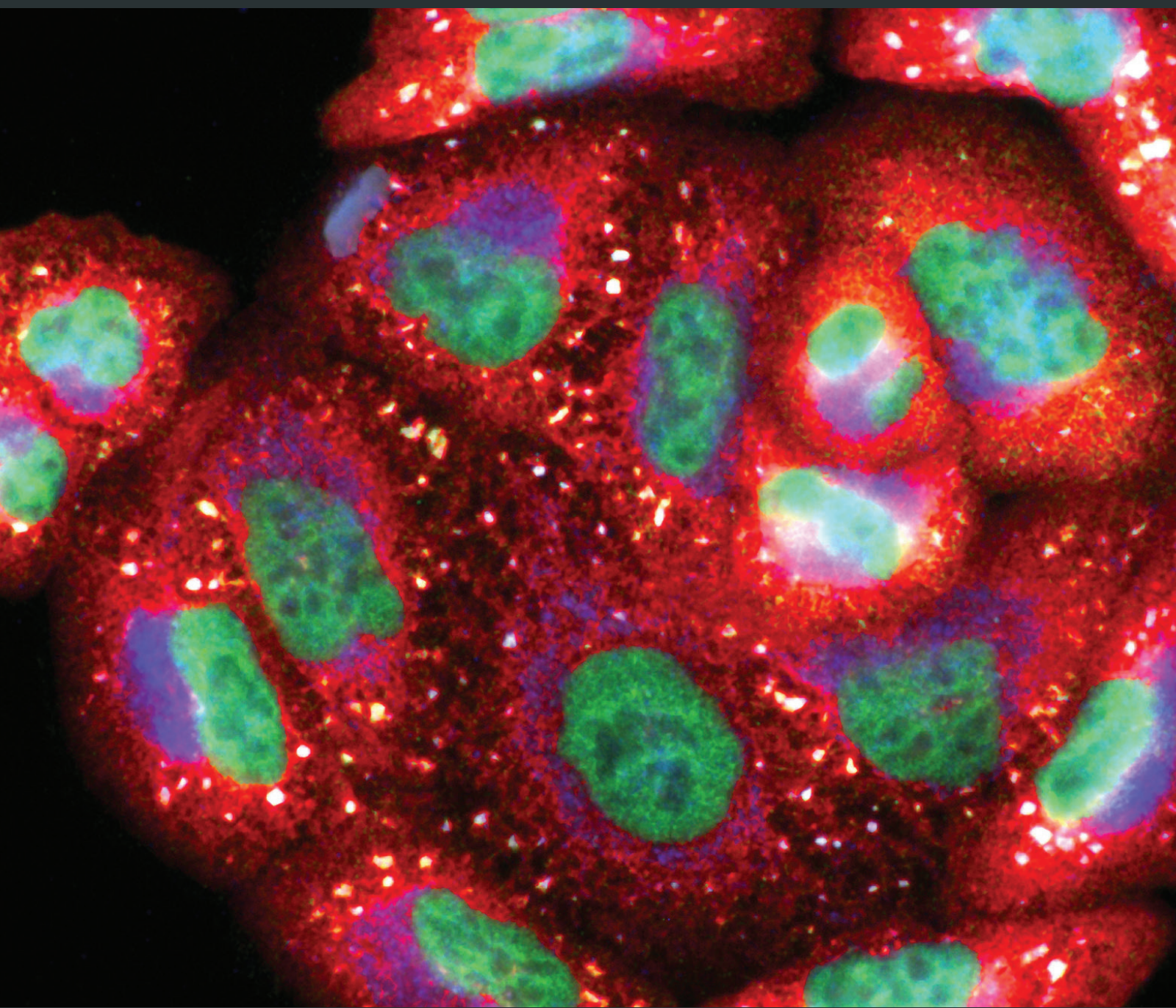
Oxidative Medicine and Cellular Longevity

mTOR Signaling in Cardiometabolic Disease, Cancer, and Aging 2018

Lead Guest Editor: Anindita Das
Guest Editors: Flávio Reis and Paras Kumar Mishra



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Editorial Board

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Editorial

mTOR Signaling in Cardiometabolic Disease, Cancer, and Aging 2018

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The mammalian target of rapamycin (mTOR), an atypical multidomain serine/threonine kinase of the phosphoinositide 3-kinase (PI3K)-related kinase family, elicits a significant role in diverse signaling cascades responsive to changes in intracellular and environmental conditions. Activation of mTOR has been implicated in an increasing number of pathological conditions, including cancer, obesity and diabetes, cardiovascular diseases, and neurodegenerative disorders. Based on its pathophysiological importance, the mTOR signaling pathway has attracted unprecedented attention among basic scientists and clinicians. Growing empirical evidences demonstrate the pivotal role of mTOR signaling in oxidative stress, aging, proliferative disorders, and metabolic abnormalities. The current special issue is aimed at bringing together both original research papers (7 articles) and review articles (4 articles) to advance our understanding of mTOR signaling pathways in metabolic and cardiovascular diseases, cancer, muscle toxicity, and aging (Figure 1). Internationally recognized experts highlighted the distinct role of mTOR signaling in cardiovascular and metabolic diseases as well as cancer and neuronal tissue with insightful presentations to enrich our knowledge in emerging therapeutic application of mTOR inhibitors. Specific contributions to this special issue are summarized below.

In the previous special issue on "mTOR Signaling in Cardiometabolic Disease, Cancer, and Aging, 2017," Dr. Pulakat's group reported the chronic treatment with rapamycin (Rap, a mTORC1 inhibitor) reduced the obesity and cardiac fibrosis in Zucker obese rats (ZO-C), while increasing their blood glucose levels. In contrast, rapamycin treatment induced cardiac fibrosis in heathy Zucker lean (ZL) rats, suggesting that mTORC1 inhibition exerts differential effects on diabetic versus healthy hearts. In the present study, A. M. Belenchia et al. demonstrated differential expression profiles of cardiac miRNAs between control and rapamycin-treated ZO and ZL rats to evaluate the mechanisms underlying adverse effects of rapamycin. They reported that 47% of rapamycin-induced cardiac miRNA transcriptome in healthy rats (ZL-Rap) are identical to 80% of the diabetes-induced cardiac miRNA transcriptome (ZO-C), which might be responsible for the rapamycin-induced insulin resistance. Using *in silico* analyses, the authors presented the interactions between differentially expressed cardiac cytokines and miRNAs, which might reflect both diabetes- and rapamycin-induced immune suppression. Several differentially expressed miRNA transcriptomes also serve as an adaptive mechanism to regulate cardiac fibrosis. This study provides a new insight for developing novel drugs, which can ameliorate the adverse effects of long-term treatment with rapamycin.

H. Merino and D. K. Singla reported the molecular mechanism underlying doxorubicin-induced apoptosis in soleus muscle using C57BL/6 mice. Their results suggest that

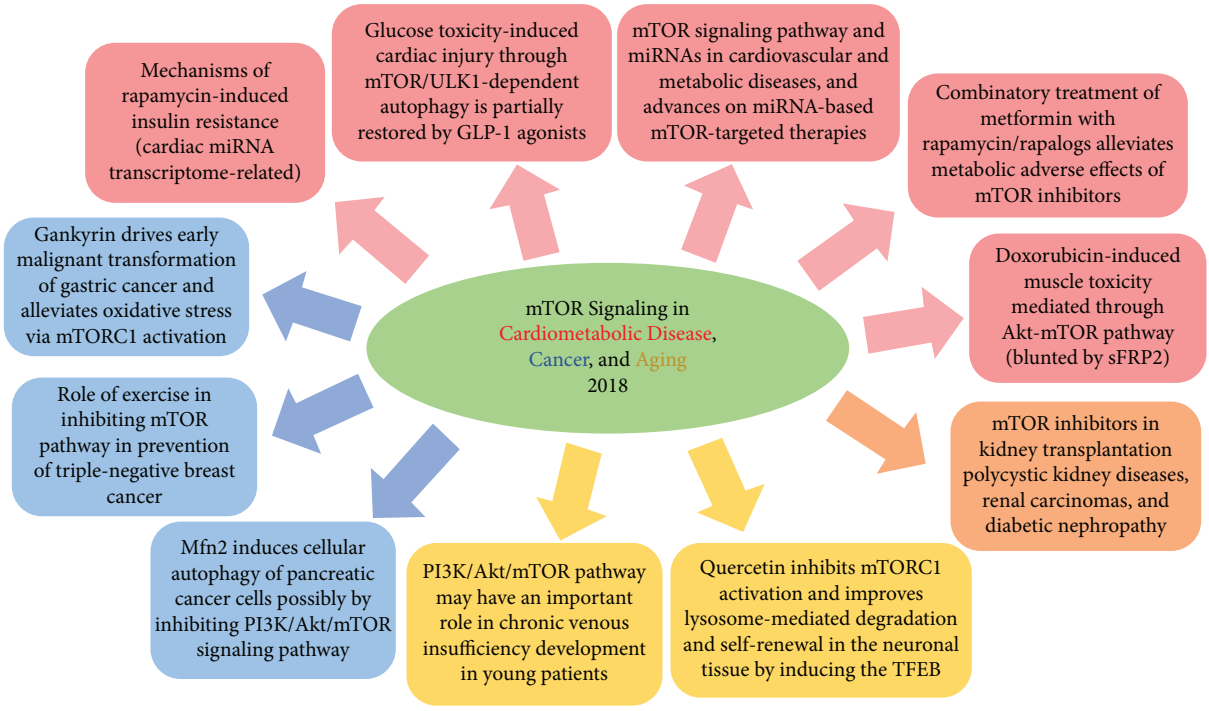

Figure 1: Key contents of all articles in the special issue on "mTOR Signaling in Cardiometabolic Disease, Cancer, and Aging 2018."

doxorubicin treatment increases oxidative stress and apoptosis. Notably, it decreases antioxidants and antiapoptotic proteins, which are mediated through the Akt-mTOR pathway. Interestingly, tail vein injections of secreted frizzled-related protein-2 (sFRP2) blunt the detrimental effects of doxorubicin. Accordingly, they concluded that sFRP2 might be a valuable therapeutic candidate for doxorubicin-induced muscle toxicity.

Y. Huang et al. provided a mechanistic evidence of the beneficial effects of quercetin, a natural polyphenolic compound, in the neuronal tissue. They found that quercetin improves lysosome-mediated degradation and self-renewal in the neuronal tissue by inducing the nuclear translocation of transcription factor EB (TFEB). TFEB controls lysosome biogenesis, autophagy, and cellular trafficking in the phagocytic cells, like the retinal pigment epithelium (RPE). mTOR phosphorylates TFEB at its C-terminal serine-rich motif and thereby sequesters TEFB in the cytoplasm. Quercetin directly inhibits mTORC1 activation possibly by acting as a competitive mTOR kinase inhibitor at the ATP-binding motif; however, it does not influence the activity of Akt.

B. Huang et al. evaluated the relevance of gankyrin, a molecular chaperone that acts on assembly of 26S proteasome, specifically the 19S regulatory complex, in gastric cancer, a malignant epithelial tumor usually asymptomatic until late diagnosis associated with poor overall survival. Using samples of malignant infiltrating gastric cancer tissues and paired noncancerous tissues obtained from patients, as well as two gastric cancer cell lines, they suggested that gankyrin drives early malignant transformation of gastric cancer and alleviates oxidative stress via mTORC1 activation. The authors suggested that increased gankyrin expression could be a biomarker for early diagnosis of gastric cancer, which could be the risk factor of gastric cancer in patients with precancerous lesions such as dysplasia and intestinal metaplasia.

M. A. Ortega et al. performed an observational, analytical, and prospective cohort study on young (less than 50 years) and aged (more than 50 years) patients with and without valvular incompetence (venous reflux, which leads to chronic venous insufficiency, CVI). Their study was focused on the PI3K/Akt/mTOR pathway and inflammatory process by measuring the levels of CD4+, CD8+, and CD19+ cells. They also measured the levels of hypoxia-inducible factor-1α (HIF-1α) and HIF-2α expressions, which are induced in the heart deprived of oxygen supply. Their results showed an increased activity of the PI3K/Akt/mTOR pathway and upregulation of HIF-1α, CD4+, and CD8+ in young patients with valvular incompetence. It suggests that the PI3K/Akt/mTOR pathway may have an important role in CVI development in young patients.

W. Yu et al. determined the effect of exendin-4 and liraglutide, two glucagon-like peptide-1 (GLP-1) agonists, on glucose toxicity-induced cardiac injury through mTOR/ULK1-dependent autophagy. They treated primary cardiomyocytes from adult mice and H9C2 cardiomyocytes with high or normal dose of glucose with or without exendin-4 or liraglutide. They found that high-glucose treatment decreased cardiomyocyte contractility, which was partly restored by GLP-1 agonist treatment. GLP-1 agonist also rescued cardiomyocytes from glucose toxicity by inducing autophagy.

Mitofusin 2 (Mfn2), an outer mitochondrial membrane GTPase, is critical for mitochondrial fusion, which controls mitochondrial dynamics, distribution, and function within the cell. Intriguingly, emerging evidences identify the key role of Mfn2 in the onset/progression of different pathological conditions, including cancer. In this special issue, R. Xue

et al. demonstrated that the overexpression of Mfn2 in pancreatic cancer cells inhibits proliferation and ROS generation, while inducing apoptosis. Mfn2 induces cellular autophagy of pancreatic cancer cells possibly by inhibiting the PI3K/Akt/mTOR signaling pathway. Authors found that pancreatic cancer patients with Mfn2-positive expression have significantly longer survival time than those with Mfn2-negative expression. Based on the bioinformatics analysis, they suggested that Mfn2 might be a potential therapeutic target in pancreatic cancer.

In the minireview article, A. Kezic et al. briefly summarized the metabolic adverse side effects (hyperglycemia, insulin resistance, and dyslipidemia) of chronic treatment with mTOR inhibitors (like macrolide rapamycin or other rapalogs), especially in patients with organ transplantation or cancer. The chronic pharmacological inhibition of activated mTOR may deteriorate the systemic metabolism in diabetes mellitus due to the pleiotropic effects of mTOR. Acute treatment with rapamycin or rapalogs specifically inhibits mTORC1 activity, without interfering the mTORC2 activity. However, a prolonged exposure of rapamycin or rapalogs leads to the suppression of mTORC2/Akt signaling, with consequent insulin resistance and insufficient immunosuppression. The authors compared the metabolic consequences of the chronic treatment with mTOR inhibitors with the metabolic profile provoked by metformin, a widely prescribed antidiabetes drug. Based on the literature, the authors proposed to use rapamycin/rapalogs in combination with metformin to induce AMPK activity, which might be a better therapeutic intervention to reduce the dose of rapamycin/rapalogs as well as associated adverse metabolic effects after solid organ transplantations.

A. Samidurai et al. comprehended our recent knowledge in the mechanisms of interactions between the mTOR signaling pathway and miRNAs (a class of short noncoding RNA) in cardiovascular diseases, like myocardial infarction, vascular remodeling and hypertrophy, heart failure, arrhythmia, and atherosclerosis. The authors also summarized the critical roles of miRNAs in the regulation of mTOR signaling in cardiovascular disease-associated risk factors, including diabetes and obesity. The review highlighted the latest advances on mTOR-targeted therapy and interactions of mTOR with miRNAs in clinical trials, which encourages us in exploring the novel therapeutics for heart disease with a unique perspective. Advancing our knowledge in the interplay between mTORC1 and mTORC2 complexes and its association with miRNAs could lead to the development of an efficient miRNA-based therapeutics and diagnostics for cardiovascular diseases.

S. D. Viana et al. focused their review article on the advances, drawbacks, and challenges regarding the use of mTOR inhibitors in four major classes of renal interventions/diseases: (1) kidney transplantation, (2) polycystic kidney diseases, (3) renal carcinomas, and (4) diabetic nephropathy. In this comprehensive review, the authors briefly revisited the mTOR components and signaling pathways and then addressed the pharmacological armamentarium targeting the mTOR pathway currently available in research and development stages, covering different generations of mTOR inhibitors and complementary approaches (allosteric mTOR inhibitors: rapamycin/rapalogs; dual PI3K/mTOR inhibitors; ATP-competitive inhibitors: mTOR kinase inhibitors; and new-generation drugs, namely, RapaLink-1). After a concise revision on the physiological role of mTOR in the kidney, S. D. Viana et al. critically reviewed the therapeutic use of mTOR inhibitors in the aforementioned renal conditions, using a translational perspective from preclinical data to current clinical applications. The authors concluded that although mTOR inhibitors (specifically rapamycin and everolimus) have been successfully used as immunosuppressive therapy for the prevention of allograft rejection, namely, in renal transplantation, further preclinical (and particularly clinical) data are still needed to understand the putative benefits of mTOR inhibitors against polycystic kidney diseases, renal carcinomas, and diabetic nephropathy.

D. Agostini et al. present a review article with the updated discoveries regarding the role of exercise in inhibiting the mTOR pathway in triple-negative breast cancer (TNBC), which is an aggressive carcinoma and has poor response to available chemotherapies. TNBC is associated with early recurrences. The authors focused on the biological mechanisms putatively involved in TNBC, including microRNAs. They also discussed the benefits evoked by distinct exercise and training protocols as well as nutrients on mTOR signaling that could be involved in TNBC initiation and progression. They suggested that exercise could ameliorate the TNBC risk and reduce the tumor burden by inhibiting PI3K-Akt-mTOR signaling, when canonical radio-, chemotherapies or chemical mTOR inhibitors are largely ineffective to prevent and manage the TNBC. In this sense, prescription and implementation of active lifestyles, including exercise/training and healthy nutritional habits, could have wide-ranging implications for society, which might improve conventional cancer treatment, including emotional and social wellbeing, in TNBC patients.

In conclusion, we believe that our series of special issues on this research topic published several new findings, which advanced our knowledge of the pivotal roles of mTOR signaling in developing an effective and safe therapeutic strategy for the growing prevalence of multiple pathological disorders.

Conflicts of Interest

There is no conflict of interest regarding the publication of this article.

Acknowledgments

We would like to thank all the authors and reviewers of this special issue for their contributions and participation. We hope that the readers of this special issue would appreciate the development of the new mTOR-targeted therapeutic strategies to combat pathogenesis of various human diseases.

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Flávio Reis
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Research Article

Comparison of Cardiac miRNA Transcriptomes Induced by Diabetes and Rapamycin Treatment and Identification of a Rapamycin-Associated Cardiac MicroRNA Signature

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Rapamycin (Rap), an inhibitor of mTORC1, reduces obesity and improves lifespan in mice. However, hyperglycemia and lipid disorders are adverse side effects in patients receiving Rap treatment. We previously reported that diabetes induces pansuppression of cardiac cytokines in Zucker obese rats (ZO-C). Rap treatment (750 μg/kg/day for 12 weeks) reduced their obesity and cardiac fibrosis significantly; however, it increased their hyperglycemia and did not improve their cardiac diastolic parameters. Moreover, Rap treatment of healthy Zucker lean rats (ZL-C) induced cardiac fibrosis. Rap-induced changes in ZL-C's cardiac cytokine profile shared similarities with that of diabetes-induced ZO-C. Therefore, we hypothesized that the cardiac microRNA transcriptome induced by diabetes and Rap treatment could share similarities. Here, we compared the cardiac miRNA transcriptome of ZL-C to ZO-C, Rap-treated ZL (ZL-Rap), and ZO (ZO-Rap). We report that 80% of diabetes-induced miRNA transcriptome (40 differentially expressed miRNAs by minimum 1.5-fold in ZO-C versus ZL-C; $p \leq 0.05$) is similar to 47% of Rap-induced miRNA transcriptome in ZL (68 differentially expressed miRNAs by minimum 1.5-fold in ZL-Rap versus ZL-C; $p \leq 0.05$). This remarkable similarity between diabetes-induced and Rap-induced cardiac microRNA transcriptome underscores the role of miRNAs in Rap-induced insulin resistance. We also show that Rap treatment altered the expression of the same 17 miRNAs in ZL and ZO hearts indicating that these 17 miRNAs comprise a unique Rap-induced cardiac miRNA signature. Interestingly, only four miRNAs were significantly differentially expressed between ZO-C and ZO-Rap, indicating that, unlike the nondiabetic heart, Rap did not substantially change the miRNA transcriptome in the diabetic heart. *In silico* analyses showed that (a) mRNA-miRNA interactions exist between differentially expressed cardiac cytokines and miRNAs, (b) human orthologs of rat miRNAs that are strongly correlated with cardiac fibrosis may modulate profibrotic TGF-β signaling, and (c) changes in miRNA transcriptome caused by diabetes or Rap treatment include cardioprotective miRNAs indicating a concurrent activation of an adaptive mechanism to protect the heart in conditions that exacerbate diabetes.

1. Introduction

Obesity and diabetes are metabolic diseases that increase risks for cardiovascular, immune, and inflammatory disease. Chronic inflammation in patients with obesity and diabetes is characterized by an impaired immune response and increased risk of infections [1, 2]. We recently reported that diabetic Zucker obese (ZO) rats exhibit an intracardiac

cytokine protein expression profile that reflects deficient host defense compared to that of age-matched healthy Zucker lean (ZL) rats [3]. Moreover, we observed that host defense deficiency entailed suppression of both proinflammatory and anti-inflammatory cytokines. We also reported that ZL and diabetic ZO rats exhibited differential metabolic, cardiac structural, functional, and immune responses to rapamycin, an immunosuppressive agent that inhibits the mechanistic target of rapamycin complex 1 (mTORC1) [3].

Rapamycin (Rap) is a macrolide antibiotic and is used as an effective immunosuppressant during solid organ transplantation [4–6]. It is used as an anticancer drug because mTORC1 signaling is hyperactivated in up to 70% of human cancers [7–12]. mTORC1 inhibition has been proposed as an effective strategy for stabilization of atherosclerotic plaques [13]. The mTOR signaling network is implicated in cellular senescence, aging, and lifespan regulation, and rapamycin treatment improves lifespan in different model organisms [14–16]. In brief, Rap and rapalogues (new inhibitors of mTORC1) exert several beneficial effects in the treatment of chronic diseases. On the other hand, accumulating evidence from clinical trials indicates that adverse metabolic side effects of Rap treatment include new onset diabetes and lipid disorders [17–19]. While Rap reduced mortality in healthy mice, paradoxically, long-term rapamycin treatment increased mortality in diabetic mice [20]. We recently reported the suppression of intracardiac expression of GM-CSF, IL-2, IFN-γ, and IL-10, as well as increased decorin and prolactin in diabetic rats and rapamycin-treated nondiabetic rats, indicating a similarity in cardiac cytokine signaling associated with both diabetes and Rap treatment [3]. These observations underscore the need for a better understanding of the molecular regulators that mediate the effects of rapamycin in the diabetic heart.

We have shown previously that when diabetic ZO rats were treated for 12 weeks with a low dose of Rap (750 μg/kg/day delivered via subcutaneous injection), they exhibited significant increase in their fasting glucose levels [3]. While Rap treatment suppressed cardiac fibrosis in ZO rats, it induced cardiac fibrosis in heathy ZL rats, suggesting that mTORC1 inhibition exerts differential effects in diabetic versus healthy animals [3]. Recent studies have identified several microRNAs that mediate the effects of Rap treatment in different cancers [21–26]. MicroRNAs are short (~23 nt) noncoding RNA molecules that function as master regulators of networks of gene expression by virtue of their ability to bind hundreds or thousands of mRNAs [27]. The human genome has over 2000 microRNAs that are predicted to regulate one-third of the genes in the genome [28]. To date, there are no reports that describe how Rap treatment modulates the microRNA expression profiles in healthy and diabetic hearts.

Given the similarities between the intracardiac cytokine expression patterns of Rap-treated and diabetic hearts and that induction of diabetes is one of the main adverse effects of Rap treatment, we hypothesized that there could be significant similarities in the cardiac microRNA transcriptome induced by diabetes and Rap treatment. We further hypothesized that Rap treatment may induce a shift towards increased expression of miRNAs implicated in fibrosis in healthy rat hearts. This hypothesis was tested in the present study using the same four rat models (and corresponding individual rats within those four groups) that we used to characterize how diabetes and Rap treatment modulate intracardiac cytokines. ZL rats (ZL-C) served as baseline controls for miRNA expression in the healthy heart. ZO rats (ZO-C) served as the controls for obesity- and diabetes-induced changes in cardiac miRNA expression. Parallel groups of ZL and ZO rats were treated with Rap for 12 weeks to evaluate the effects of Rap treatment on cardiac miRNA expression in healthy and diabetic hearts, respectively.

We used the GeneChip miRNA 4.0 Array (Thermo Fisher Scientific) to profile miRNA expression in placebo or Rap-treated ZL and ZO rat hearts. Here, we report that changes in intracardiac miRNA transcriptomes induced by rapamycin treatment and diabetes shared significant similarities and provide new insights into mechanisms underlying adverse effects of rapamycin. Differentially expressed miRNAs showed significant correlation with cardiac fibrosis in Rap-treated healthy ZL and diabetic ZO rats. This analysis also uncovered a new, Rap-induced cardiac microRNA signature. Additionally, our *in silico* analysis indicated that human orthologs of rat miRNAs that were highly correlated with cardiac fibrosis in the rats used in this study are involved in modulating the profibrotic TGF-β pathway.

2. Methods

2.1. Rapamycin Treatment of Rats. Rap treatment of 8-week-old ZL and ZO rats was performed as described previously [3]. All animal procedures used in this study were approved by the Harry S. Truman Memorial Veterans Hospital (HSTMVH) Subcommittee for Animal Safety and University of Missouri IACUC before commencing. All animals were cared for in accordance with the guidelines for the care and use of laboratory animals (National Institutes of Health publication 85-23). Briefly, 8-week-old ZO (fa/fa) and lean (ZL) rats (Charles River Laboratories) were maintained on ad libitum food and water and housed singly at the HSTMVH animal housing facility under standard laboratory conditions at room temperature 21–22°C. Animals were entrained to have dark cycle (12 hr: awake time) during the day and light cycle (12 hr: sleep time) during the night so that all interactions with animals matched their awake time. Placebo pellets or rapamycin pellets designed to deliver Rap at a concentration of 750 μg/kg/day for 21 days (from Innovative Research of America Inc., Sarasota, FL) were surgically placed under the skin behind the shoulder blades under brief isoflurane anesthesia, and this procedure was repeated 3 times to achieve a 12-week treatment. ZL and ZO rats that received placebo pellets are referred as ZL-C and ZO-, and those received Rap pellets are referred as ZL-Rap and ZO-Rap, respectively.

2.2. Cardiac miRNA Isolation, Microarray Analysis, and Quantitative Real-Time PCR. Frozen heart tissue from saline- and Rap-treated ZL and ZO rats stored at −80°C was powdered under liquid nitrogen, and miRNA isolation was performed using mirVana miRNA isolation kit (Ambion)

Table 1: MicroRNAs differentially expressed in ZO-Rap versus ZL-C and their similarity to microRNAs that were differentially expressed in ZL-Rap versus ZL-C.

MicroRNAs	ZO-Rap/ZL-C	ZL-Rap/ZL-C	Link to diabetes, fibrosis, and/or CVD		
			Diabetes	Fibrosis	CVD
miR-200c/b	4.71/4.099	2.98/4.99	Increased [73, 74]	Suppressed [75]	Increased in familial hypercholesterolaemia [76]
miR-7a-1-3p	4.599	6.844	—	—	—
miR-138-1-3p	4.580	4.238	—		Suppresses cardiac hypertrophy [67, 77]
miR-21	4.533	5.305	Increased [59]	Increased [60]	Reduced in hypertension; cardioprotective [61–63]
miR-26b	4.428	5.147	—	—	Increased in hypertensive patients [78]
miR-434	4.345	9.322	Induces insulin resistance [79]		Increases atrial fibrillation, cardiac damage, heart disease [79–82]
miR-155	4.125	4.55	Increased in T1DM & T2DM [31, 32]	Increased [35]	Increased in cardiac hypertrophy and CHD [33, 34]
miR-30e-3p	4.06	4.80	Suppressed by insulin [45]	—	Suppression associates with cardiac injury [46]
miR-328b-3p	3.734	4.807	—	—	—
miR-505-3p	3.652	4.938	—	—	Increased in familial hypercholesterolaemia [83]
miR-382	3.407	4.56	Increased [84]	Increased [85]	—
miR-499	3.338	3.86	Induces insulin resistance [79]		Increases atrial fibrillation, cardiac damage, heart disease [79–82]
miR-872-5p	3.255	5.519	—	—	Associated with cardiac oxidative stress and atherosclerosis [86, 87]
miR-217	2.996	2.3409	Increased [88]	Increased [89]	Increased in cardiovascular aging [90]
miR-92b-3p	2.942	3.128	—	—	Suppressed in response to hypoxia [47]
miR-362-3p	2.907	6.316	Suppressed in DM patients [91]	Increased in cardiac fibroblasts [92]	Increased in acute myocardial infarction [93]

Please see Supplemental Table 4 for the exact gene expression levels and p values.

following the manufacturer's protocol and quantified using NanoDrop (Thermo Scientific) as described previously [3]. FlashTag™ Biotin HS RNA Labeling Kit for GeneChip® miRNA Array was used for generating miRNA probes as per manufacturer's instructions using 300 ng of miRNA as input per reaction. Hybridization and scanning of the arrays were performed at the Microarray Core Lab at the University of Colorado, Denver, for a fee. GeneChip miRNA 4.0 Arrays are designed to interrogate all mature miRNA sequences in miRBase release 20. CEL files generated from the scanning of arrays were analyzed using the miRNA microarray data QC analysis as described in the Affymetrix Expression Console Software 1.4 user manual for data normalization. The robust multichip analysis (RMA) + DBAG workflow (Rat), that performs quantile normalization and has a general background correction, was used to generate CHP files. Threshold test showed that all CHIP files were within bounds. Significance of differentially expressed miRNAs between different pairs (ZL-C versus ZO-C, ZL-C versus ZL-Rap, and ZL-C versus ZO-Rap, as well as ZO-C versus ZO-Rap) was determined using unpaired two-tailed t-test. cDNA was generated from the previously isolated miRNA using the miScript II RT Kit (Qiagen, Valencia, CA). Real-time PCR reactions were performed in triplicate using miScript II SYBR Green PCR Kit and prevalidated Qiagen miScript Primer Assays for miR-21-5p (cat. #MS00013216), miR-144-3p (cat. #MS00021833), miR-155-5p (cat. #MS0001701), miR-101b-3p (cat. #MS00012964), miR-26b-3p (cat. #MS00000140), miR-30e-3p (cat. #MS00013426), and miR-34b-3p (cat. #MS0027468). Reactions were performed using the Bio-Rad IQ5 (Bio-Rad, Hercules, CA) under cycle conditions specified by the manufacturer. The expression levels of target miRNAs relative to endogenous control (RNU6-2; cat. #MS00033740, Qiagen) were quantified by a comparative quantitation cycle method. Relative quantification (RQ) values were obtained by determining ΔCt values followed by determining ΔΔCt values and then RQ values via the equation $2(-\Delta\Delta Ct)$.

2.3. Principal Component Analysis (PCA). RMA + DBAG workflow identified 1218 rat miRNAs. Of the 1218 miRNAs, one-way ANOVA (SAS 9.4, PROC ANOVA) revealed 70 miRNAs that were differentially expressed ($p < 0.05$) by at least 1.5 log2-fold between one or more groups. To identify

similarities in miRNA expression patterns between the groups, PCA was performed on these 70 miRNAs using SAS 9.4 software, PROC PRINCOMP.

2.4. In Silico Analysis for Identifying Differentially Expressed Cytokines Targeted by Differentially Expressed miRNAs. NCBI gene database was used to retrieve complete mRNA sequence data for differentially expressed cytokines identified from pairwise comparison of ZO-C or ZO-Rap versus ZL-C that we reported previously [3]. RegRNA software [29] was used to retrieve predicted miRNA binding sites for each of the cytokines. miRNA binding sites for each cytokine mRNA in the differentially expressed cytokine list for a given pairwise comparison (e.g., ZO-C versus ZL-C) were compared with the list of differentially expressed miRNAs in the same pairwise comparison. Then, the data were compiled, and the list of different cytokines targeted by a given miRNA was organized and presented in Table 1.

2.5. In Silico Analysis to Determine Correlation of Fibrosis with Differentially Expressed miRNAs. Human orthologs for miRNAs demonstrating a significant relationship ($p < 0.05$) with cardiac fibrosis were entered into DIANA-miRPath v3.0 software [30] to determine associated pathways in humans, using both Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology-Biological Processes (GO-bp) analyses, and target genes associated with those pathways. Secondary KEGG pathway enrichment analyses and subsequent identification of target genes along significantly enriched pathways were performed using miRNet (http://www.miRNet.ca/).

2.6. Statistical Analysis. Results are reported as means ± SE. Statistical analysis was performed using SigmaStat or SAS 9.4 software. Unpaired two-tailed t-test was performed for pairwise comparisons. A p value < 0.05 was deemed significant. Spearman correlation coefficients were obtained for miRNA expression and measures of cardiac fibrosis, independent of the treatment group, using PROC REG (SAS 9.4).

3. Results

One-way ANOVA of the 1218 rat miRNAs used as probes in this study showed that 70 miRNAs exhibited statistically significant ($p < 0.05$) differential expression by at least 1.5 log2-fold between one or more groups (Figure 1). Principal component analysis (PCA) of these 70 miRNAs showed that ZL-C and ZL-Rap were distinct groups whereas ZO-C and ZO-Rap groups clustered together. This observation suggested that while Rap treatment had strong effects on the miRNA transcriptome of ZL rats, Rap did not alter the miRNA transcriptome of ZO-rats to the same extent.

3.1. Comparison of Cardiac miRNA Transcriptome in ZL-C and ZO-C. We previously reported metabolic and cardiac parameters of ZL-C and ZO-C used in this study and differences in their intracardiac cytokine profiles [3]. In this study, miRNA transcriptomes of the same heart tissues were analyzed. Diabetes induced significant differential expression of a total of 177 cardiac microRNAs in ZO-C (Supplemental Table 1). Specifically, 105 miRNAs showed an increase, and 72 miRNAs showed a reduction in their expression in ZO-C compared to ZL-C. Among them, 40 microRNAs showed differential expression ≥ 1.5 log2-fold (Figure 2). A literature search provided evidence that 20 of these miRNAs are associated with diabetes and/or cardiac fibrosis and/or different cardiovascular diseases (Table 2). The microRNA miR-155-5p, a biomarker that is increased in the plasma of patients with type 1 diabetes [31, 32] and in gingival crevicular fluid of patients with periodontitis and type 2 diabetes [33] and contributes to cardiac hypertrophy and coronary heart disease [34, 35], was increased by 5.21-fold in the ZO rat heart, consistent with the cardiovascular detrimental effects of diabetes. Other miRNAs that have defined roles in cardiovascular damage included miR-872-5p, miR-350, miR-362-3p, miR-223-3p, miR-204, miR-98, miR-217, miR-379, and miR-181-3p (Table 2 and references therein). MicroRNAs that contribute to fibrosis such as miR-21, miR-382, miR-155, miR-223-3p, and miR-217 were also increased in ZO-C hearts compared to ZL-C hearts (Table 2). However, miRNAs that suppress fibrosis (miR-200b/c, miR-411-5p, miR-140, miR-322, and miR-98) and render cardiovascular protective effects (miR-21 and miR-140) were also simultaneously increased in the ZO-C heart indicating the activation of an adaptive mechanism to regulate fibrosis induced by high glucose and insulin resistance.

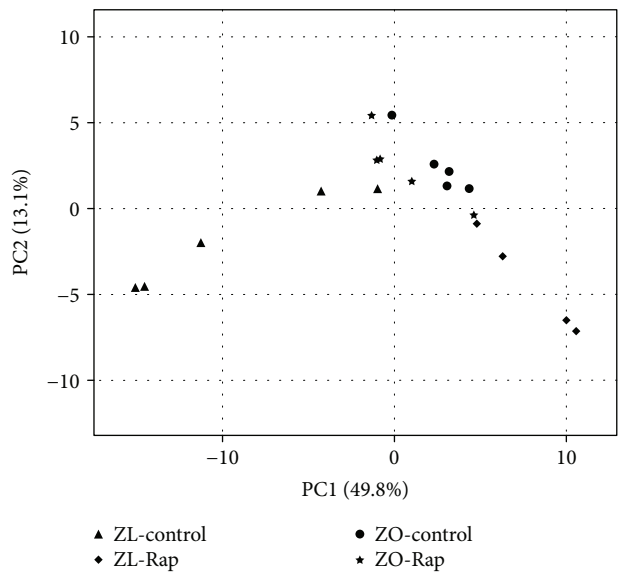


Figure 1: PCA analysis of the 70 miRNAs determined to be differentially expressed ($p < 0.05$) by at least 1.5 log2-fold between one or more groups. Comparisons revealed three moderately distinct clusters (1) ZL-control (ZL-C), (2) ZL-Rap, and (3) ZO-control (ZO-C) and ZO-Rap.

3.2. Comparison of Cardiac miRNA Transcriptome in ZL-C and ZL-Rap. A total of 221 cardiac miRNAs were differentially expressed between ZL-C and ZL-Rap that showed statistical significance. Out of these, 131 cardiac miRNAs had increased expression and 90 miRNAs had decreased expression in ZL-Rap compared to ZL-C (Supplemental Table 2).

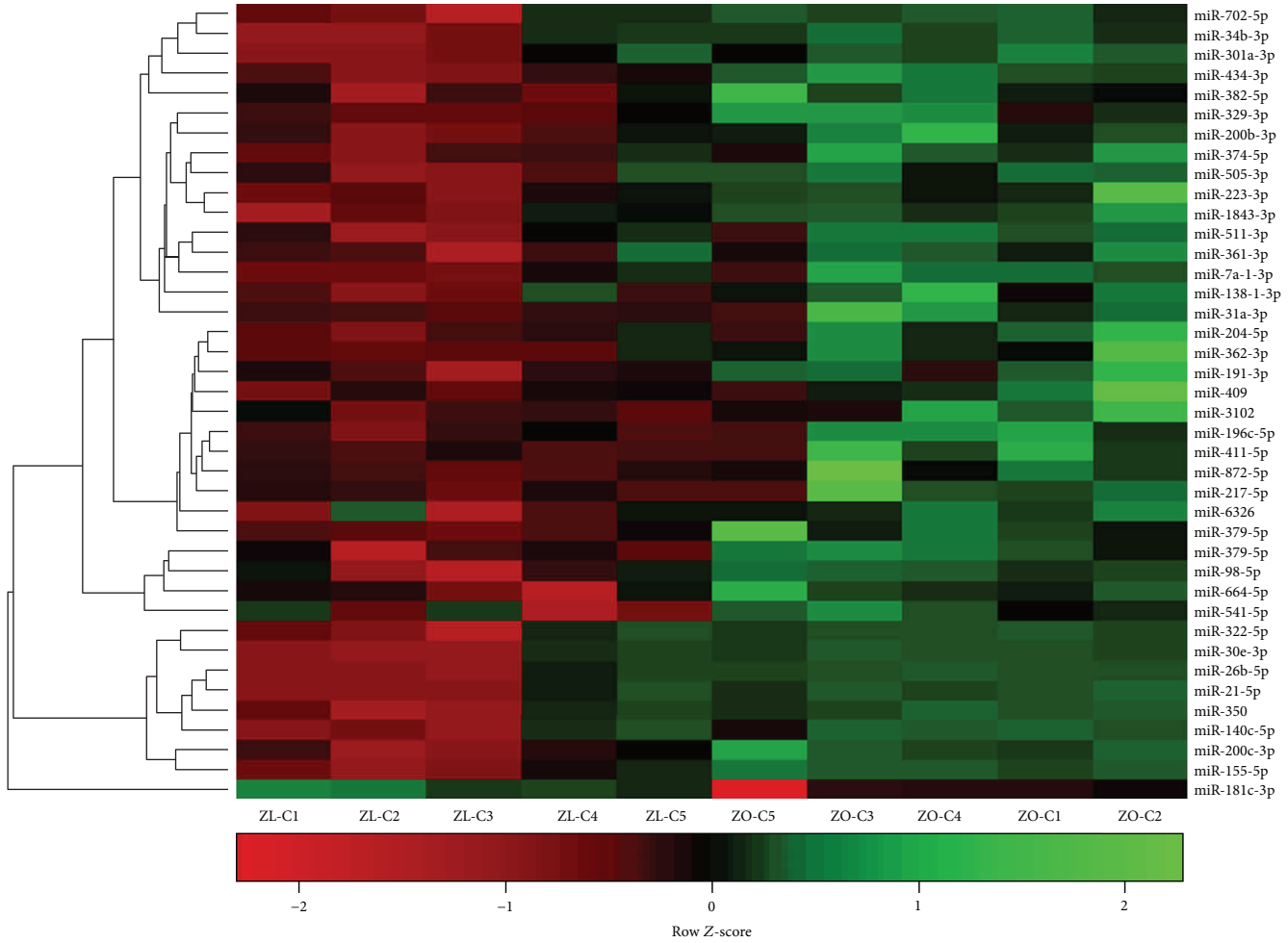


Figure 2: Hierarchal cluster heat map of differentially expressed cardiac miRNAs in ZL-C ($n = 5$) vs. ZL-Rap ($n = 4$) that exhibited a 2.25-fold change in expression in either direction. Red signals indicate lower expression levels, and green signals indicate higher expression levels.

