10 nM, 10 μM and 100 μM) decreased $^{Thr37/Thr46}$P-4E-BP1/4E-BP1 ratio in HepG2 cells (Fig. 3A). The effect on this ratio was restricted to high drug concentration in Huh7 cells (Fig. 3B). However, the regulation of the $^{Thr37/Thr46}$P-4E-BP1/4E-BP1 ratio did not fully reflect the impact of polysome profile. As shown

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in Supplementary Fig. 1A, doses of the drugs below 100 µM did not significantly alter the polysome profiles in HepG2 cells (for all supplemental material see www.cellphysiolbiochem.com). In contrast, both Tacrolimus and mTOR inhibitors administered at high concentration (100 µM) resulted in abnormal polysome profiles with a dramatic increase of the 80S peak and a reduction of actively translating polysomes in HepG2 (Fig. 4A) and Huh7 (Fig. 4B). This result is consistent with a reduction of global translation in treated cells. Interestingly, salt treatment was not able to dissociate the 80S peak from treated cells in 40S and 60S subunits, strongly suggesting that this peak does not correspond to 80S vacant ribosome but to monosomes, in clear contrast to the result found for control cells (Supplementary Fig. 1B). Altogether, these results suggest that immunosuppressive drugs at high concentration halts early translation elongation phase in HepG2 cells. Differently, in Huh7 cells the polysome profile appeared to be already slightly
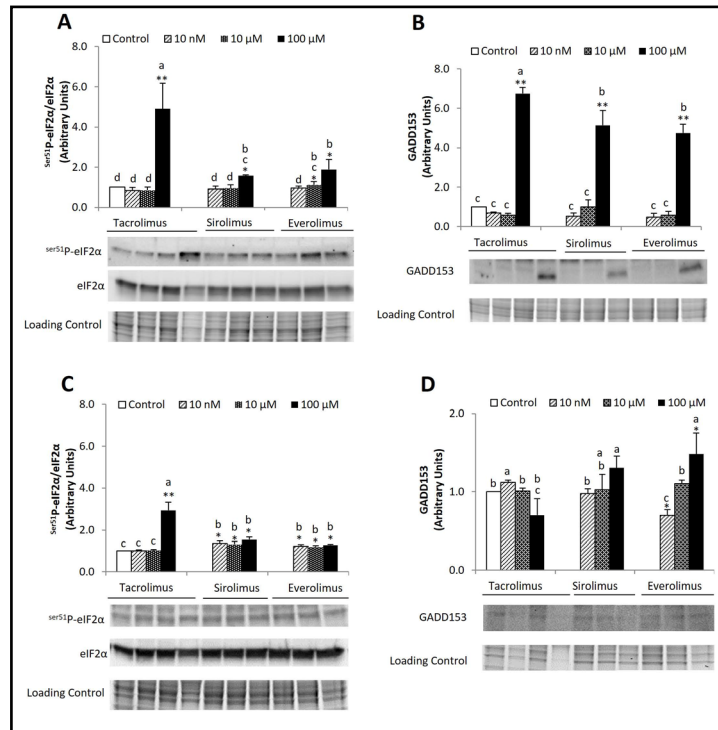
Fig. 2. ^{Ser51}P-eIF2α/eIF2α ratio and GADD153 protein expressions in Tacrolimus-, Sirolimus- and Everolimus-treated HepG2 (A and B, respectively) and Huh7 (C and D, respectively) cells. Treatments were administered at different concentrations (0, 10 nM, 10 µM, and 100 µM). The expression of ^{Ser51}P-eIF2α and eIF2α (3 h), and GADD153 (12 h) was evaluated by Western-blot analysis as described in Material and Methods. Results are expressed as mean ± SEM, and blots are representative of five independent experiments. *p ≤ 0.05 and **p ≤ 0.01 between control and immunosuppressant-treated cells. The groups with different letters (a, b, c or d) were significantly different (p ≤ 0.05).
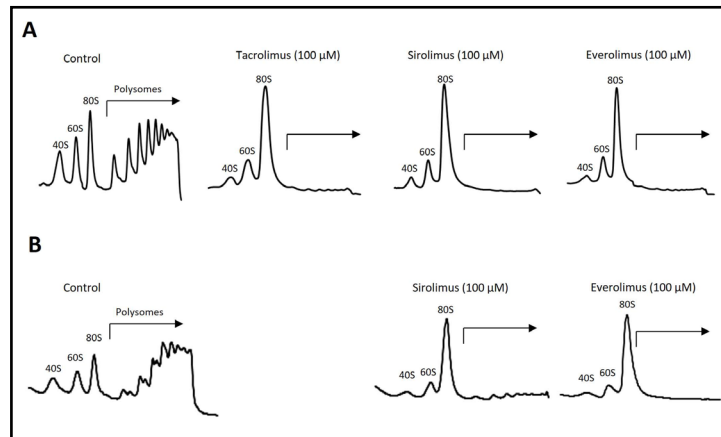
Fig. 3. $^{Thr37/Thr46}$P-4E-BP1/4E-BP1 ratio in Tacrolimus-, Sirolimus- and Everolimus-treated HepG2 (A) and Huh7 (B) cells. Treatments were administered at different concentrations (0, 10 nM, 10 µM, and 100 µM). The protein expression of $^{Thr37/Thr46}$P-4E-BP1/4E-BP1 (6 h) was determined by Western-blot analysis as
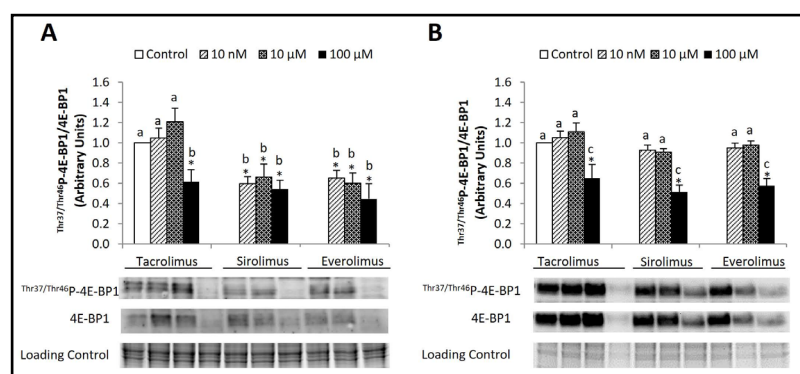

described in Material and Methods. Results are expressed as mean ± SEM, and blots are representative of four independent experiments. *p ≤ 0.05 between control and immunosuppressant-treated cells. The groups with different letters (a, b or c) were significantly different (p ≤ 0.05).

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Fig. 4. Polysome profile in Tacrolimus-, Sirolimus- and Everolimus-treated HepG2 (A) and Huh7 (B) cells. Treatments were administered at 100 μM. Polysome profiles (12 h) were obtained following the procedure described in Material and Methods. No polysome profile was observed in Tacrolimus-treated Huh7 cells. Results are expressed as mean ± SEM of three to four independent experiments.

Fig. 5. LC3II/LC3I ratio and Beclin protein expressions in Tacrolimus-, Sirolimus- and Everolimus-treated HepG2 (A and B, respectively) and Huh7 (C and D, respectively) cells. Treatments were administered at different concentrations (0, 10 nM, 10 μM, and 100 μM). The protein expression of LC3II, LC3I and Beclin (6 h) was determined by Western-blot analysis described in Material and Methods. Results are expressed as mean ± SEM, and blots are representative of three to five independent experiments. *p ≤ 0.05, **p ≤ 0.01, and ***p ≤ 0.001 between control and immunosuppressant-treated cells. The groups with different letters (a, b, c, d, or e) were significantly different (p ≤ 0.05).
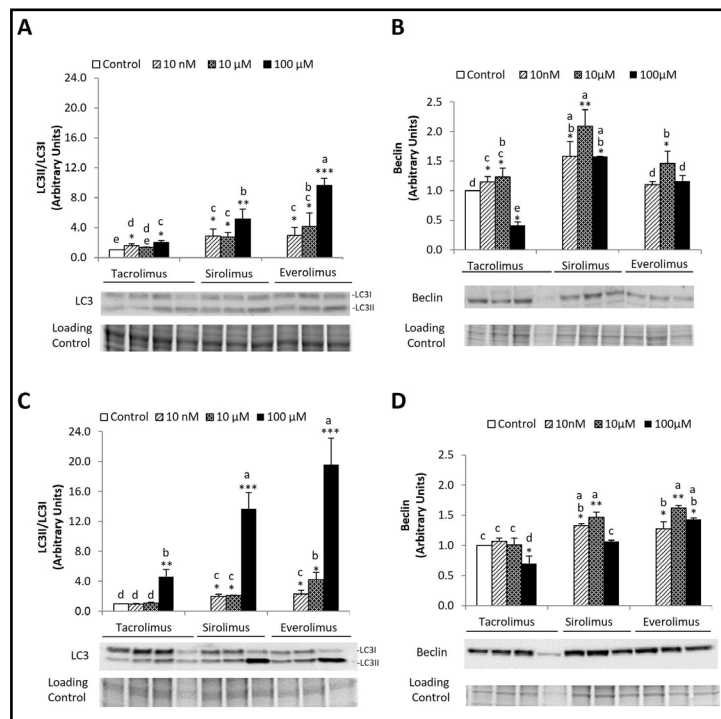

downregulated in the presence of low doses of mTOR inhibitors (10 μM) while still not altered at low doses of Tacrolimus (Supplementary Fig. 1C). No profile could be obtained for Huh7 cells treated with Tacrolimus at 100 μM concentration probably reflecting a relevant induction of cell death in these conditions (Fig. 4B).