Further selection of miRs that exhibited at least a 1.5 log2-fold difference showed that 68 miRNAs were differentially expressed between ZL-C and ZL-Rap (Figure 3). We found that 32 of these miRNAs (47%) were the same and showed the same directionality of expression as those that were found to be differentially expressed between ZL-C and ZO-C (Figure 4). This accounted for 80% of the differentially expressed cardiac miRNAs in ZO-C. Therefore, there is a remarkable similarity between the cardiac transcriptome activated by Rap treatment in healthy hearts and that activated in response to diabetes. Table 2 lists 20 of these miRNAs that showed the same expression pattern in ZL-Rap and ZO-C and that are also implicated in diabetes and/or fibrosis and/or cardiovascular damage.

Table 3 lists those miRNAs that were significantly differentially expressed by at least 1.5 log2-fold in either direction in ZL-Rap compared to ZL-C and show some relationship with diabetes and/or fibrosis and/or cardiovascular damage according to literature. These miRNAs were not differentially expressed by 1.5 log2-fold in ZO-C compared to ZL-C and therefore were unique to the ZL-Rap hearts. Several miRNAs that are diabetes markers and contribute to insulin resistance, pancreatic beta cell death, and progression of diabetes are included in this list. For example, miR-29 family miRNAs are significantly increased in patients with T1DM and T2DM and involved in pancreatic beta cell death and progression of diabetes [36–38]. MicroRNAs miR-19a, miR-499, miR-539, miR-363, miR-495, miR-7a, and miR-429, which are reported to be increased in diabetic patients or animal models and/or contribute to insulin resistance, were increased in ZL-Rap hearts suggesting that Rap treatment induces a diabetes-associated miRNA transcriptome in ZL-Rap hearts. However, miR-455 and miR-451, which are reported to be suppressed in diabetes, were found to be increased in ZL-Rap hearts, whereas miR-144 that is shown to be increased in diabetes was suppressed in ZL-Rap hearts [39–41]. Additionally, a miRNA implicated in promoting diabetic wound healing, miR-335, was also among the miRNAs that had increased transcription in ZL-Rap hearts [42].

Several of these miRNAs are also linked to CVD (Table 3). miRNAs that are associated with exacerbating different CVDs according to literature and increased in ZL-Rap hearts include miR-451, miR29 family, miR-19a, miR-499, miR-539, miR-363, miR-495, and miR-429 (Table 3 and references

Table 2: Similarities between differentially expressed microRNAs that modulate diabetes, cardiac fibrosis, and other cardiovascular diseases in ZO-C versus ZL-C and ZL-Rap versus ZL-C.

MicroRNA	ZO-C/ZL-C	ZL Rap/ZL-C	Nature of association with human or animal model pathology		
			Diabetes	Fibrosis	CVD
miR-155	5.21	4.55	Increased in T1DM & T2DM [31, 32]	Increased [35]	Increased in cardiac hypertrophy and CHD [33, 34]
miR-200b/c	5.17/4.00	4.99/2.98	Increased [73, 74]	Suppressed [75]	Increased in familial hypercholesterolaemia [76]
miR-21	4.59	5.28	Increased [59]	Increased [60]	Reduced in hypertension; cardioprotective [61–63]
miR-26b-5p	4.41	5.13	—	—	Increased in hypertensive patients [78]
miR-872-5p	4.32	5.52	—	—	Associated with cardiac oxidative stress and atherosclerosis [86, 87]
miR-411-5p	3.89	7.16	—	Suppresses fibrosis [94]	Increased in abdominal aortic aneurism [95]
miR-382	3.84	4.56	Increased [84]	Increased [85]	—
miR-301a	3.81	4.86	Increased [96]		Increased in the diabetic heart [96]
miR-329	3.81	5.50	—	—	Increased in ischemia [97]
miR-350	3.68	3.61	—	—	Induces pathological cardiac hypertrophy [98]
miR-505-3p	3.66	4.92	—	—	Increased in familial hypercholesterolaemia [83]
miR-140	3.58	4.00	Reduced in platelets [99]	Suppresses fibrosis [100]	Suppresses pulmonary arterial hypertension [101]
miR-322	3.56	3.84	Suppressed by high glucose [102]	Suppresses fibrosis [103]	Improved cardiac function [103]
miR-362-3p	3.48	6.28	Increased [104]	—	Associated with atherosclerosis [105]
miR-374	3.34	5.94	—	—	Increased in cardiac hypertrophy and aneurism [106, 107]
miR-223-3p	3.27	3.56	Suppressed in DM patients [91]	Increased in cardiac fibroblasts [92]	Increased in acute myocardial infarction [93]
miR-204	3.20	7.01	Suppresses insulin [108]	—	Increases endoplasmic reticulum stress [109]
miR-98	3.20	3.41	Increased [110]	Reduces collagen [111]	Reduced in myocarditis [112]; increased in postheart transplant [113]
miR-34b	3.10	3.29	—	—	Reduced in diabetic ischemic heart failure [114]
miR-217	2.99	2.98	Increased [88]	Increased [89]	Increased in cardiovascular aging [90]
miR-541	2.97	3.18	Involved in pancreas development [115]	—	Suppresses cardiac hypertrophy [116]
miR-379	2.85	2.91	—	—	Apoptosis of VSMC [117]

Please see Supplemental Tables 1 and 2 for the exact gene expression levels and p values.

therein). Additionally, loss of miR-144 and miR-542 also exacerbated CVD. Conversely, miR-101b and miR-7a, which are cardioprotective (Table 3 and references therein), are also among the cardiac miRNAs that are increased by Rap treatment.

3.3. Comparison of Cardiac miRNA Transcriptome in ZO-C and ZO-Rap. Rap treatment of ZO-C resulted in differential myocardial expression of 128 miRNAs that included 84 miRNAs with increased expression and 44 miRNAs with suppressed expression (Supplemental Table 3). However, only four miRNAs from this group met the ±1.5 log2-fold change threshold (Figure 5). Associations between these miRNAs to specific diseases are shown in Table 4. miR-743a-5p that mediates mitochondrial oxidative stress was increased in the ZO-Rap heart, but not in the ZL-Rap heart, indicating that this is a unique effect caused by the combination of diabetes and Rap treatment (Table 4) [43]. miR-511-3p was the only miRNA that showed similar expression changes in Rap-treated ZL and ZO rats (suppressed in both cases), and loss of miR-511-3p is associated with minimally oxidized low-density lipid-associated increase in atherosclerosis (Table 4) [44]. There is very little information regarding miR-1843-3p and miR-409b in the literature; however, Rap treatment had opposing effects on the expression of these miRNAs in healthy ZL-C and diabetic ZO-C (Table 4).

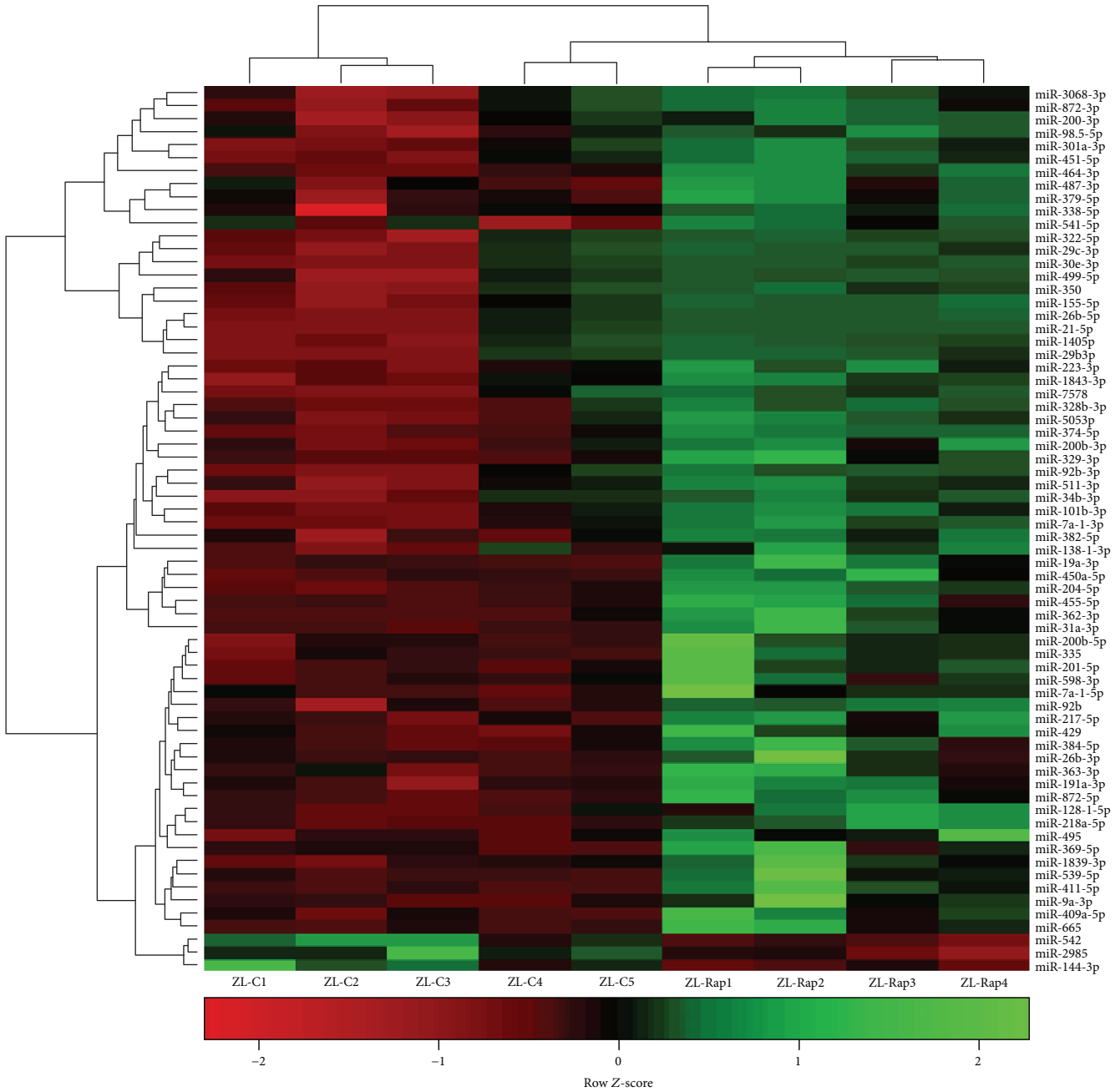


FIGURE 3: Hierarchal cluster heat map of differentially expressed cardiac miRNAs in ZL-C ($n = 5$) vs. ZO-C ($n = 5$) that exhibited a 2.25-fold change in expression in either direction. Red signals indicate lower expression levels, and green signals indicate higher expression levels.

3.4. Comparison of Cardiac miRNA Transcriptome in ZL-C and ZO-Rap. Next, we compared the cardiac miRNA transcriptome of ZO-Rap with that of ZL-C. Of the 116 miRNAs that were differentially expressed between these two groups (Supplemental Table 4), 83 had increased expression and 33 exhibited suppressed expression in ZO-Rap hearts. However, only 27 of these miRNAs met the ±1.5 log2-fold change threshold (Figure 6). Importantly, 17 of these miRNAs were the same as those that were increased in ZL-Rap (Table 1). Among these miRNAs, miR-30e-3p is shown to be suppressed by insulin [45]; however, another study showed that its suppression is associated with myocardial injury induced by coronary microembolization via autophagy activation [46]. Moreover, miR-92b-3p is shown to be suppressed by hypoxia [47], but its expression is elevated in the heart tissues of both Rap-treated groups. One miRNA in this group, miR-7a-1-3p, is not known to associate with diabetes, fibrosis, or CVD. The remaining miRNAs in this group are associated with either diabetes, fibrosis, or cardiovascular diseases as shown in Table 1.

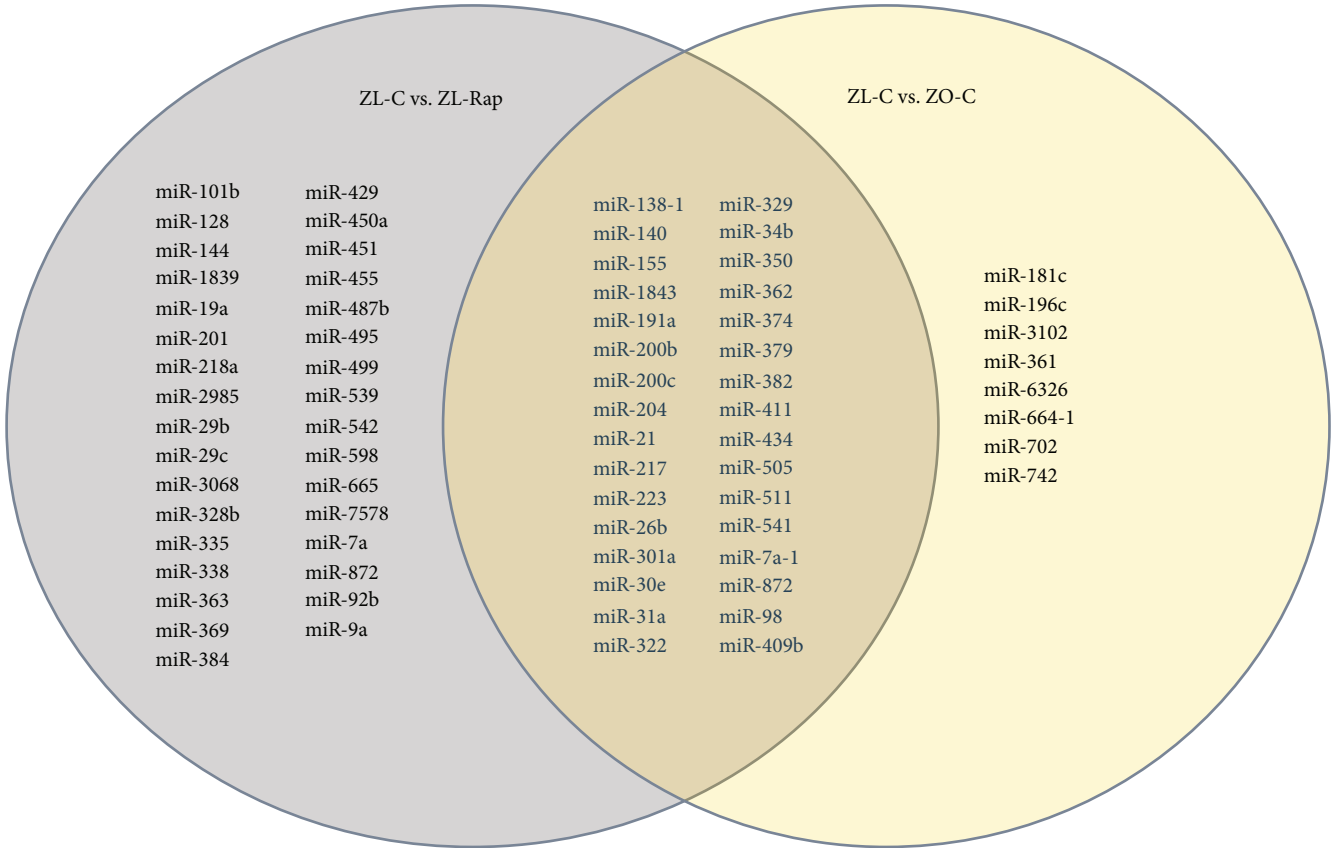


Figure 4: Venn diagram demonstrating miRNA that exhibited a 2.25-fold change in expression in either direction between ZL-C vs. ZL-Rap (left) and ZL-C vs. ZO-C (right), as well as those that were differentially expressed in both comparisons (center).

Table 3: MicroRNAs that modulate diabetes, cardiac fibrosis and other cardiovascular diseases and differentially expressed in ZL-Rap versus ZL-C.

MicroRNAs	ZL-Rap/ZL-C	Link to diabetes and/or CVD	
		Diabetes	CVD
miR-455	6.76	Suppressed [39]	—
miR-451	5.1076	Suppressed [40]	Induces cardiac hypertrophy [40]
miR-101b	5.0625	—	Suppresses cardiac hypertrophy [67, 77]
miR-29b/c	4.75/3.83	Increased in diabetes [36–38]	Correlates with CVD progression in diabetes [36–38, 118]
miR-19a	4.7089	Increased in diabetes [119]	Induces heart failure and vascular inflammation [120–122]
miR-499	3.8636	Induces insulin resistance [79]	Increases atrial fibrillation, cardiac damage, heart disease [79–82]
miR-539	3.7249	Increased in diabetes [123]	Induces mitochondrial fission, cardiomyocyte apoptosis [123, 124]
miR-363	3.7249	Urinary marker for diabetes [125]	Inhibition protects cardiomyocytes from apoptosis [126]
miR-335	3.2761	Improves diabetic wound healing [42]	—
miR-92b	3.0976	—	Increased in heart failure patients [127]
miR-495	3.0976	Urinary marker for diabetes [125]	Involved in causing hypertrophy [128, 129]
miR-7a	2.8224	Inhibits glucose-stimulated insulin secretion [130]	Protects against cardiomyocyte injury [131, 132]
miR-429	2.56	Impairs intestinal barrier in diabetic mice [133]	Causes cardiomyocyte apoptosis [134]
miR-487b	2.3409	—	Mitigates chronic heart failure [135]
miR-144	−2.28	Increased in diabetes [41]	Loss of miR-144 impairs cardioprotection [136]
miR-542	−2.28	—	Involved in aortic calcification [137]

Please see Supplemental Table 2 for the exact gene expression levels and p values.

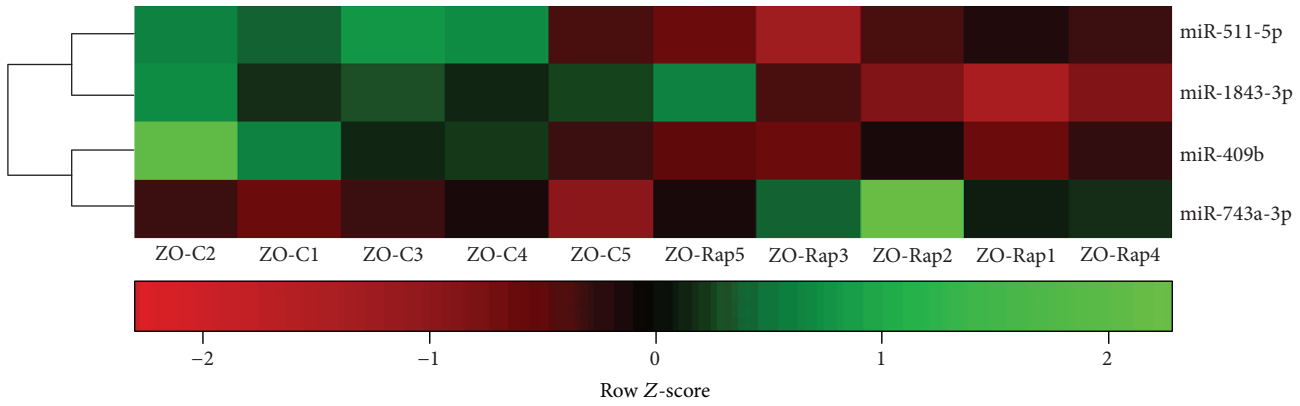


Figure 5: Hierarchal cluster heat map of differentially expressed cardiac miRNAs in ZO-C ($n = 5$) vs. ZO-Rap ($n = 5$) that exhibited a 2.25-fold change in expression in either direction. Red signals indicate lower expression levels, and green signals indicate higher expression levels.

Table 4: MicroRNAs differentially expressed in ZO-Rap versus ZO-C and their similarity to microRNAs that were differentially expressed in ZL-Rap versus ZL-C.

MicroRNA	ZO-Rap/ZO-C	ZL-Rap/ZL-C	Nature of Association with human or animal model pathology		
			Diabetes	Cardiac fibrosis	CVD
miR-743a-5p	2.25	1.09; p = N.S.	—	—	Mediates mitochondrial oxidative stress [43]
miR-1843-3p	−2.25	2	—	—	—
miR-511-3p	−2.25	−1.7	—	—	Suppressed in moX-LDL-induced VSMC transformation in atherosclerosis [44]
miR-409b	−2.25	1.45; p = N.S.	—	—	—

N.S.: not significant. Please see Supplemental Table 3 for the exact gene expression levels and p values.

Because myocardial expression of these 17 miRNAs is increased by Rap treatment in both ZL and ZO rats, we propose that these miRNAs constitute a Rap-induced cardiac microRNA signature.

3.5. Cytokines Targeted by the Differentially Expressed miRNAs. We previously reported which intracardiac cytokines were differentially expressed in response to diabetes (ZL-C versus ZO-C) and Rap treatment (ZL-C versus ZL-Rap) [3]. Because there was significant overlap between the miRNAs that were differentially expressed between these two groups, we hypothesized that these miRNAs would play a role in regulating differentially expressed cytokines in these groups. To determine if the differentially expressed miRNA transcriptome exerts the regulation of the corresponding cytokine profile, we performed an *in silico* analysis using miRbase miRNA target analysis. The miRNAs that had binding sites on the 3′ untranslated sites of the mRNAs expressing differentially expressed cytokines from each pairwise comparison were selected. Table 5 shows miRNAs that exhibited increased expression and their target cytokines that carried binding sites for the corresponding miRNAs on the 3′ untranslated regions of their mRNAs. As shown in Table 5, differentially expressed miRNAs had binding sites on the 3′ untranslated regions of most of the differentially expressed cytokines. Therefore, changes in expression patterns of intracardiac cytokines in response to diabetes and rapamycin treatments in rat hearts were associated with changes in their miRNA transcriptomes.

3.6. Differentially Expressed miRNAs That Correlated with Fibrosis and Validated by Real-Time PCR. We have previously published that ZL rats treated with rapamycin have increased cardiac fibrosis compared with untreated ZL rats [3]. However, in ZO rats, treatment with rapamycin attenuated cardiac fibrosis [3]. In order to determine which specific miRNA may be contributing to cardiac fibrosis, we performed a correlation analysis using all differentially expressed miRNAs that exhibited at least 1.5 log2-fold change in pairwise comparisons. Analysis of the combined data from all treatment groups showed that seven miRNAs were positively correlated (miR-140-5p, miR-155-5p, miR-21-5p, miR-26b-5p, miR-30e-3p, miR-34b-3p, and miR-379-5p; $p < 0.05$) and one miRNA, miR-144-3p, was inversely correlated (miR-144; $p < 0.05$) with the degree of cardiac fibrosis (Figure 7). Of the seven positively correlated miRNAs, there is evidence supporting strong profibrotic activity for miR-21 and miR-34b [48–52]. In contrast to the positive correlation with fibrosis observed here, miR-140 is considered antifibrotic [53, 54]. miR-155 and miR-34b demonstrate both pro- and antifibrotic activities [55, 56]. miR-140, the only one demonstrating an inverse association, has well-documented antifibrotic activity [57, 58]. To further validate differential expression of some of these miRNAs, we performed quantitative RT-PCR. As shown in Figure 7, miRNAs

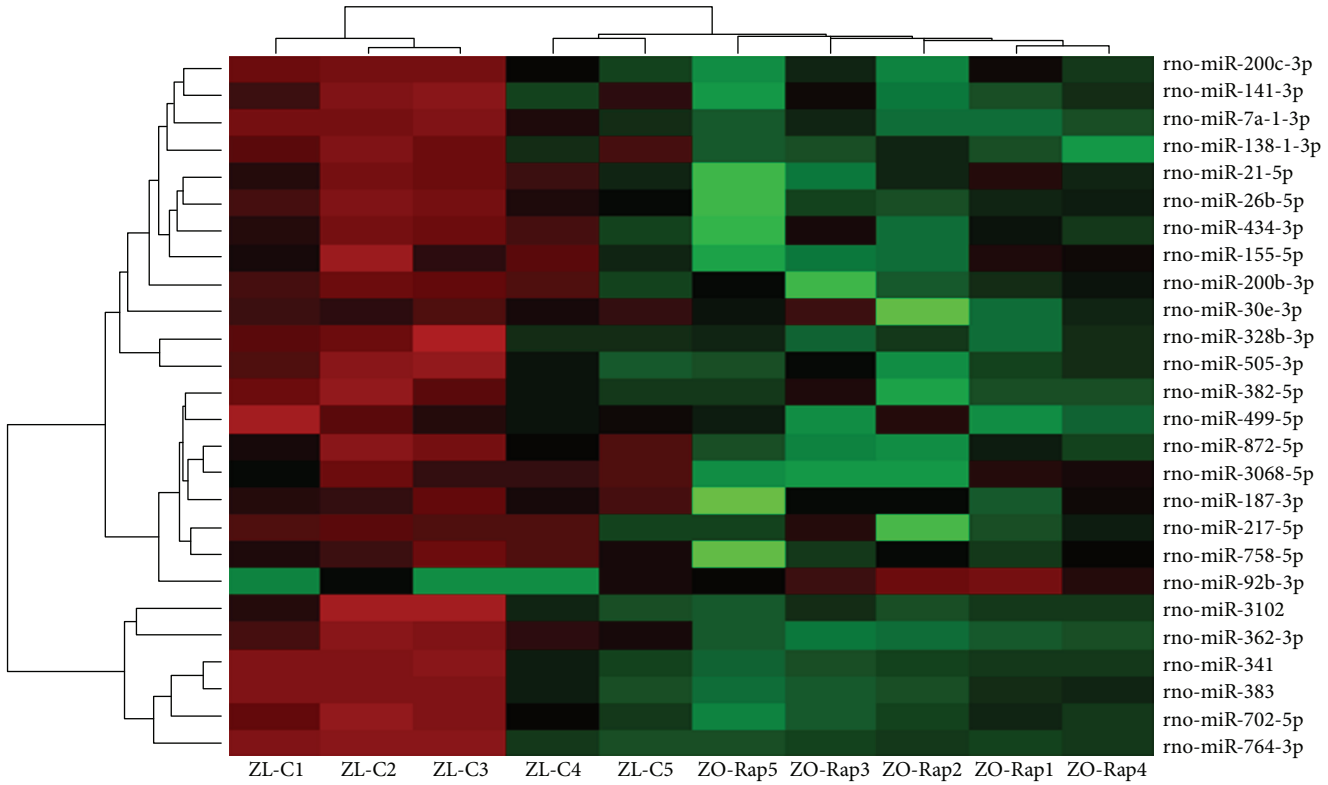


Figure 6: Hierarchal cluster heat map of differentially expressed cardiac miRNAs in ZL-C ($n = 5$) vs. ZO-Rap ($n = 5$) that exhibited a 2.25-fold change in expression in either direction. Red signals indicate lower expression levels, and green signals indicate higher expression levels.

(miR-34b-3p, miR-26b-3p, miR-140-5p, miR-155-5p, miR-21-5p, and miR-379-5p) exhibited differential expression consistent with the data obtained from microarray analysis.

4. Discussion

In this investigation, we have identified an identical subset of the cardiac miRNA transcriptome, comprised of 32 miRNAs, which are differentially expressed in the same direction in the hearts of diabetic ZO rats (ZO-C) and nondiabetic ZL rats treated with Rap (ZL-Rap). Since ZL rats did not develop diabetes after 3 months of Rap treatment [3] and Rap treatment is reported to mitigate aging [14–16], this high similarity between diabetes- and Rap-induced alterations to the cardiac miRNA transcriptome was surprising. Our recent findings that diabetes suppresses both inflammatory and anti-inflammatory intracardiac cytokines may shed some light in this regard [3]. As such, we posit that the similarity in the altered expression of identical miRNAs in the cardiac microRNA transcriptome between diabetic ZO-C and Rap-treated ZL rats might reflect that fact that both diabetes and Rap treatment cause significant immune suppression. Importantly, these studies also identified an identical subset of 17 miRNAs that exhibited increased expression in response to Rap treatment in both ZL and ZO hearts (Table 1). To our knowledge, this is the first evidence of a Rap-induced cardiac microRNA signature common to both healthy and diabetic hearts. It is noteworthy that most of these miRNAs seem to be associated with increasing CVD. This information is clinically relevant and provides new targets for developing drugs that can be coadministered with Rap to reduce potential detrimental effects of long-term Rap treatment in patients with comorbidities.

Previous studies to identify Rap-induced changes in the miRNA transcriptome in cell models of tuberous sclerosis (TSC) and lymphangioleiomyomatosis (LAM) have identified microRNAs 29b, 21, 24, 221, 106a, and 199a as candidate "RapamiRs" [21]. Among these miRNAs, miR-21 and miR-29b were found to be differentially expressed in ZL-Rap. miR-21 is a key miRNA biomarker of diabetes that causes fibrosis and has been characterized as a "mechano-miR" due to its response to arterial stress and hypertension [59–64]. A direct connection between miR-21 and mTOR has also been reported since miR-21 promotes mTORC1-driven tumorigenesis [65]. Additionally, miR-155 has been identified as a potent autophagy inducer that targets the mTOR signaling pathway [66]. miR-29 family miRNAs are involved in pancreatic beta cell death and exacerbate cardiomyocyte loss [36–38, 67]. It was also reported that long-term Rap treatment induced the upregulation of miR-17–92 and related clusters and downregulation of tumor suppressor miRNAs (miR-7a, miR-706, and miR-320) in rapamycin-resistant tumors [68]. Consistent with this, 3-month Rap treatment increased miR-19a, a member of this cluster. However, Rap treatment actually increased miR-7a expression in ZL-C hearts.

Consistent with the pansuppression of cardiac cytokines in the ZO-C heart [3], we observed an increase in miRNAs

Table 5: Differentially expressed miRNAs in ZL-C versus ZO-C and ZL-C versus ZL-Rap pairwise comparisons that target differentially expressed cytokines in the corresponding pairwise comparisons and as determined by binding sites on the 3′ untranslated region of their mRNAs by *in silico* analysis in the corresponding sets.

miRNA	Targeted cytokines increased in			miRNA	Targeted cytokines	
	ZO-C	ZL-Rap	Both		ZO-C	ZL-Rap
miR-21	CTACK, PDGFAA, CINC2	Notch2		miR-350		Notch2
miR-411	IL-2, IL-1α, TNF-α (2 sites)		IL-2, IL-10	miR-505	CINC2	Gas1
miR-7a*	PDGFAA	IL-2, decorin, IL-22	IL-10	miR-217		Notch 2, IL-1α
miR-384-5p		IL-2, IL-10, Notch2		miR-541		Notch 2, CINC2
miR-30e*		IL-22, Notch2	IL-10, IFNγ	miR-328b-3p		Notch 2, Gas1
miR-322			IFNγ	miR-29b		Notch 2
miR-382		Notch2	IFNγ, Prolactin	miR-455		Notch2
miR-742			IFNγ	miR-34b		Gas1
miR-539		IFNγ		miR-19a		Gas1
miR-329*	PDGFAA	Notch2	IL-10	miR-26b-3p		Gas1
miR-140		Notch2	IL-10, decorin	miR-429		Gas1, Tim1
miR-204	TNF-α	Gas1	IL-10	miR-128-1-5p		Gas1
miR-98	IL-1α		IL-10	miR-144		Gas1
miR-665		IL-10, Notch 2 (3 sites), Tim1		miR-2985		Gas1
miR-335		IL-10, Notch2, Tim 1		miR-301a		Prolactin
miR-495		IL-10 (2 sites), Notch 2, Gas1 (2 sites)		miR-362*		Tim1
miR-338*		IL-10, Notch2		miR-451*		Tim1
miR-200b, c			Decorin	miR-409-5p		Tim1
miR-223			Decorin	miR-218a		Tim1
miR-499		Decorin (2 sites), Gas1		miR-369-5p		Tim1
miR-542-3p		Decorin, Tim1		miR-434		IL-22
miR-138-1*	B7–1/CD80	Notch2		miR-379	TREM1, FGF-BP	
miR-196c	Decorin, TNF-α			miR-217	Notch 2, IL-1α	

∗ indicates a functional but nonpredominant miRNA as indicated by miRbase.

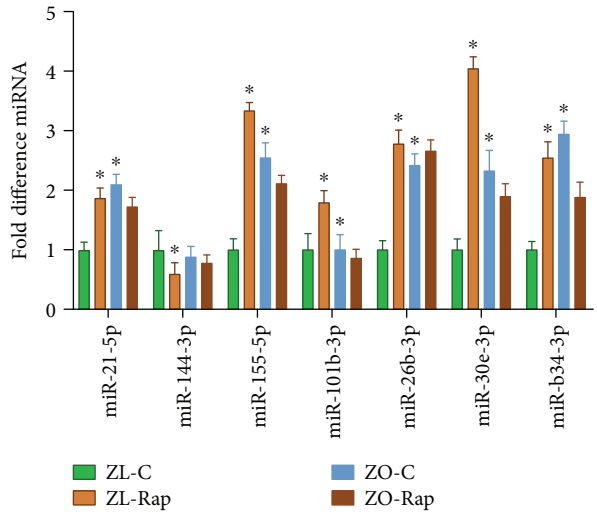
that target these cytokines in our *in silico* analysis (Table 5). miRNAs that have predicted binding sites on the 3′ untranslated regions of mRNAs coding for the cytokines, CTACK, PDGFAA, CINC2, IL-2, IL-1α, TNF-α, IL-10, IFNγ, prolactin, and decorin were among the miRNAs that were upregulated in ZO-C hearts over 1.5 log2-fold. It is noteworthy that Notch2, decorin, and prolactin were also suppressed in ZO-Rap hearts [3]. However, in ZL-Rap hearts, while IL-2, GM-CSF, IL-10, and IFNγ were suppressed, decorin, Notch2, Gas1, prolactin, Tim1, IL-22, and TWEAK-R were upregulated by Rap treatment. Interestingly, analysis of predicted miRNA binding sites on the 3′ untranslated regions of these mRNAs uncovered binding sites for many of the cardiac miRNAs that exhibited increased expression in ZL-Rap hearts. mRNAs encoding decorin, Notch2, Gas1, prolactin, Tim1, and IL-22 (but not TWEAK-R) carried binding sites for multiple miRNAs that were upregulated by Rap treatment. Although additional experiments are warranted to verify the validity of these miRNA binding sites, collectively, these observations suggest that the presence of a tightly regulated posttranscriptional gene expression pattern is present in the ZL-Rap heart for these cardiac cytokines.

We reported that in the ZO-Rap group, eight intracardiac cytokines were differentially expressed compared to ZO-C. However, only four miRNAs met the criteria of 1.5 log2-fold change in ZO-Rap compared to ZO-C. These miRNAs did not seem to have any predicted binding sites on the eight differentially expressed cytokines in the ZO-C heart. Importantly, cardiac miRNA transcriptome of ZO-C was already 80% similar to that of ZL-Rap and that may be why Rap treatment did not result in any additional major changes in their cardiac miRNA transcriptome that met the criteria of 1.5 log2-fold change.

4.1. Correlation of miRNA Transcriptome with Fibrosis. In humans and animals alike, myocardial fibrosis is associated with nearly all forms of cardiovascular disease. Myocardial fibrosis is a condition of multiple etiologies, characterized by the transformation of cardiac fibroblasts to a myofibroblast phenotype. The cardiac remodeling that takes place

MicroRNA	Correlation coefficient	Adjusted p-value
miR-34b-3p	0.74157	0.0084
miR-30e-3p	0.70627	0.0149
miR-26b-5p	0.68714	0.0196
miR-140-5p	0.64252	0.0344
miR-155-5p	0.63933	0.0357
miR-21-5p	0.63272	0.0385
miR-379-5p	0.61126	0.0484
miR-144-3p	−0.83065	0.0023

(a)



(b)

Figure 7: (a) Correlation coefficients and p values of fibrotic scores and differentially expressed miRNA from all groups. (b) Comparative miRNA expression levels of several miRNAs that were associated with cardiac fibrosis scores. Data represents means ± SEM. $n = 4$ for all groups. $^*p < 0.05$ vs. the ZL-C group.

Table 6: miRNAs associated with cardiac fibrosis, their overall effect on fibrosis/TGF-β signaling, and the predicted target genes along the TGF-β signaling pathway.

MicroRNA	Assoc. with fibrosis (r^2, p value)	Fibrotic effect	Predicted gene targets in the TGF-beta signaling pathway[†]
hsa-miR-140	**0.6443**, **0.034**	Anti [1]	*PITX2*, *BMP2*
hsa-miR-144	**−0.83**, **0.002**	Anti [2, 3]	**ACVR2B**, *SMAD9*, *ROCK1*, *BMPR1B*, CDKN2B, ID4
hsa-miR-155	**0.639**, **0.036**	Pro/anti* [4, 5]	*ACVR1C*, *BMPR2*, *SMAD5*, *SMAD2*, PPP2CA, *GDF6*, *SP1*, *RPS6KB1*
hsa-miR-21	**0.633**, **0.039**	Pro [6–8]	**ACVR2A**, *BMPR2*, SMAD7, TGFB2
hsa-miR-26b	**0.687**, **0.02**	Pro/anti* [9, 10]	*ACVR1C*, *BMPR1B*, *BMPR2*, *CREBBP*, *EP300*, IFNγ, INHBA, INHBB, *SMAD1*, *SMAD2*
hsa-miR-34b	**0.742**, **0.008**	Pro [11]	**ACVR2A**, *ACVR1C*, FST, *SMAD5*, SMAD7, SMURF1, *RPS6KB1*

*Physiological conditionally based effects. †Emphasis indicates the predicted effect that miRNA inactivation of that gene would have on fibrosis. Italic emphasis refers to decreased fibrosis. Underlined emphasis refers to increased fibrosis. Bold emphasis refers to effect dependent on ligand milieu. ACVR1C: activin receptor type-1C; ACVR2A: activin receptor type-2A; ACVR2B: activin receptor type-2B; BMP2: bone morphological protein-2; BMPR2: bone morphological protein receptor type-2; CDKN2B: cyclin-dependent kinase inhibitor 2B; CREBBP: cAMP response element-binding protein; EP300: E1A-associated protein p300; FST: follistatin; GDF6: growth differentiation factor 6; ID4: inhibitor of DNA-binding protein 4; IFNγ: interferon-gamma; INHBA: inhibin beta A subunit; INHBB: inhibin beta B subunit; PITX2: paired-like homeodomain transcription factor 2; PPP2CA: protein phosphatase 2 catalytic subunit alpha; ROCK1: Rho-associated coiled-coil-containing protein; RPS6KB1: ribosomal protein S6 kinase B1; SMURF1: SMAD-specific E3 ubiquitin protein ligase 1; TGFB2: transforming growth factor beta 2.

during this phenotypic change is attributable to several pathologies including but not limited to LV dilation, ventricular stiffening, and cardiomyocyte death; all of which play pivotal roles in the progression to heart failure. We reported that ZL rats treated with rapamycin had greater degrees of cardiac fibrosis than untreated ZL rats. However, in ZO rats, treatment with rapamycin attenuated cardiac fibrosis [3]. Data presented here indicates that changes in the miRNA transcriptome induced by diabetes and Rap treatment shared significant similarities. Interestingly, while some of the miRNAs that were differentially expressed in ZO-C and ZL-Rap compared to ZL-C were profibrotic or increased in conditions of fibrosis, others were involved in suppressing fibrosis (Tables 2 and 3). This observation suggests that while cardiac fibrosis develops in response to diabetes (ZO-C) or Rap treatment of healthy animals (ZL-Rap), a concurrent adaptive mechanism to regulate fibrosis via modulating miRNA transcriptome is also activated. Several signaling pathways have been implicated in the transformation of fibroblasts to myofibroblasts (fibrotic remodeling). Among the best understood of these signaling pathways are transforming growth factor-β, which is thought to be the primary regulatory pathway of pathological fibrosis [69, 70].