The initiation of autophagy is controlled by the unc-51 like autophagy activating kinase 1 (ULK1)/ULK2 complex, which remains inhibited by mTOR, and is essential for the formation of the phagophore [17]. Thus, as a second readout of the mTOR pathway in hepatoma cells treated with Tacrolimus, Sirolimus and Everolimus, we determined the ratio of LC3II/LC3I and the levels of Beclin, two classical autophagy markers. The administration of mTOR inhibitors increased more significantly than Tacrolimus both parameters in HepG2 (Fig. 5A and 5B) and Huh7 (Fig. 5C and 5D). The LC3II/LC3I ratio appeared to be moderately increased in Huh7 (Fig. 5C) than that observed in HepG2 cells (Fig. 5A).

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Fig. 6. ^{Thr172}P-Cdk4/Cdk4 (A) and ^{Ser15}P-p53/p53 (B) ratios in Tacrolimus-, Sirolimus- and Everolimus-treated HepG2 cells; as well as p21 protein expression in Tacrolimus-, Sirolimus- and Everolimus-treated HepG2 (C) and Huh7 (D). Treatments were administered at different concentrations (0, 10 nM, 10 μM, and 100 μM). The protein expression of ^{Thr172}P-Cdk4 and Cdk4 (6 h), ^{Ser15}P-p53 and p53 (12 h), and p21 (12 h) was determined by Western-blot analysis. Results are expressed as mean ± SEM, and blots are representative of three to five independent experiments. *p ≤ 0.05, **p ≤ 0.01 and ***p ≤ 0.001 between control and immunosuppressant-treated cells. The groups with different letters (a, b, c, d or e) were significantly different (p ≤ 0.05).
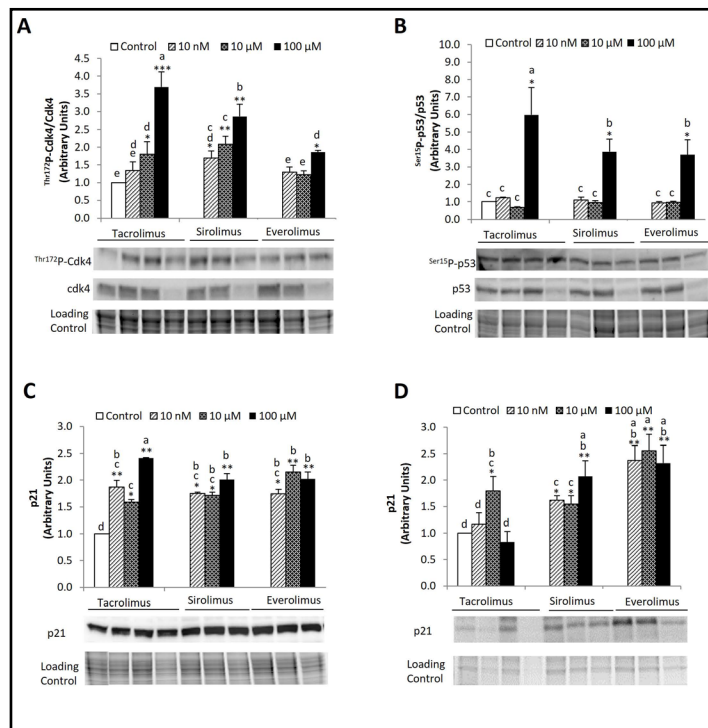

Regulation of cell cycle by Tacrolimus and mTOR inhibitors

We have previously shown that the anti-proliferative properties of Tacrolimus were related to a strong cell cycle arrest at G0/G1 phase in HepG2 cells [13]. The regulation of Cdk4/cyclin D complex and the phosphorylation of retinoblastoma protein (pRB) plays a fundamental role in activating E2F transcription factors, G1/S-phase gene expression and growth control [18, 19]. Calcineurin downregulates Cdk4 phosphorylation and its kinase activity [20]. The administration of Tacrolimus drastically upregulated the ^{Thr172}P-Cdk4/Cdk4 ratio in HepG2 cells (Fig. 6A). Sirolimus and Everolimus were also able to upregulate ^{Thr172}P-Cdk4/Cdk4 but to a lower extent than Tacrolimus (Fig. 6A). p53 is a key transcriptional factor involved in cell cycle arrest mainly through p21 upregulation leading to induction of apoptosis [21]. The expression of wild type p53 was lower in Huh7 compared to that observed in HepG2 cells (Supplementary Fig. 2). The activation of p53 involves its phosphorylation at Ser15. The ^{Ser15}P-p53/p53 ratio was increased after administration of the high concentrations of Tacrolimus and mTOR inhibitors in HepG2 cells (6- and 4-folds, respectively) (Fig. 6B). The pattern of inducibility of p21 by treatments were similar in HepG2 and Huh7 cells (Fig. 6C and 6D). The increase of ^{Ser15}P-p53/p53 ratio (Fig. 4B) and p21 (Fig. 4C) expression may counterbalance the potential pro-proliferative effect caused by ^{Thr172}P-Cdk4/Cdk4 upregulation (Fig. 4A) in HepG2 cells. The expression of p21 in Huh7 (Fig. 6D) was lower than that observed in HepG2 (Fig. 6C). However, a similar p21 pattern of expression upon treatments was observed in both liver cancer cell lines (Fig. 6C and 6D).

Role of FKBPs in the regulation of cell death and proliferation in drug-treated liver cancer cells

FKBPs play a relevant role in physiopathology, cancer and chemoresistance [5]. The administration of low (10 nM-10 μM) doses of Tacrolimus and mTOR inhibitors moderately but significantly increased the protein expression of FKBP12 (Fig. 7A) and FKBP51 (Fig. 7B) in HepG2 cells. However, FKBP12 (Fig. 7C) and FKBP51 (Fig. 7D) remained close to control levels upon the administration of low doses of Tacrolimus and mTOR inhibitor in Huh7 cells. The expression of FKBP38 and FKBP52 did not changed with the treatments (data not shown). In order to further identify the role of FKBP12 and FKBP51 in our system siRNA

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Fig. 7. FKBP12 and FKBP51 protein expressions in Tacrolimus-, Sirolimus- and Everolimus-treated HepG2 (A and B, respectively) and Huh7 (C and D, respectively) cells. Treatments were administered at different concentrations (0, 10 nM, 10 µM, and 100 µM). The protein expression of FKBP12 and FKBP51 was evaluated by Western-blot analysis as described in Material and Methods. Results are expressed as mean ± SEM, and blots are representative of four to six independent experiments. *$p \le 0.05$ between control and immunosuppressant-treated cells. The groups with different letters (a, b, c or d) were significantly different ($p \le 0.05$).
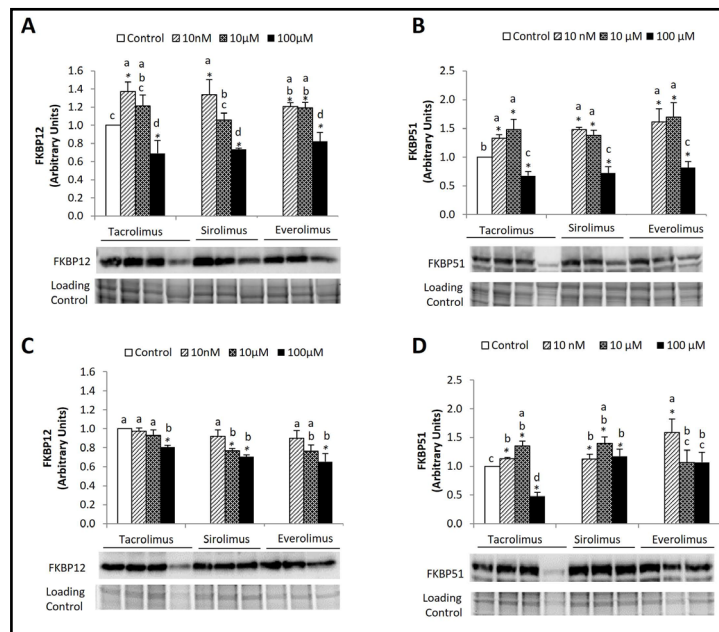