While some interspecies variation exists in the expression and target specificity of miRNA, previous studies have demonstrated high degrees of similarity [71, 72]. With the goal of exploring how our differentially expressed miRNA panel may be associated with cardiac fibrosis in humans, we utilized the DIANA software [30] and human orthologs for the miRNA that were significantly correlated with cardiac

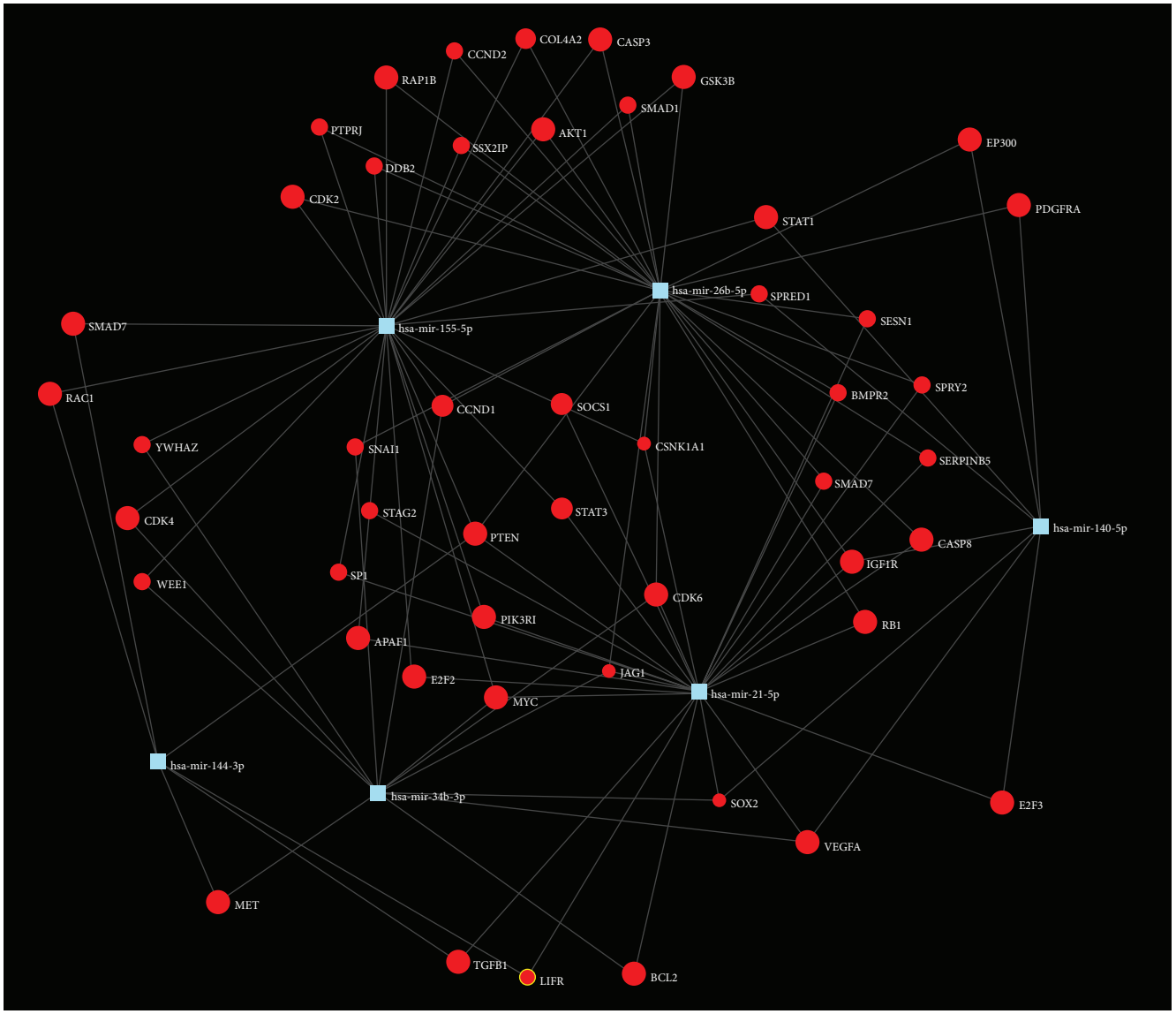


Figure 8: KEGG pathway enrichment analyses and subsequent identification of target genes along significantly enriched pathways associated with cardiac fibrosis. Generated using miRNet software.

fibrosis in our rats were used as input. KEGG analysis returned multiple interactions between our miRNA set and genes involved in the TGF-β signaling pathway (Table 6; Figure 8). Profibrotic TGF-β signaling involves numerous cell surface receptors in addition to TGF-β receptors, including those of the bone morphological protein and activin subfamilies (BMPRs and ACVRs, respectively). SMAD proteins are the primary signal transducers of these receptors and therefore are profibrotic with the exception of SMAD7 which inhibits the action of other SMADs. As with most biological pathways, the TGF-β pathway contains several internal feedback loops. Collectively, these data suggest that human orthologs of rat miRNAs that showed the highest correlation with cardiac fibrosis are involved in modulating the profibrotic TGF-β pathway.

In summary, the data presented here show that 47% of miRNA transcriptome activated in the hearts of healthy rats in response to Rap treatment are identical to 80% of the miRNA transcriptome activated in diabetic rat hearts. In diabetic rat hearts, the miRNA transcriptome could have played a significant role in inducing the pansuppression of anti-inflammatory and proinflammatory intracardiac cytokines. However, while several miRNAs had predicted profibrotic effects, others had antifibrotic effects, suggesting that the miRNA transcriptome may serve as an adaptive mechanism to regulate the progression of cardiac fibrosis. Moreover, human orthologs of rat cardiac miRNAs that exhibited the highest correlation with cardiac fibrosis have the potential to modulate the profibrotic TGF-β pathway.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Supplementary Materials

Supplemental Table 1: differentially expressed miRNAs that were significant ($p < 0.05$) in ZO-C vs. ZL-C hearts. Supplemental Table 2: differentially expressed miRNAs that were significant ($p < 0.05$) in ZL-Rap vs. ZL-C hearts. Supplemental Table 3: differentially expressed miRNAs that were significant ($p < 0.05$) in ZO-Rap vs. ZO-C hearts. Supplemental Table 4: differentially expressed miRNAs that were significant ($p < 0.05$) in ZO-Rap vs. ZO-C hearts. Supplemental Table 5: KEGG pathway enrichment analysis of pathways corresponding to differentially expressed miRNA*. *(Supplementary Materials)*

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Review Article

Therapeutic Use of mTOR Inhibitors in Renal Diseases: Advances, Drawbacks, and Challenges

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The mammalian (or mechanistic) target of rapamycin (mTOR) pathway has a key role in the regulation of a variety of biological processes pivotal for cellular life, aging, and death. Impaired activity of mTOR complexes (mTORC1/mTORC2), particularly mTORC1 overactivation, has been implicated in a plethora of age-related disorders, including human renal diseases. Since the discovery of rapamycin (or sirolimus), more than four decades ago, advances in our understanding of how mTOR participates in renal physiological and pathological mechanisms have grown exponentially, due to both preclinical studies in animal models with genetic modification of some mTOR components as well as due to evidence coming from the clinical experience. The main clinical indication of rapamycin is as immunosuppressive therapy for the prevention of allograft rejection, namely, in renal transplantation. However, considering the central participation of mTOR in the pathogenesis of other renal disorders, the use of rapamycin and its analogs meanwhile developed (rapalogues) everolimus and temsirolimus has been viewed as a promising pharmacological strategy. This article critically reviews the use of mTOR inhibitors in renal diseases. Firstly, we briefly overview the mTOR components and signaling as well as the pharmacological armamentarium targeting the mTOR pathway currently available or in the research and development stages. Thereafter, we revisit the mTOR pathway in renal physiology to conclude with the advances, drawbacks, and challenges regarding the use of mTOR inhibitors, in a translational perspective, in four classes of renal diseases: kidney transplantation, polycystic kidney diseases, renal carcinomas, and diabetic nephropathy.

1. Introduction

The mechanistic (formerly mammalian) target of rapamycin, was discovered almost simultaneously by three independent groups in the mid-1990s and coined as rapamycin and FK506-binding protein-12 (FKBP-12) target 1 (RAFT1), FKBP–rapamycin-associated protein (FRAP), and mTOR [1–3]. These names reflected the fact that mTOR was identified as the target of rapamycin (etymol.: Rapa- (Rapa Nui = Easter Island), -mycin (related to the antifungal properties)), which is a natural antibiotic macrolide firstly isolated from bacterium (Streptomyces hygroscopicus) extracts found on Easter Island soil samples [4].

mTOR is a member of the phosphatidylinositol 3-kinase-related kinase (PIKK) family, which is one of the key players of cellular metabolism that is coupled with nutrient availability, energy, and homeostasis [5, 6]. It plays a prominent role as a molecular sensor of gene transcription and protein synthesis, tissue regeneration and repair, immunity, oxidative stress, and cell proliferation/cell death (e.g., autophagy and apoptosis) upon environmental and cellular cues (nutrients (e.g., glucose, amino acids, and fatty acids), growth factors (e.g., insulin-like growth factor-1, IGF-1; vascular endothelial growth factor, VEGF), hormones (e.g., insulin), and cytokines) [7–9]. Given the ubiquitous distribution of mTOR in distinct cell types throughout the body, mTOR pathway

control several anabolic and catabolic processes in distinct organs/tissues including (but not restricted) the liver, lymphocytes, white and brown adipose tissue, skeletal muscle, brain, heart, and kidney [8]. Hence, impaired mTOR activity has been associated in widespread human diseases, including cancer, type 2 diabetes, cardiovascular pathology, and neurodegeneration as well as during aging [10–12].

Notably, accumulated evidence suggests mTOR signaling deregulation as a central player in the pathophysiology of distinct kidney diseases. Herein, we will critically discuss the advances, drawbacks, and future challenges of mTOR pharmacological inhibition in distinct renal conditions and in a bench-to-bedside perspective.

2. Overview of mTOR Components and Signaling Pathways

mTOR is a 289 kDa protein kinase encoded in humans by the *MTOR* gene (1p36.2). It interacts with several proteins to form two evolutionary conserved complexes among eukaryotes—mTORC1 and mTORC2. There are two common proteins shared by mTORC1/mTORC2 multimeric complexes: the positive regulator mLST8 (mammalian lethal with Sec13 protein8, also known as GβL) and the negative regulator Deptor (DEP domain-containing mTOR-interacting protein). Yet, there are unique proteins coupled to each complex: mTORC1 is associated with raptor (regulatory-associated protein of mTOR), fundamental for mTORC1 stability and a positive regulator of downstream effectors recruitment and with PRAS40 (proline-rich Akt substrate), a protein which blocks mTORC1 activity; mTORC2 is coupled with mSIN-1 (mammalian stress-activated protein kinase-interacting protein), PROTOR 1/2, and Rictor (rapamycin-insensitive companion of mTOR), a scaffold protein that displays chief roles for mTORC2 assembly, stability, and substrate recognition (e.g., Akt and SGK1) [7, 11].

The mTOR-containing complexes also differ in terms of upstream modulators, substrate specificity, functional outputs, and sensitivity to rapamycin [13]. mTORC1 broadly senses nutrients, growth factors, mitogens, and stress signals, thus being generally associated with cell growth by regulating important cellular processes, including the translation of mRNAs into the synthesis of key proteins for proliferation, lipid synthesis, mitochondrial biogenesis, and autophagy [14, 15]. Examples of mTORC1 downstream effectors are the lipin 1/SREBP (sterol regulatory element-binding proteins), the p70S6 kinases (S6K1 and S6K2), and the EIF4EBP1 (eukaryotic translation initiation factor 4E-binding protein 1) [16, 17]. In contrast to mTORC1, the control of mTORC2 by upstream modulators and downstream effector proteins is not as well understood, even though insulin and related pathways have been suggested as the main activators [11]. Nevertheless, plasma membrane localization as well as ribosome-binding through insulin-stimulated phosphatidylinositol 3-kinase (PI3K) signaling seem to have a chief role in mTORC2 regulation [18, 19]. Phosphorylation of protein kinase B (Akt) and other AGC-family kinases (e.g., serum- and glucocorticoid-induced protein kinase 1, SGK1; protein kinases C, PKC) has been linked with mTORC2 activation, with important consequences on cell survival, cytoskeleton organization, and cycle progression [14, 20, 21]. Interestingly, Akt appears to have a complex dual role on mTOR, being both an (i) upstream regulator of mTORC1 (indirect activation through phosphorylation and inactivation of TSC1/TSC2 complex, who constitutively suppress mTORC1 activity through Rheb GTPase inhibition) and a (ii) downstream target of mTORC2 [10, 22]. The activity of the two complexes is finely and mutually tuned through some feedback circuits promoted not only by upstream regulators of mTORC1 (e.g., Akt) but also by other downstream effectors of mTORC1, such as the p70S6K1 [23–25]. For instance, p70S6K1 phosphorylates mSIN-1 at both Thr86 and Thr389 residues and dissociates mSIN-1 from mTORC2, thus providing a negative feedback mechanism downstream of mTORC1. The loss of such reciprocal mechanistic feedback loops is observed in some mutational loss-of-function in mTOR key components, as in the case of the R81T Sin1 mutation identified in ovarian cancer patients, highlighting their clinical relevance [25]. Hence, impairments of constitutive feedback mechanisms and unexpected mTOR hyperactivation are particularly relevant when mTOR signaling modulation is envisaged. In this regard, the fact that PI3K/Akt/mTOR pathway critically regulates a plethora of physiological processes that become deregulated in a wide spectrum of pathologic conditions prompt the design of several pharmacological agents that target distinct components of this signaling cascade, as outlined in Section 3 [22, 26–28].

Finally, the sensitivity to rapamycin is another important feature that distinguishes mTORC1 and mTORC2 complexes. Rapamycin does not directly inhibit the catalytic (kinase) activity of mTOR; instead, it binds to the immunophilin FKBP12 (FK506-binding protein of 12 kDa), which is a protein that couples with mTOR FKBP-rapamycin-binding domain (FRB). Even though the FRB domain is present in both mTORC1/2 complexes, it is only exposed in mTORC1, as Rictor blocks the access of FKBP12-rapamycin complex to FRB domain in mTORC2 [25, 29]. Hence, mTORC2 is relatively resistant to the effects of rapamycin both *in vitro* and *in vivo*, although this phenomenon can be disrupted by chronic treatments [13, 30, 31].

3. Pharmacological Advances and Challenges within mTOR Inhibition

The recent breathtaking advances in up- and downstream targets of mTOR, reciprocal feedback mechanistic loops, and mutational loss-of-function in mTOR key components (e.g., TSC1/2, PIK3CA, and Akt), the most common cause of mTOR signaling hyperactivity, provided new rationales for translating the mTOR basic science to the clinic. In fact, pharmaceutical companies have discovered impressive arrays of small molecules targeting PI3K/Akt/mTOR cascade elements which are currently undergoing evaluation in preclinical and clinical studies mainly in cancer and transplantation, even though mTOR inhibitors are being also considered for other pathological conditions such as

rheumatoid arthritis, atherosclerosis and a wide spectrum of neurologic disorders where aberrant mTOR pathway activity is consistently observed [22, 27, 28, 32]. Herein, it will be focused on the different classes of mTOR inhibitors currently undergoing preclinical/clinical studies aimed at providing new pharmacological agents with increased efficacy and a lower side effect profile.

3.1. Allosteric mTOR Inhibitors: Rapamycin/Rapalogues. Rapamycin (or sirolimus), a macrocyclic lactone, was initially described as an antibiotic agent. Nevertheless, this molecule also exhibits immunosuppressant, cytostatic, antiangiogenic, and antiproliferative properties, expanding the clinical applications to transplantation and oncology fields [10]. Rapamycin acts as an allosteric inhibitor of mTORC1, which, together with FKBP12, interacts with the FRB domain of mTORC1 blocking some of the functions of this complex (see Figure 1). The data suggest that rapamycin impairs mTORC1 activity mainly by preventing the association and phosphorylation of substrates into the kinase complex [33, 34]. However, not all mTORC1 downstream targets are equally inhibited by rapamycin, with potency varying for weak versus strong substrates [25]. Moreover, and even though rapamycin does not interact with mTORC2, some studies have shown that this molecule is able to indirectly modify mTORC2 complex in a dose-, time-, and cell-type dependent manner, probably by preventing mTOR molecules from the interaction with mTORC2-specific partner protein Rictor [3, 31, 35].

The fact that rapamycin has limited bioavailability led to the development of semisynthetic analogs, named rapalogues, with superior aqueous solubility and improved pharmacokinetic properties. Examples of this first-generation of mTOR inhibitors are temsirolimus (CCI-779), everolimus (RAD001), and ridaforolimus/deforolimus (MK-8669/AP23573) who share a central macrolide chemical structure yet differ in the functional groups added at C40 that significantly alter bioavailability, half-life, and administration routes (oral versus intravenous) [22]. In contrast with everolimus and ridaforolimus, temsirolimus is a prodrug that requires removal of the dihydroxymethyl propionic acid ester group after administration, becoming sirolimus in its active form [36]. Rapalogues exhibit a safe toxicity profile, with side effects such as skin rashes and mucositis being dose-dependent. Other symptoms described are fatigue, anemia, neutropenia, and metabolic disorders such as hypertriglyceridemia, hypercholesterolemia, and hyperglycemia [22]. In this regard, it should be highlighted that rapamycin prevented insulin-mediated suppression of hepatic gluconeogenesis and impaired *in vitro* basal and insulin-stimulated glucose uptake in adipocytes from human donors [37, 38]. Temsirolimus and sirolimus are also associated with pulmonary toxicity, being interstitial lung disease, risk of secondary lymphoma, and reactivation of latent infections rare side effects [39].

Since mTORC1 and mTORC2 control events intimately related to cell growth and survival, rapalogues have been extensively studied in the oncology field, with several works conducted to analyze the effectiveness of these class of molecules alone and/or in combination with standard chemotherapy in the treatment of several types of cancers [26]. Although clinically promising, the results of such studies are quite disappointing, and some putative explanations have been hypothesized. Rapalogues have some serious drawbacks in terms of the desired molecular effects, and the efficacy may be partially limited by their drug action (cytostatic rather than cytotoxic). Moreover, as rapamycin and rapalogues act only on mTORC1, treatment with any of the molecules can elicit long-term feedback loops deregulation in mTOR network, therefore leading to aberrant activity of compensatory prosurvival pathways, including the PI3K/Akt signaling network itself. This phenomenon can seriously compromise the anticancer efficacy as well as the acquisition of chemoresistant phenotypes [22, 23, 25]. Since mTOR is a member of PIKK-related family sharing a high degree of similarity/sequence homology within the catalytic domain with PI3K, the next logical approach was the development of ATP-competitive dual PI3K/mTOR inhibitors.

3.2. Dual PI3K/mTOR Inhibitors. As highlighted above, rapamycin and rapalogues are incomplete inhibitors of mTORC1 and elicit feedback activation of PI3K/Akt mitogenic pathways. This argues for a theoretical therapeutic advantage of dual PI3K/mTOR inhibition in terms of better efficacy and less likelihood to induce drug resistance. These new agents are a class of catalytic ATP competitive inhibitors that exert their effects by binding indiscriminately to the ATP-binding site on both mTORC1/2 and PI3K catalytic domains (see Figure 1), which are two crucial signaling hubs [26, 40]. The prototype molecule in this class is the pyridofuropyrimidine PI-103, even though it was never translated into the clinic mainly because of its rapid *in vivo* metabolism [41, 42]. Over the next few years, other dual PI3K/mTOR inhibitors were discovered and advanced into the clinical evaluation (phase 1 and 2 trials), namely, the imidazoquinoline derivative NVP-BEZ235 (dactolisib), GDC-0980 (apitolisib), and PKI-587 (gedatolisib) [26, 40]. Although the appealing prospects of simultaneously targeting PI3K/mTOR, clinical studies have revealed a limited efficacy and important toxicity concerns (e.g., nausea, diarrhea, vomiting, decreased appetite, hyperglycemia, mucositis, cutaneous rash, elevated liver enzyme levels, renal failure, and hypertension). Moreover, it was proposed that dual PI3K/mTOR inhibitors suppressed a yet unidentified negative feedback loop mediated by mTORC2, which could partially explain the *in vitro* resistance and limited efficacy *in vivo* [25, 43].

3.3. ATP Competitive Inhibitors: mTOR Kinase Inhibitors (TOR-KIs). More recently, a second-generation of pharmacological mTOR inhibitors have been developed. In contrast to the rapamycin analogs, these molecules exert their effects by directly blocking the ATP catalytic site that is integral to both mTOR complexes (see Figure 1), resulting in widespread inhibition of the mTOR signal [36, 44, 45]. These agents exhibit a much lower half-maximal inhibitory concentration (IC_{50}) against mTOR activity than PI3K [26]. Hence, they are more discerning in their function: the main target is the mTORC1/2 catalytic domain without substantial effect on

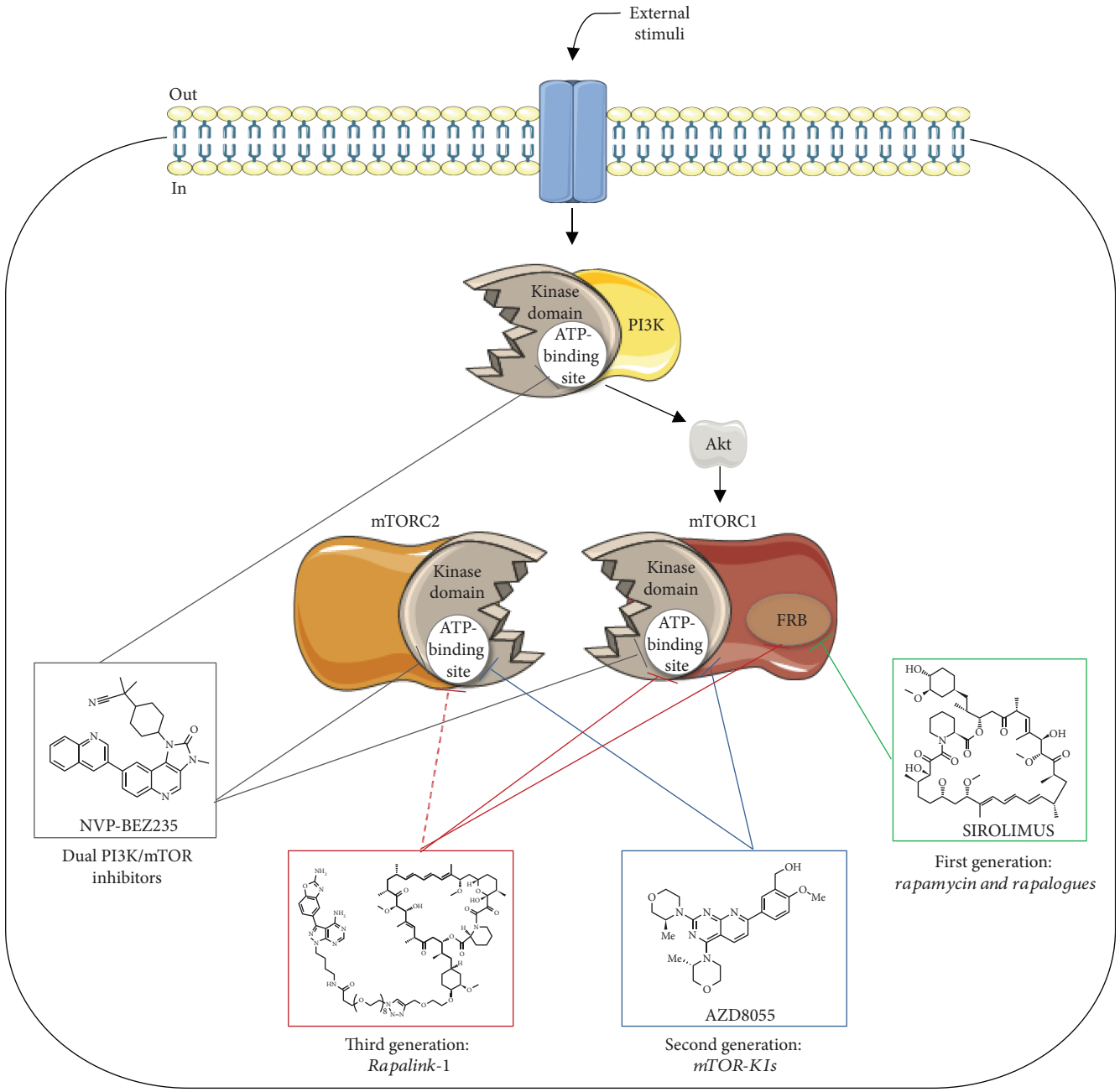

Figure 1: Mechanisms of action of three distinct generations of mTOR inhibitors and dual PI3K/mTOR inhibitors. The first generation of mTOR inhibitors (sirolimus chemical structure selected as an example) interacts with FRB domain of mTORC1 and partially inhibits mTOR downstream signaling events. Dual PI3K/mTOR inhibitors indiscriminately bind to the ATP-binding site of mTOR and PI3K catalytic domains, thus blocking the activity of both kinases (NVP-BEZ235 chemical structure selected as an example). The second generation of mTOR inhibitors act as ATP analogs and compete with ATP only in mTORC1/2 catalytic domains without substantial effect on PI3K (AZD8055 chemical structure selected as an example). Finally, the third generation of mTOR inhibitors combines a mTOR kinase inhibitor with rapamycin within the same molecule, which allows compounds to interact with the FRB domain and also to reach mTORC1 kinase domain, acting as an ATP-competitive inhibitor (Rapalink-1 chemical structure selected as an example). Dashed arrow represents the ability of Rapalink-1 to inhibit mTORC2 kinase activity, even though the precise molecular mechanism remains to be fully addressed (mTOR, mammalian target of rapamycin; PI3K, phosphatidylinositol 3-kinase; Akt, protein kinase B; FRB, FKBP-rapamycin-binding domain). Elements of the scheme were drawn using the website https://smart.servier.com/.

PI3K, with an expected reduction of toxicological events associated with dual PI3K/mTOR inhibitors [43]. Remarkably, mTOR kinase inhibitors (TOR-KIs) were effective antiproliferators in cell models displaying insensitivity to the first-generation of mTOR inhibitors [46, 47]. The first such compound was PP242, with numerous other TOR-KIs subsequently discovered, including Torin 1 and its sister Torin 2, AZD8055, TAK-228, and CC-223, some of them

currently undergoing phase 1/2 clinical evaluation in neoplastic disorders [25, 26]. Nevertheless, mechanisms of resistance were already reported for these second generation of compounds, highlighting the many adaptive skills of PI3K/Akt/mTOR network upon modulation of any key component [25]. Among several reasons that may concurrently explain such discouraging results are feedback loops dysregulation as well as a wide range of mTOR mutations responsible for the increased catalytic activity of both mTORC1/2 complexes, rather than a direct active-site mutation interfering with drug binding [48–50].

3.4. New Generation: RapaLink-1. Considering the poor efficacy, resistance mechanisms, and severe side effects described for the class of drugs previously mentioned, an attempt to develop a third generation of mTOR inhibitors have been recently outlined. Through exploitation of both ATP- and FRB-binding sites of mTOR, the new molecule RapaLink-1 combine the high affinity of rapamycin for mTORC1 with the effective kinase inhibition of the TOR-KI MLN0128, which is a highly selective structural analog of PP242 that is currently in clinical trials [50]. The linker portion between these two molecules—a polyethylene glycol unit—does not disrupt rapamycin binding to FKBP12 or the FRB domain of mTOR, thus leveraging the high selectivity and affinity of rapamycin for mTORC1 and the "deliver" of MLN0128 to the ATP site of mTORC1 [50, 51]. Notably, RapaLink-1 was effective in the inhibition of both mTORC1 and mTORC2 downstream targets (mTORC1 (S6K, 4EBP1) and mTORC2 (Akt)) at doses between 1 and 3 nM, suggesting that it is also able to suppress the catalytic activity of both mTORC2 components through direct or indirect mechanisms that remain to be elucidated (see Figure 1). This drug was found effective in reversing resistance of breast cancer due to mTOR FRB or kinase domain mutations [50]. Despite its size, Rapalink-1 can cross blood-brain barrier and has shown increased efficacy in a glioblastoma cell model as well as in a genetically engineered *in vivo* model of brain cancer, when compared with earlier mTOR inhibitors [52]. Moreover, this compound did not display significant toxicity events when given intraperitoneally in mice and was also recently suggested as a possible new alternative to treat and prevent the development of alcohol use disorder (AUD) [53]. Overall, Rapalink-1 shows an appealing potency profile compared with earlier mTOR inhibitors which encourage next clinical evaluation. Nevertheless, further preclinical studies aimed at establishing whether Rapalink-1 has immunosuppressive properties is an inductor of autophagy and/or disrupts homeostatic mTOR feedback loops deserve to be better exploited.

4. The Role of mTOR in the Kidney

As previously mentioned, mTOR plays a major role in the regulation of cell proliferation and growth, mainly acting as a metabolic sensor, while low cellular energy supply suppresses mTOR activation and high metabolic input fuels mTOR activation. Whereas, the precise roles played by mTORC1 and mTORC2 complexes in the different types of renal cells is not fully unveiled during development nor in the adulthood, it is suggested that mTOR signaling pathways impact glomerular and tubulointerstitial renal physiological processes [54].

The same also holds truth under conditions of kidney injury. Podocytes, the most vulnerable elements of all kidneys, can adapt to stressful conditions (e.g., metabolic, immunological, and toxic) acquiring a hypertrophic phenotype [55, 56]. Noteworthy, this compensatory mechanism related to size control seems to be mTOR-mediated [57, 58]. In fact, features like podocyte damage and proteinuria are observed in both animal models and transplanted patients upon rapamycin treatment, strengthening the concept that mTOR activity is paramount for adaptive compensatory mechanisms in response to glomerular insult [59–63]. Moreover, studies using genetic models revealed that besides mTORC1 complex, mTORC2 and its downstream target Akt2 also play a role in renal glomerular functions, including podocyte stress surveillance and survival of remaining podocytes in conditions of nephron mass reduction [58, 64]. Furthermore, prevention of mTORC2-Akt2 activation by rapamycin in biopsy tissue from kidney transplant patients was accompanied by increased glomerular apoptosis [64], reinforcing the notion that mTORC2 (along with mTORC1) might contribute to rapamycin-induced proteinuria.

Regarding kidney tubules, much less is known concerning the physiological (and pathological) role of mTORC1 and mTORC2. Apart from proteinuria, subjects under sirolimus therapy may develop hypophosphatemia and hypokalemia; since phosphaturia is a reliable outcome, and considering that *in vivo* mTORC1 inhibition does not seem to affect the apical phosphate reabsorption machinery [65], it could be conjectured whether mTORC1 could affect the basolateral efflux pathways in proximal tubular cells or other unknown hormonal components of phosphate homeostasis. Further research, namely, using mTORC1 ablation in the proximal tubule, is advisory to clarify the precise mechanisms. Concurrently, *in vitro* data have been suggested on the involvement of mTORC2 in renal tubular Na^+ balance regulation [66]. This hypothesizes, if further confirmed *in vivo*, might be important for some clinically relevant conditions, such as salt-sensitive hypertension or volume overload occurring with congestive heart failure.

5. mTOR Inhibition and Renal Diseases

5.1. Kidney Transplantation. The true challenge of transplantation research, in addition to the specific advances in surgery, was to improve knowledge about the complexity of the immune system and to design and synthesize drugs able to counteract acute rejection. In the early 1950s, even without effective solutions to prevent rejection, the first successful kidney transplant between genetically related donors was performed, thereby minimizing the role of HLA system, which would ultimately be discovered in 1958. In fact, in 1954, the kidney transplant performed by the Boston group between identical twins was crowned by huge success, with the kidney receptor surviving eight years posttransplant. However, it was imperative to extend the transplantation to

unrelated living donors and deceased donors; however, in those cases, the incidence of rejection, with consequent organ loss, remained very high. With the discovery of the first calcineurin inhibitor and its use in clinical practice in 1983, a new era dawned for graft and patient survival. Later, in 1994, a more potent calcineurin inhibitor came into use: tacrolimus. Tacrolimus, in combination with mycophenolate mofetil or mycophenolate sodium (MMF/MPA), showed a remarkable impact on the incidence of acute rejection, which declined to around 5% and 15%, respectively, with a significant improvement in graft and patient survival to over 90% in the first year after transplantation [67].

This advance notwithstanding intensive experience with calcineurin inhibitors has progressively shown their "dark side", with side effects frequently related to high drug blood concentrations. Adverse effects such as acute and chronic nephrotoxicity, worsening risk of cardiovascular disease, new onset diabetes after transplantation, increased incidence of neoplasms, and viral infections such as CMV, BKV, and oncogenic viruses have been and still are the Achilles' heel of these drugs, and even today continue to fill discussion forums. At the heart of the controversy remains the permanent search for the balance between receiving adequate immunosuppression to prevent graft rejection and minimizing adverse effects, especially nephrotoxicity and cardiovascular events, which continue to be the main cause of death.

Despite all the undisputable therapeutic progress, improvement in long-term graft survival remains lacking. Several factors have been identified to this end, namely, graft quality (older donors, and/or with expanded criteria) and alloantibody-mediated chronic rejection [68]. This multifactorial problem stimulated research on new drugs, alternatives to calcineurin inhibitors, and/or novel immunosuppression strategies which could simultaneously provide two key transplantation objectives: a better long-term graft survival and fewer toxic and adverse effects on the graft and receptor.

The use of mTOR inhibitors in kidney transplantation started in 1990 with the discovery of rapamycin (sirolimus). Exciting results were observed when sirolimus was combined with cyclosporine and prednisone, leading to a significant reduction in the incidence of acute rejection when compared to azathioprine or placebo, despite persistent high triglyceride levels [69, 70]. It was readily observed that the use of these new immunosuppressant drugs could be an attractive alternative to the calcineurin inhibitors, and thus two immunosuppressive strategies were proposed: either the use of mTOR inhibitors without calcineurin inhibitors or maintenance of calcineurin inhibitors in the early posttransplant period with a switch to mTOR inhibitors shortly thereafter (early conversion). The exclusion of calcineurin inhibitors was tested in some studies [71], but with disappointing results due to the high number of acute rejection episodes. Only one center achieved satisfactory results when comparing sirolimus and IL2R antibody induction, calcineurin inhibitor-based regimen [72]. Induction with lymphocyte-depleting antibodies in two therapeutic strategies comparing sirolimus with cyclosporine also showed no advantage, and there was no difference in graft and receptor survival in the first year. This showed that immunosuppression without calcineurin inhibitors was not a good alternative and suggested that sirolimus alone was less potent in controlling the immune response in the early posttransplant period. This disadvantage was not resolved by increasing the dose, a strategy which was associated with more adverse effects [73].

Considering early conversion to mTOR inhibitors, a study on conversion from cyclosporine A (CsA) to sirolimus at three months posttransplantation, combined with MMF and oral steroids, showed that eGFR in the first year was significantly higher in the sirolimus group (68.9 vs. 64.4 mL/min), with no statistically significant difference regarding receptor and graft survival [74]. The incidence of acute rejection occurred mainly after the suspension of corticosteroids, but the difference was not statistically significant. It should be noted that the sirolimus group had higher serum triglycerides as well as more cases of diarrhoea, aphthous ulcers, and acne. Another randomized study with early conversion of cyclosporine to a different mTOR inhibitor—everolimus—while maintaining mycophenolate mofetil (MMF) observed that the everolimus group showed a significant improvement in eGFR (71.8 vs. 61.9 mL/min). These patients, however, also had a higher incidence of biopsy-proven acute rejection (10 vs. 3%) [75]. The follow-up of these patients at five years confirmed that in the first year posttransplant grafts presented better function, but also a higher incidence of acute rejection [76]. Another study following the same line of research found a better eGFR in the sirolimus group compared to the calcineurin inhibitor at one-year posttransplantation, but after two years this difference disappeared [77]. Biopsy-proven acute rejection biopsy was similar in both groups, but the number of deaths was higher in the calcineurin inhibitor group. In both studies, however, a higher incidence of adverse effects was observed, leading to the discontinuation of the mTOR inhibitor.

In the ZEUS study, designed to analyze the incidence of anti-HLA antibodies (specific to the donor), we found significantly higher levels in patients undergoing mTOR inhibitors. It is unknown whether this effect was drug-related or due to corticosteroid suspension [78].

Faced with somewhat disappointing results from the isolated use of mTOR inhibitors and admitting the undisputed superiority of calcineurin inhibitors (CNI) in the control of rejection, researchers sought to explore the complementarity of both drugs, while minimizing their drawbacks and enhancing their advantages. It should be noted that the combination of mTOR inhibitors and CsA had previously been tested in the 1990s, with a low incidence of acute rejection at the expense of a large number of adverse reactions, mainly related to the high doses that were practiced at the time [79]. Sirolimus doses should vary according to the type of calcineurin inhibitor. In fact, the combined administration of sirolimus with cyclosporine increases its toxicity, implying that a lower dose should be used than with tacrolimus [80]. For these reasons, clinical trials started testing the combination of mTOR inhibitors and calcineurin inhibitors using lower doses. In the work by Langer et al., the combination of everolimus (whole blood concentration target blood level > 3 ng/mL) with very low dose tacrolimus (target blood level 2–4 ng/mL) resulted in a low incidence

of acute rejection episodes, without compromising graft function [81]. The combination of sirolimus with a reduced exposure to tacrolimus also showed a low incidence of acute rejection and a trend towards better graft function [82]. A meta-analysis focusing on this topic concluded that the association of mTOR inhibitors with low-dose tacrolimus effectively preserves graft function without a significant impact on patient survival and graft rejection when compared to the standard dose of tacrolimus [83]. The most frequently found adverse events in patients were dyslipidaemia and new-onset diabetes after transplantation (about 60 and 38%, respectively), followed by surgical wound complications and hypertension. In accordance with current experience, the combination of mTOR inhibitors with tacrolimus in low dose appears to be a very potent immunosuppressive regimen, considering that the former adverse effects are dose-dependent.

The challenge of combining efficacy and safety while preventing episodes of acute rejection, and maintaining good long-term graft function, is well present in the ongoing TRANSFORM trial (Advancing renal TRANSplant efficacy Outcomes with an eveRoliMus-based regimen) (NCT01950819), whose final conclusions are expected in 2018 [84]. In this trial, the mTOR inhibitor everolimus combined with a low-dose calcineurin inhibitor is compared to mycophenolate with standard CNI exposure, and the long-term effects are observed. The significant number of patients enrolled and three-year follow-up period makes this the largest randomized study ever undertaken in kidney transplantation and is expected to clarify the advantages or disadvantages of utilizing the combined strategy. In the preliminary results published at 12 months, eGFR was similar in both arms [85], and the study also met its key secondary endpoint showing noninferiority with respect to the composite endpoint of tBPAR, graft loss, and death [85]. A decrease in the incidence of viral infection by cytomegalovirus (3.5 vs. 12.5%) and BK virus (3.9 vs. 7.2%) was observed [85]. The preliminary analysis was able to demonstrate the noninferiority of this therapeutic regimen, with the advantage of a lower incidence of viral infections.

According to current knowledge, it is possible to conclude that mTOR inhibitors in kidney transplantation may be satisfactory and effective when applied in the following two strategies: in combination with low-dose calcineurin inhibitors or in early conversion that provided patients with moderate-to-high immunological risk are excluded.