Fig. 8. Impact of FKBP12 downregulation on LC3II/LC3I ratio (A) and caspase-3 activity (B) in Tacrolimus-, Sirolimus- and Everolimus-treated HepG2 cells. The downregulation of FKBP12 was carried using siRNA technologies. Autophagy and apoptosis were determined using the measurement of LC3II/LC3I ratio and commercial caspase-3 activity assay, respectively as described in Materials and Methods. The protein expression of LC3II and LC3I was evaluated by Western-blot analysis as described in Material and Methods. Results are expressed as mean ± SEM of six independent experiments. *$p \le 0.05$ and **$p \le 0.01$ between control and immunosuppressant-treated cells. The groups with different letters (a, b, c, d, e or f) were significantly different ($p \le 0.05$).
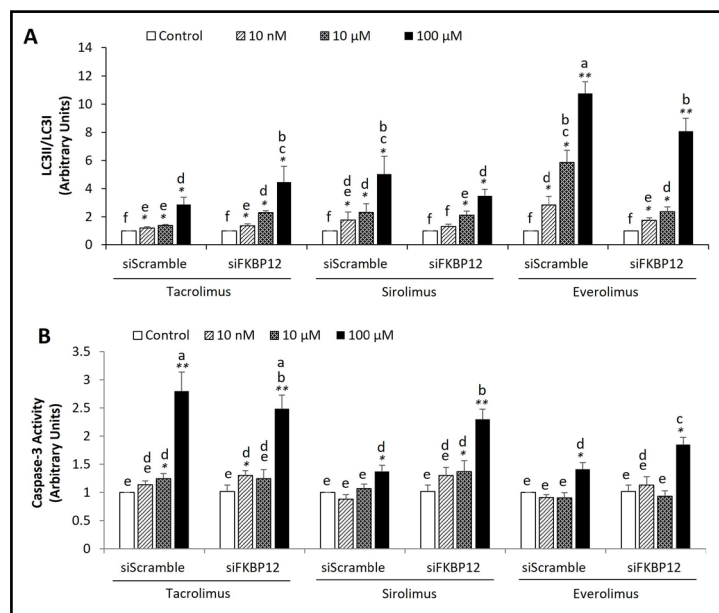

technology was applied in HepG2 cells. Interestingly, the knockdown expression of FKBP12 by siRNA increased the LC3II/LC3I ratio (Fig. 8A) in Tacrolimus-treated HepG2 cells, while reduced this ratio and increased caspase-3 activity (Fig. 8B) in HepG2 cells treated with Sirolimus and Everolimus. The knockdown expression of FKBP51 by siRNA reduced BrdU incorporation (Fig. 9A) in control and drug-treated HepG2 cells, and increased caspase-3 activity (Fig. 9B) in drug-treated HepG2 cells. The application of siRNA strategies showed that FKBP12 and FKBP51 mediate cell survival in immunosuppressive-treated HepG2 cells.

Fig. 9. Impact of FKBP51 downregulation on BrdU incorporation (A) and caspase-3 activity (B) in Tacrolimus-, Sirolimus- and Everolimus-treated HepG2 cells. The downregulation of FKBP51 was carried using siRNA technologies. Cell proliferation and apoptosis were determined using commercial BrdU incorporation and caspase-3 activity assays respectively as described in Material and Methods. Results are expressed as mean ± SEM of six independent experiments. *p ≤ 0.05 and **p ≤ 0.01 between control and immunosuppressant-treated cells. The groups with different letters (a, b, c, d, e or f) were significantly different (p ≤ 0.05).
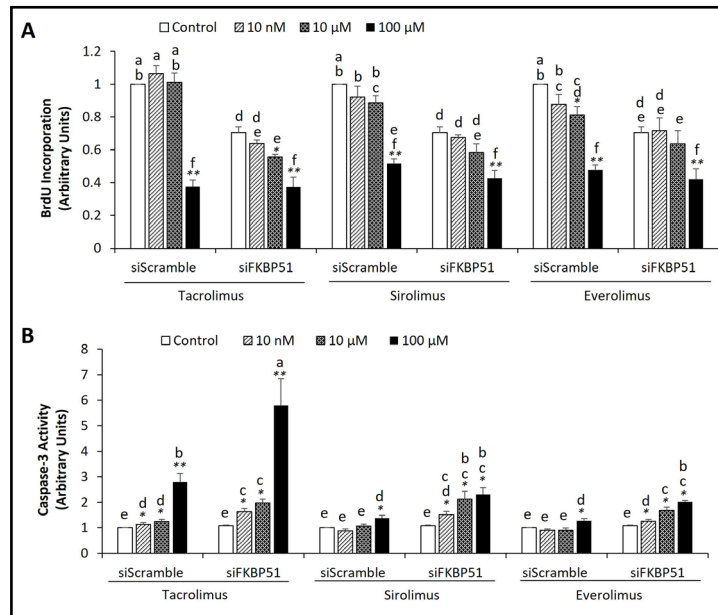
Fig. 10. Up- (red) and down-regulation (green) of miRNA expression in Tacrolimus-, Sirolimus- and Everolimus-treated HepG2 cells. Treatments were administered at 10 μM final concentration. The expression of miRNA was analysed using a commercial TaqMan® OpenArray® Human miRNA Panel as described in Material and Methods. miRNA data are obtained from three independent experiments.
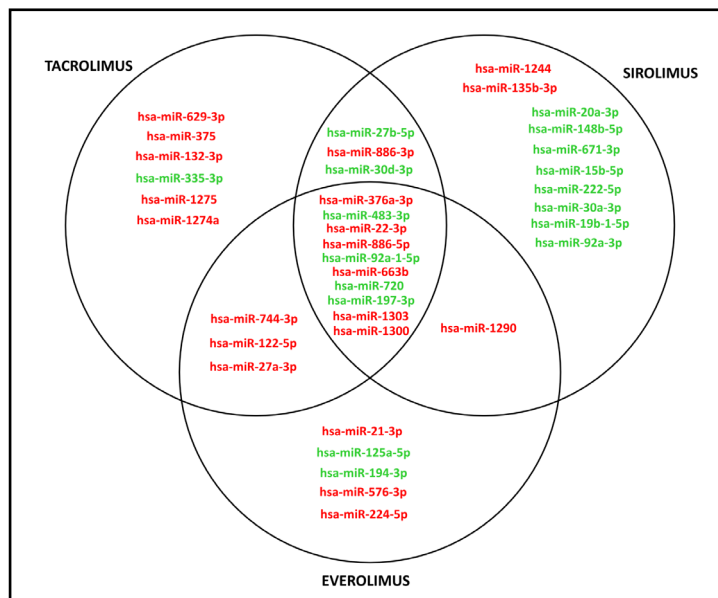
The immunosuppressants drastically altered miRNA expression pattern in HepG2 cells but not in primary human hepatocytes

miRNAs are non-coding short RNA sequences that normally destabilize and/or prevent translation of complementary target mRNAs [22]. The level of expression and activity of critical components of miRNA biogenesis and miRNAs themselves are profoundly altered in all types of cancers [23]. We assessed whether Tacrolimus, Sirolimus and Everolimus were able to alter miRNA expression pattern in primary human hepatocytes and liver cancer cells. As shown in Fig. 10, the induction of apoptosis and reduction of cell proliferation by Tacrolimus, Sirolimus and Everolimus were associated with a decrease of miR-92a-1-5p, miR-197-3p, miR-483-3p and miR-720, and an increase of miR-22-3p, miR-376a-3p, miR-663b, miR-886-5p, miR-1300 and miR-1303 in HepG2 cells (Fig. 10 and Supplementary Table 1). Differently, primary human hepatocytes were highly unresponsiveness to alteration

of miRNA expression by the treatments. In this sense, Tacrolimus only downregulated miR-151a-5p expression (0.369 fold-change, $p \leq 0.042$), while Sirolimus upregulated miR-1291 expression (2.838 fold-change, $p \leq 0.038$) in primary human hepatocytes.

Discussion

HCC is the primary indication for OLT, and the tumor recurrence, which is estimated 8 %-20 % in different studies, is the main drawback for the survival of patients [24]. The administration of immunosuppression therapies, such as calcineurin inhibitors (Cyclosporin A and Tacrolimus) or mTOR inhibitors (Sirolimus and Everolimus), are required to avoid graft rejection in organ transplantation. Tumor-related variables appear to be widely related to recurrence [25]. However, controversial reports exist in relation to the involvement of immunosuppression regimes in tumor recurrence. Cyclosporine A and Tacrolimus have been suggested to be related to increased risk of tumor recurrence post-OLT [26, 27], in contrast to the immunosuppression based on mTOR inhibitors [28-32]. However, the five-year disease-free survival between two cohorts receiving or not Sirolimus regimes following OLT showed no differences in a prospective phase III multicenter randomized-controlled trial [33]. The induction of apoptosis and cell cycle arrest induced by drugs are key features for their antitumoral properties. In this sense, we [13], and others [34-37], have showed that Tacrolimus and mTOR inhibitors promote apoptosis and reduce cell proliferation in cancer cells. In concordance, the present study showed that the administration of Tacrolimus, and to a lesser extent of Sirolimus and Everolimus, greatly increased apoptosis and reduced cell proliferation in HepG2 and Huh7 cells (Fig. 1). Interestingly, this pro-apoptotic and anti-proliferative properties were limited to liver cancer cells, as primary human hepatocytes were unresponsiveness to the different immunosuppressant treatments (Fig. 1).