5.2. Polycystic Kidney Disease. Polycystic kidney disease (PKD) is a clinically and genetically heterogeneous group of monogenic disorders. This pathologic entity comprises several Mendelian diseases including autosomal dominant polycystic kidney disease (ADPKD), autosomal recessive polycystic kidney disease (ARPKD), and atypical PKD forms [86, 87]. ADPKD is the most common life-threatening hereditary renal disease, with an incidence of 1 : 400 to 1 : 1000 individuals [88]. Disease severity is highly variable, displaying distinct phenotypes ranging from manifestations *in utero* or during infancy (very early onset (VEO) disease) to clinically silent disease well into the second or third decade of life [89, 90]. In contrast, ARPKD typically presents much earlier (1 : 20000 live births among Caucasians). With advancing clinical course, ARPKD pathophysiological features often resemble the pattern of ADPKD, even though a more severe phenotype is often observed [87].

ADPKD is a chronic entity characterized by the appearance of cysts in both kidneys, which may also occur in other organs such as the liver, ovary, pancreas, spleen, and the central nervous system [91]. It is the most frequent hereditary kidney disease that progresses to end-stage kidney disease by the 5th or 6th decade of life, reaching a prevalence of around 8–10% in patients on dialysis [91]. Kidney size can reach significant dimensions as a consequence of the progressive increase in the volume of cysts in about 5% to 8% of the nephrons, leading to a gradual decline of renal function [91]. Renal capsule distension and compression of surrounding renal tissue may lead to complications such as hypertension and chronic pain, whereas the accumulation of urine can precipitate parenchymal infection. The CRISP study (Consortium for Radiologic Imaging Studies of Polycystic Kidney Disease) showed that renal volume and cysts increase at an exponential rate of about 5% per year and that this increase, as detected by magnetic resonance imaging, is accompanied by progressive deterioration in renal function [92].

About 85% of ADPKD is caused by mutations in the PKD1 gene which encodes polycystin-1, a large glycosylated integral membrane protein receptor present in the plasma membrane and in the renal tubular epithelium as well as in the bile and pancreatic ducts [93]. The remaining 15% are the result of mutations in the PKD2 gene encoding polycystin-2 [94]. Polycystin-1 is an adhesion molecule thought to be involved in cell-cell and cell-matrix interactions, whereas polycystin-2 is similar to a voltage-gated calcium channel. Both interact to regulate calcium influx [95]. Mechanisms of cystogenesis are not fully understood, but disruption of ciliary structure and changes in the cyclic AMP (cAMP) secondary to changes in intracellular calcium are responsible for cell proliferation, fluid secretion, and extracellular matrix composition [91]. These pathophysiological changes are mainly due to the overactivation of EGFR, cAMP, and mTOR pathway, leading to great interest in research regarding the inhibition of this signaling pathway in the treatment of this disease [96, 97]. Given that ADPKD patients carry deletions in adjacent genes such as PKD1 and tuberous sclerosis complex 2 (TSC2) which are responsible for the polycystin 1 and tuberin proteins, the hypothesis of a common cystogenic pathway has been advanced [98]. In fact, the TSC2 gene is responsible for the modulation or inactivation of the cell growth signals and proliferation promoted by serine-threonine kinase mTOR, which is abnormally activated in the cystic epithelium of patients with ADPKD. Polycystin 1 inhibits mTOR signaling through its interaction with tuberin. In the absence of this regulatory function, hyperactivity of the mTOR pathway results in a translational increase of the protein through the phosphorylation of S6K and 4EBP1, leading to proliferation, cell growth, and progression of cystogenesis [98, 99].

Initial studies conducted in preclinical models aimed at establishing whether mTOR inhibition through rapamycin

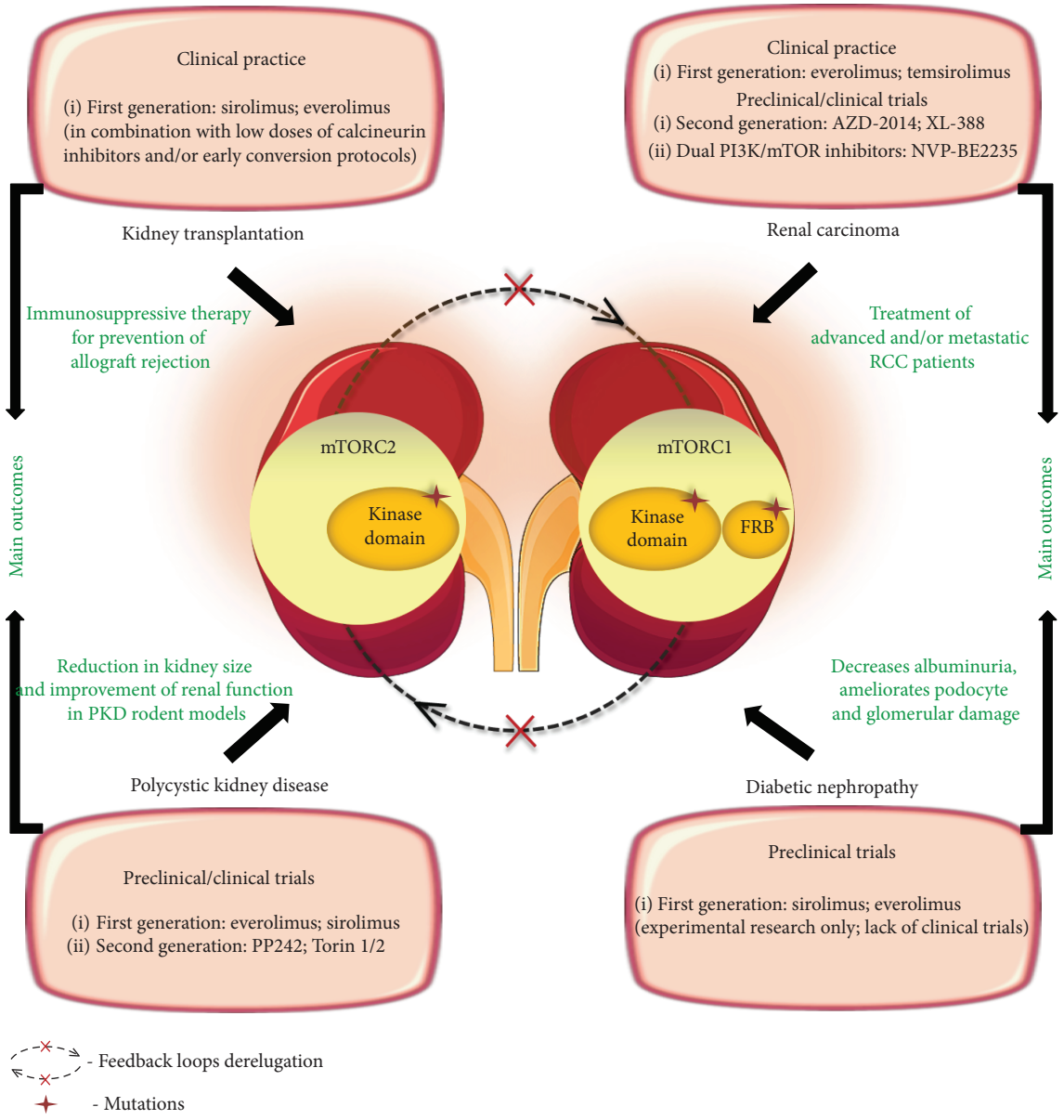

Figure 2: Pharmacological inhibition of mTOR network in renal diseases. A plethora of evidence highlights mTORC1 and/or mTORC2 hyperactivation through deregulation of feedback mechanisms that constitutively regulate mTOR network as well as acquired mutations on mTOR key components, as exemplified in the figure. Herein, it is summarized distinct classes of mTOR inhibitors that are currently available in clinical practice and/or in R&D trial stages in four classes of renal diseases: kidney transplantation, polycystic kidney disease, renal carcinoma, and diabetic nephropathy. The main outcomes from mTOR inhibition are highlighted with green color. Elements of the scheme were drawn using the website https://smart.servier.com/.

or everolimus (first generation of mTORC1 inhibitors) could ameliorate PKD (see Figure 2). The majority of these studies have reported that these agents elicited a long-lasting reduction in kidney size and an improvement of renal function in rodent models of ADPKD, late-stage nephronophthisis, and models that are not orthologous to any known human mutation [98, 100–104]. Nevertheless, a lack of efficacy was observed in the PCK rat model of ARPKD, Han:SPRD female rats, and early-stage nephronophthisis pcy mice [97, 101, 105]. Rodent models limitations along with a more prominent role of mTOR activity in later phases of the disease were possible explanations suggested by former authors. In light of these studies, mTOR activity inhibition has shown promising results as a therapy to retard the PDK course [104]. Advances in animal models have been recently established in the PKD field. One example is the Vil-Cre;Pkd2$^{f3/f3}$ mice, a ADPKD standardized model showing an important temporal cystic phenotype similar to what occurs in human ADPKD. Interestingly, this new preclinical tool has provided new insights into translational medicine, corroborating the involvement of mTOR pathway (mTORC1–CDK1/cyclin axis) in ADPKD pathophysiology and the efficacy of rapamycin treatment protocols in the improvement of mice survival, cystic phenotype, and renal

function [106]. Finally, it is important to emphasize that both mTORC1 (rapamycin-sensitive) and mTORC2 (rapamycin-insensitive) complexes are hyperactivated in PKD [97, 98, 107]. Hence, the use of mTOR kinase inhibitors (that target both mTORC1 and mTORC2) have been hypothesized as a promising strategy to slow cystic kidneys proliferation and improve kidney function. Interestingly, a high-throughput phenotypic screening of kinase inhibitors showed a potent inhibitory activity in cyst size inhibition for most mTOR inhibitors, and a most notable profile was found for Torins 1 and 2 [108]. Additionally, a preclinical study using the Cy/+ rat model of ADPKD highlighted that PP242, another mTOR kinase inhibitor, is able to slow cyst growth and improve kidney function [109]. The influence of mTOR tissue concentration on cyst volume was also evaluated by Novalic et al. which conducted an animal model study using low (3 ng/mL) vs. high (30–60 ng/mL) sirolimus concentrations at different stages of the disease. Only the high-dose group, at the early stage, showed histologically proven inhibition of cystogenesis and regression of cysts, pointing out that effective mTOR inhibition leads to a delay in cyst development and renal volume stabilization, but require higher doses and longer exposure to the drug [110]. Overall, an abundance of preclinical evidence suggests that mTOR inhibitors effectively slow cyst growth, even though the specific role of mTOR complexes is still poorly understood [104].

Because inhibition of mechanistic target of rapamycin (mTOR) effectively slows cyst growth expansion and preserves kidney function in PKD preclinical models, the next logical step was to test the effects of mTOR inhibitors (currently in clinical use as immunosuppressants) on cyst growth in human clinical trials. However, results from large randomized clinical trials testing both sirolimus and everolimus in ADPKD patients are still controversial. In the human randomized study conducted by Serra et al., and after 18 months of observation, patients with eGFR > 70 mL/min and kidney volume of about 1000 mL, rapamycin did not modify the eGFR, nor the total renal volume, compared to the control group, while albuminuria increased in the treated group [111]. In another study, higher doses of sirolimus seemed to stabilize cyst volume, comparing to the conventional therapy-treated patients [112]. The evaluation of the effects of another mTOR inhibitor-everolimus on ADPKD was also performed in a 2-year study that included placebo controls, but the treated group consisted of patients at an advanced disease (stage II or III), and an average kidney volume greater than 1500 mL. It was observed that in treated patients, cysts volume growth rate and renal parenchyma decreased; however, at the end of the study, no eGFR significant difference was found [113]. Stallone et al. also conducted a prospective and randomized study to evaluate the effects of rapamycin on type 1 ADPKD. Patients with eGFR between 40 and 80 mL/min/1.73 m^2 were divided into three groups receiving ramipril. In two of those groups, a low dose of rapamycin (through levels of 2–4 ng/mL) and a high dose (through levels of 6–8 ng/mL) were given. At 24 months, the authors did not observe any significant difference between treated patients, either in total kidney volume, cystic volume, or estimated creatinine clearance, and it was found that patients receiving rapamycin showed increased urinary protein excretion [114].

Overall, these clinical results were largely disappointing, taking into account the promising effects of mTOR inhibition in PKD animal models and retrospective studies of kidney transplant recipients undergoing immunosuppression with mTOR inhibitors who displayed reduced liver cystic phenotype [115, 116]. Some hypothesis has been figured out to explain such discouraging clinical results. Divergent approaches in terms of sample acquirements, use of different mTOR inhibitors/doses and biomarkers evaluation between experimental groups, may help to explain the lack of clinical efficacy of this class of drugs. In fact, kidney volumes have been extensively used as a surrogate endpoint of disease progression. However, therapeutic strategies that halt kidney enlargement does not necessarily improve renal function, and this is particularly relevant in ADPKD patients who constitutively display enlarged kidneys, even though the renal function is maintained for many years. Hence, from the clinical viewpoint, more adequate biomarkers to assess the efficacy of mTOR inhibition in ADPKD have been proposed, namely, the measurement of changes in GFR, serum creatinine level, and the urinary protein : creatinine ratio [116–118]. Another important feature may rely on the fact that mTOR inhibitors used in these trials may exhibit inadequate tissue penetration at clinically tolerable doses [119, 120]. In this regard, the mTOR kinase inhibitors appear to have a low side effect profile besides their ability to inhibit both mTORC1 and 2 complexes [109, 121]. Taken together, and until now, the results of mTOR inhibition therapy in ADPKD in humans, contrary to the impression left by animal model studies, does not consistently confirm the beneficial impact on renal volume or function. On the other hand, the high dose required to show some efficacy increases the adverse effects incidence, namely, the increase in urinary protein excretion.

5.3. Renal Carcinomas. Renal cell carcinoma (RCC) accounts for 2 to 3% of all adult malignancies and is the most common type of kidney cancer [89]. It develops from the proximal tubular cells and is histologically classified as clear cell RCC (ccRCC, ~85%) and nonclear cell RCC (nccRCC, ~15%). ccRCC is frequently associated with the von Hippel-Lindau (VHL) tumor suppressor mutational loss of function and subsequent accumulation of hypoxia-inducible factor (HIF) proteins, leading to the aberrant activation of HIF target genes that regulate angiogenic factors (vascular endothelial growth factor A, epidermal growth factor receptor type 1, platelet-derived growth factor B chain, and transforming growth factor), glycolysis, and apoptosis [122]. Yet, other driver mutations are also involved in the ccRCC development, including those responsible for the constitutive increase in mTOR activation [123, 124]. For example, loss-of-function mutations of PTEN, a negative regulator of mTOR through the PI3K/Akt pathway, are found in nearly 5% of RCC patients. Moreover, loss-of-function mutations of TSC1/TSC2 genes that lead to the inactivation of TSC—a negative regulator of mTOR—are present in patients with

tuberous sclerosis, a population particularly predisposed to the development of RCC [125, 126].

RCC is a highly vascularized malignancy and has been relatively resistant to traditional chemotherapy; therefore, the focus of current treatments relies in (i) cytokine-based immunotherapy (e.g., IFN-α), (ii) VEGF receptor-associated tyrosine kinase inhibitors (e.g., sorafenib, sunitinib, and axitinib), (iii) anti-VEGF monoclonal antibody, and (iv) mTORC1 inhibitors, taking into account their potential to simultaneously inhibit both tumor cell proliferation and angiogenesis [122, 127]. In fact, mTOR has presented itself as a valid target for the treatment of RCC, and both everolimus and temsirolimus (first generation of mTOR inhibitors) have EMA- and FDA-approved indications for the treatment of RCC particularly in advanced and/or metastatic RCC patients as well as in patients refractory to anti-VEGF therapy (see Figure 2) [128, 129]. Retrospective studies carried out to compare efficacies of everolimus and temsirolimus in mRCC patients suggest that everolimus treatment appears more favourable than temsirolimus, even though prospective trials are needed to confirm these results [122]. The five-year survival of metastatic RCC has been improved after application of mTORC1 inhibitors, even though clinical data is somewhat mixed and the utility of these agents in advanced and/or metastatic RCC (alone or combined with VEGF inhibitors) is currently controversial based on the results from more recent clinical trials (e.g., METEOR and Checkmate 025) [127, 129–132]. These observations are aligned with the poor efficacy of rapalogues in other pathological conditions as they only partially block mTOR signaling. Furthermore, incomplete inhibition of mTORC1 often induces feedback activation of procancerous signaling cascades (e.g., PI3K/Akt and ERK/MAPK).

Recent research efforts have been placed in other classes of mTOR inhibitors [128, 133]. Cho and colleagues tested the antitumor efficacy of NVP-BEZ235, a dual PI3K/mTOR inhibitor, alone or in combination with sorafenib in renal cancer xenografts. The combined protocol showed positive results with enhanced apoptosis and reduction of renal cancer cell proliferation [134]. Another preclinical study focused on AZD2014, a dual mTORC1/2 inhibitor, showed higher *in vitro* efficiency in the inhibition of RCC cell survival and growth as well as RCC cell apoptosis when compared with conventional mTORC1 inhibitors (rapamycin), providing evidence for clinical trials using AZD2014 in RCC treatment [135]. Nevertheless, a randomized phase II study of AZD2014 versus everolimus in anti-VEGF-refractory metastatic RCC showed inferior progression-free survival (primary endpoint) and overall survival with this TOR-KI, despite favourable toxicity and pharmacokinetic profiles (secondary endpoints) [136]. More recently, a novel, selective, and orally available mTOR-KI—XL388—was found to inhibit the survival and proliferation of both established and primary human RCC cells. XL388 was significantly more potent in RCC cell death than rapalogues and showed efficacy in 786-0 RCC tumor growth in nude mice. Moreover, this molecule was also able to elicit HIF-1α/2α downregulation in RCC cells with putative antiangiogenic effects, strengthening the value of XL388 for future clinic evaluation [137]. Overall, future studies are needed to translate new evidence from basic research into novel multitargeted agents of mTOR network modulation within RCC.

5.4. Diabetic Nephropathy. Diabetic nephropathy (DN) is a common complication of type 1 and type 2 diabetes mellitus and is the leading cause of end-stage renal disease (ESRD) worldwide. Clinically, DN is characterized by gradually worsening of albuminuria and GFR decline, in a process that seems to start by glomerular podocyte damage and loss, then progressing to fibrosis of renal glomerulus and of tubulointerstitial region cells. All kidney cell types, including podocytes and mesangial, endothelial, and tubulointerstitial cells, are affected. In opposition to the thesis that DN progression is mainly caused by glomerular protein leakage, it is currently accepted that the glomerular filtration barrier and the tubulointerstitial compartment are an entire dynamic unit that participates in disease evolution.

mTOR pathway signaling abnormalities seem to be present in all the key steps of DN progression, including (i) podocyte damage and loss, an early event in DN that further causes glomerulosclerosis; (ii) overactivation of mesangial cells that promotes increased ECM synthesis and decreased degradation of damaged podocytes; (iii) glomerular endothelial cells and mesangial cell crosstalk that precedes glomerulosclerosis; and (iv) fibrosis and epithelial-to-mesenchymal transition in tubulointerstitial cells [11]. Although the precise mechanisms remain to be clarified, accumulating experimental and clinical evidence supports a major role of mTOR pathway disturbances in DN progression.

Collectively, diabetes is closely linked with conditions that cause mTOR activation, namely, excessive caloric intake, even when preceding obesity, insulin resistance, and overt hyperglycemia development. Activation of mTOR complexes 1 and 2 promotes fat deposition in the adipose tissue [138, 139], which is in agreement with the rapamycin-induced hyperlipidemia seen clinical practice [140]. In conditions of overt diabetes, hyperglycemia further exacerbates mTORC1 activation due to inhibition of AMPK phosphorylation [141]. Concerning the kidney tissue, mTOR activation by diabetic conditions is related to both glomerular and tubulointerstitial changes of DN. Podocyte hypertrophy is a pivotal and early step in the glomerular hypertrophy that precedes proteinuria development and irreversible structural changes, culminating in glomerulosclerosis and nephron loss in DN [142]. Importantly, accumulating evidence from animal models of DN has suggested that mTORC1, via S6K1, participates in such process of renal hypertrophy. The role played by mTOR in podocyte function in conditions of DN was better clarified by the results of two experimental studies based on podocyte-specific genetic deletion of critical components of the mTOR signaling pathway [58, 143]. Briefly, these studies make use of two distinct models to show that mTORC1 overactivation in nondiabetic mice caused a glomerular disease closely resembling DN, while podocyte-specific inhibition of mTORC1 activity protected mice from DN development [58, 143]. Altogether, these studies strongly supported the idea that mTORC1 inhibition could be an

effective therapeutic strategy against DN development. A drawback of this approach was the development of proteinuria when raptor expression was ablated in podocytes, which is in line with the known proteinuric effect of rapamycin treatment in both animal models and humans [59–63]. Other relevant metabolic side effects of rapamycin should be also noticed at this point, including hyperglycemia, insulin resistance, and dyslipidemia, which seem to be related to glucose and lipids metabolism in the pancreas and in the peripheral insulin resistant tissues (liver, adipocyte tissue, and muscle), as previously reported in animal and human studies, some of them from our own group [37, 144–150].

Apart from impaired mTOR signaling in podocytes that contributes to podocyte loss, mTORC1 activation seems to be associated with renal hypertrophy and matrix expansion, overexpression of type IV collagen, fibronectin, and laminin [11]. mTOR inhibition by rapamycin prevents these effects and ameliorates the key glomerular changes found in DN, such as hypertrophy, basement membrane thickening, and mesangial matrix accumulation, accompanied by a decrease in albuminuria [142, 151]. Regarding interstitial fibrosis, mTOR seems to be able to stimulate fibroblasts proliferation, collagen synthesis, and expression of profibrotic cytokines, such as TGF-β1 and CTGF, which are pivotal players in the tubulointerstitial damage, a crucial feature of DN [142, 151]. Finally, mTOR seems also to participate in the epithelial-to-mesenchymal transition, a mechanism that is inhibited by rapamycin [142, 152].

To conclude, accumulating evidence, mostly from animal models, shows that mTOR activation might have a role on DN progression by acting on different kidney cell types and mechanisms, suggesting that mTOR inhibition could be, in theory, an attractive therapeutic strategy to overcome DN. However, the recognition of relevant side-effects in transplanted patients treated with rapamycin, such as hyperglycemia, insulin resistance, and dyslipidemia, may explain the scarceness of preclinical studies and lack of clinical trials using mTOR inhibitors to prevent or modify DN course (see Figure 2).

6. Conclusions and Future Directions

The mTOR pathway is an exciting area of research in many biomedical areas of knowledge, including aging, metabolism, neurobiology, oncobiology, and cardiovascular and renal diseases. Regarding the kidney, activation of mTOR complexes (mainly mTORC1) has been recognized to participate in a multiplicity of renal processes underlying the development of glomerular and tubular damage/fibrosis, such as regulation of podocyte size (hypertrophy and/or proliferation), epithelial-to-mesenchymal transition, and tubulointerstitial inflammation.

Inhibition of mTOR using rapamycin (sirolimus) or everolimus (a rapalogue), alongside with other immunosuppressive agents and depending on the immunological risk, has been a well succeeded strategy to improve outcomes in renal transplanted patients, regardless of the possibility of drug-induced proteinuria and other metabolic side-effects, which should be closely monitored and controlled. However, further clinical data is still needed to understand the putative benefits of mTOR inhibitors against the development of certain types of cancers and viral infections in transplanted patients.

Concerning PKD, in particular, the autosomal dominant form (ADPKD), the few clinical data available with mTOR inhibition was unable to confirm the preclinical studies in animal models. Therefore, clinical trials with sirolimus and everolimus have not improved renal volume or function at doses that do not cause significant adverse effects, namely, the increase in urinary protein excretion. Currently, there are not enough data to propose mTOR inhibition in PKD clinical practice. Further disclosure of (i) mechanistic insights of mTOR complexes in PKD pathophysiology, (ii) assessment of more potent and specific mTOR inhibitors, and (iii) careful systematization of clinical trials is paramount to overcome current drawbacks that postpone the translation of mTOR modulation from the benchside to PKD clinical practice.

As regards to RCC, in particular, in advanced and/or metastatic forms, the first generation of mTOR inhibitors (temsirolimus and everolimus) is already in clinical use and has been showing some efficacy, particularly when combined with VEGF modulators. However, the clinical data available remains controversial, namely, due to resistance-acquired phenomena and activation of prooncogenic pathways that limit the long-term use and outcome. Therefore, new pharmacological strategies targeting the mTOR network are currently under preclinical evaluation, which is focused on mTOR-KIs and dual PI3K/mTOR inhibitors.

Regarding the possibility of using mTOR inhibitors to prevent the progression of DN, rapamycin has been shown an ability to ameliorate mesangial expansion, glomerular basement thickening, and release of proinflammatory cytokines or chemokines by monocytes and macrophages. In spite of this amount of promising preclinical data, rapamycin is associated with some metabolic and renal side-effects, namely, insulin resistance and proteinuria, which could compromise its wide-spread use in some conditions. It should be noted that most of the actual knowledge on mTOR pathway in DN was obtained by using pharmacological inhibition of mTORC1 with rapamycin; nevertheless, it has been suggested by studies using animal models that mTORC2 activation also has a role in DN, which should be further exploited.

Although remarkable insights have been achieved over the last years, there is an ample room to improve our knowledge regarding the roles played by mTOR complexes and pathways in kidney physiology and pathogenesis of several renal diseases. In particular, further studies are required to disclose the precise mechanisms underlying the glomerular and tubulointerstitial actions of mTORC1 and mTORC2 in order to improve management of renal diseases and to reduce glomerular side effects and proteinuria reported with the traditional mTOR inhibitors currently available. Further insights are also still needed concerning the upstream regulation of mTOR, the identification of downstream mTOR targets, and, importantly, the specific role played by the regulatory proteins that interact with mTOR in both mTORC1 and mTORC2 complexes, such as Deptor, in order to unveil

the impact of the mTORC1-mTORC2 interactome. Likewise, further research, particularly in the clinical setting, is required regarding the impact of mTOR inhibition in immune cells and the ability to ameliorate age-related cellular decline. Finally, the insights hopefully coming in the near future from the studies ongoing with new pharmacological approaches targeting the intricate mTOR network, such as dual PI3K/mTOR inhibitors and new-generation inhibitors (namely, mTOR-KIs), might be able to open new avenues in the treatment of renal diseases in which the impaired mTOR pathway plays a relevant pathological role.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors' Contributions

Flávio Reis and Rui Alves contributed equally to this work.

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Research Article

Enhancing TFEB-Mediated Cellular Degradation Pathways by the mTORC1 Inhibitor Quercetin

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Signaling pathways mediated by the mechanistic target of rapamycin (mTOR) play key roles in aging and age-related diseases. As a downstream protein of mTOR, transcription factor EB (TFEB) controls lysosome biogenesis and cellular trafficking, processes that are essential for the functions of phagocytic cells like the retinal pigment epithelium (RPE). In the current study, we show that a naturally occurring polyphenolic compound, quercetin, promoted TFEB nuclear translocation and enhanced its transcriptional activity in cultured RPE cells. Activated TFEB facilitated degradation of phagocytosed photoreceptor outer segments. Quercetin is a direct inhibitor of mTOR but did not influence the activity of Akt at the tested concentration range. Our data suggest that the dietary compound quercetin can have beneficial roles in neuronal tissues by improving the functions of the TFEB-lysosome axis and enhancing the capacities of cellular degradation and self-renewal.

1. Introduction

Transcription factor EB (TFEB) is a member of the MiTF/TFE protein family that contains a basic helix-loop-helix domain for DNA binding and a leucine-zipper domain for heterodimerization [1]. TFEB controls lysosomal biogenesis and autophagy by positively regulating genes in the Coordinated Lysosomal Expression and Regulation (CLEAR) network [2–5]. Activation of TFEB leads to a coordinated upregulation of CLEAR genes, which collectively improves the efficiency of vesicular trafficking and promotes the eventual substrate degradation at the lysosome. The transcriptional activity and nuclear-cytoplasmic shuttling of TFEB are controlled by mechanistic target of rapamycin (mTOR) complex 1 (mTORC1) [3, 6], which phosphorylates TFEB at its C-terminal serine-rich motif and thereby sequesters TEFB in the cytoplasm [6]. Synthetic chemical inhibitors of mTORC1, such as torin 1 and torin 2, are known activators of TFEB [7–10]. TFEB has been considered a therapeutic target with implications in various human diseases that are associated with defects in autophagy and lysosome-mediated degradation [1, 11]. However, most of the commonly used protein kinase inhibitors such as torins have relatively low substrate specificity and may inhibit other pathways, particularly the Akt-mediated cell survival signaling pathway [12, 13]. Their applications in chronic human degenerative diseases are limited.

Quercetin (3,3′,4′,5,7-pentahydroxyflavone) is a plant-derived polyphenolic compound and is present in a number of dietary components [14]. It is a broad-spectrum protein kinase inhibitor, and a phase I clinical trial of quercetin has demonstrated its tyrosine kinase inhibitory effect [15]. Quercetin has been used as a lead compound for synthesizing derivatives of commonly used kinase inhibitors, such as LY294002 [16]. Whether quercetin influences the activities of mTOR and its downstream proteins like TFEB is not well understood.

The main objective of our current study was to examine the biological effects of quercetin on TFEB in cultured retinal pigment epithelial (RPE) cells. RPE cells provide essential

support to the functions of the neurosensory retina [17]. They are phagocytic and have high activity in cellular trafficking and lysosome-mediated degradation processes [17]. Our data show that quercetin dose-dependently activated the transcriptional activity of TFEB and elevated its downstream gene expression. Cells with enhanced TFEB activity had increased autophagy and higher efficiency to degrade phagocytosed photoreceptor outer segments (POS). Quercetin effectively suppressed amino acid-induced mTORC1 activation and likely functioned as a competitive mTOR kinase inhibitor at the ATP-binding motif. These findings provide mechanistic support for the beneficial effects of quercetin as a nutritional supplement to improve the capacity of lysosome-mediated degradation processes in the neuronal tissue.

2. Materials and Methods

2.1. Materials. Quercetin either was purchased from Sigma-Aldrich (St. Louis, MO, USA) (≥95% HPLC, catalogue number Q4951) or was provided by USANA Health Sciences (Salt Lake City, UT, USA). Cells were treated with 0.5 to 20 μM of quercetin. No difference was observed between the activities of the compounds from the two sources. Torin 1 was purchased from Tocris (Minneapolis, MN). E64d and pepstatin A were purchased from Sigma-Aldrich. A rhodopsin antibody (RET-P1) and TFEB antibody were purchased from Abcam (Cambridge, MA, USA). All other primary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Fluorophore-conjugated secondary antibodies were purchased from Invitrogen (Carlsbad, CA, USA) and LI-COR Biosciences (Lincoln, NE, USA).

2.2. Cell Culture. ARPE-19 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and were cultured in Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 50/50 mix supplemented with 10% fetal bovine serum (Sigma-Aldrich). For quercetin treatment, the compound was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) as a stock solution. The final DMSO concentration in the medium of treated cells was less than 0.1%. Cell viability after 20 μM quercetin treatment was assessed by measuring trypan blue exclusion with a Countess automated cell counter (Invitrogen).

2.3. Measurement of TFEB Transcriptional Activity by a Reporter Assay. TFEB reporter construct was generated by inserting four tandem copies of the CLEAR motif (5′-G TCACGTGAC-3′) in the pGL3-promoter luciferase reporter vector (Promega, Madison, WI) [18], between KpnI and XhoI sites. The insertion was sequence-verified. For measuring the transcriptional activity of TFEB, ARPE-19 cells in 6-well plates were transiently transfected with 1 μg of the TFEB reporter plasmid per well, using Lipofectamine 2000 Transfection Reagent (Invitrogen). To normalize the transfection efficiency, 30 ng of control reporter construct of Renilla luciferase (pRL-CMV Vector, Promega) was cotransfected [19]. Six hours after transfection, the culture medium was refreshed. One day after transfection, cells were incubated with indicated concentrations (0.5 to 20 μM) of quercetin for additional 16 hr. Afterward, the luciferase activities were measured using a Dual-Luciferase® Reporter Assay System (Promega) following the manufacturer's instructions [19].

2.4. Immunofluorescence Staining. For imaging LC3, cultured ARPE-19 cells were seeded on the cover glass and exposed to 10 μM quercetin either in the absence or in the presence of 10 μM chloroquine (CQ). At the end of treatment, cells were rinsed with Tris-buffered saline (TBS) and fixed with 4% paraformaldehyde for 15 min. After permeabilization with methanol for 5 min, the cells were incubated in blocking buffer of 10% FBS and 0.5% Triton X-100 in TBS for 1 hr at room temperature. The cells were then incubated with primary antibodies diluted in the blocking buffer, followed by appropriate fluorophore-conjugated secondary antibodies. After stringent washes, the nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). The slides were mounted with Fluoro-Gel (Electron Microscopy Service, Hatfield, PA, USA). Fluorescence microscopy was performed using a Zeiss Axio Observer fluorescence microscope equipped with the ApoTome imaging system. The same staining procedures were used for examining TFEB nuclear translocation, in experiment where cells were treated with either 20 or 50 μM quercetin for 2 hr. At least fifty cells were randomly scored per condition per experiment. Image quantification was performed by ImageJ (NIH) [20], by comparing the fluorescence intensity of areas with and without DAPI staining.

2.5. RNA Isolation and Quantitative Reserve Transcription PCR (RT-PCR). Cells were treated with 0.5 to 10 μM quercetin for 16 hr. Total RNA was isolated using TRIzol™ Reagent (Invitrogen) and treated with the DNA-free™ kit (Ambion, Austin, TX, USA) to remove contaminating genomic DNA. The yield and purity of the RNA were determined using a spectrophotometer (NanoDrop ND-1000; Thermo Fisher Scientific, Waltham, MA, USA). Complementary DNA was reversely transcribed from 1 μg of total RNA using oligo$(dT)_{15}$ Primer (Promega). The relative abundance of mRNA expression of CLEAR network genes was determined by quantitative RT-PCR using primers designed with the Universal Probe Library approach (Roche Diagnostics, Indianapolis, IN, USA) [21]. The primers used were as follows: TFEB: 5′-CGG CAG TGC CTG GTA CAT-3′ and 5′-CTG CAT GCG CAA CCC-3′, ATP6V0C: 5′-AGT CCA TCA TCC CAG TGG TC-3′ and 5′-CAG CTG GAG GAA GCT CTT GT-3′, MCOLN1: 5′-AAG GCG ATG GTG TTC TCT TC-3′ and 5′-GCT GCA AGT GGT CAA GAT CC-3′, UVRAG: 5′-CAG AAG GAA TCC CTA AAT GAG C-3′ and 5′-TGC AAC GAA TTG TCA ACT GAG-3′, ATP6V0D2: 5′-ACA AGT CTT ACC TTG AGG CAT TCT-3′ and 5′-TCT GTC GGC CTC AAA CTC A-3′, and PPARGC1a: 5′-TGA GAG GGC CAA GCA AAG-3′ and 5′-ATA AAT CAC ACG GCG CTC TT-3′.

2.6. Subcellular Fractionation. Confluent ARPE-19 cells were treated with 20 μM quercetin for 2 hr. At the end of incubation, cells were collected by gentle trypsinization and were

washed once with ice-cold TBS. Cytosolic and nuclear extracts were prepared by using the Subcellular Protein Fractionation Kit for Cultured Cells (Thermo Fisher), following the manufacturer's instructions.

2.7. Western Blot Analyses. Cells were harvested and lysed in buffer containing a 1 : 1 (*v*/*v*) ratio of CelLytic™ M Cell Lysis Reagent (Sigma-Aldrich) and 2X Laemmli Sample Buffer (Bio-Rad, Hercules, CA, USA), supplemented with 10 mM glycerophosphate, 10 mM pyrophosphate, 1 mM NaF, 1 mM Na_3VO_4, and protease inhibitor cocktail [20]. Cell lysates were sonicated, and samples were resolved on SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad). The membranes were probed with specific primary antibodies followed by appropriate fluorophore-conjugated secondary antibodies. The fluorescent signals were detected by the Odyssey Infrared Imaging System (LI-COR). Detection and quantification of band intensities were performed using Odyssey imaging software version 3.0 (LI-COR) [20].

2.8. Measurement of the Turnover Rate of Photoreceptor Outer Segments (POS). Cultured ARPE-19 cells were incubated with purified porcine POS [22], at a 10 : 1 ratio (POS : cell) for 3 hr. At the end of incubation, unbound POS were removed by washing with phosphate-buffered saline (PBS) containing 1 mM $MgCl_2$ and 0.2 mM $CaCl_2$ [23]. Cells were kept in the refreshed culture media and chased for up to 4 hr. The amount of rhodopsin in ARPE-19 cells was assessed by Western blot analyses [21]. For experiments on lysosome inhibitors, cells were pretreated for 16 hr with either 10 μM chloroquine or 10 μM E64d and 10 μM pepstatin A, before POS loading.

2.9. In Vitro Kinase Assay. Inhibition of mTOR kinase activity by quercetin was determined using LanthaScreen™ kinase assay technology from Invitrogen. The LanthaScreen assay is based on time-resolved fluorescence resonance energy transfer (TR-FRET). Kinase reactions were performed in a 10 μL volume in Corning 4513 white 384-well assay plates. The testing compound was diluted into reaction buffer (50 mM HEPES (pH 7.5), 0.01% polysorbate 20, 1 mM EGTA, 2 mM DTT, and 10 mM MnCl2). Kinase (PV4753, mTOR, 114.51 ng/mL final concentration), substrate (GFP-4E-BP1, PV4759, 0.4 μM final concentration), and ATP (PV3227, half Km value was used, 10 μM final concentration) were mixed with inhibitors, and the reaction was allowed to proceed for 1 hr at room temperature. The reaction was stopped by adding antibodies (Tb-anti-p4EBP1, pThr46, PV4757) at a final concentration of 2 mM, diluted in antibody dilution buffer (PV3574) and EDTA (10 mM final concentration). After 30 min incubation with the antibody, the TR-FRET emission ratios were acquired on a Tecan Spark 10M plate reader. A known inhibitor of mTOR, LY294002 (PHZ1144), was used as a positive control and tested using the same conditions.

2.10. Statistical Analysis. Data analyses were performed using GraphPad Prism 5. The data given in the text were representatives from at least three independent experiments and were presented as mean ± SEM. $p < 0.05$ (Student's t-test or one-way ANOVA) was considered statistically significant.

2.11. Computational Modeling. Docking simulation studies of quercetin with mTOR were carried out using the modeling software MOE (Chemical Computing Group Inc., Montreal, Quebec, Canada). Crystal structure of mTOR (PDB ID: 4JT5) was retrieved from the Protein Data Bank (PDB). All molecular visualizations were produced by MOE.

3. Results

3.1. Induction of TFEB-Mediated Gene Transcription by Quercetin. We used a luciferase reporter assay [24] to screen for nutritional compounds that can potentially activate TFEB. Cultured human ARPE-19 cells were transiently transfected with a reporter plasmid with the CLEAR elements inserted in the enhancer region of the luciferase gene and were treated with various doses of testing compounds for 16 hr. The luciferase activities were measured as an indicator of the transcriptional activity of TFEB. Among the compounds screened, quercetin was identified as a reliable inducer of TFEB. APRE-19 cells exposed to 0.5 to 20 μM quercetin showed a dose-dependent increase in TFEB-driven luciferase activity (Figure 1(a)). At 20 μM, quercetin achieved 2.5-fold induction (1.5–3.3, 95% confidence interval) of the reporter activity. As a validation of the reporter assay, torin 1, which is a well-established mTOR inhibitor [7], was used to treat the ARPE-19 cells. The transcriptional activity of TFEB was elevated by torin 1 (Figure 1(b)), at a concentration range of 2 to 50 nM that suppressed the phosphorylation of S6 but not the phosphorylation of Akt (Figure 1(c)). No sign of cytotoxicity was observed at 20 μM of quercetin. After 48 hr treatment, the viability of vehicle- and quercetin-treated cells was 91.3 ± 2 and 91.3 ± 5.7, respectively (mean ± SD, $N = 3$).