The ER is highly sensitive to alterations in cellular homeostasis and provides quality control program ensuring that only correctly folded proteins transit to the Golgi and unfolded or misfolded proteins are degraded. The selective upregulation of ER transmembrane proteins PERK, ATF6 or IRE1α induces the activation of unfolded protein response (UPR) that involves transcriptional recovery response, transcriptional induction response of ER chaperones, transcriptional induction response of amino acid transporter and activation of nuclear factor kappa light chain enhancer of activated B cells (NF-κB), and apoptotic response [38]. The main effector of PERK-mediated apoptosis is the transcription factor GADD153 (CHOP) which can be induced through all three ER branches [39]. In our conditions, the administration of Tacrolimus, and mTOR inhibitors to a lesser extent, induced a significant PERK activation measured by ATF4-dependent increase of ^{Ser51}P-eIF2α/eIF2α that led a potent upregulation of GADD153 in HepG2 cells (Fig. 2A and 2B, respectively). Huh7 cells showed a lower responsiveness to these drugs compared to that observed in HepG2 cells (Fig. 2C and 2D versus Fig. 2A and 2B, respectively). The increased upregulation of PERK activation of Tacrolimus versus mTOR inhibitors (Fig. 2) was related to the higher pro-apoptotic and anti-proliferative properties in HepG2 and Huh7 cells (Fig. 1).

FK506-binding domain (FKBD) of FKBP12 binds to Tacrolimus and mTOR inhibitors [6]. The complex generated upon the binding of Sirolimus and Everolimus to FKBP12 interacts with the FKBP-rapamycin binding domain in mTOR, adjacent to the catalytic kinase domain, and blocks its function [40]. The activation of mTOR induces phosphorylation of p70S6K1 and 4E-BP1, and promotes mRNA translation and cell cycle progression [41]. Although mTOR inhibitors reduced around 50 % control $^{Thr37/Thr46}$P-4E-BP1/4E-BP1 in HepG2 cells at any dose tested (Fig. 3A), the global mRNA translation visualized via the analysis of the polysome profiles was only altered at high concentration of the drugs (100 μM) (Fig. 4A) when apoptosis were sharply induced (Fig. 1A). In Huh7 cells, translation is already moderately impaired at a low concentration of mTOR inhibitors but not of Tacrolimus (Supplementary Fig. 1), despite the lack of inhibitory effect on the $^{Thr37/Thr46}$P-4E-BP1/4E-BP1 ratio (Fig. 3B). Interestingly, the negative effect of Tacrolimus or mTOR inhibitors on mRNA translation was characterized by

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an increase of the 80S peak and a reduction of actively translating polysomes in HepG2 and Huh7 (Fig. 4A and 4B, respectively). The lack of effectiveness of salt treatment suggested the absence of vacant 80S ribosomes that it is compatible with the blockage of translation at early elongation phase instead of alteration within the initiation of the translation phase in drugs (100 µM)-treated HepG2 cells (Supplementary Fig. 1B). Altogether, these results strongly suggest that other mechanisms different than the activation of the 4E-BP1 repressor protein might be responsible of the impairment of translation.

mTOR inhibits the formation of the initial membrane nucleation of autophagic process through the regulation of a protein complex composed of ULK1, autophagy-related gene 13 (ATG13), and focal adhesion kinase family-interacting protein of 200 kDa (FIP200) [42-44]. The inhibition of mTOR by Sirolimus and Everolimus upregulated the expression of LC3II/LC3I ratio and Beclin in both HepG2 (Fig. 5A and 5B) and Huh7 (Fig. 5C and 5D) cell lines. Interestingly, Tacrolimus was also able to moderately increase autophagic markers which is consistent with the PERK/eIF2α activation signaling and ER-stress-induced autophagy in the two different hepatoma cells lines tested (Fig. 2 and Fig. 5) [45].

Tacrolimus, and to a lesser extent mTOR inhibitors, increased ^{Thr172}P-Cdk4/Cdk4 (Fig. 6A) that could be explained by the Calcineurin inhibition by the immunosuppressive agents [20]. This observation might highlights the potential common impact of Tacrolimus and mTOR inhibitors on Calcineurin activity in contrast to the previous observation in which it was suggested a relevant antagonism of Sirolimus and Everolimus on Tacrolimus-induced Calcineurin inhibition via saturation of FKBP12 in peripheral blood mononuclear cells [46]. The inhibition of Calcineurin-dependent phosphatase activity by Tacrolimus and Cyclosporine increased Cdk4-related activity and cell cycle progression in Jurkat cells [20]. In our experimental setting, the potential risk of pro-proliferative effect of Tacrolimus and mTOR inhibitors by upregulation of Cdk4 phosphorylation, can be counterbalance by the up-regulation of p21 protein expression in the both cell hepatoma cell lines tested (Fig. 6C and 6D). p21 is a universal Cdk inhibitor whose gene expression is upregulated by p53 [47]. It is worthy to mention that p21 expression was also upregulated in Huh7 (Fig. 6D) although a strong reduction of p53 expression was observed in Huh7 compared to that observed in HepG2 (Supplementary Fig. 2).