Phosphorylation of TFEB by mTOR leads to its sequestration in the cytoplasm near lysosomes [6]. Upon quercetin treatment, the amount of TFEB protein in the nucleus was increased, as determined by both immunofluorescence staining (Figures 1(d) and 1(e)) and subcellular fractionation (Figures 1(f) and 1(g)). The distribution of TFEB in the cytoplasm, however, was not influenced by quercetin under the experimental conditions.

The mRNA levels of known TFEB target genes, including the ones involved in cellular vesicular trafficking and lysosome functions [2, 3, 5, 25], were measured in cells treated with 0.5 to 10 μM quercetin (Figure 2). Quercetin treatment at 5 and 10 μM concentrations significantly upregulated *UVRAG*, *MCOLN1*, *ATP6V0C*, and *ATP6V0D2*. The expression of *PPARGC1A* did not respond to quercetin treatment. TFEB itself was upregulated by 2 μM quercetin treatment. Thus, by measuring the transcriptional activity of TFEB, its nuclear translocation, and its downstream gene expression, the data collectively demonstrate that quercetin treatment activates TFEB in the ARPE-19 cells.

3.2. Quercetin Enhanced TFEB-Mediated Cellular Degradation Capacity in the RPE. A specialized function of

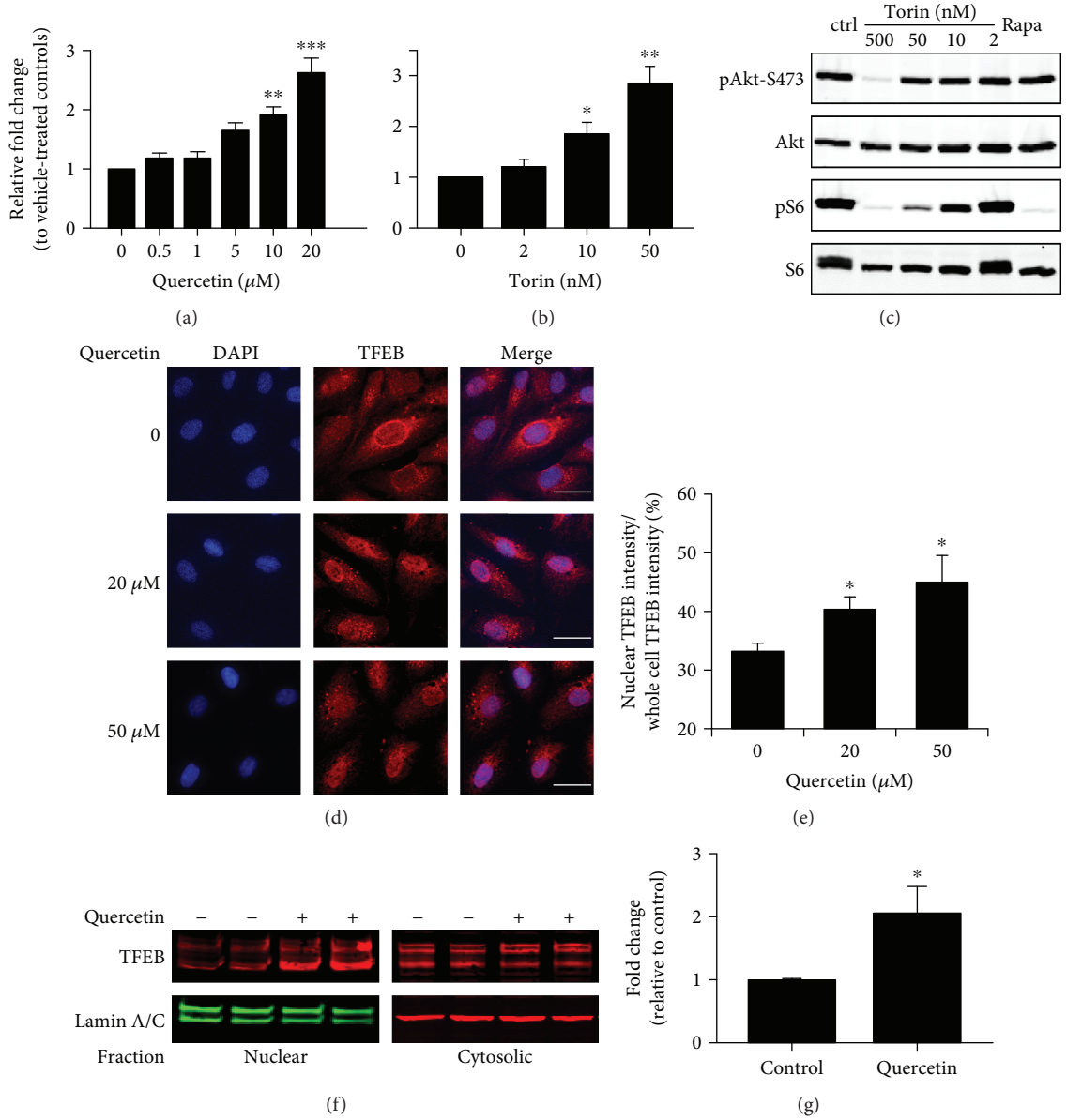

Figure 1: Activation of TFEB by quercetin in cultured ARPE-19 cells. (a, b) Transcriptional activity of TFEB as measured by the luciferase reporter assay. ARPE-19 cells were transfected with the CLEAR-Luc plasmid and measured for dose-dependent responses to quercetin (a) or torin 1 (b) treatment after 16 hr exposure. Data presented are averages from 5 to 6 independent experiments (mean ± SEM). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. One-way ANOVA and Dunnett's post hoc test. (c) Western blot showing the dose-dependent effects of torin 1 on Akt and S6 phosphorylation. Cells were treated with the indicated concentrations of torin for 16 hr. The last lane was the sample from cells treated with 20 nM rapamycin (Rapa) for 16 hr. (d) Immunofluorescence staining of TFEB nuclear translocation after 2 h exposure to 20 or 50 μM quercetin. Quantification data are presented in (e). Scale bar: 10 μm. (f) Measurement of TFEB nuclear translocation after subcellular fractionation. RPE cells were treated with 20 μM quercetin for 2 hr, and the amount of TFEB in the nuclear and cytosolic fractions was determined by Western blot analyses. Quantification data are presented in (g). Data presented are averages from 3 independent experiments (mean ± SEM). $^{*}p < 0.05$. Student's t-test.

the RPE is phagocytosis of shed POS from photoreceptor neurons [17]. The turnover and recycling processes of POS are part of the visual cycle and are critical for retinal health and function [26]. Protein components of the internalized POS, such as rhodopsin, are eventually degraded in lysosomes. Inhibiting lysosome proteolysis with E64d and pepstatin effectively protects rhodopsin from degradation [27]. Because TFEB controls cellular trafficking and lysosome function, we examined whether ARPE-19 cells treated with quercetin had increased degradation capacity for POS. Cells were first loaded with purified porcine POS for 3 hours. Afterward, the unbound POS were removed and the remaining POS in cells were monitored by measuring the level of rhodopsin, the major protein component that is unique to POS. As shown in Figure 3(a), cells treated with 10 μM quercetin had accelerated POS degradation. Two hours after

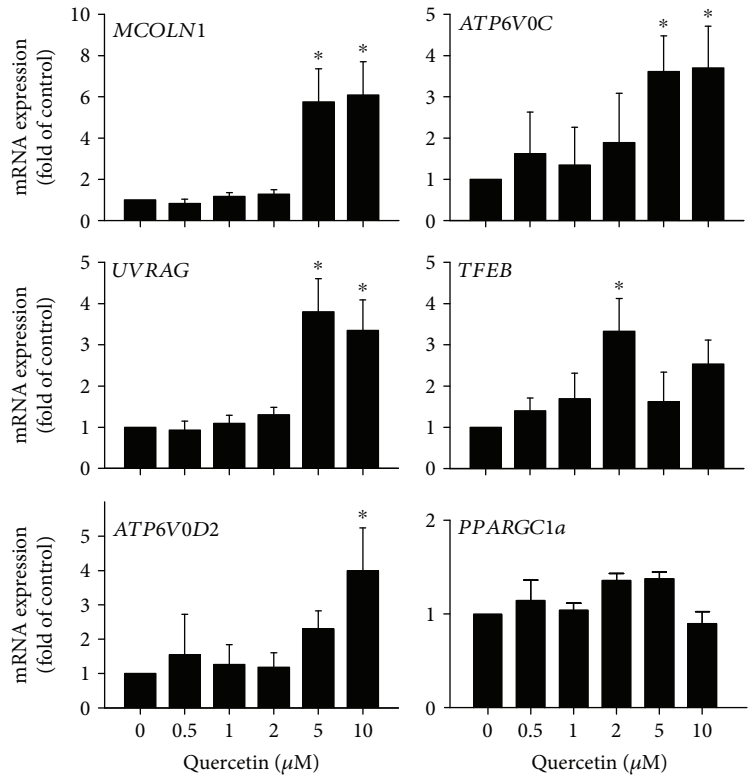

Figure 2: Induction of TFEB downstream genes by quercetin. Dose-dependent upregulation of TFEB in downstream genes in cultured ARPE-19 cells. Cells were treated with the indicated concentration of quercetin for 16 hr and analyzed by quantitative RT-PCR. Data presented are averages from 4 independent experiments (mean ± SEM). $^*p < 0.05$. One-way ANOVA and Dunnett's post hoc test.

chasing, most of the rhodopsin associated with ingested POS were degraded in quercetin-treated RPE cells, while control cells still had a notable amount of rhodopsin between 2 and 3 hr. POS degradation in the ARPE cells is dependent on lysosome functions. Inhibitors of lysosome acid proteases, chloroquine (CQ), E64d, and pepstatin A, effectively suppressed the turnover of ingested POS (Figure 3(a)).

Next, we examined the effects of quercetin on markers of autophagy. RPE cells are highly efficient in autophagy, and CQ treatment is required to visualize autophagosome and LC3-II under the experimental conditions (Figure 3(b)). We found that in the presence of CQ, 5 and 10 μM quercetin treatment increased the level of LC3-II, the lipidated form of LC3 and a marker protein of autophagosomes [28, 29], as compared to CQ treatment alone (Figures 3(b) and 3(c)). The ratio of LC3-II/LC3-I was also increased (Figure 3(c)), while the level of autophagy substrate protein p62 was decreased (Figures 3(d) and 3(e)). Both changes after exposure to quercetin indicated the enhanced autophagy. When examined by immunofluorescent staining, the number of LC3-positive puncta was markedly increased by quercetin in the presence of CQ (Figures 3(f) and 3(g)). Similar results were obtained when cells were treated with vinblastine, a compound that disrupts autophagic trafficking on microtubules (Figures 3(h) and 3(i)) [30]. Thus, with multiple independent measurements, we showed that quercetin treatment can elevate the cellular trafficking and degradation capacity in the RPE.

3.3. Inhibition of mTOR Kinase Activity by Quercetin. A key upstream regulator of TFEB is mTORC1 [6, 9]. In RPE cells, mTORC1 can be activated by various stimuli including nutrient and growth factors [21]. In ARPE-19 cells treated with 10 μM quercetin, there was a selective suppression of amino acid-induced mTORC1 activation, as measured by the phosphorylation status of its downstream ribosome protein S6 (Figure 4(a)). Insulin- or serum-induced mTOR activation was not influenced by quercetin at this dosage (Figure 4(b)). Furthermore, quercetin did not alter the phosphorylation of Akt (Figure 4(b)), suggesting that it did not inhibit mTOR complex 2 that phosphorylates Akt at serine 473 [31]. Phosphorylation of Thr308, an indicator of Akt activity [32, 33], was not influenced either.

To assess whether quercetin is a direct inhibitor of mTOR, we performed an *in vitro* kinase assay with purified mTOR protein. As shown in Figure 5(a), quercetin suppressed the kinase activity of mTOR in a dose-dependent way, and the IC50 was 7.8 μM, which is consistent with the concentration range for the cell-based assays (Figure 1).

To further understand the mechanism of inhibition at the structural level, computer-based molecular docking modeling was utilized to explore the interaction of quercetin with mTOR at the ATP-binding site. The modeling revealed that the most apparent interactions were the two hydrogen bonds between the benzene rings of quercetin and Tyr225 and Met2345 of mTOR. The carbonyl group of quercetin serves as a potential backbone acceptor for the interaction with

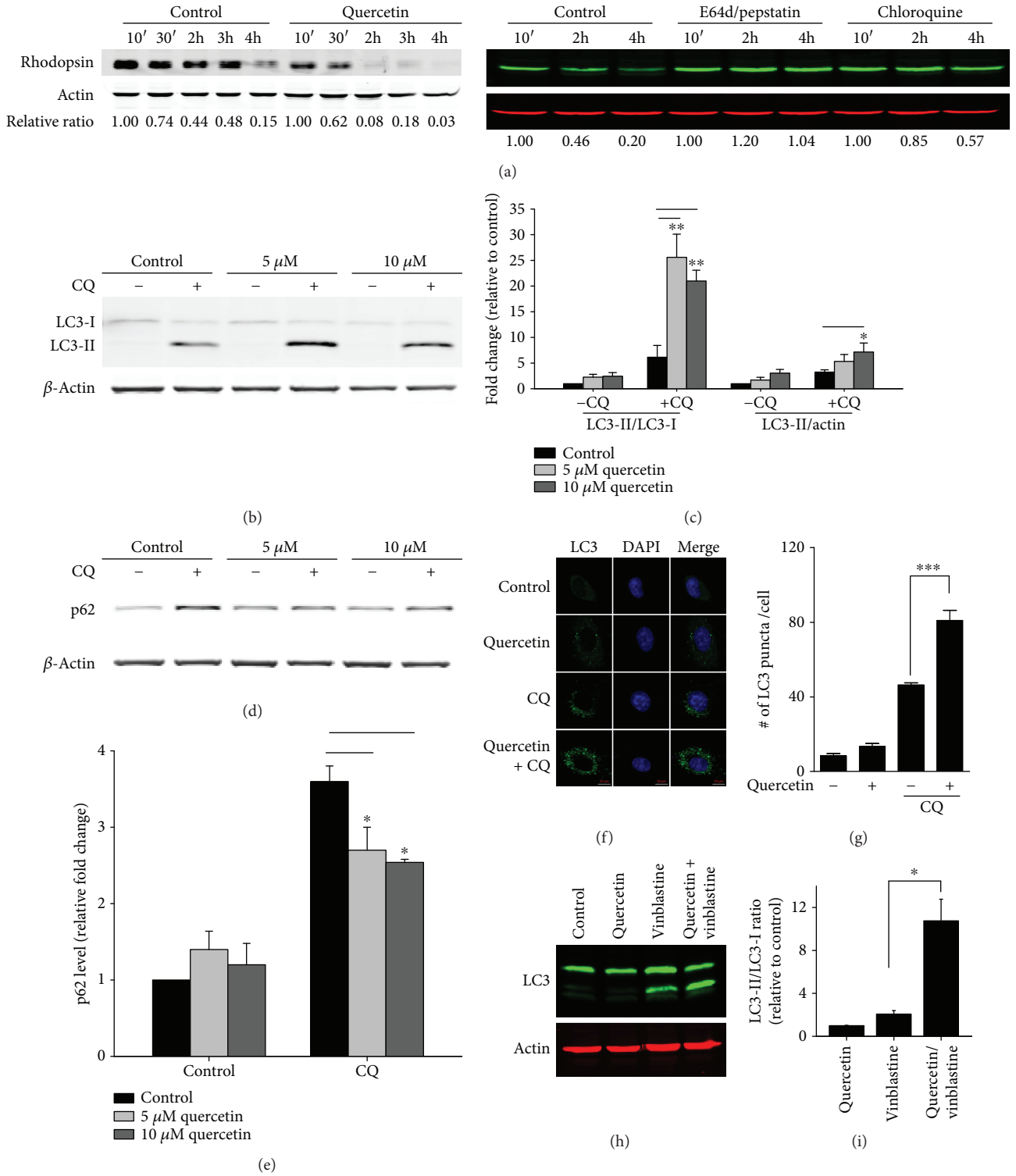

FIGURE 3: Enhancing RPE cell degradation capacity by quercetin. (a) Measurement of the POS turnover rate. ARPE-19 cells were treated with 10 μM quercetin, or vehicle control, for 16 hr and subsequently were loaded with purified POS (5 : 1 ratio, POS : RPE) for 3 hr. After stringent washes, the rates of degradation of engulfed POS were measured by Western blot analyses of rhodopsin. To study the effects of lysosome inhibitors, cells were treated with 10 μM of E64d and pepstatin A or 10 μM CQ for 16 hr and then loaded with POS. (b) Effects of quercetin on LC3 lipidation. Cells were treated with the indicated concentration of quercetin, with or without 10 μM CQ, for 16 hr. Quantification data are presented in (c). (d, e) Western blot analyses of p62 protein in ARPE-19 cells treated with quercetin alone or with CQ. (f) Immunostaining of LC3 punctum formation after quercetin and CQ treatment; quantification data are presented in (g). (h, i) Effects of quercetin and vinblastine treatments on LC3 lipidation. Data presented are averages from 3–5 independent experiments (mean ± SEM). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. One-way ANOVA and Dunnett's post hoc test.

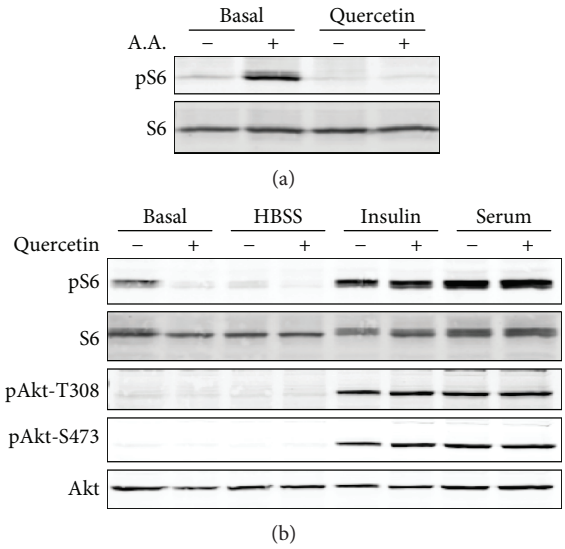

Figure 4: Effects of quercetin on mTOR activity in RPE cells. Cells were pretreated with 10 μM quercetin for 16 hr and stimulated with either amino acids (a), insulin, or serum (b) for 30 min. Phosphorylation of S6 and Akt was measured by Western blot analyses as indicators of mTOR kinase activity. Amino acids (almost all) used were a mixture of essential amino acids used in Minimum Essential Medium (MEM), including glutamine. Basal: cells incubated in serum-free medium.

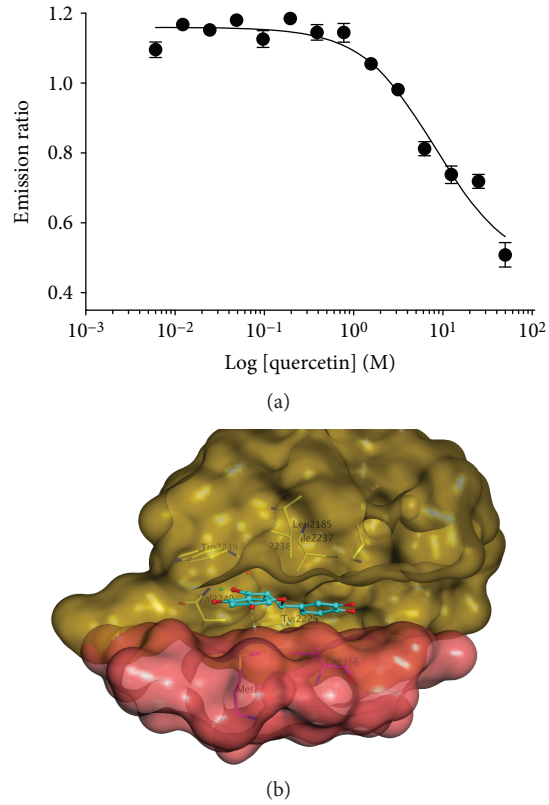

Figure 5: Quercetin as a direct inhibitor of mTOR kinase. (a) *In vitro* kinase assay showing a dose-dependent inhibition of mTOR activity by quercetin. (b) Structural modeling of interactions of quercetin with the ATP-binding pocket of mTOR.

Val2240, and the methoxyl group as a backbone donor for Gly2238 (Figure 5(b)).

4. Discussion

In the current study, we investigated the effects of a naturally occurring polyphenolic compound, quercetin, on the functions of the transcription factor TFEB. Using a combination of immunostaining, subcellular fractionation, luciferase reporter assay, and quantitative RT-PCR approaches, we demonstrate that quercetin exposure elevated the transcriptional activity of TFEB (Figure 1(a)), promoted its nuclear translocation (Figures 1(d) and 1(f)), and upregulated the downstream gene expression (Figure 2). Consistent with our findings, a structurally related polyphenolic compound, fisetin, was recently reported to activate TFEB and induce autophagy [34, 35].

Nutritional supplementation has been viewed as a promising interventional approach for delaying the progression of age-related neurodegenerative disorders [36, 37]. Quercetin was previously reported as an effective treatment against tauopathy and β-amyloidosis in a mouse model of Alzheimer's disease and preserved cognitive functions [38]. A number of protective mechanisms have been proposed, and enhancing TFEB-mediated autophagy and lysosome functions likely contributes to the beneficial effects of quercetin in the central nervous system. The retina and RPE are highly active in metabolism and are susceptible to proteolytic and ER stress [39]. Similar to the effects in the brain, enhancing the degradation capacity of RPE can be beneficial for the improvement of the health of the retina under chronic disease conditions. Whether quercetin can exert long-term protective effects can be explored by future *in vivo* studies.

Quercetin was used as a model compound to develop kinase inhibitors [16]. Our data from cell-based assays (Figure 4) and *in vitro* kinase assay (Figure 5(a)) demonstrated that quercetin is a direct inhibitor of mTOR. Recently, quercetin has been reported as being involved in Akt-mTOR and HIF-1α signaling [40]. Noticeably, quercetin did not inhibit Akt phosphorylation in RPE cells at the dose range that effectively suppressed mTORC1 (Figure 4(b)). The relative substrate selectivity is a unique advantage of quercetin. Rapamycin or its analogs are prototypical inhibitors of mTORC1, but whether they can effectively activate TFEB remains unclear [8]. The second-generation mTOR inhibitors, torin 1 and torin 2, are highly potent and have well-established roles in TFEB activation [8] (Figure 1(b)). However, they can also have potent inhibitory effects on the mTORC2 pathway, and the suppression on the Akt activity leads to severe cytotoxicity [12, 13].

The limitation of our study is that the concentration of quercetin that effectively inhibits mTORC1 is relatively higher than the reported blood concentration of quercetin from previous human clinical trials. For example, one study showed that healthy volunteers who had taken 1 gram of quercetin per day for 28 days reached about 1.5 μM quercetin in the plasma [41]. Future studies on the pharmacokinetic and pharmacodynamic properties can be performed

to define whether quercetin can be pursued as a nutritional supplement for intervening human diseases. The tissue concentration can be different from the blood quercetin concentration, and the dose and safety ranges will have to be further examined.

In summary, our work showed that quercetin is a naturally occurring compound that exerts beneficial effects on the central nervous system via TFEB-dependent mechanisms. Genes in the CLEAR network encode proteins that are involved in trafficking, autophagy, and lysosome degradation. Other than the translational implications, further exploring the genes that function downstream of TFEB and control RPE trafficking and lysosomal degradation can reveal novel mechanistic information of RPE cell biology.

Data Availability

The data used to support the findings of this study are included within the article.

Disclosure

The funding sponsors had no role in the design of the study, in the collection, analyses, or interpretation of the data, in the writing of the manuscript, and in the decision to publish the results.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Research Article

Gankyrin Drives Malignant Transformation of Gastric Cancer and Alleviates Oxidative Stress via mTORC1 Activation

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Gastric cancer, as a malignant epithelial tumor, is a major health threat leading to poor overall survival and death. It is usually diagnosed at an advanced stage due to asymptomatic or only nonspecific early symptoms. The present study demonstrated that gankyrin contributes to the early malignant transformation of gastric cancer and can be selected to predict the risk of gastric cancer in those patients harboring the precancerous lesions (dysplasia and intestinal metaplasia). In addition, a new insight into gastric cancer was provided, which stated that gankyrin alleviates oxidative stress via mTORC1 pathway activation. It can potentiate the mTORC1 by PGK1-AKT signaling that promotes the tumor process, and this phenomenon is not completely consistent with the previous report describing colorectal cancer.

1. Introduction

Gankyrin (also named p28, p28GANK, or PSMD10) acts as a molecular chaperone during the assembly of the 26S proteasome, specifically the 19S regulatory complex [1–3], and is commonly overexpressed in human malignancies [4–9]. It is involved in the negative regulation of pRB1 and p53/TP53 to execute the oncoprotein function [3, 10]. The competitive binding with CDKN2A triggers gankyrin to regulate the CDK4-mediated phosphorylation and further proteasomal degradation of RB1 [11, 12]. Similarly, gankyrin binds to MDM2, a major E3 ubiquitin ligase for p53, and increases the MDM2-mediated mono- or polyubiquitination of p53 in order to exhibit the antiapoptotic activity in the cells that were injured by DNA-damaging agents [4, 10, 12, 13]. Moreover, oxidative stress disorder is also speculated to be involved in the development of various human diseases including cancer [14, 15]. In hepatocellular carcinoma, overexpressed gankyrin inhibits the proteasomal degradation of Nrf2 by blocking the interaction between Nrf2 and Keap1 and plays an antioxidative role via the feedback regulation of Nrf2 [15]. Beyond the function of mediating protein degradation, gankyrin can directly bind to the NF-κB component RelA and accelerate its chromosomal region maintenance-1- (CRM-1-) mediated nuclear export in hepatocellular carcinoma [16]. This phenomenon might be attributed to the gankyrin-mediated attenuation of the acetylation of RelA and its retention in the cytoplasm to suppress NF-κB transactivation [17]. This evidence reveals that gankyrin is involved in the multiple biological and physiological processes in cells and contributes to the development of cancer. The most recent data from our group showed that gankyrin mediates TSC2 for degradation and regulates mTOR signaling via a p53-independent pathway in colorectal cancer [18]. However, whether gankyrin plays an analogical function or regulatory role in the cell signaling pathway in gastric cancer is yet an enigma.

Gastric (stomach) cancer is the third leading cause of deaths from cancer accounting for 7% of the cases and 9% deaths after lung and liver cancer [19]. It is primarily caused by *Helicobacter pylori* infection, which accounts for >60% of the cases [20, 21], smoking [21], diet [22], and genetics [23, 24]. Due to the absence of early typical clinical signs, gastric cancer has often been diagnosed at an advanced stage and may have occurred as distant metastasis before the

symptoms onset. These manifestations might be the primary cause of the relatively poor prognosis of the disease [25]. However, increasing evidence has revealed that gankyrin is a promising target for the diagnosis and treatment of several cancers [8, 26–29]. Nevertheless, compelling proofs to describe and illustrate the functional role or clinical significance of gankyrin in the development of gastric cancer are yet lacking.

Gastric intestinal metaplasia (GIM) and dysplasia, precursor lesions to and gastric cancer, are usually observed in the milieu of long-standing nonatrophic gastritis (NAG) and chronic atrophic gastritis (CAG) [30, 31]. Herein, we demonstrated that gankyrin is transcriptionally activated in tissue cells since patients harbored chronic atrophic gastritis, precancerous lesion (GIM and dysplasia), or gastric cancer and, thus, might be a preeminent candidate target for the early diagnosis of gastric cancer. In addition, we also found that gankyrin restricted the oxidative stress by stimulating the mTORC1 signaling in gastric cancer.

2. Materials and Methods

2.1. Tissues and Immunohistochemistry. Deidentified tissues from 262 patients were included. 77 malignant infiltrating gastric cancer tissues and paired noncancerous tissues were collected from the hospital and developed into tissue array by OUTDO Biotech (Shanghai, China). 120 noncancerous tissues, including nonatrophic gastritis (NAG), chronic atrophic gastritis (CAG), CAG with intestinal metaplasia (IM), and CAG with dysplasia (dys) gastric tissue, were acquired by endoscopy, 30 cases for each group. 65 gastric cancer samples with complete follow-up data were collected for survival analysis. Follow-up time and survival time were calculated from the day of the operation to the end of the follow-up or the date of death due to recurrence and metastasis. The study protocols were approved by the SJTUSM (Shanghai Jiao Tong University School of Medicine) Ethics Committee. All procedures adhere to the BRISQ Guidelines reporting research on human biospecimens. Immunohistochemical detection of gankyrin was performed using a streptavidin-biotin complex method as described previously [18]. For quantitative analysis, a histoscore (H-score) was calculated using Aperio Scan Scope systems (Vista, CA, USA) as previously described, by multiplying the intensity score and the fraction score, producing a total range of 0–300 [18]. Tissue sections were examined and scored separately by two independent investigators blinded to the clinicopathologic data.

2.2. Cell Culture and Reagents. The gastric cancer cell lines, MKN45 and MKN74, were purchased from the Shanghai Institute for Biological Sciences (SIBS, Shanghai, China) and cultured in RPMI 1640 medium (HyClone, Los Angeles, CA, USA) containing 10% fetal bovine serum (FBS; HyClone) and 100 U/mL penicillin/streptomycin under conditions of 5% CO_2 and humidified air at 37°C. The lentiviral pCDH-EF1-MCS-T2A-copGFP gankyrin plasmid was constructed, packed, and purified by Sunbio (Shanghai, China).

2.3. MTT Assay. The activity of MKN45 and MKN74 cells overexpressing gankyrin or vector control (NC) was determined by MTT assay. Briefly, the cells were seeded in quintuplicate in 96-well culture plates and cultured for up to 96 h, followed by an addition of 20 μL of 5 mg/mL MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide; Sigma-Aldrich, St. Louis, MO, USA) solution per well. After incubation for 4 h at 37°C, the supernatant was replaced with 100 μL DMSO. The absorbance per well was measured by a Microculture Plate Reader at 570 nm and 630 nm after 20 min agitation at room temperature. The data represent the means ± standard deviation (SD) from three independent triplicate experiments.

2.4. Colony Forming Assay. Cells were seeded in triplicate in 12-well plates to form colonies for up to 7–10 days. The medium containing selective antibiotics was replaced every 3–5 days. The colonies were stained with methylene blue and counted. Data represent the means ± SD from three independent experiments performed in triplicate.

2.5. Soft Agar Assay. The anchorage-independent growth of MKN45 and MKN74 cells overexpressing gankyrin or NC was determined by soft agar assays. Briefly, 0.7% basal-layer agar was prepared with 1.4% low-melting-point agarose and 2 × cell medium (1 : 1, $v : v$). 1 mL of basal layer agar was added to each well of a 6-well plate. The exponentially growing cells were harvested by trypsinization to 5000 cells/mL single-cell suspension. 0.35% top-layer agar was prepared with 0.7% low-melting point agarose and 2 × cell medium (1 : 1, $v : v$). Subsequently, 1 mL top-layer agar was blended with 100 μL single-cell suspension/well (500 cells/well). The cells were incubated for up to 1–2 weeks at 37°C after solidification at room temperature. Cultures were stained with p-iodonitrotetetrazolium violet (Sigma-Aldrich) for 2 h and then inspected and photographed using a MiniCount Colony Counter. The colonies containing more than 50 cells were counted and imaged. The data represent the means ± SD from three independent experiments in triplicate.

2.6. Transwell Invasion Assay. The cell suspension was prepared in a blank culture medium containing 5×10^5 cells/mL for 24-well invasion chambers. The upper surface of the membrane was scrubbed carefully with a cotton swab to remove the remaining cells, and Matrigel matrix after chambers was incubated for up to 16–18 h at 37°C. The cells on the lower surface of the membranes were fixed with 100% methanol and stained with 0.5% crystal violet. The invaded cells were imaged and counted in several fields under the microscope at approximately 40x–100x magnification. Data represent the means ± SD from three independent triplicate experiments.

2.7. Immunoblotting. Total protein was extracted by RIPA lysis Buffer (Sigma-Aldrich) and subjected to immunoblotting as described previously [18]. Reagents were obtained from the following sources: antibodies for gankyrin (Santa Cruz, Dallas, Texas, USA); antibodies for PGK1 (Abcam, Cambridge, MA, USA); antibodies for p-S6K (T398), S6K1, S6, p-S6 (S235/236), mTOR, p-AKT (S473), AKT, p-4E-BP1 (T37/46), and 4E-BP1; and HRP-conjugated secondary antibody (Cell Signaling Technology; Danvers, MA, USA).

2.8. Reactive Oxygen Species (ROS) Fluorescent Probe. MKN45 cells overexpressing gankyrin or NC were treated with DMSO or 10 nM rapamycin for 1 h. Oxidative stress of treated cells was determined by 2′,7′-dichlorodihydrofluorescein diacetate (H2DCF-DA) and dihydroethidium (DHE) that indicate the level of ROS. DHE or H2DCF-DA probe solution was diluted to an appropriate concentration by culture medium, and the cell culture medium was replaced by the diluted probe solution. After incubation at room temperature for 10–90 min in the dark light, the cells were washed with fresh solution and imaged by green or blue filters using a fluorescence microscope.

2.9. Statistical Analysis. Statistical analysis was performed with the SAS for Windows and GraphPad Prism V6 (GraphPad Prism Inc., USA); $P < 0.05$ was considered to be statistically significant. The results were expressed as the mean ± SD. The correlation between gankyrin expression and clinicopathological parameters was analyzed by Fisher's exact test. The comparisons were analyzed using Student's *t*-test. The correlation between gankyrin and PGK1 expressions was tested by Pearson's correlation analysis. The cancer-specific survival curves were estimated by Kaplan-Meier plots and log-rank test.

3. Results

3.1. Gankyrin Contributes to the Early Malignant Behavior of Gastric Cancer. To explore the relationship between gankyrin and the risk of harboring gastric cancer, we investigated the expression of gankyrin in a large panel of gastric precancerous and cancerous clinical samples. Compared to nonatrophic gastritis (NAG) tissue, the expression of gankyrin was elevated in chronic atrophic gastritis (CAG) and significantly higher in CAG with intestinal metaplasia (CAG + Im) or dysplasia (CAG + dys) (Figures 1(a) and 1(b) and Table 1). The gankyrin staining-positive rate and median H-score in dysplasia (positive rate = 90.00%, median H-score = 150) were similar to those in gastric cancer tissues (positive rate = 92.78%, median H-score = 145). Since the CAG-metaplasia/dysplasia-cancer sequence represents the process by which most gastric cancers arise, the data indicated that gankyrin overexpression is involved in the very early stage of gastric carcinogenesis; this finding was consistent with that in human colorectal precancerous and cancerous lesions [18]. In tissue array analysis, the overall gankyrin staining was stronger in tumors (median H-score = 185) as compared to the paired noncancerous tissues (median H-score = 140) (Figures 1(c) and 1(d)). This phenomenon was further validated in the published dataset (GEO access number: GSE26942), wherein gastric cancer tissues showed significantly higher gankyrin mRNA level as compared to the noncancerous tissues ($P = 0.022$) (Figure 1(e)). In the tissue array comprising 77 cases, gankyrin overexpression was associated with lymph node metastasis ($P = 0.019$), distant metastasis ($P = 0.013$), and vascular invasion ($P = 0.037$) (Table 2). The log-rank test revealed that high gankyrin expression was significantly correlated with poor survival ($n = 65$, $P = 0.024$) (Figure 1(f)). Taken together, gankyrin contributes to the early malignant behavior of gastric cancer.

3.2. Gankyrin Promotes the Oncogenic Properties of Gastric Cancer Cell. To inspect the phenotypes induced by gankyrin in gastric cancer cells, we firstly identified the basal levels of human normal gastric epithelial cell (GSE-1) and several gastric cancer cell lines using Western blot analysis and the results revealed that high gankyrin expression was observed in all cancer cell lines, but it was undetectable in GES-1 (Figure 2(a)). To explore the function of gankyrin in gastric cancer development, MKN74 (low-gank expression) and MKN45 (high-gank expression) cells were chosen to establish the gankyrin overexpression cell lines. The MTT assays showed that overexpressed gankyrin significantly accelerated the cell growth as compared to that by background expression (Figure 2(b)). Both foci formation assay (Figures 2(c) and 2(d)) and soft agar assay (Figures 2(e) and 2(f)) measured the anchorage-dependent or anchorage-independent cell growth and revealed that gankyrin promoted the characteristics of transformed cells with marked differences in either MKN74 or MKN45 cells. To assess the gankyrin-mediated invasion ability of gastric cancer cell, the Transwell trails showed that gankyrin significantly facilitated the motility and invasiveness of cancer cells as compared to the negative controls (Figures 2(g) and 2(h)). Taken together, gankyrin possesses the oncogenic properties to promote the malignant behavior of gastric cancer cells.

3.3. Gankyrin Potentiates mTORC1 Signaling via PGK1/AKT. In a previous report, we showed that gankyrin significantly enhanced the mTOR activity in colorectal cancer (CRC) through targeting TSC2 for degradation, independent of AKT signaling [18]. Intriguingly, gankyrin can also activate the mTORC1 signaling pathway with enhanced the levels of phosphorylated S6K1 and 4E-BP1 in gastric cancer cells (Figure 3(a)). Unlike that in CRC, gankyrin activated the AKT signaling and its upstream regulator PGK1 in gastric cancer as assessed by Western blot analysis (Figure 3(a)); this phenomenon was in agreement with the studies, wherein PGK1 activates AKT/mTOR in lung cancer [32] and regulates autophagy to promote tumorigenesis via the mTOR pathway [33]. Moreover, the mRNA level of gankyrin and PGK1 was found to be correlated in 414 gastric cancer samples according to the TCGA cancer genome database by Pearson's correlation analysis (Figure 3(b)). Taken together, gankyrin can potentiate the mTORC1 signaling via a PGK1-AKT pathway in gastric cancer.

3.4. Gankyrin Alleviates Oxidative Stress in Gastric Cancer Cell by Activating mTORC1. Oxidative stress may be the cause of direct damage to DNA and, therefore, mutagenic [34]. It may also suppress apoptosis and promote cancer cell proliferation, invasiveness, and metastasis [34]. The production of reactive oxygen and nitrogen species increased by *Helicobacter pylori* infection in the stomach is also crucial for the development of gastric cancer [25, 35]. To investigate whether gankyrin affected the process of oxidative stress, the mTORC1 signaling was successfully suppressed using

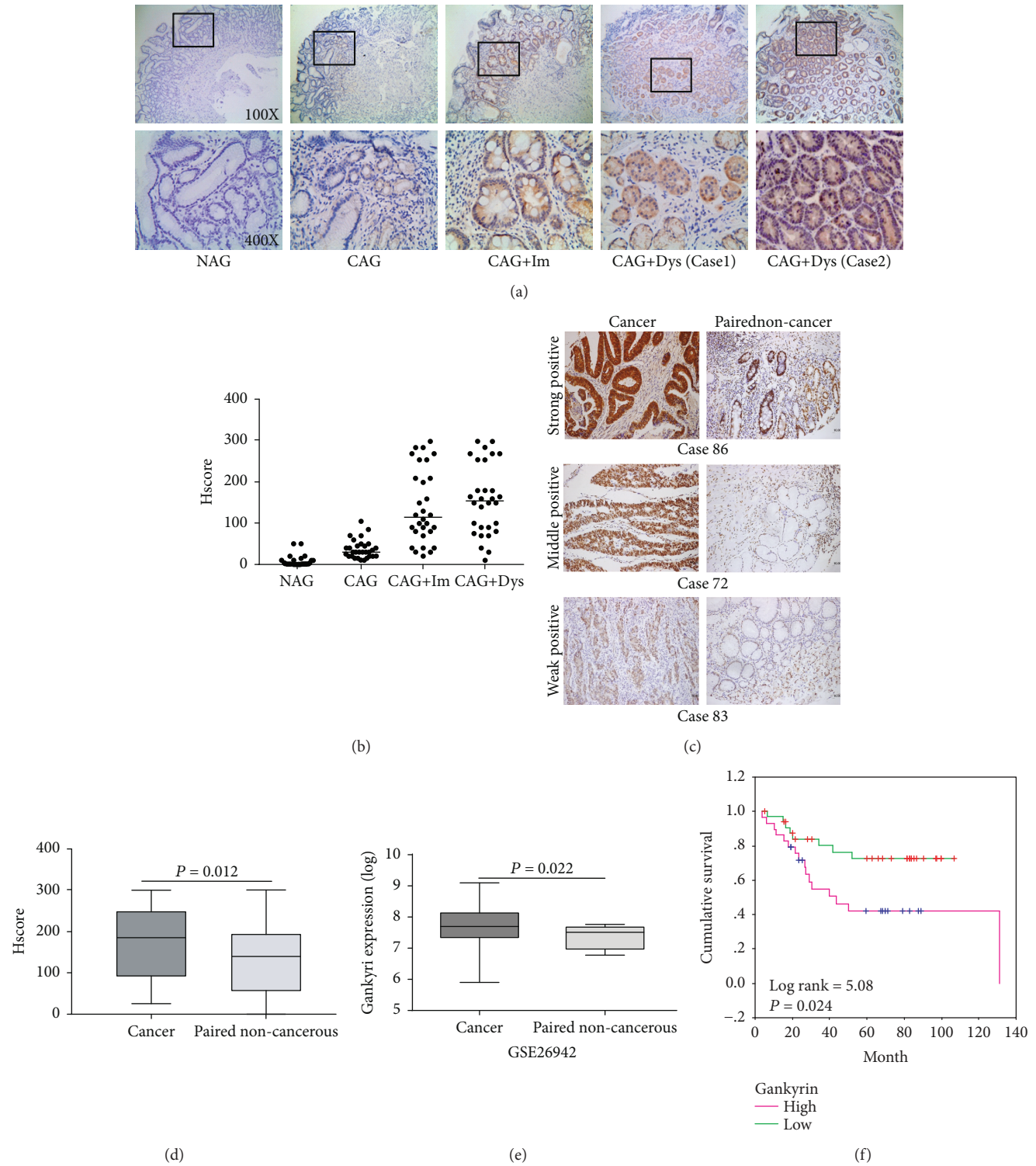

Figure 1: Gankyrin contributes to the early malignant transformation of gastric cancer. (a) Immunohistochemistry (IHC) staining of gankyrin in noncancerous and precancerous gastric tissue sections. Representative images of stained nonatrophic gastritis (NAG), chronic atrophic gastritis (CAG), CAG with intestinal metaplasia (IM), and CAG with dysplasia (dys) gastric tissue sections (original magnification, 100x or 400x) are shown. (b) Scatter plot showing gankyrin staining level in individual noncancerous and precancerous gastric tissue sections, 30 cases for each group. (c) IHC staining of human gastric cancer tissue and paired noncancerous tissues. Representative images of the stained tumor and paired noncancerous tissue are shown with strong, moderate, and weak positivity for gankyrin expression. (d) Box plot graph showing the statistical analysis of gankyrin expression in 77 gastric cancers and paired noncancerous tissues. (e) Gankyrin mRNA expression was significantly upregulated in gastric cancer tissues as compared to normal gastric tissues based on microarray data of GSE26942 ($n = 217$). (f) Kaplan-Meier survival analysis of gastric cancer cases divided into two groups by the median value (H-score = 145) for gankyrin staining. The P value was calculated by the log-rank test.

Table 1: Gankyrin expression in noncancerous and precancerous gastric tissues.

Gankyrin	NAG	CAG	CAG + Im	CAG + Dys
Positive rate	2 (6.67%)	7 (23.33%)	24 (80.00%)	27 (90.00%)
Median (QR)	1 (1, 10)	30 (20, 45)*	115 (80, 210)**	150 (90, 236.25)**

120 noncancerous and precancerous gastric tissues were analyzed for gankyrin expression. Positive gankyrin staining was defined as the H-score higher than the median 47.5. For each group, the median, 1st quartile, and 3rd quartile were shown. The P value was calculated by Mann–Whitney U-test vs. NAG (*$P < 0.05$, **$P < 0.01$). NAG: nonatrophic gastritis; CAG: chronic atrophic gastritis; Im: intestinal metaplasia; Dys: dysplasia.

Table 2: Association between gankyrin expression and clinicopathological features.

Clinicopathological features	No. of patients (n)	Relative gankyrin expression		T	P
		High	Low		
Age				0.785	0.435
<45	9	5 (55.6%)	4 (44.4%)		
≥45	68	39 (57.4%)	29 (42.6%)		
Gender				1.898	0.062
Male	52	34 (65.4%)	18 (34.6%)		
Female	25	11 (44.0%)	14 (56.0%)		
Lymph node metastasis				2.401	0.019
No	20	9 (45.0%)	11 (55.0%)		
Yes	57	35 (61.4%)	22 (38.6%)		
Distant metastasis				2.532	0.013
No	55	27 (49.1%)	28 (50.9%)		
Yes	22	17 (77.3%)	5 (22.7%)		
Pathological grade				2.557	0.084
I–II	9	7 (77.8%)	2 (22.2%)		
III	62	34 (54.8%)	28 (45.2%)		
IV	6	3 (50.0%)	3 (50.0%)		
Vascular invasion				2.122	0.037
No	20	9 (45.0%)	11 (55.0%)		
Yes	57	35 (61.4%)	22 (38.6%)		

Patients were divided by high gankyrin staining (H-score ≥ 145) and low gankyrin staining (H-score < 145).

rapamycin in both MKN45 and MKN74 cells with or without gankyrin overexpression (Figure 4(a)). Representative DHE staining and quantification data showed that overexpressed gankyrin significantly inhibited the reactive oxidative species (ROS) as compared to the negative control (Figures 4(b), 1st and 3rd panels, and 4(c)). However, ROS was sustained by rapamycin treatments either with or without gankyrin overexpression (Figures 4(b), 2nd and 4th panels, and 4(c)), which was in agreement with DCF staining and quantification data (Figures 4(a) and 4(e)). Altogether, gankyrin can alleviate the oxidative stress by activating mTORC1 in the gastric cancer cell.

4. Discussion

Gastric cancer, as a malignant epithelial tumor, continues to be a major health threat related to death and poor overall survival > 5 years in both sexes worldwide [25]. In the early stages, gastric cancer either is often asymptomatic or causes only nonspecific symptoms, which might be associated with indigestion, abdominal discomfort, anorexia, or a burning sensation. Without the endoscope detection, the occurrence of gastric cancer at an early stage cannot be diagnosed easily for a timely treatment [25]. Gastric carcinogenesis is a multistep process that develops from chronic gastritis, atrophy, gastric intestinal metaplasia (GIM), and dysplasia and finally leads to gastric cancer [36]. GIM and dysplasia are speculated as the premalignant stage of gastric cancer in a population of patients [37, 38], guiding the appropriate clinical recommendations for reducing the risk of gastric cancer [39].

Herein, we revealed that Gankyrin, an oncoprotein and a potential therapeutic target in multiple cancer diseases [7, 8, 18, 24, 26, 27], was a great biomarker for the early diagnosis of gastric cancer according to the detection and analysis of its expression in a large cohort of gastric precancerous and cancerous clinical samples (Figure 1). The significantly enhanced expression of gankyrin in CAG with intestinal metaplasia or dysplasia indicated an increased risk of gastric carcinoma. Moreover, high level of gankyrin expression in gastric tumor samples or cell lines demonstrated its

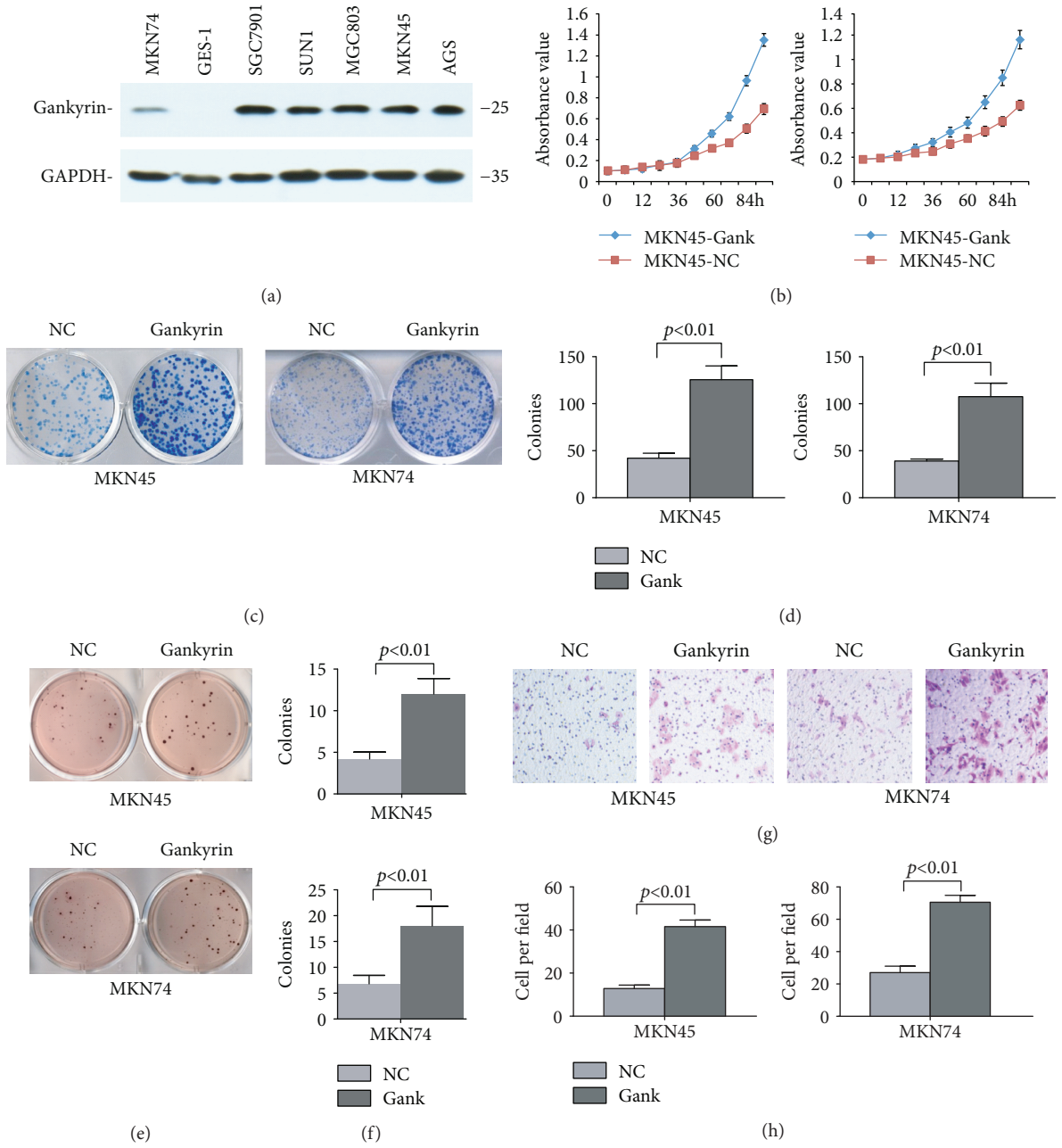

FIGURE 2: Gankyrin promotes the oncogenic properties of gastric cancer cell. (a) The basal level of gankyrin in different cell lines was detected by Western blot analysis. (b) The viability of MKN45 and MKN74 cells stably expressing gankyrin or vector control (NC) was analyzed by MTT assay. Absorbance was measured using a microculture plate reader at 490 nm. (c) MKN45 (upper panel) and MKN74 (lower panel) cells overexpressing gankyrin (-Gank) or carrying a control vector (-NC) were assessed for focus formation. The colonies were stained with methylene blue and enumerated by Image-Pro Plus (Media Cybernetics). Representative images are shown. (d) Quantification of focus formation in the experiment in (c). Data represent the means ± SD from three independent experiments in triplicate. (e) MKN45 (upper panel) and MKN74 (lower panel) cells overexpressing gankyrin (-Gank) or carrying a control vector (-NC) were plated in soft agar to determine anchorage-independent growth. The colonies were stained with p-iodonitrotetrazolium violet and enumerated by Image-Pro Plus (Media Cybernetics). Representative images are shown. (f) Quantification of soft agar colony formation in (e). Data represent the means ± SD from three independent experiments in triplicate. (g) Transwell assay was used to measure the cell invasion of MKN45 (upper panel) and MKN74 (lower panel) cells overexpressing gankyrin (-Gank) or a control vector (-NC). (h) Quantification of cell migration in (g). Data represent the means ± SD from three independent experiments in triplicate.

role in promoting the process of gastric cancer, including tumor growth, metastasis, or vascular invasion *in vivo* (Figure 1) and *in vitro* (Figure 2); also, it was significantly correlated with poor survival in clinics (Figure 1(f)). In addition to environmental risk factors (infection, smoking, or diets), a number of molecular and genetic aberrations also

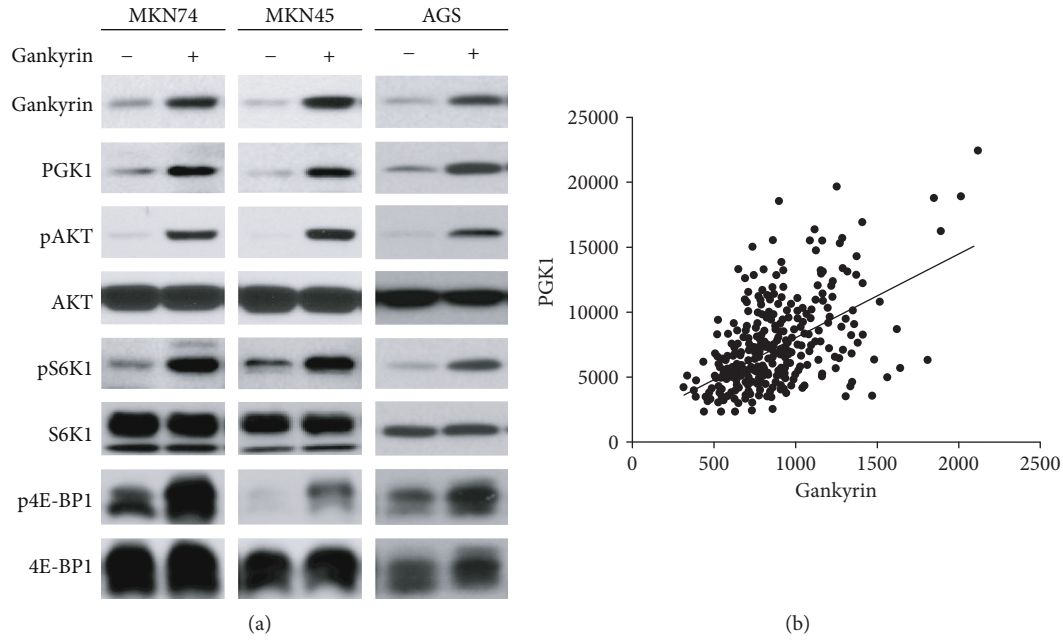

Figure 3: Gankyrin potentiates mTORC1 signaling via PGK1/AKT. (a) Overexpressed gankyrin activates AKT/mTORC1 signaling in gastric cancer cell lines, MKN45, MKN74, and AGS. The protein levels of PGK1, pAKT, AKT, pS6 K1, S6 K1, p4E-BP1, and 4E-BP1 were analyzed by immunoblotting. (b) The correlation plot of gankyrin and PGK1 mRNA level in 414 gastric cancer samples was presented by analyzing the TCGA cancer genome database.

contribute to gastric carcinogenesis, including changes in p53, KRAS, CDH1, cyclin E, Her2, and MET [40]. The genetic alteration in p53 leading to the development of gastric cancer is also seen in *H. pylori*-associated conditions such as chronic gastritis, intestinal metaplasia, and dysplasia [41–43]. Intriguingly, p53 can also be negatively regulated by gankyrin in multiple cancer types [3, 10], which is similar to the observation in the current study that gankyrin involves the precancerous lesions of gastric cancer. To compare the oncogenic properties between cell lines, MKN45 (poorly differentiated adenocarcinoma) with high-gank expression seemed showing higher proliferation abilities than MKN74 (highly differentiated adenocarcinoma) with low-gank expression (Figures 2(a)–2(d)), which also hinted that gankyrin might promote the process of gastric cancer development. Thus, the data strongly suggested that gankyrin contributes to early malignant transformation and later processes of gastric cancer (Figures 1 and 2).

In a previous report, we demonstrated that gankyrin regulates the mTORC1 signaling pathway in CRC via a PI3K/AKT-independent and TSC-dependent mechanism [18]. On the other hand, the AKT activation was found to be involved in the gankyrin-induced mTORC1 signaling according to our findings in gastric cancer (Figure 3(a)), which indicated that gankyrin might be associated with or regulated by an alternative molecule to potentiate the mTORC1 signaling pathway. The analysis of the TCGA cancer genome database also revealed that the mRNA expression of gankyrin was clinically correlated to PGK1, an upstream protein kinase of AKT (Figure 3(b)). This evidence introduced a novel insight into gankyrin biology in gastric cancer. However, additional studies are essential to support that gankyrin activates PGK1/AKT signaling to enhance the mTORC1 activation. High ROS levels leading to oxidative stress limit cancer cell survival during certain windows of cancer initiation and progression [44, 45]. Recently, it has been reported that overexpressed gankyrin amplifies the antioxidant capacity of HCC cells, reduces oxidative stress-induced mitochondrial damage, inhibits apoptosis, and promotes the development of HCC [15]. Consistently, the present study demonstrated that gankyrin can significantly impede ROS through the activation of mTORC1 signaling in gastric cancer (Figure 4). Moreover, we found that gankyrin affected not only the production of hydrogen peroxide (Figures 4(d) and 4(e)) but also the superoxide anion (Figures 4(b) and 4(c)), which supplied an interesting cue that gankyrin may exert its function through superoxide anion. However, it still needs further study on it. Therefore, gankyrin might also exhibit effects of accelerating the cancer process by regulating oxidative stress and maintaining cell homeostasis through the mTORC1 signaling pathway.

5. Conclusions

In conclusion, the current study revealed that increased gankyrin expression could be a risk factor of harboring gastric cancer. It potentially drove malignant transformation and behaviors of gastric cancer cell, as well as alleviated oxidative stress through the mTORC1 pathway. These characteristics provide a new insight into gankyrin biology with respect to gastric cancer.

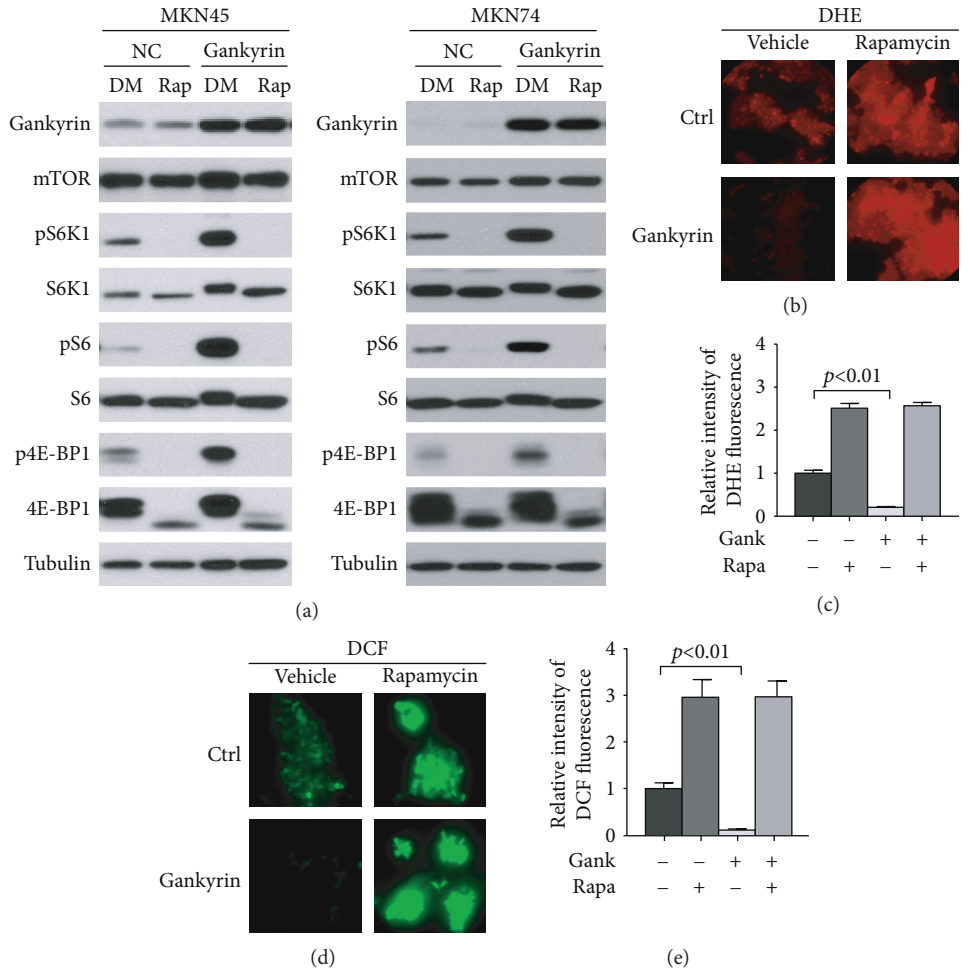

Figure 4: Gankyrin alleviates oxidative stress in gastric cancer cell by activating mTORC1. (a) Both MKN45 and MKN74 cells with or without overexpressed gankyrin were treated with DMSO or 10 nM rapamycin for 1 h. The protein levels of pS6 K1, S6 K1, pS6, S6, p4E-BP1, and 4E-BP1 were analyzed by immunoblotting. (b) Oxidative stress was determined by measuring reactive oxygen species (ROS) levels using dihydroethidium (DHE) staining (red) in living cells. Representative images are shown (cell number > 100). Scale bars = 10 μm. (c) Quantification of fluorescence intensity in the experiment in (b). Data represent the means ± SD from three independent experiments in triplicate. $P < 0.01$ represents a significant difference. (d) Oxidative stress was determined by measuring the levels of ROS using 2′,7′-dichlorodihydrofluorescein diacetate (H2DCF-DA) staining (green) in living cells. Representative images are shown (cell number > 100). Scale bars = 10 μm. (e) Quantification of fluorescence intensity in the experiment in (d). Data represent the means ± SD from three independent experiments in triplicate. $P < 0.01$ represents a significant difference.

Data Availability

The datasets analyzed during the current study are available in the TCGA (https://cancergenome.nih.gov/), GEO (https://www.ncbi.nlm.nih.gov/gds), PrognoScan dataset (http://www.prognoscan.org/), and HPA dataset (http://www.proteinatlas.org/). All data generated during this study are included in this article.

Conflicts of Interest

All authors declare that they have no potential conflicts of interests.

Authors' Contributions

Bo Huang and Weiyang Cai are the co-first authors.

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Review Article

New Insights into the Role of Exercise in Inhibiting mTOR Signaling in Triple-Negative Breast Cancer

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Triple-negative breast cancer (TNBC) does not express estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 and is characterized by its aggressive nature, lack of targets for targeted therapies, and early peak of recurrence. Due to these specific characteristics, chemotherapy does not usually yield substantial improvements and new target therapies and alternative strategies are needed. The beneficial responses of TNBC survivors to regular exercise, including a reduction in the rate of tumor growth, are becoming increasingly apparent. Physiological adaptations to exercise occur in skeletal muscle but have an impact on the entire body through systemic control of energy homeostasis and metabolism, which in turn influence the TNBC tumor microenvironment. Gaining insights into the causal mechanisms of the therapeutic cancer control properties of regular exercise is important to improve the prescription and implementation of exercise and training in TNBC survivors. Here, we provide new evidence of the effects of exercise on TNBC prevention, control, and outcomes, based on the inhibition of the phosphatidylinositol-3-kinase (PI3K)/protein kinase B (PKB also known as Akt)/mammalian target of rapamycin (mTOR) (PI3K-Akt-mTOR) signaling. These findings have wide-ranging clinical implications for cancer treatment, including recurrence and case management.

1. Introduction

Breast cancer (BC) is one of the most common carcinomas and one of the main causes of cancer-related death worldwide [1]. Among the various subtypes, triple-negative BC (TNBC) accounts for approximately 20% of BC cases. The absence of estrogen and progesterone receptors and human epidermal receptor 2 (HER2) in malignant cells reduces treatment options and increases the risk of recurrence and death, especially in the first 3–5 years of follow-up after surgery [2]. Thus, TNBC exhibits a more aggressive clinical course than non-TNBC. Most TNBC cases are diagnosed in women under the age of 60, and in 20% of diagnosed cases, there is a mutation of the germinal BC (BRCA) gene [3–7]. In patients with metastatic TNBC, there are currently no available targeted therapies and chemotherapy is the only possible treatment option. In addition to the biological-molecular aspects associated with prognosis and BC development, a growing body of evidence highlights the impact of lifestyle on disease-related outcomes. Unhealthy lifestyles with low levels of physical activity (PA) result in overweight and obesity, which appear to have a negative impact on BC [8], increasing the risk of recurrence and death in all subtypes, including TNBC [9]. Conversely, proper diet, weight loss, and increased PA lead to more favourable outcomes in the short and long term [10, 11]. The mechanisms underlying the effects of exercise on breast carcinogenesis are not clear, but experimental evidence suggests that PA

induces phosphatidylinositol-3-kinase (PI3K)/protein kinase B (PKB also known as Akt)/mammalian target of rapamycin (mTOR) (PI3K-Akt-mTOR) signaling inhibition and slows TNBC tumor cell growth [12–14]. Physiological adaptations to exercise occur primarily in skeletal muscle, but the effects of exercise and training also impact other tissues through systemic control of energy homeostasis and metabolism, thus influencing the TNBC tumor microenvironment and mTOR inhibition [15].

Given the scope of this review, we summarise recent discoveries related to the underlying biology of exercise-induced modulation of the mTOR pathway in TNBC, examining the benefits induced by different exercise and training protocols.

We also consider how exercise affects the level of microRNAs (miRNAs) linked to the mTOR pathway involved in TNBC initiation and progression [16, 17], and how nutrients can influence mTOR signaling.

Finally, we discuss how exercise induces beneficial adaptations and why it should be prescribed as a coadjuvant "medicine," which has the potential to improve TNBC outcomes.

2. mTOR Signaling

2.1. mTOR Pathway and mTOR Activation in BC. mTOR is a serine-threonine kinase that interacts with several proteins to form two distinct complexes, mTORC1 and mTORC2, which show different sensitivities to rapamycin [18]. mTORC1 is acutely sensitive to rapamycin and responds to growth factors, stress, amino acids, and energy, promoting protein translation and synthesis, cell growth, mass, division, and survival. mTORC1 comprises mTOR, the regulatory associated protein of mTOR (Raptor), the G-protein β-subunit-like protein (GβL), also known as mLST8, DEP domain-containing mTOR-interacting protein (Deptor), proline-rich Akt substrate of 40 kDa (PRAS40), and Tti1/Tel2 complex. mTORC2 is insensitive to acute rapamycin treatment and contains mTOR, the rapamycin-insensitive companion of mTOR (Rictor), the mammalian stress-activated map kinase-interacting protein 1 (mSIN1), GβL, Deptor, protein observed with Rictor-1/2 (Protor 1/2), and Tti1/Tel2. Raptor and PRAS40 are unique to mTORC1, while Rictor, mSIN1, and Protor 1/2 are unique to mTORC2 [18].

The various components of mTORC1, which is the most widely studied complex, have several regulatory effects: Raptor, Tti1, and Tel2 are positive regulators, whereas PRAS40 and Deptor are negative regulators [19]. Several factors regulating mTORC1 activation converge in the tubular sclerosis complex (TSC), consisting of hamartin (TSC1), tuberin (TSC2), and TBC1 domain family member 7 (TBC1D7) [20]; the complex works via the Ras homolog enriched in brain (Rheb) GTPase, negatively regulating mTORC1 [21].

An upstream regulator of TSC is the PI3K/Akt pathway activated by growth factors such as insulin-like growth factor 1 (IGF-1) and insulin. PI3K phosphorylates phosphatidylinositol (3,4)-bis-phosphate (PIP2) lipid to phosphatidylinositol (3,4,5)-tris-phosphate (PIP3), which recruits phosphoinositide-dependent kinase-1 (PDK1) and Akt. Akt phosphorylates TSC2 and PRAS40 inactivating them and inducing, in turn, mTORC1 activation [22]. TSC2 can also be phosphorylated and inactivated by the activated Ras/extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) signaling pathway [19].

Another critical regulator of mTORC1 is the adenosine monophosphate-activated protein kinase (AMPK), which is activated when cellular energy level is low. AMP linking to AMPK allows its phosphorylation (while ATP availability prevents it) triggering repression of energy-consuming processes, also inhibiting mTOR, and enhancing energy-producing processes. AMPK phosphorylates TSC2 in different sites than Akt, activating rather than inactivating TSC2, and phosphorylates Raptor, thus achieving mTORC1 repression [23].

mTORC1 activation requires sufficient amino acid levels, though it is not clear how these levels are sensed. Amino acid regulation requires the formation of a Rag GTPase complex, which binds Raptor, in order to translocate mTORC1 to the lysosome allowing its association with Rheb, and thus its activation [24].

The activation of mTORC1 leads to several downstream effects, including protein synthesis promotion. Raptor binds to the eukaryotic translation initiation factor 4E- (eIF4E-) binding protein 1 (4E-BP1) and the ribosomal protein S6 kinase beta-1 (S6K1), recruiting them to the mTORC1 complex and allowing their phosphorylation [25, 26]. Hyperphosphorylation of 4E-BP1 by mTOR prevents the association of 4E-BP1 and eIF4E, allowing eIF4E to bind eIF4G to begin translation. Phosphorylation of S6Ks, including several S6K1 isoforms and S6K2, by mTOR promotes their activation and thus the phosphorylation of their targets involved in mRNA translation. S6K1 is also involved in negative feedback on mTORC1 and mTORC2 [27].

The mTORC1 complex and AMPK also regulate the autophagic process, a cellular mechanism through which cells eliminate damaged components associated with a wide range of diseases, including cancer. After glucose deprivation, AMPK associates with, and directly phosphorylates, the serine/threonine Unc-51-like autophagy activating kinase (ULK1), an upstream component of the autophagy mechanism. By contrast, when nutrients are plentiful, mTORC1 phosphorylates ULK1, preventing its association with and activation by AMPK, inhibiting autophagy [28].

Aberrant activation of the PI3K/Akt/mTOR pathway is often found in human cancers and promotes cell proliferation [29]. Activation has been shown in the lung, head, and neck and breast, gynaecologic, colorectal, and prostate cancers and glioblastoma multiforme [30] and also in B-lineage acute lymphoblastic leukemia [31]. PI3Ks are pivotal molecules in this pathway and possess eight isoforms grouped into class I, class II, and class III. Class I PI3Ks (PI3Kα, β, γ, and δ), stimulated by Tyr kinases, G protein-coupled receptors, and Ras, are currently the focus of research in drug development. Mutation of the PIK3CA gene, which encodes the catalytic subunit α (p110α), one of the class I PI3K isoforms, is found in several cancers [32]. The signaling and biological

roles of class II and III PI3Ks are not clear, and they have not been implicated in oncogenesis [32].

In TNBC, the activation of the PI3K/Akt/mTOR pathway is induced by an overexpression of upstream regulators (i.e., growth hormone receptors), mutations of the PIK3CA gene, and by decreased activity of the phosphatase and tensin homolog (PTEN) and of the proline-rich inositol polyphosphatase, which are downregulators of PI3K [33–35]. By contrast, activation of downstream effectors of PI3K (e.g., Akt and mTOR) and activation of downstream effectors of parallel pathways (MAPK and Ras) are rare events in TNBC [36]. Furthermore, other oncogenic pathways (i.e., FGFR, cMET, and RAF) regulated by P53 inactivation converge to activate the PI3K pathway [37].

Due to the frequent activation of the PI3K/Akt/mTOR pathway in human cancers, more than 50 inhibiting drugs are in development, and several clinical trials are ongoing [38]. The first established therapeutic anticancer agents targeting this pathway are everolimus and temsirolimus, which abrogate mTOR signaling, and have been approved by the U.S. Food and Drug Administration. Based on the results obtained with everolimus in pancreatic neuroendocrine tumors [39], and temsirolimus for advanced renal cell cancer [40], these agents are now approved for treatment of these diseases.

Therapies targeting other pathway members have been described. Monotherapy using pan-class I PI3K, which inhibits all class I PI3K isoforms, has effects at dose-limiting toxicity, leading to prolonged disease stabilization in some patients with advanced solid tumors (especially the lung) during phase I clinical trials [41]. Isoform-specific PI3K inhibitors have also been tested and have shown an antitumor activity in tumors such as p110δ- (isoform δ-) driven hematologic malignancies [42] or PIK3CA-mutant HR-positive BC [43]. Akt inhibitors and mTORC1/2 inhibitors aimed to suppress not only mTORC1, but also the feedback activation of Akt by mTORC2 [44], are currently being investigated in clinical studies [45]. The use of PI3K/Akt/mTOR pathway inhibitors is often associated with MAPK inhibitors, growth factor receptor inhibitors, and endocrine therapy. Furthermore, they might sensitize tumors to chemotherapy synergistically inducing apoptosis, as showed in sarcomas [46].

These promising strategies are now under investigation for the treatment of several tumors, including nonsmall cell lung cancer [47], colorectal cancer [48], nonmedullary thyroid carcinoma [49], and B-lineage acute lymphoblastic leukemia [31].

Although these strategies have been shown to be effective, there is great variability in the duration and quality of their benefits and the long-term side effects for patients. Thus, the identification of protein and/or genetic biomarkers to recognize subjects that will benefit the most from these therapeutic strategies is essential [50]. In TNBC, the development of PI3K/Akt/mTOR-targeted therapies, taking into account the inhibitors of this pathway alone or in combination with other strategies, will provide new tools to control disease progression and improve outcomes [51]. In a recent phase 2 clinical trial, the efficacy of ipatasertib (an Akt inhibitor) in association with paclitaxel (an antineoplastic agent used in TNBC treatment) was shown [52].

2.2. MicroRNAs and mTOR Signaling in BC. Several studies highlight the role of circulating microRNAs (miRNAs), in different tumors, including BC and the TNBC subtype [16]. In particular, recent evidence has shown that miR10a is downregulated in triple-negative BC cells [53]. Furthermore, overexpression of miR-10a decreases the proliferation and migration of TNBC cell lines via PI3K/Akt/mTOR signaling and through the mitochondrial apoptotic pathway [53]. Recently, Phua et al. [54] demonstrated that miR-184 is also downregulated in TNBC patients and that miR-184 overexpression in TNBC cells leads to a reduced expression of mTOR. The decreased cancer cell proliferation, due to mTOR reduction, has been confirmed *in vivo*: mice injected with mir-184-transfected MDA-MB-231 cells showed a delayed primary tumor formation and reduced metastatic burden. Emerging evidence points to epigenetic silencing by hypermethylation as a possible mechanism through which these tumor suppressor/growth inhibitor miRNAs are downregulated in TNBC [55]. In metastatic breast tumors, miR-184 has been found to be hypermethylated compared to the methylation status of miR-184 in normal breast tissue, suggesting a selective pressure in silencing this miRNA during the metastatic process [54].

Upregulation of miR-21 was detected in TNBC tissues and in MDA-MB-468 cells by Fang et al. [56]. Inhibition of this miRNA resulted in decreased proliferation, viability, and invasiveness of TNBC cells and enhanced apoptosis. Experiments to identify miR-21 targets have shown that PTEN is downregulated, suggesting an activation of mTOR and the oncogenic properties of miR-21 in TNBC, with increased proliferation and invasion by TNBC cells. Another miRNA that has been found to be upregulated in TNBC tissues in comparison to non-TNBC or adjacent tissues is miR-146a. Indeed, it has been reported to be significantly related to tumor size and histological stage: patients with elevated miR-146a expression have lower survival rates and worse prognoses than low-expression individuals [57]. In addition, miR-146a has been shown to bind the 3′-UTR region of BRCA1, inhibiting its expression; the BRCA1 protein is absent or present at very low levels in about one-third of sporadic BCs [58]. Evidence suggests that downregulation of BRCA1 expression leads to Akt/mTOR oncogenic pathway activation [59]. Hence, strategies that could modify the deregulated status of these miRNAs in TNBC could have a pivotal role in inhibiting the Akt/mTOR pathway and could affect TNBC initiation and progression. It is not yet known how these miRNAs might be modulated by exercise and whether they can be associated positively or negatively with TNBC progression, for which there are no reliable prognostic factors.

2.3. Autophagy and mTOR Signaling. Autophagy is the cellular mechanism responsible for the degradation of cytoplasmic components. It is through this mechanism that cells maintain cellular homeostasis by eliminating damaged proteins and organelles and by providing substrates for

energy generation and biosynthesis under stress conditions. The mTOR complex is a major negative regulator of autophagy. It suppresses autophagy in response to nutrients, growth factors, and hormone availability, promoting protein synthesis, cell division, and metabolism. The mTOR signaling pathway is frequently activated in tumor cells, resulting in the activation of its growth-promoting functions and the inhibition of autophagy [60]. In cancer, the cytoprotective role of autophagy could prevent tumorigenic transformation by inhibiting chronic tissue damage. By contrast, once cancer occurs, cancerous cells could utilize autophagy to enhance fitness and survive in the hostile tumor microenvironment, providing energy via substrate degradation. Autophagy could therefore be tumor suppressive (for example, via elimination of damaged cellular components), as well as tumor promoting in established cancers [61]. In addition, autophagy has recently been shown to play a role in necroptosis, and, together with apoptosis, autophagy also regulates other death pathways, including immunogenic cell death, entosis, and pyroptosis [62]. It has been demonstrated that suppression of autophagy in epidermal growth factor receptor- (EGFR-) driven nonsmall cell lung adenocarcinoma xenografts promotes cell proliferation, tumor growth, and dedifferentiation, as well as resistance to EGFR tyrosine kinase inhibitor therapy [63]. Moreover, autophagy suppresses early oncogenesis in lung adenocarcinoma through effects on regulatory T cells [64], and autophagy genes are often required for the cytotoxic effects of chemotherapy [65]. In view of the complex- and context-dependent role of autophagy in cancer progression and response to therapy, it could be hypothesized that the inhibition of the mTOR pathway and the consequent induction of autophagy may be useful in certain cancers through autophagy-dependent antitumor immunity, autophagy-dependent cytotoxic effects, or other tumor-suppressor effects [66]. In addition to its effects on skeletal muscle, exercise has also been found to induce autophagy in the liver, pancreas, adipose tissue, and cerebral cortex in transgenic mouse models [67, 68]. Whether exercise-induced stress activates autophagy in healthy cells (or cells primed for malignant transformation), or cancer cells themselves, and whether such effects inhibit or potentiate tumorigenesis, is not known and needs further investigation [15].

3. Evidence of mTOR Modulation by Exercise in TNBC

3.1. mTOR and Exercise. PA reduces mortality for all diseases, including tumors [69], reducing the incidence of primary development and ameliorating the prognosis [15]. Hence, it should be prescribed like a medication indicating the correct typology, dose, and timing, i.e., the type, intensity, duration, and frequency of exercise as described in Exercise Prescription in BC Survivors. Physiological adaptations to exercise occur not only in skeletal muscle but also systemically in other metabolically active tissues involved in the exercise response (such as the bone, heart, adipose, endothelium tissue, and brain) profoundly altering the systemic milieu, in turn influencing the tumor microenvironment and cancer hallmarks [15]. In order to understand the effect of PA on mTOR and BC, muscular, systemic, and microenvironment effects should be considered.