FKBPs are involved in relevant cellular functions, and play a role in cancer and chemoresistance [5]. The attenuation of NFAT transcriptional activity by Tacrolimus downregulates the expression of downstream target genes such as cyclooxygenase-2 and c-MYC, thus resulting in decreased cell viability/colony formation, cell migration/invasion, and increased apoptosis in bladder cancer cell lines [48]. Several reports have suggested that Sirolimus exerts potent anticancer effects in addition to is immunosuppressant activity [49, 50]. Our study showed that the expression of FKBP12 and FKBP51 was increased in HepG2 cells (Fig. 7A and 7B), but at lower extend in Huh7 (Fig. 7C and 7D), by the administration of low concentrations of the treatments. Tacrolimus and mTOR inhibitors can bind FKBP12 through FKBD [6]. The downregulation of FKBP12 by siRNA strategy increased autophagy in Tacrolimus-treated HepG2 cells, while reduced autophagy and increased apoptosis in mTOR inhibitor-treated HepG2 cells (Fig. 8). The downregulation of FKBP51 reduced cell proliferation in control and immunosuppressant-treated HepG2 cells, while increased apoptosis in immunosuppressant-treated HepG2 cells (Fig. 9). These results suggest that FKBP12 and FKBP51 play a general pro-survival role in liver cancer cells.

miRNAs are small non-coding RNAs of ~22-nucleotides which mediate destabilization and/or translational suppression of target mRNAs bearing partially complementary sequences [22]. The level of expression and activity of different components of the miRNA biogenesis pathway are often found to be dysregulated in cancer [23]. No reports exist in the literature identifying the alteration of miRNA expression caused by Tacrolimus and mTOR inhibitors. The induction of apoptosis and reduction of cell proliferation by Tacrolimus, Sirolimus and Everolimus (10 µM) were associated with a decrease in the expression of miR-92a-1-5p, miR-197-3p, miR-483-3p and miR-720 expressions, while an increase in the expression of miR-22-3p, miR-376a-3p, miR-663b, miR-886-5p, miR-1300 and miR-1303 expression in

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HepG2 cells (Fig. 10 and Supplementary Table 1). Very few reports exist identifying the role of the above miRNAs in other settings. In this sense, miR-483-3p is an oncogenic miRNA that affects Wnt/β-catenin, TGF-β, and TP53 signaling pathways by targeting several genes as *CTNNB1*, *SMAD4*, *IGF1*, and *BBC3* in different tumor cells [51]. The downregulation of miR-483-3p by immunosuppressants might be relevant for its antitumoral properties. By contrast, the upregulation of miR-663b [52], miR-22-3p [52] and miR-1303 [53] has been related to oncogenic properties in non-hepatic tumor cells. More studies are required to understand the precise contribution of miRNAs in the cellular response observed upon the treatments with Tacrolimus and mTOR inhibitors in hepatoma cells.

Conclusion

Tacrolimus exerted a more potent pro-apoptotic and anti-proliferative properties than mTOR inhibitors that appeared to be associated with an increased PERK-related induction of ER stress and activation of the p53-p21 cell signaling. FKBP12 and FKBP51 appeared to be the most relevant FKBP partners of immunosuppressants being both related to pro-survival effect in HepG2 cells. FKBP12 and FKB51 appeared to be related to the regulation of cell death or cell proliferation, respectively. A pattern of miRNA expression was observed independently of the administered immunosuppressants in liver cancer cells.

Abbreviations

Autophagy-related gene 13 (ATG13); Barcelona Clinic Liver Cancer (BCLC); Bromodeoxyuridine (BrdU); C/EBP homologous protein (CHOP/GADD153); Endoplasmic reticulum (ER); FK506-binding domain (FKBD); FK506-binding protein (FKBP); Focal adhesion kinase family-interacting protein of 200 kDa (FIP200); Hepatocarcinoma (HCC); Inositol-requiring enzyme 1 α (IRE1α); Mammalian target of rapamycin (mTOR); MicroRNAs (miRNAs); Nuclear factor of activated T cells (NFAT); Nuclear factor kappa light chain enhancer of activated B cells (NF-κB); Ortothopic liver transplant (OLT); Peptidylprolyl cis/trans isomerase (PPIase); Performance status (PS); Protein kinase RNA-like endoplasmic reticulum kinase (PERK); Retinoblastoma protein (pRB); Unfolded protein response (UPR); Unc-51 like autophagy activating kinase 1 (ULK1).

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Disclosure Statement

The authors have no conflicts of interest to declare.

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