3.1.1. Aerobic Exercise and Muscular Effects. In skeletal muscle, aerobic exercise activates several adaptive pathways, including protein kinases, transcription, and coregulatory factors that, by gene expression modification, increase mitochondrial biogenesis and stimulate metabolic reprogramming [70]. Exercise induces a depletion of nutrients, energetic substrates, and nicotinamide adenine dinucleotide (NAD)H that elevate the ratios of AMP : ATP and NAD+ : NADH, directly activating AMPK and other metabolic sensors, including NAD-dependent protein deacetylase sirtuin 1 (SIRT1) and kinases, such as ERK1/2, p38 MAPK, and Jun N-terminal kinase (JNK) [71]. These energy sensors trigger the transcriptional regulator peroxisome proliferator-activated receptor-γ coactivator 1α (PGC1α), which regulates the expression of mitochondrial biogenesis, increase the expression of mitochondrial transcription factor A (TFAM), which, once transferred to the mitochondria, controls transcription of mitochondrial DNA [71]. Moreover, aerobic exercise, through PGC1α phosphorylation, influences other transcription factors, including peroxisome proliferator-activated receptor-γ (PPARγ), an important regulator of fatty acid oxidation and estrogen-related receptor-α (ERRα) and ERRγ, which directly regulate mitochondrial energy metabolism by oxidative phosphorylation, fatty acid oxidation, and the tricarboxylic acid (TCA) cycle [72, 73]. In this regard, the reactive oxygen species (ROS) and reactive nitrogen species produced by exercise also directly or indirectly regulate contraction-induced mitochondrial biogenesis [74] and skeletal muscle metabolic reprogramming via AMPK and PGC-1α [75]. AMPK-mediated cell survival requires inhibition of mTOR. Therefore, AMPK and mTOR play antagonistic roles in cells and inhibition of mTOR is essential for AMPK-mediated metabolic homeostasis [76].

3.1.2. Resistance Exercise and Muscular Effects. In skeletal muscle, resistance exercise causes an increase in muscle size and strength via mTOR activation. In canonical growth factor signaling, mTOR is activated by PI3K/Akt, through IGF-1 and insulin signaling, but a considerable body of evidence suggests that mTORC1 is also likely activated by a growth factor-independent movement of proteins to and from the lysosome, via resistance exercise-induced phosphorylation of TSC2 [77]. Cellular trafficking of mTOR and its association with positive regulators that occur in human skeletal muscle leading to protein synthesis after resistance exercise, in fed condition, were recently confirmed by Song and colleagues [78].

3.1.3. Systemic and Microenvironment Effects of Exercise. Exercise stimulates the release of molecular signals such as muscle-derived regulatory RNAs, metabolites, and myokines with autocrine, paracrine effect on energetic substrate oxidation, hypertrophy, angiogenesis, inflammation, and regulation of the extracellular matrix. To better evaluate the systemic response to PA, a distinction must be drawn

between long term (training) and acute exercise. Training induces a reduction of basal concentration of circulatory sex hormones and lowers adiposity, both recognized risk factors [79], while acute exercise causes a sharp increase in circulating hormones, cytokines, and immune cells [80–82]. Both the systemic adaptations to training and the strong response to acute exercise support plausible mechanisms that inhibit carcinogenesis by suppressing the activation of mTOR signaling network. Hence, exercise may improve BC outcomes [14] (Figure 1). Moreover, both long-term training and a single bout of exercise control energy availability and induce a hormetic response that accounts for the physiological cellular stress adaptation [83, 84].

Hormesis is a process whereby exposure to a low dose of a potential stress favours adaptive changes in the cell that enables it to better tolerate subsequent stress [85, 86]. This type of stress is often related to reactive oxygen species (ROS) originating from the mitochondrial respiratory chain [87]. The accumulation of transient low doses of ROS through exercise influences signaling from the mitochondrial compartment to the cell [88]. Remarkably, this coordinated response to mild mitochondrial stress appears to induce mitochondrial metabolism, increase stress resistance, stimulate various long-lasting cytoprotective pathways, and favour the establishment of an oxidant-resistant phenotype, hence preventing oxidative damage and chronic diseases. Accordingly, low levels of ROS elicit positive effects on physiological cellular and systemic responses and ultimately increase lifespan [83, 88–93]. The hormetic nature of exercise, which produces low levels of ROS, emerges as a key feature for cancer control. Indeed, in the tumor microenvironment, the activation of exercise-induced hormesis of the AMPK-p38-PGC1-α axis supports oxidative metabolism maintaining the cellular ATP pool and conserving cellular energy and viability during the metabolic stress condition: AMPK regulates metabolism and energy homeostasis [94, 95]. Exercise-induced mitochondrial biogenesis improves mitochondrial function in addition to the upregulation of antioxidant defenses that function as back regulators of intracellular ROS levels, and leads to improved redox homeostasis [96, 97] as well as significantly improved insulin sensitivity. By contrast, high levels of ROS cause functional oxidative damage to proteins, lipids, nucleic acids, and cell components, induce a significant increase in intracellular Ca^{2+}, and promote signaling cascades for apoptosis or autophagy via NF-κB or forkhead box sub group O (FoxO) pathways. High ROS levels are therefore reputed to act as etiological, or at least exacerbating factors in chronic/aging-related diseases.

The typical hormetic response modulated by exercise involves kinases, deacetylases, and transcription factors; many of which have also been shown to be involved in the carcinogenic process [86]. The most studied are sirtuins (SIRT), which are histone deacetylases, and the FoxO family of transcription factors. The pathways in which NF-kappaB and the Nrf-2/ARE are components are also involved in hormetic responses and implicated in carcinogenesis and are modulated by exercise [86].

FoxO transcription factors play a critical role in cell cycle control and cellular stress responses. FoxOs are known to be regulated by the insulin signaling pathway; however, recently, the research group of Burnet demonstrated that AMPK phosphorylates 6 specific residues on FoxO and opposes the phosphorylation of other FoxO sites by Akt [98]. Phosphorylation of FoxO by AMPK affects the conformation of the protein in such a way that sirtuin-mediated deacetylation is also modified [99]. The dependence of sirtuins on nicotinamide adenine dinucleotide (NAD(+)) links their activity to cellular metabolic status. Emerging evidence indicates that deacetylation of FoxO by SIRT1 favours expression of cell survival/stress resistance and the downregulation of proapoptotic genes [85, 100, 101]. Sirtuins therefore protect against cancer development as they regulate the cellular stress responses and ensure that damaged DNA is not propagated and that mutations do not accumulate [99]. However, how FoxO activation is influenced by exercise remains unclear. In addition, cytokines such as those that we and others have found to be regulated by exercise and training [14, 102–104] have been reported to have direct and indirect effects on cellular stress responses modulated by acetylation/deacetylation reactions, and these effects can be further modified by cortical steroids, which exercise dramatically induces [105].

Similarly, various chemical mimetics of PA and caloric restriction (CR) such as AICAR, PPARδ agonist, resveratrol, and metformin can trigger a beneficial response by activation of key regulators of stress tolerance at the level of transcription, posttranscriptional modifications, and regulation of energy metabolism [92, 106]. Cross talk between major CR hormesis-induced pathways, especially AMPK/PPAR and antioxidant systems, IGF-1, and homeostatic energy balance, reveals the correlation between CR and exercise mimetics [107].

Likewise, depending on the exercise, the level/persistence could induce an adaptive response that might turn the same process from "physiologic" into "pathologic," as in the case of inflammation. Careful titration of ROS levels within specific tumor microenvironments may lie at the crossroads between the prevention, protection, and/or initiation and progression of disease, in particular, as regards the induction of mitochondrial functionality, cellular homeostasis, and more generally, cellular metabolic health.

Considering the type of exercise, both aerobic and resistance training increase glucose uptake in skeletal muscle via insulin-independent mechanisms, with a subsequent decrease in circulating levels of insulin, IGF-1, and glucose [108]. In a model of mammary carcinogenesis, PA caused a delay in carcinogenesis with a concomitant activation of AMPK and reduction in Akt and mTOR activation and reduction in insulin and IGF-1 in circulation [12]. Reduction of insulin levels is an important aspect given that hyperinsulinemia and insulin resistance are commonly observed in obesity with adipokine alterations, conditions associated with increased risk of BC and poor prognosis [8]. Insulin resistance is a condition in which the target tissues of insulin such as skeletal muscle, adipose tissue, and liver show a reduction in their response to physiological concentrations of the insulin hormone. As a consequence, the pancreatic β-cells produce more of the hormone to compensate for the

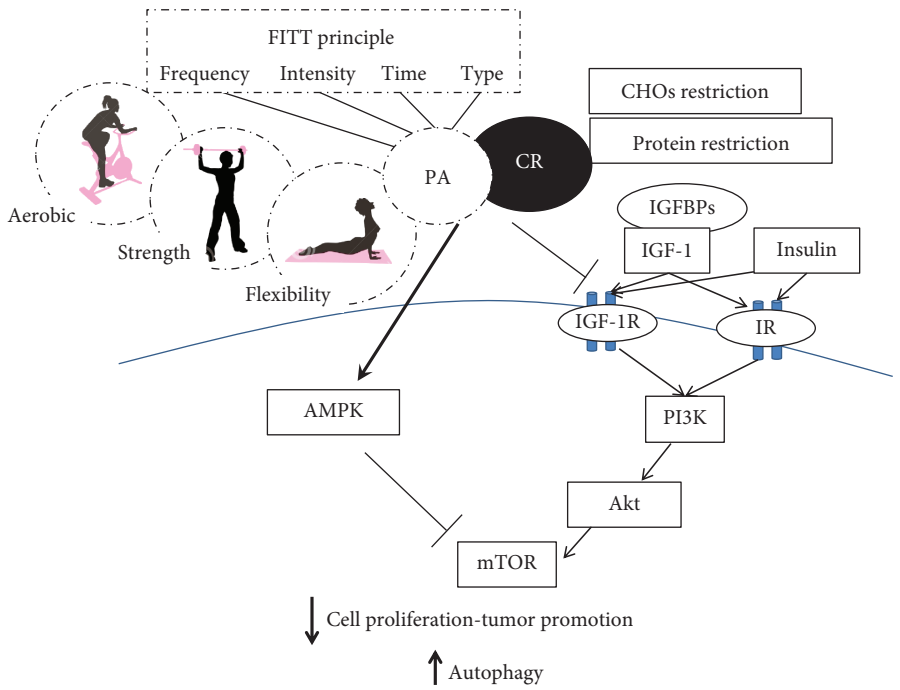

Figure 1: In this figure, we consider potential mechanisms regulated by physical activity and caloric restriction in inhibiting the mTOR pathway. Both refer to energy availability inhibiting carcinogenesis by suppressing the activation of the mTOR signaling network in this subtype of mammary carcinoma. The mTOR inhibition is mediated through the effects of vigorous PA or long-term exercise on systemic response such as concentrations of the circulating growth factors and hormones (i.e., IGF-1 and insulin) that regulate the mTOR network. The network is controlled through the PI3K/Akt signaling pathway, the glycaemia and glutamine levels, inducing apoptosis and reversing malignancy-associated metabolic programming. Moreover, the control of energy availability by both exercise and CR induces a mitohormetic response that accounts for a physiological cellular stress adaptation through AMPK activation inducing mTOR inhibition. In this context, exercise should be considered in terms of its four components: frequency, intensity, time, and type; however, dose-dependent effects of each component on cancer protection via mTOR inhibition have not yet been clarified. Most data indicate that vigorous PA, either long-term or in adulthood, may reduce a woman's risk of mammarian cancer, especially TNBC relapse. The inhibition of the mTOR complex and its cell growth-promoting functions leads to a reduction of cell proliferation, control of cancer progression, and consequent autophagy induction probably involved in tumorigenesis prevention. Thus, we hypothesized that the exercise-induced inhibition of the mTOR pathway may be useful in the control of cancer progression, including TNBC. PA: physical activity; CR: caloric restriction; CHOs: carbohydrates; mTOR: mammalian target of rapamycin; IGF-1: insulin-like growth factor 1; IGF-1R: insulin-like growth factor receptor 1; IR: insulin receptor; IGFBPs: insulin-like growth factor binding proteins; PI3K: phosphatidylinositol-3-kinase; AMPK: adenosine monophosphate-activated protein kinase; TNBC: triple-negative breast cancer. FITT-VP principle, which reflects the frequency (F), intensity (I), time (T), and type (T) of exercise, and its volume (V) and progression (P) over time, in an individualized exercise training program.

defective response of target tissues, thus leading to hyperinsulinemia. BC cells express high levels of the insulin receptor (IR), and increased circulating insulin is associated with BC recurrence and death [109]. In contrast, PA has a fundamental role in reducing muscle insulin resistance and normalizing circulating insulin levels. Regular exercise in both healthy and oncological conditions ameliorates glycemic control including glycated hemoglobin (HbA1c) and insulin sensitivity in a "dose"-dependent manner according to duration and intensity [110, 111]. Skeletal muscle in virtue of its mass and high rate of insulin and exercise-stimulated glucose transport, represents the most important tissue in glucose uptake. Exercise per se increases trafficking of glucose transporter 4 (GLUT4) to the plasma membrane through insulin-independent mechanisms [112]. Under normal physiological conditions, in skeletal muscle, insulin actions are mediated by the IR-catalyzed phosphorylation of the IR substrates 1 and 2 (IRS1 and IRS2). The tyrosine-phosphorylated IRS proteins then interact with and activate PI3K, a critical player in insulin signaling, particularly with regard to glucose homeostasis. Activation of PI3K generates PIP3 that induces membrane translocation of the serine/threonine kinase Akt. PIP3 activation of PDK1 and the Rictor/mTOR complex 2 leads to phosphorylation and subsequent activation of Akt [113]. Akt phosphorylates TBC1D4 (also known as Akt substrate of 160 kDa, AS160) and TBC1D1 promoting the translocation of GLUT4 vesicles from intracellular compartments to membrane for glucose uptake [114].

Although recent findings help to better understand the effect of exercise on glycemic control, the specific exercise-induced signaling mechanisms leading to the acute and long-term adaptations favouring enhanced glycemic control are less clear [112, 115].

Endurance and, to a lesser extent, resistance exercise represent a significant metabolic stress, activating AMPK

and thus inhibiting mTOR also in nonmuscular tissue such as liver, fat, and tumor tissues. In order to better evaluate the impact of exercise on mTOR in the BC microenvironment, not only AMPK, but also other circulating factors, should be considered. IGF-1, as well as insulin, activates the MAPK pathway and the PI3K pathway, which are both involved in cancer development and progression. The importance of IGF-1 axis in the development and progression of BC has been clearly shown [116]. The overexpression of IGF-1R in BC has been reported and related to poorer survival rates [117].

The IGF signaling system is composed by IGF-1 and IGF-2, insulin-like growth factor binding proteins (IGFBPs), a family of binding proteins regulating IGF half-lives and available in circulation and extracellular fluids, IGF receptors, and insulin receptors. Furthermore, we recently evaluated the complexity of the IGF-1 gene [118] and the biological activity of IGF-1 isoforms in BC cell lines [119] showing that the IGF-1 isoforms induced cell proliferation via IGF1R phosphorylation. Some studies have reported conflicting results regarding the regulation of IGF-1. Such studies report an increase, no difference or a decrease in circulating IGF-1 levels associated with PA [120–123]. These results are not surprising because the IGF-1 levels are influenced by several clinical factors such as gender, age, body mass index (BMI), sex steroid concentrations, nutrition, stress, level of PA, and intervening illness. Thus, exercise prescription should take into consideration most of these variables.

Another process through which exercise might regulate tumor metabolism is the autophagic machinery [15], as described in Autophagy and mTOR Signaling.

It is clear that exercise can ameliorate the BC microenvironment and can be very important in reducing BC risk and tumor burden when canonical radiochemotherapies or chemical mTOR inhibitors are not working, as in TNBC. Exercise workouts for these subjects will be explained in Exercise Prescription in BC Survivors. Ex vivo experimental data, using TNBC cell lines stimulated with sera collected before and after a single aerobic exercise bout (pre- or postexercise serum/a), are described in Experimental Evidence of mTOR Inhibition.

3.2. Experimental Evidence of mTOR Inhibition. As regards the mechanisms involved in the exercise-induced reduction of TNBC risk and tumorigenesis, few data are available. Ex vivo experiments, working with TNBC cells stimulated with sera collected before and after a single aerobic exercise bout (pre- or postexercise serum/a), are a good starting point to understand how exercise could affect the progression and recrudescence of TNBC. The research group of Dethlefsen has demonstrated that incubation of MCF-7 estrogen-responsive BC cells and MDA-MB-231 TNBC cells treated with postexercise serum, from both healthy volunteers [124] and operated cancer patients [14, 124], resulted in a reduction of BC cell viability in comparison with BC cells incubated with preexercise sera. In particular, it has been demonstrated that MCF-7 and MDA-MB-231 stimulation with sera leads to a viability reduction of 11% in MCF-7 cells and 9% in MDA-MB-231 cells in the case of supplementation with postexercise serum from operated cancer patients receiving adjuvant chemotherapy compared to preexercise serum [124]. Furthermore, the viability of both BC cell lines supplemented with sera from healthy women was also significantly reduced by the exercise-conditioned sera, resulting in a 10% and 19% reduction in MCF-7 viability and a 14% and 13% reduction in MDA-MB-231 viability by 1 h and 2 h postexercise sera, respectively. The reduced viability of MDA-MB-231 supplemented with 5% of healthy women 2-hour postexercise serum has also been confirmed by a pilot study that we performed working with culture medium with a physiological concentration of glucose (80mg/dl), resulting in a statistically significant reduction in cell proliferation of about 10% compared to cells supplemented with preexercise human serum [103]. Promising data on the tumorigenic potential of cancer cells in mice are also available. As reported by Dethlefsen et al. in 2017 [124], different outcomes in incidence and growth of tumors were detected inoculating NMRI-Foxn1nu mice with MCF-7 or MDA-MB-231 BC cells preincubated for 48 hours with pre or postexercise sera from healthy volunteers. In particular, only 45% of the mice inoculated with MCF-7 supplemented with postexercise human serum formed tumors compared with 90% of mice inoculated with MCF-7 preincubated with at rest sera, and the volume of tumors was reduced by 76%. Moreover, tumor incidence in mice inoculated with MDA-MB-231 cells preincubated with postexercise sera tended to be lower than it was in mice inoculated with MDA-MB-231 cells preincubated with rest sera, but no difference in tumor volume was observed between the two groups. These results show that exercise-stimulated changes suppress BC cell proliferation and reduce the tumorigenic potential of BC cells, also in the case of TNBC cells. Another important aspect to be considered is the fact that PA has been reported to lead to an increased level of the catecholamines epinephrine (EPI) and norepinephrine (NE) [82]; this result has also been confirmed in BC survivors two hours after a single exercise session [124]. Moreover, by blocking the β-adrenergic signaling pathway in BC cells, the effects of postexercise sera in BC cell viability is completely blunted, indicating the crucial role of catecholamines in inhibiting BC cells viability and tumor growth [124]. Their role in exercise-induced effects on BC cell viability has also been confirmed by MCF-7 and MDA-MB-231 treatment with different doses of EPI and NE, resulting in a dose-dependent growth inhibitory effect in both BC cell lines. Catecholamines have been shown to induce a dose-dependent phosphorylation of yes-associated protein (YAP) in MDA-MB-231 cells [125]; YAP is the main downstream target of the mammalian Hippo pathway and, when phosphorylated, it is retained in the cytoplasm. Hippo pathway is a tumor suppressor signaling cascade that regulates cell growth, and it has been shown to be a dysregulated pathway in several types of cancers, including BC, in which there is an activation of YAP oncoproteins and transcriptional coactivators with the PDZ-binding motif (TAZ) associated with tumor formation, growth and progression, metastasis, and drug resistance [126]. Dethlefsen et al. showed that

the Hippo pathway is regulated by exercise-conditioned sera: incubation of BC cells with postexercise sera led to a time-dependent phosphorylation of YAP in MCF-7 BC cells and to a decreased expression of YAP target genes, due to phosphorylated-YAP cytoplasmic retention, in both MCF-7 BC cells and MDA-MB-231 TNBC cells [124]. Studies performed by Tumaneng et al. demonstrated that Hippo pathway is related to the mTOR signaling cascade: YAP mediates the effects of the Hippo pathway regulating target genes, including the miR-29; this miRNA family has been proven to inhibit PTEN, an upstream activator of mTOR [127]. In summary, the Hippo pathway can be activated by exercise through the production of the catecholamines EPI and NE and can inhibit BC cell growth through the action of YAP and miR-29, inactivating the mTOR pathway.

As mentioned above, several miRNAs have been found to be deregulated in TNBC cells and patients; evidence suggests that different types of exercise can regulate these miRNAs in different ways. One of these miRNAs is miR-21, which has been found to be upregulated in TNBC patients; it has an oncogene activity and plays a crucial role in tumor cell proliferation and invasion, repressing PTEN [128].

Nielsen et al. [129] showed how miR-21 level significantly decreased 3–5 days after endurance training (60 min of cycle ergometer exercise at 65% of P_{max}, 5 times a week for 12 weeks), at rest. However, levels of miR-21 were also found to be upregulated immediately after a single exhausting cycling exercise at a low heart rate, just as it was after a training period of 90 days [130]. Discrepancies between data obtained by these two studies could be explained by the different types of exercise considered, as confirmed by Wardle et al. [131].

The microRNA precursor miR-146a has also been found to be an upregulated miRNA in TNBC tissues, and its level is related to tumor size and survival rate. Nielsen et al. [129] showed that miR-146a levels significantly decreased immediately after a single session of pedaling exercise performed at 65% of the maximal power output. In this case, depending on the different exercise considered, miR-146 levels can be dysregulated: after a single exhausting cycling exercise at a low heart rate, it has been found to be upregulated [130]. Variations in miR-146 levels when comparing strength or endurance exercise groups to controls were observed; levels increase in the endurance group, while they decrease in the strength group [131]. The downregulation of miR-146a after strength exercise was also confirmed by a study that involved a single strength exercise session performed at 70% of one-repetition maximum [132] in which the miR-146a level was found to have decreased 3 days after exercise.

In short, a subset of circulating miRNAs, including miR-21 and miR-146a, are associated with the whole-body adaptive response to differential forms of exercise and training. These miRNAs have been found to be upregulated in TNBC patients and related to the repression of PTEN or BRCA1 with consequent mTOR pathway activation. Hence, their downregulation with specific types of exercises could be a very promising approach to control TNBC initiation and progression.

4. Energy Intake in TNBC and mTOR Modulation

mTORC1 is a key regulator of cell growth and proliferation, and at the same time, it is also at the centre of nutrient regulation and utilization. In this regard, a large number of studies have demonstrated the role of excessive energy intake on cancer development, and by contrast, the protective effects of CR [133]. While the antitumorigenic effects of CR are well established, the mechanism behind this relationship is not completely clear, though it is believed that the tumor suppressive effects are mediated, as they are for exercise, by enhanced apoptosis, modulation of systemic signals such as IGF-1, insulin, metabolic, and inflammatory pathways, as well as by reduced angiogenesis [134]. Specifically, a large quantity of data points to the role of mTOR activation in cancer development through protein-induced IGF-1 signaling and to the beneficial effects of caloric and protein restriction not only on aging-associated diseases such as cancer but also on life span [135, 136] (Figure 1).

CR increases the level of the circulating adiponectin, which can exert anticancer effects through mechanisms that include an increase in insulin sensitivity, a decrease in insulin/IGF-1 and mTOR signaling via AMPK activation as well as a reduction in the proinflammatory cytokine expression via inhibition of the nuclear factor κ-light-chain-enhancer of activated B-cells (NF-κB) [136, 137].

AMPK, as mentioned above, is an important mediator in the maintenance of cellular energy homeostasis, and recently, it has gained attention for its possible role as a metabolic tumor suppressor and in cancer prevention and control. Since AMPK phosphorylation is regulated by energy availability (AMP : ATP ratio), AMPK activators, such as metformin, CR, and aerobic exercise, reduce the incidence of cancer.

Leptin is a peptide hormone produced by white adipose tissue. It affects several tissues and acts on the hypothalamus to regulate appetite and energy expenditure. It also impacts carcinogenesis, angiogenesis, immune responses, cytokine production, and other biological processes [138, 139].

Intermittent CR is associated with the suppression of murine mammary tumor incidence and a decrease in the leptin-to-adiponectin ratio [139]. This ratio, when elevated, is related to metabolic syndrome and some cancers [140, 141]. In TNBC metastases, CR decreases proliferation, increases apoptosis, and downregulates the IGF1-1R pathway, coadiuvating canonical therapies [142]. Taken together, these findings show that dietary interventions can ameliorate the systemic milieu and tumor microenvironment. Chronic CR is not suitable for cancer patients at risk for weight loss, cachexia, and immunosuppression, but it can be substituted with intermittent CR, fasting-mimicking diets, low carbohydrate/ketogenic diets, or CR mimetic drugs.

Fasting and low carbohydrate diets have been shown to reduce side effects and to enhance the effectiveness of chemotherapy and radiation therapy in animal models, and there is a great deal of interest in the potential clinical value of these interventions.

Protein consumption has different effects on cancer mortality, which vary according to age, with an increased risk in middle age and a reduction in the elderly [143].

Protein restriction (PC) for the middle-aged followed by moderate protein intake in elderly subjects may increase longevity and health span since protein restriction is sufficient to reduce growth hormone receptor (GHR)-IGF1 activity and can reduce cancer incidence in model organisms regardless of energy intake [144].

Moreover, L-type amino acid transporter 1 (LAT1), which transports large quantities of neutral amino acids, was found highly expressed in human BC tissues. The upregulation of LAT1 plays an important role in BC progression because more amino acids are required for protein synthesis and cellular proliferation [145].

The activation of the mTOR/S6K1 signaling pathway depends on the availability of amino acids (AA), particularly branched chain AA, such as leucine, and also glucose [106]. Growth factor signals, which usually activate mTORC1 signaling, have little or no impact in the absence of AA.

Leucine deprivation causes an upregulation of insulin-like growth factor binding protein 1 through transcriptional activation and mRNA stabilization, probably decreasing the effects of IGF1 and thus lowering cell proliferation [146].

However, in most BC cell lines with constitutively activated Akt/mTOR signaling, leucine restriction is not efficient in inhibiting mTOR signaling since it is associated with activation of survival molecule Akt, making leucine deprivation an undesirable approach for BC therapy [146].

Glutamine is another AA involved in the regulation of the mTOR pathway inducing the uptake of leucine [147]. Tumor cells are more sensitive to amino acid deprivation than normal cells; thus, glutamine restriction and/or transporter inhibition decrease mTOR activity [147].

A novel therapeutic approach based on whey protein concentrate (WPC) supplementation for BC treatment has been suggested by Cheng et al. [148]. WPC is rich in bioavailable cysteine, which can be used for glutathione synthesis, and contains all nine essential AAs. WPC promotes muscle protein synthesis [149] and can be used as a nutritional supplement during chemotherapy [150]. WPC has also been shown to enhance rapamycin sensitivity in MDA-MB-231 TNBC cells, a cell line resistant to rapamycin and other mTOR inhibitors [148].

The combination of conventional therapies and *n*-3 polyunsaturated fatty acid (PUFA) supplementation (nutritional interventions) increases the sensitivity of tumor cells to conventional therapies, possibly improving their efficacy especially against cancers resistant to treatment, as suggested by D'Eliseo and Velotti [151]. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have anticancer effects on different cancer types by inducing apoptotic cell death in human cancer cells either alone or in combination with canonical therapies. EPA and/or DHA also have proapoptotic effects in both triple-negative [152] and ER+ BC subtypes [153], although when compared at the same dose, DHA appears to be more effective. This might be due to the structural differences between DHA and EPA. The proapoptotic effects occur with increases in plasma membrane incorporation and decreases in cell viability [152–154], PI3K/Akt pathway [155], and pEGFR activation [152].

In agreement, CR and other nutritional interventions could play an important role in support of conventional therapies to improve TNBC outcomes.

5. Exercise Prescription in BC Survivors

In general, reviews and meta-analyses tend to group PA and exercise interventions into general categories and rarely examine the specific exercise protocols employed in the studies. Therefore, which characteristics make an exercise protocol safe and effective for BC survivors and, particularly, for TNBC patients?

Since the 2009 roundtable consensus statement on exercise guidelines for cancer survivors [156], which outlined the situations in which deviations from the 2008 US Physical Activity Guidelines for Americans (PAGA) were appropriate and included relevant implementation strategies [157], exercise recommendations from several internationally recognized institutions, such as the American Cancer Society [158] and the National Comprehensive Cancer Network [159], have been published for BC survivors. Fortunately, all of the abovementioned publications have recently been reviewed within the framework for exercise prescription of the American College of Sports Medicine (ACSM) [160], along with others providing practical guidance for exercise prescription in these patients [161, 162]. ACSM's framework for exercise prescription employs the so-called FITT-VP principle [160], which considers the frequency (*F*), intensity (*I*), time (*T*), and type (*T*) of exercise and its volume (*V*) and progression (*P*) over time in an individualized exercise training program.

A detailed description of the FITT-VP principle for each type of exercise—i.e., aerobic, resistance, and flexibility—adapted to BCS needs is provided in Tables 1, 2, and 3. Note that the following guidelines should not be regarded as specific for BC patients because no studies, to date [163], have adopted (and/or reported) the proper application of the principles of specificity, progression, overload, initial values, and adherence, within their exercise interventions. Therefore, although specific exercise guidelines for cancer survivors still need to be outlined, particularly for TNBC survivors, the following information represents the most up-to-date adaptations of the PAGA to BCS, including TBNC patients. Improving the reporting of exercise prescriptions will also allow for more specific recommendations regarding types and doses of exercise for BCS (and, hopefully, for the TNBC subgroup), in order to identify effective exercise interventions to be delivered to this growing community.

6. Benefits of Exercise Pre- and Postdiagnoses

Humans have not been "designed" for a sedentary lifestyle. The absence of an adequate level of PA puts us at increased risk of developing cancer. This has been highlighted by the European Breast Cancer Conference [164], issued an important statement: regular PA reduces the risk of BC for woman of any age and body weight by 12%.

Table 1: Aerobic (cardiorespiratory endurance) exercise recommendations.

Intensity (I)	Frequency (F)	Time (T) (duration)	Type (T) (mode) (examples)	Volume (V) (quantity)	Progression (P) (rate of)	Specific notes
Light: 30–39% VO_2R/HRR; 57–63% HR_{max}; 9–11 RPE.	At least 5 d wk^{-1}.	30 to 60 min each session (i.e., at least 150 min wk^{-1}).	Continuous and rhythmic exercises that involve major muscle groups (walking, cycling, slow dancing, jogging, running, rowing, stepping, fast dancing, etc.).	≥500–1,000 MET min wk^{-1}.	Increase gradually any of the FITT components as tolerated by the patient (gradual progression is required to minimize the risks of muscular soreness, injury, undue fatigue, and the long-term risk of overtraining). Initiate increasing exercise duration (as tolerated): an example for healthy people is adding 5–10 min every 1–2 wk over the first 4–6 wk and adjusting upward over the next 4–8 months to meet the recommended FITT components, but slower progression may be needed for BCS.	If tolerated without adverse effects of symptoms or side effects, moderate to vigorous intensity and 3–5 d wk^{-1} frequency are recommended, but lower (light) intensities and frequencies are still beneficial when the current physical activity level is low. Avoid prescribing and monitoring intensity using %HRR (using %HR_{max} or RPE is recommended in BCS). Be aware of fracture risk, because bone is a common site of metastases in breast cancer: BCS with metastatic disease to the bone will require modification of their exercise program (e.g., reduced impact, intensity, and volume) given the increased risk of bone fragility and fractures.
Moderate: 40–59% VO_2R/HRR; 64–75% HR_{max}; 12-13 RPE.	At least 5 d wk^{-1}.	30 to 60 min each session (i.e., at least 150 min wk^{-1}).				
Vigorous: 60–89% VO_2R/HRR; 76–95% HR_{max}; 14–17 RPE.	At least 3 d wk^{-1}.	20 to 60 min each session (i.e., at least 75 min wk^{-1}).				

Modified from [160]. VO2R: oxygen uptake reserve, calculated as the difference between maximal oxygen uptake and resting oxygen uptake; HRR: heart rate reserve, calculated as the difference between maximal heart rate and resting heart rate; HR_{max}: maximal heart rate; RPE: rate of perceived exertion on the 6–20 scale; MET-min: metabolic equivalents (MET) of energy expenditure for a physical activity performed for a given number of minutes (min), calculated as MET × min; FITT: frequency, intensity, time, and type of exercise.

Table 2: Resistance (strength) exercise recommendations.

Intensity (I)	Frequency (F)	Time (T) (duration)	Type (T) (mode) (examples)	Volume (V) (quantity)	Progression (P) (rate of)	Specific notes
Light: 30–49% 1-RM.	2-3 d wk^{-1}.	Depends on exercise volume (number of sets, repetitions for each set, and rest intervals in-between) and is not associated with effectiveness.	Any form of movement designed to improve muscular fitness by exercising a muscle or a muscle group against external resistance: exercise and breathing techniques are of paramount importance and symptom-limited ROMs should be adopted according to BCS responses to exercise (free weights, resistance machines, weight-bearing functional tasks, etc.).	2–4 sets of 8–15 repetitions (at least 1 set of 8–12 repetitions can be effective in BCS) with 2-3 min rest between sets.	BCS should start with a supervised program of at least 16 sessions and very low resistance (<30% 1-RM), and progress with smallest increment possible (e.g., 2–10% 1-RM, depending on muscular size and involvement, is recommended for healthy adults). If a break is taken, lower the level of resistance by 2 wk worth for every week of no exercise.	No upper limit on the account of weight to which BCS can progress. Individuals with lymphedema should wear a compression sleeve during resistance training activity. Watch for arm/shoulder symptoms including lymphedema and reduce resistance or stop specific exercises according to symptom response. Be aware of risk of fracture (see aerobic exercise for details).
Moderate: 50–69% 1-RM.	2-3 d wk^{-1}.					
Vigorous: 70–84% 1-RM.	2-3 d wk^{-1}.					

Modified from [160]. 1-RM: one-repetition maximum, i.e., the load that can be lifted one time only; ROM: range of motion; BCS: breast cancer survivors.

Table 3: Flexibility (stretching) exercise recommendations.

Intensity (I)	Frequency (F)	Time (T) (duration)	Type (T) (mode) (examples)	Volume (V) (quantity)	Progression (P) (rate of)	Specific notes
Stretch to the point of feeling tightness or slight discomfort.	≥2-3 d wk^{-1} (stretching on a daily basis is most effective).	Hold a static stretch for at least 10–30 s (30–60 s may confer greater benefit). Accumulate a total of 60 s of stretching for each flexibility exercise by adjusting time/duration and repetitions (see volume) according to individual needs.	Stretching exercise that increases the ability to move a joint through its complete ROM (provided individual specific conditions are accounted for) (static active flexibility, static passive flexibility, dynamic flexibility, ballistic flexibility, proprioceptive neuromuscular facilitation, etc.).	Repeat each exercise 2–4 times in order to attain the goal of 60 s stretch time (e.g., two 30 s stretches or four 15 s stretches). A stretching routine can be completed approximately in ≤10 min.	Optimal progression is still unknown.	BCS should focus on joints in which a loss of ROM occurred because of surgery, corticosteroid use, and/or radiation therapy. Flexibility exercises are most effective when the muscles are warm.

Modified from [160]. ROM: range of motion; BCS: breast cancer survivors.

PA as a nonpharmacological treatment to combat the collateral effects associated with BC is under considerable scientific attention [160, 165, 166]. To allow physicians to prescribe PA to patients before and after treatment, scientific clarity and evidence supporting the thesis that PA programs reduce the damaging effects of cancer and its treatment are needed. Very little is known about the effect of exercise on TNBC outcomes, but data suggest that pre- and postdiagnosis PA may be one of the factors, which, if appropriately prescribed, could bring benefits to patients.

Generally, TNBC has poor treatment outcomes because of a lack of receptor targets for conventional drugs to act upon. However, there is irrefutable evidence of the effectiveness of regular PA in primary and secondary prevention of premature death from any cause, including BC. Thus, different types of exercise can influence the prevention and progression of disease through several common mechanisms such as reduction of insulin resistance and improvement in immunity and cardiovascular function. Research in humans shows that exercise can regulate inflammation [13, 167], oxidation [168, 169], and gene expression [170].

Together with the potential mechanisms underlying the effects of exercise on breast carcinogenesis, Thompson [12] proposed three interesting hypotheses: (i) the hormesis hypothesis: oncological response to exercise is antithetical to a physiological cellular stress response; (ii) the metabolic reprogramming hypothesis: exercise reduces the glucose and glutamine available to mammary carcinomas, inducing apoptosis and reversing tumor-associated metabolic program; and (iii) the mTOR network hypothesis: exercise inhibits carcinogenesis by suppressing the activation of the mTOR signaling network in mammary carcinomas.

Recent investigations have revealed that the most active women had, on average, a 25–30% lower BC risk than women in the lowest category of recreational PA [171]. Data from the California Teachers Study (CTS) suggest that PA has a protective role in prediagnoses and may reduce a woman's risk of BC, especially the TNBC subtype. An analysis of the risk index (HR) associated with variations in the amount of PA hours among TNBC women yielded significant results. The HR results show significant associations when moderate-intense activity is considered as the only variable. When they are considered as separate variables, there are no statistically significant associations between moderate activity and TNBC, whereas intense activity is inversely associated with TNBC [172]. The reduction risk associated with baseline strenuous recreational PA was statistically significant among overweight or obese pre- or postmenopausal women, but not among their leaner counterparts.

In patients with BC postdiagnosis, acute and chronic symptoms, such as muscle mass loss, fatigue, weight gain, hormone alterations, bone loss, cachexia, and adverse psychological effects, may all be favourably influenced by regular exercise. A prospective cohort study analysed modifiable lifestyle factors, including exercise, associated with total mortality and recurrence/disease-specific mortality in patients with TNBC [173]. The association between TNBC prognoses and exercise postdiagnosis yielded important results: women who engaged in exercise regularly during the first 6 months postdiagnosis had a lower risk of total mortality and recurrence/disease-specific mortality, with adjusted HRs of 0.58 and 0.54, respectively. In addition, those who engaged in PA for a long time (2.5 h/wk) or women who exercised ≥7.6 metabolic equivalent hours/wk had a reduced risk of all causes and recurrence/disease-specific mortality compared with nonexercisers. Survivors who maintain a healthy weight and stay physically active have a better response to treatment and better survival outcomes. Thus, it is necessary to identify an appropriate promotion and prescription of regular PA for BC survivors in order to improve their prognosis, response to therapy, and quality of life. As previously described, the mTOR signaling pathway is differentially regulated by different exercise modalities, and it represents one of the main key regulators of the protective effects of exercise.

7. Conclusions

In this review, we presented new insights into the downregulation of mTOR signaling in TNBC by exercise and CR. It has been shown that mTOR network inhibition is mostly mediated through the effects of CR and vigorous PA as well as long-term exercise, which decrease the level of circulating growth factors and hormones.

During exercise, the body is exposed to different types of stressors, including temperature, metabolism, hypoxic, oxidative, and mechanical stress. These stressors initiate biochemical targets, which in turn actuate different signaling pathways that regulate gene expression and adaptive responses. Beneficial adaptation likely depends on the basal state of oxidative stress and inflammation at the beginning of exercise training. In turn, this basal state may depend on the periodization of training and recovery, together with age, health status, and diet.

Exercise, as a hormetic agent, has the potential for beneficial energy upregulation. The dose response effects are complex and reflect activation of major defensive pathways in both systemic and local environments. A mitohormetic stimulus that occurs through a physiological cellular stress adaptation and AMPK activation across hormetic control circuits, such as increase of oxidative metabolism, mitochondrial biogenesis, angiogenesis, immune regulation and a decrease in BMI, and insulin secretion, are induced by exercise. Moreover, PA increases glucagon, catecholamines, and other hormones and influences miRNAs involved in cancer. Exercise as well as CR limit glycaemia and glutamine availability to mammary carcinomas, inducing apoptosis and reversing malignancy-associated metabolic programming. It is also known that intratumoral metabolism is regulated by exercise, but how this affects tumor growth and metastatic rate is not clearly understood. Although the signal for these hormonal and autonomic changes has been partially described in ex vivo experiments, such changes are difficult to transfer in vivo. Currently, there is an agreement in the literature that there is a role for exercise as a coadiuvant "medicine" in canonical therapies and that it has an increasingly protective tumorigenic effect. In this context, PA needs to be broken down into its main components:

frequency, intensity, time, and type; however, the dose-dependent effects of each of these components on cancer protection via mTOR inhibition are still unclear. Most data suggest that both vigorous and long-term PA in adulthood may reduce a woman's risk of mammalian cancer, especially the TNBC subtype.

Finally, we can assert that there is a sufficient evidence showing that sedentary behaviour and nutritional risk factors for TNBC are modifiable. Hence, the suggestions regarding the modification of such risk factors highlighted in this review could have wide-ranging implications for society and may improve public healthcare cancer management. Accordingly, we would like to emphasize the importance of promoting physically active lifestyles to reduce the risk of relapse in TNBC. Fostering active lifestyles can provide important support during conventional cancer treatment, preventing the potential negative impacts on patients' physical condition, as well as their emotional and social well-being.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Authors' Contributions

Deborah Agostini, Valentina Natalucci, and Giulia Baldelli contributed equally to this work.

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Review Article

Emerging Role of mTOR Signaling-Related miRNAs in Cardiovascular Diseases

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Mechanistic/mammalian target of rapamycin (mTOR), an atypical serine/threonine kinase of the phosphoinositide 3-kinase-(PI3K-) related kinase family, elicits a vital role in diverse cellular processes, including cellular growth, proliferation, survival, protein synthesis, autophagy, and metabolism. In the cardiovascular system, the mTOR signaling pathway integrates both intracellular and extracellular signals and serves as a central regulator of both physiological and pathological processes. MicroRNAs (miRs), a class of short noncoding RNA, are an emerging intricate posttranscriptional modulator of critical gene expression for the development and maintenance of homeostasis across a wide array of tissues, including the cardiovascular system. Over the last decade, numerous studies have revealed an interplay between miRNAs and the mTOR signaling circuit in the different cardiovascular pathophysiology, like myocardial infarction, hypertrophy, fibrosis, heart failure, arrhythmia, inflammation, and atherosclerosis. In this review, we provide a comprehensive state of the current knowledge regarding the mechanisms of interactions between the mTOR signaling pathway and miRs. We have also highlighted the latest advances on mTOR-targeted therapy in clinical trials and the new perspective therapeutic strategies with mTOR-targeting miRs in cardiovascular diseases.

1. Introduction

Cardiovascular disease (CVD) is one of the leading cause of mortality and morbidity in the world and is a global pandemic threat to human health [1, 2]. Coronary artery diseases (CAD) such as ischemia reperfusion injury (I/R) and acute myocardial infarction (AMI) are the primary forms of CVD that account for the majority of the deaths. Apart from this, additional comorbid factors like diabetes [3, 4], obesity [5], inflammation [6], and atherosclerosis [7] escalate the complication associated with heart disease and increase the incidence of death. The underlying mechanisms involved in cardiovascular complication are complex and multifactorial. The existence of several metabolic perturbations in diseases like diabetes and inflammation further pose a tough challenge in understanding the mechanism and pathology of CVD. These obstacles largely impede our goal to develop an effective treatment against progression of CVD and its prevention. However, current therapies for heart diseases have been substantially improved using integrated genome-based evidences and molecular clues. Our recent understanding of genomics and the regulation of gene expression by noncoding RNAs (ncRNA) during both normal and pathological conditions encourage us in exploring the novel therapies for heart disease with a unique perspective.

In the cardiovascular system, the mechanistic target of rapamycin (mTOR) pathway regulates both physiological and pathological processes in the heart [8]. mTOR is an evolutionarily conserved signaling pathway found in various species including yeast [9, 10], Caenorhabditis elegans [11, 12], drosophila [13, 14], and mammals [15–18]. mTOR is a master regulator of cell metabolism and plays a central role in integrating various signaling network [19]. mTOR participates in the fundamental aspect of cell function and is therefore indispensable for cellular life. It governs several key cellular processes such as nutrient sensing [20–22], protein synthesis [23, 24], cell proliferation [14], and apoptosis [25, 26]. mTOR is also actively involved in the epigenetic

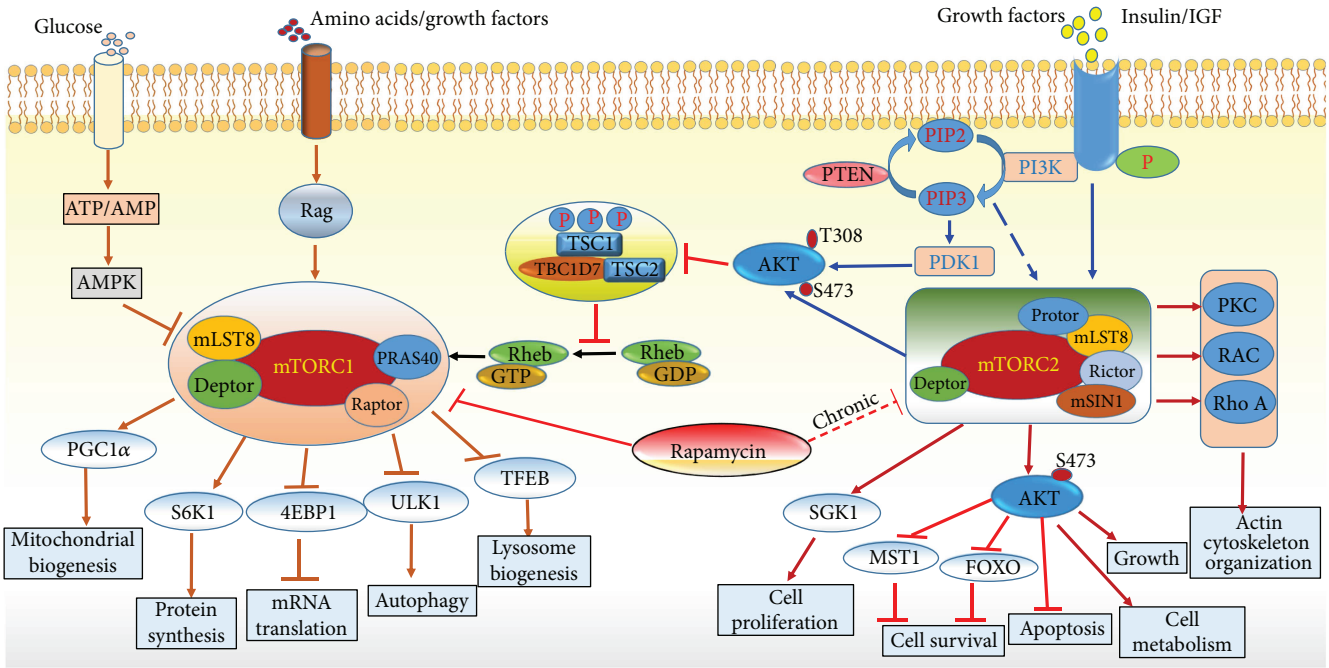

FIGURE 1: Schematic representation of various subunits of mTORC1 and mTORC2 complex and its upstream signaling regulators and cellular function. The mammalian target of rapamycin (mTOR); insulin growth factor (IGF); adenosine monophosphate activated protein kinase (AMPK); eukaryotic translation initiation factor 4E- (eIF4E-) binding protein 1 (4EBP1); proline-rich AKT substrate 40 (PRAS40); tuberous sclerosis protein ½ (TSC1/2); Ras homolog enriched in brain (Rheb); phosphoinositide 3 kinase (PI3K); Unc-51 like autophagy activating kinase (ULK); ribosomal protein S6 kinase beta-1 (*S6K1*); forkhead box O transcription factor (FOXO); serum/glucocorticoid-regulated kinase 1 (SGK1); peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α).

regulation of gene expression and control process like aging [27] and autophagy [28]. However, aberrant regulation of mTOR is known to play a significant role in various maladies including cancer [29], diabetes [30], aging [31], and cardiovascular diseases [32]. mTOR plays an important role in normal cardiac development [33–36] and during cardiac pathophysiologic condition [37, 38].

Recent studies have demonstrated that mTOR signaling pathway is profoundly influenced by small noncoding RNAs, and an interplay between these two molecules define a synergistic regulation of gene expression [39–41]. The unique combination and cross talk between mTOR and miRs have opened up research interest from a distinct perspective and to revisit mTOR signaling in the light of miR. The intention of this review article is to highlight our recent understanding on mTOR pathway in cardiovascular system and its coordinated interaction with miRs to fine-tune the regulation of gene expression under both normal and pathological conditions.

1.1. Structure, Mechanism, and Function of mTOR Complexes. The mTOR macromolecular complex is a serine/threonine protein kinase of 289 kDa that belongs to phosphatidylinositol-3-kinase (PI3K) family of proteins and governs several cellular processes including protein synthesis and metabolic regulation [42]. Hall and colleagues first identified target of rapamycin 1 (TOR1) and TOR2 in yeast *Saccharomyces cerevisiae* [43, 44], which was subsequently characterized in mammalian cells and hence called mTOR [42, 45, 46]. Discovered in the year 1970 for its antifungal property [47], rapamycin played a bigger role in elucidating the cellular function of mTOR [42, 48]. The mTOR consists of two major distinct complexes termed as mTORC1 and mTORC2 (Figure 1) and have different sensitivity towards its inhibitor rapamycin [49]. mTORC1 and mTORC2 are similarly large, weighing in at ~1.2 and ~1.4 MDa, respectively [50]. The central core catalytic subunit, mTOR, is common to both complexes and characterized by their own unique subunits. TOR proteins contain ~2500 amino acids and comprise several distinct domains [51], including 32 tandem HEAT (huntingtin, elongation factor 3, protein phosphatase 2A, and Tor1) repeats towards their N-termini, followed by FAT (FRAP, ATM, and TRRAP) domain consist of multiple antiparallel α-helical features, termed tetratricopeptide repeats (TPRs) [51].

mTORC1 consist of five components (1) mTOR (mammalian target of rapamycin), the central catalytic subunit, (2) raptor (regulatory-associated protein of mTOR) [52, 53], (3) mLST8 or GβL (mammalian lethal with Sec13 protein 8) [54, 55]. (4) PRAS40 (proline-rich AKT substrate 40 kDa) [56–58], and (5) Deptor (DEP-domain-containing mTOR-interacting protein) [59, 60]. The cryoelectron microscopy structure of human mTORC1 revealed that mTORC1 maintains an obligate dimer with an overall rhomboid shape and a central cavity by interlocking mTOR-raptor interactions [61]. The principal interaction between raptor and mTOR

consists of an α-solenoid stack formed between the horn and bridge domains of mTOR and the raptor armadillo domain [62].

The precise function of all mTOR-interacting proteins in mTORC1 complex remains to be understood. It was shown that rapamycin forms complex with FKBP12 and interacts with mTOR subunit and inhibits mTORC1 activity [53, 63, 64]. Experimental evidences also suggest that raptor might affect mTORC1 activity by regulating assembly of the complex and by recruiting substrates for mTOR [11, 21, 60]. The role of mLST8 in mTORC1 function is partially understood. Studies using raptor-, rictor-, or mLST8-deficient mice demonstrate that mLST8 is required for mTORC1 activity and is necessary to maintain the rictor-mTOR, but not the raptor-mTOR, interaction [55]. The interaction among different proteins in mTORC1 complexes is crucial and determines its active state. For example, the subunits of mTORC1, PRAS40 and Deptor, are known to interact with each other and docked to the core complex, resulting in the inhibition of mTORC1. PRAS40 and Deptor are phosphorylated by mTORC1, which blocks their interaction leading to the activation of mTORC1 [56–59].

Relatively, much less is known about the regulation of mTORC2 compared to mTORC1. Essentially, mTORC2 contains six different proteins, many of which are components of mTORC1: (1) mTOR; (2) rictor (rapamycin-insensitive companion of mTOR), unique to mTORC2; (3) mammalian stress-activated protein kinase-interacting protein (mSIN1) [65]; (4) protein observed with Rictor-1 (Protor-1); (5) mLST8; and (6) Deptor, an important interacting protein in mTORC1. mTORC2 contains its unique subunit rictor, which is insensitive to rapamycin. This is due to the fact that rapamycin when forms a complex with FKBP12 subunit, it does not bind to mTORC2 and losses it ability to block its activity [66–68]. The N-terminal region of rictor is composed of helical repeat clusters, which binds to mTOR as well as makes multiple contacts with mSin1. The FRB domain of mTOR also shows multiple cross-links with mSin1 and C-terminal regions of rictor. Rictor and mSin1 together generate a steric hindrance to inhibit binding of FKBP12-rapamycin to mTOR, revealing the mechanism for rapamycin insensitivity of mTORC2 [50, 69].

Recent evidences suggest that rapamycin inhibits mTORC1 at low concentration, and a prolonged chronic inhibition leads to inactivation of mTORC2 [70, 71]. Moreover, interaction between rictor and mSIN1 is essential for their own stability and to form the mTORC2 complex, since the deletion of SIN1 blocked the phosphorylation of AKT at serine 473 residue leading to the disruption of rictor-mTOR [72]. Deptor is common to both mTORC1 and C2 and acts as an endogenous inhibitor of mTORC2 [59]. mLST8 is also shown to be a crucial element in the mTORC2 complex formation since the ablation of this protein destabilize mTORC2. Interestingly, several of the subunits in mTORC1 and 2 are common to each other but they interact in an exclusive mechanism that characterize the individual complex. Even though they are unique in many aspects, mTORC1 and mTORC2 phosphorylate entirely different substrates and consequently have distinct function [55].

Several external stimuli such as nutrient, insulin, growth factors, leptin, and stress signals regulate mTOR complexes. However, mTORC1 and C2 respond to these factors differently and have exclusive downstream effects. The primary effector pathway of mTORC1 is through activation of ribosomal proteins S6 kinase 1 and 2 (S6K1/2) by phosphorylating their hydrophobic motif (HM), on Thr389 and Thr 388, respectively, which promotes mRNA biogenesis as well as translational initiation and elongation of protein synthesis. Other substrates of mTORC1 includes 4E- (eIF4E-) binding protein1 and (4EBP1), which are also involved in the activation of gene expression and protein translation. mTORC1 complex is also very sensitive to nutrients, particularly amino acids and glucose level. Deprivation of amino acids especially leucine results in rapid dephosphorylation of S6K1 and 4EBP1 and results in the inactivation of mTORC1 [13, 20]. The energy status of the cell is also sensed by mTORC1 through AMP-activated protein kinase (AMPK). AMPK is phosphorylated in response to low cellular energy status indicated by the high AMP/ATP ratio. The activated AMPK in turn inhibits cell growth via TSC2-dependent suppression of mTORC1 activity and blocks the phosphorylation of S6K1 and 4EBP1 mediated by mTORC1 [22, 73, 74]. Apart from its role in protein synthesis, mTORC1 is also involved in catabolic processes such as apoptosis and autophagy. Under starvation, mTORC1 phosphorylates ULK1 (Unc-51-like autophagy activating kinase), thereby preventing its activation by AMPK, an important activator of autophagy [75, 76].

mTORC2 is widely recognized to play an important role in cell proliferation and response to growth factors such as insulin. Unlike mTORC1, which acts through various downstream effectors, mTORC2 mainly acts through insulin/PI3K pathway via phosphorylation of AKT at serine 473, Thr 308, and Thr 450 residues upon stimulation by insulin [77, 78]. Recent evidence also suggest that mTORC2 can phosphorylate AKT at S377/T479 residues in the C-terminal end and can regulate apoptosis [79]. The mTORC2 subunit mSin1 contains a phosphoinositide-binding PH domain that is critical for the insulin-dependent regulation of mTORC2 activity [65, 80, 81]. Insulin binding to its tyrosine kinase receptor activates IRS and recruits activated PI3K. [82] The PI3-PDK pathway phosphorylates AKT in a mTORC2-dependent manner [83]. mTORC2 phosphorylates several protein kinases including PKA, B, C, G, SGK1 (serum/glucocorticoid-induced kinase 1), and Rho1 (GDP-GTP exchange protein-2), resulting in their stabilization and activation [84–90]. Rictor enables mTORC2 to directly phosphorylate AKT at its Ser473 and facilitates Thr308 phosphorylation by PDK1 (phosphoinositide-dependent kinase 1) as part of the insulin-signaling cascade [91].

Interestingly, there is a cross talk between mTORC1 and mTORC2 and they are functionally interconnected. Apparently, mTORC1 inhibits mTORC2 through phosphorylation of rictor and mTORC2 regulates mTORC1 through phosphorylation of AKT, which controls both the activity and abundance of AKT [92]. Rictor subunit of mTORC2 complex can be phosphorylated by S6K1, a downstream effector of mTORC1, and this phosphorylation negatively regulates the mTORC2-dependent phosphorylation of AKT-S473 [93].

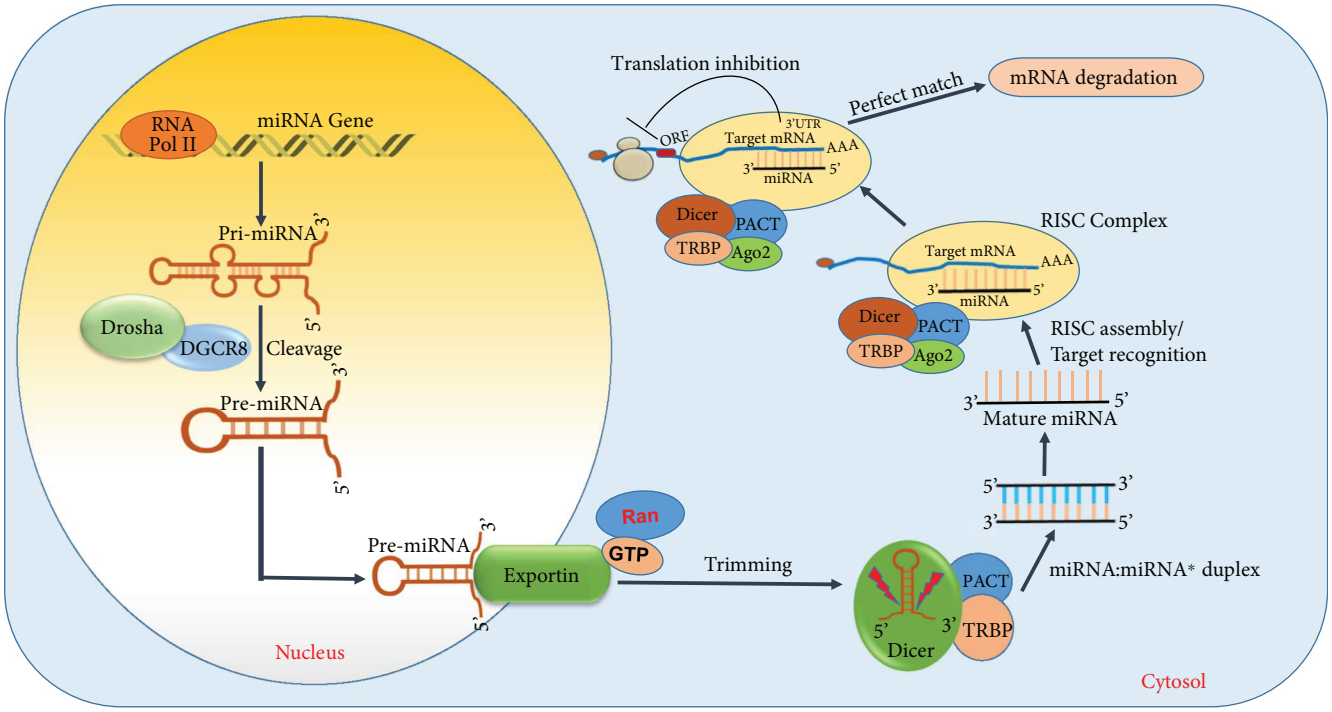

FIGURE 2: Illustration of miRNA biogenesis pathway and mode of gene regulation. RNA polymerase (RNA-Pol); Drosha; DiGeorge syndrome critical region-8 (DGCR8); guanosine triphosphate (GTP); RAs-related nuclear protein (Ran); human immunodeficiency virus trans-activating response RNA-binding protein (TRBP); argonaute 2 (Ago2); RNA-induced silencing complex (RISC); protein kinase RNA activator (PACT); open reading frame (ORF); 3′ untranslated region (3′UTR).

In contrast, upon stimulation with growth factors, mTORC2 activates AKT, which in turn enhances mTORC1 activity through the inactivation of TSC1/2 (tuberous sclerosis complex). The TSC2 is inactivated by AKT-dependent phosphorylation, which destabilizes TSC2 and disrupts its interaction with TSC1 [94, 95].

1.2. MicroRNA Biogenesis and Mode of Action. MicroRNAs (miRs) are small noncoding RNAs consisting of approximately 22 nucleotide in size and function as gene suppressors [96, 97]. They bind to the 3′ untranslated region (UTR) of mRNA and regulate their expression via either degradation of mRNA transcript or interfere in the translation process [98, 99]. The regulation of gene expression mediated by miR has now been widely recognized as a major molecular mechanism employed by cells to control various function and signaling pathway [100–103], including AKT, AMPK, JNK, and TGF-β [104–114].

miRs are encoded across genomic locations including introns and intergenic. Once synthesized and matured through several steps these miRs bind to the complementary 3′UTR of their target mRNA and either degrade or silence them [115–117]. miRs undergo a series of maturation process before they develop into a mature miR (Figure 2). They are initially synthesized from their respective genomic region by the enzyme RNA polymerase II into a hairpin structure of approximately ~400–500 bps nucleotide into a primary miR transcript [118, 119]. They are further cleaved by the enzyme Drosha into a 70 nt length nucleotide, named pre-miR [120, 121]. The pre-miR then binds with the protein exportin-5 which transports them out of the nucleus for further trimming [122]. Once in the cytoplasm, they undergo further cleavage by the enzyme ribonuclease III (RNase III) and dicer in to a 22 nt mature miR [123–125]. The mature miR, depending on the complementary nucleotide sequence of its seed region (2–7) at its 5′ end, forms complex with its target mRNA. The double stranded miR-mRNA complex induces the RNA-induced silencing complex (RISC) and targets them for degradation or gene suppression [126–128]. A near perfect match between the seed region (~8 nt) of miR and mRNA leads to a complete degradation of the mRNA, while a partial complementarity results in the suppression of the gene expression [129–133]. miRs are transcribed either as an individual miRNA (e.g., miR-1) or as a family of clusters (e.g., miR-17~92) [134]. The coding region for miRNA can arise from either strand of the DNA and can have multiple mRNA targets [135–137].

2. mTOR in Cardiovascular Diseases

The role of mTOR in cancer and aging is well recognized and documented with numerous scientific publications [35, 138–144]. However, their role in cardiovascular system is still in the early stages and yet to be elucidated. mTOR plays an important role in the normal development of cardiovascular system and is crucial during pathophysiological conditions [35, 145–150]. Nevertheless, studies have revealed a novel role for mTOR in CVDs like ischemia reperfusion (I/R)

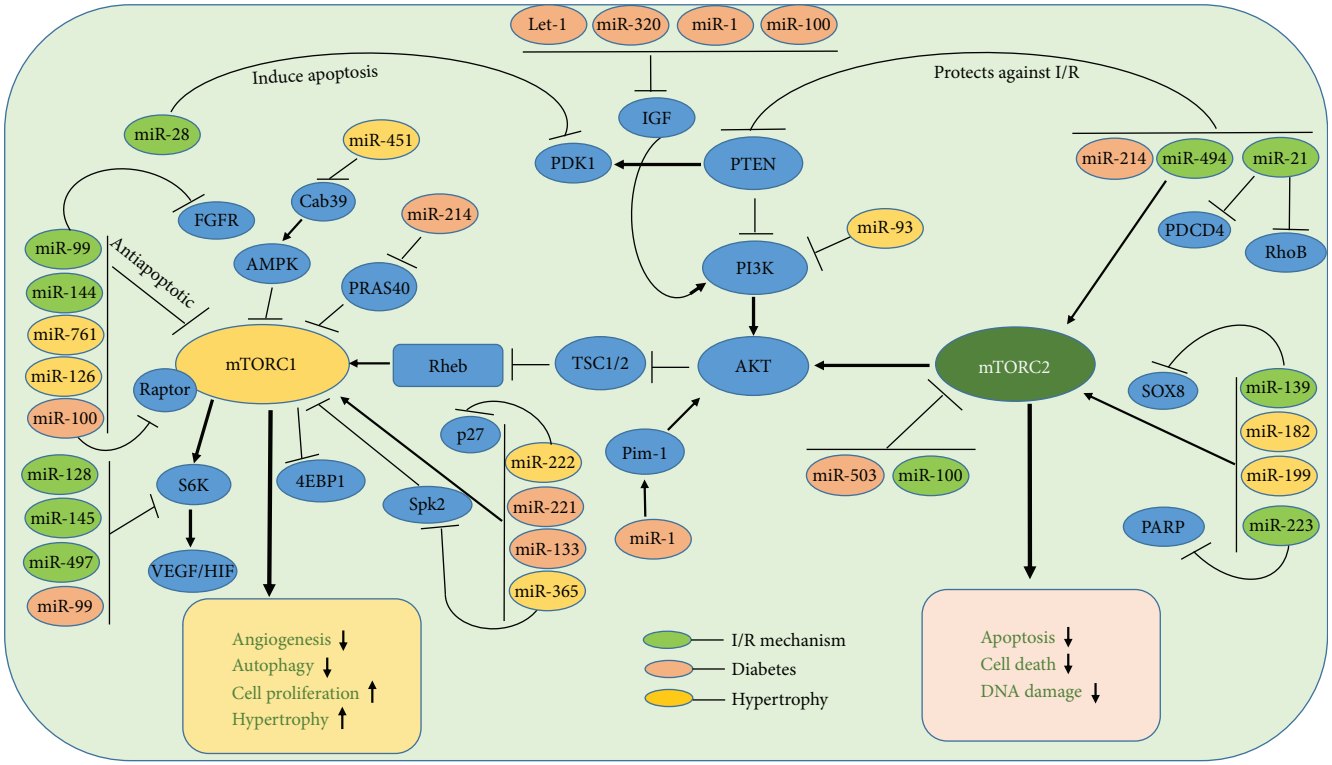

Figure 3: Diagram depicting network of coordinated interaction between miRNA and mTOR pathway in the regulation of cardiovascular diseases. Fibroblast growth factor receptors (FGFR); insulin-like growth factor 1 (IGF-1) sphingosine kinase 2 (Spk2); regulation of proline-rich AKT substrate 40 kDa (PRAS40); phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K); phosphoinositide-dependent kinase l (*PDK1*); calcium-binding protein 39 (Cab39); poly (ADP-ribose) polymerase (PARP); SRY-related HMG-box 8 (SOX8); programmed cell death protein 4 (PDCD4); Ras homolog gene family, member B (RhoB); phosphatase and tensin homolog (PTEN); vascular endothelial growth factor (VEGF); hypoxia-inducible factors (HIF); tuberous sclerosis protein (TSC1/2); ras homolog enriched in brain (Rheb); ribosomal protein S6 kinase beta-1 (S6K1); proviral integration site for Moloney murine leukemia virus-1 (Pim-1).

injury [151–153], heart failure [154–156], and its associated risk factors including diabetes [146, 157, 158] and aging [31, 159, 160]. Recent research findings also indicate that the components involved in mTOR pathway are regulated by miRs in cancer and other diseases [39, 40, 161, 162]. A comprehensive role of miRs regulating mTOR signaling in cardiovascular diseases is depicted in Figure 3.

2.1. miRNA-Dependent Regulation of mTOR in Ischemia Reperfusion Injury. Oxidative stress induced by ROS generation is a major mechanism of cell injury during myocardial I/R injury [163]. Deprivation of oxygen during I/R stress activates mTORC1 [146, 151, 158, 164] and controls several downstream kinases and leads to cellular effects such as apoptosis [165], autophagy [166, 167], and proliferation [22]. Accumulating evidences point out several different miRs are involved in the modulation of mTOR signaling via targeting mTOR-interacting partners [40, 168–171].

mTOR mediates cardiomyocyte response during ischemia and is an important determinant of cell survival [172, 173]. Inhibition of mTORC1 was shown to be beneficial for the survival of cardiomyocytes via induction of autophagy [174]. Sciarretta et al. demonstrated that selective and direct mTORC1 activation is detrimental during acute cardiac energy deprivation, whereas both pharmacological and genetic mTORC1 inhibition are protective [175]. Pretreatment with rapamycin, the mTOR inhibitor, reduced myocardial infarct size after I/R injury by attenuating necrosis and apoptosis in cardiomyocytes [158, 176]. Reperfusion therapy with rapamycin also attenuated myocardial infarction and apoptosis by activation of PI3K and ERK [177]. Activation of autophagy prevents unwanted expenditure of cellular energy to damaged cells, especially mitochondria, which results in an increased ROS generation [174, 178]. Cardiac-specific overexpression of Rheb leads to the inhibition of Atg7, a key effector protein in the autophagy cascade, and enhances cardiomyocyte cell death through activation of Rheb/mTORC1 signaling pathway [175]. Interestingly, mTOR inhibition with rapamycin promotes the survival of oxygen-deprived cardiomyocytes through activation of autophagy via inhibition of Ras homolog enriched in brain (Rheb) protein [175]. These results indicate that Rheb is a main regulator of mTORC1 during cardiomyocyte energy stress, and Rheb/mTORC1 inhibition promotes cell survival through activation of autophagy [175]. Moreover, obesity and metabolic syndrome, which are characterized by increased myocardial susceptibility to ischemic injury and cardiovascular mortality, are associated with

inadvertent activation of the Rheb/mTORC1 pathway and reduction of autophagy [175, 179–181]. Mice with high fat diet- (HFD-) induced obesity and metabolic syndrome exhibit deregulated cardiac activation of Rheb/mTORC1 and inhibition of cardiac autophagy, which lead to increased ischemic injury [175]. Rapamycin treatment before prolong ischemia (3 hours) increases autophagy and significantly reduces the myocardial infarction of both HFD-treated mice [175].

Autophagy is a delicate process that involves a closely-knit transcriptional and epigenetic regulation through miRs [182, 183]. Overexpression of microRNA-99a (miR-99a) through intramyocardial delivery improved cardiac function after MI stress and prevented cell death via enhancing autophagy in mTOR/P70/S6K-dependent signaling pathway [184]. Notably, overexpression of miR-99a in the border zone of infarct area prevented cell apoptosis, but increased autophagy via inhibiting mTOR/P70/S6K [184]. Furthermore, the expression level of miR-99a was reduced in neonatal mice ventricular myocytes (NMVMs) subjected to hypoxia. Similarly, intramyocardial delivery of lenti-miR-99a in mice showed a significant improvement in both left ventricular (LV) function and cell survival post four weeks of MI compared to sham groups [184]. Even though the study illustrated that miR-99a is cardioprotective through decreasing mTOR activity, it did not show a direct target of miR-99a. However, further evidence from the same group also showed that fibroblast growth factor receptor 3 (FGFR3) to be a direct target of miR-99a and hinted a possible role for FGFR-mTOR pathway in MI-induced hypertrophy [185]. Interestingly, multiple findings established a link between miR-99a and FGFR in cell proliferation via mTOR signaling [186–188].

Prevention of the loss of cardiomyocyte during I/R injury is the primary focal point and strategic approach to avoid cardiac dysfunction post-MI, which can potentially be achieved by regulating apoptosis and autophagy [189]. Experiments using primary neonatal cultured mouse cardiomyocytes identified a direct link between miR-28 and PDK1, an immediate upstream regulator of mTOR in the PI3K pathway [190]. Induction of oxidative stress in cardiomyocyte using hydrogen peroxide elevated the expression of miR-28 and increased apoptosis-mediated cell death [190]. Interestingly, overexpression of miR-28 downregulated p-AKT, p-p70, and p-mTOR suggesting a direct interference of mTOR signaling by miR-28 [190]. Mechanistically, PDK1 was found to be a direct target of miR-28 and target-binding assay using luciferase activity and PDK protein expression after transfection with miR-28 confirmed the prediction [190]. Similarly, miR-223 has been reported to play an important role in cell survival by regulation of autophagy and apoptosis [191]. The miR-223 was upregulated in the border zone of infarct area in rats subjected to LAD occlusion [191]. Moreover, overexpression of miR-223 protected H9c2 cells and neonatal rat cardiomyocytes (NRCMs) against hypoxia-induced apoptosis by directly targeting PARP-1 [191]. Decisively, this study showed that H9c2 and NRCMs cells treated with miR-223 mimic increased p-AKT and p-mTOR expression under hypoxic conditions and the protective effect of miR-223 was abolished upon treatment with miR-223 inhibitor [191]. Although, earlier study by van Rooij et al. reported an upregulation of miR-223 in human failing heart tissues [192], but did not explore on the mechanism. The same study showed that miR-29 family are downregulated in the region of the fibrotic scar after MI.

Phosphatase and tensin homology deleted from chromosome 10 (PTEN) are an important determinant for the activation of AKT through PI3 kinase-mTOR pathway. It enhances cardiomyocyte cell death and increases cardiac dysfunction during MI and I/R injury [193–196]. Genetic ablation or pharmacological inhibition of PTEN has been shown to be cardioprotective against MI and vascular remodeling [196, 197]. Our laboratory demonstrated that miR-21 had a powerful cardio protective effect against I/R injury [198–200]. miR-21 expression is induced in the border zone of the infarcted hearts, but it is significantly decreased in the infarcted area [181, 192]. Overexpression of miR-21 protects against I/R injury by reducing myocardial infarct size and apoptosis, by its target genes, PTEN, and programmed cell death 4 (PDCD4) [201–203]. Recent studies suggest that the therapeutic effects seen with miR-21 may be mediated through PTEN/AKT/mTOR signaling pathway [204]. It was observed that miR-21 expression was downregulated, and autophagy was remarkably increased in H9c2 cells during H/R injury. Simultaneously, increased apoptosis after H/R injury was associated with reduction of Bcl2-Bax ratio. Such an effect was abolished by overexpression of miR-21 with a miR-21 precursor, which also inhibited autophagic activity and decreased apoptosis accompanied by the activation of the AKT/mTOR pathway [204]. Thus, it appears that miR-21 plays an active role in disrupting the PTEN-AKT-mTOR pathway. Similar result was also observed in cardiac stem cells, where miR-21 reduced hydrogen peroxide-(H_2O_2-) induced apoptosis, as evidenced by the downregulation of caspase-3 and Bax and upregulation of the antiapoptotic Bcl-2 [205]. Overexpression of miR-21 suppressed the expression of PTEN, a direct target of miR-21, with simultaneous increased in the phosphorylation state of AKT. Moreover, the antiapoptotic effect of miR-21 was abolished in cells treated with miR-21 inhibitor and PI3 inhibitor, LY294002, suggesting an involvement of PTEN/PI3K/AKT signaling in miR-21-mediated antiapoptotic effect [205]. Although miR-21 has been considered to promote cellular proliferation, invasion, and migration in various types of tumors [206, 207], rapamycin treatment induced the expression of miR-21 in human umbilical vein endothelial cells (HUVECs) but attenuated endothelial cell proliferation and migration [208]. RhoB, an important partner in AKT-mTOR pathway, is a direct target of miR-21, and silencing of RhoB impairs endothelial cell migration and tubulogenesis, thus providing a possible mechanism for miR-21 to inhibit angiogenesis after rapamycin treatment [208, 209]. However, raptor knockdown, but not rictor silencing, upregulates miR-21 expression, and inhibition of miR-21 blunted the antiproliferative and antimigration effects of rapamycin treatment [208].

Interestingly, miR-21 was shown to be upregulated with rapamycin treatment in angiomyolipoma-derived cells isolated from patient with lymphangioleiomyomatosis (LAM)

[210]. Conceptually, LAM is induced by mutation in the tuberous sclerosis complex genes (*TSC1* or *TSC2*) [211] and results in hyperactivation of mTOR signaling, which is characterized by proliferation of smooth muscle-like cells and leads to the malfunction of the lungs. The study demonstrated that 19 miRNAs were differently regulated by rapamycin, and miR-21 was shown to be robustly upregulated in TSC2-deficient 621–101 cells (Renal angiomyolipoma cells). This study also suggested that rapamycin-mediated upregulation of miR-21 is independent of AKT signaling, but rather dependent on mTOR. More importantly, the study also demonstrated that rapamycin potentiates the Drosha-mediated posttranscriptional processing of pri-miR-21 to pre-miR-21. Moreover, rapamycin was clinically shown to improve pulmonary function in LAM patients. Surprisingly, a recent another study demonstrated a significant upregulation of miR-21 in Tsc2-deficient cells compared to wild type controls, which was further induced by rapamycin [212]. Experimental evidences suggest that miR-21 induced proliferation, tumor growth, and offered resistance to apoptosis in TSC2-deficient cells. Moreover, data analysis of RNA Seq implicated that miR-21 promoted mitochondrial adaptation and homeostasis in Tsc-2-deficient cells. Inhibition of miR-21 using LNA reduced the tumor size and mitochondrial function. In addition, rapamycin cotreatment with miR-21 inhibition more efficiently reduced tumorigenic growth of Tsc2-deficient cells *in vivo* xenograft model. Importantly, this study showed that rapamycin increased mitochondrial content and polarization, and these effects of rapamycin were miR-21-dependent. The study also proposed that the unexpected upregulation of miR-21 in TSC2-deficient cells was partly due to miR-21 regulation of mTOR in a noncanonical pathway via either STAT3 or Rheb [212]. Recently, mTORC1 was also reported to regulate the miRNA biogenesis pathway itself [213]. Extensive expression analysis of 752 miRs in TSC2-deficient cells, treated with Torin1 (inhibitor of both mTORC1 and C2), demonstrated an upregulation of majority of miRs in consistent with the increased activity of microprocessor (the multiprotein complex that includes Drosha (a type III RNAse) and DGCR8) in TSC2-deficient cells. Microprocessor activity is regulated in part by GSK3β, which is phosphorylated at S9 and subsequently inhibited by mTORC2 via AKT. Inhibition of mTORC1 impaired the microprocessor activity through regulation of Drosha and GSK3β-dependent pathways via mTORC2 [213].

Numerous studies reported that miR-21 is involved in a variety of disorders and is highly upregulated during cardiac remodeling [201, 214–216]. However, genetic deletion of miR-21 or acute inhibition of miR-21 did not alter the pathological responses of the heart to pressure overload or other stresses, which suggests that miR-21 is not required for cardiac hypertrophy, fibrosis, or loss of contractile function in response to acute or chronic injury in mice [217]. However, the precise role of miR-21 regulating mTOR signaling in cardiovascular system is still not well evolved, and the effect of miR-21 on proliferative cells like endothelial, smooth muscle cell, and nonproliferative cells like cardiomyocytes may be different. However, several reports in cancer biology strongly suggest an active role for miR-21 in regulating mTOR signaling largely through PTEN/PI3K/AKT pathway [218–222].

Angiogenesis is an important process that restores blood supply to the infarct area post-MI and I/R injury and improves cardiac function [223, 224]. In this context, miR-100 was reported to be an antiangiogenic miR and functioned through repressing mTOR signaling after induction of hind-limb ischemia in mice [162]. miR-100 modulated proliferation, tube formation, and sprouting activity of endothelial cells and migration of vascular smooth muscle cells and functions as an endogenous repressor of mTOR. Inhibition of miR-100 by specific antagomirs stimulated angiogenesis with functional improvement of perfusion after femoral artery occlusion in mice. Moreover, the stimulatory effect of antagomir therapy was abolished by simultaneous rapamycin treatment, demonstrating that the angiogenic effect of miR-100 inhibition in hind-limb ischemia was dependent on its target gene mTOR [162]. Nevertheless, this study did not address the specific role of mTORC1 or C2 in blocking the angiogenic response and did not use a long-term treatment with rapamycin [162].

In tumor glioma, miR-128 is downregulated and acts as a tumor suppressor by directly targeting p70S6K1 [225]. Overexpression of miR-128 attenuated cell proliferation, tumor growth, and angiogenesis by suppressing p70S6K1 and its downstream signaling molecules such as HIF-1 and VEGF expression. Similarly, the expression of miR-145 is downregulated in colon and ovarian cancer, and overexpression of miR-145 inhibits tumor growth and angiogenesis by targeting p70S6K1 and suppressing its downstream angiogenic factors HIF-1 and VEGF [226]. Another miR, miR-497 is downregulated in breast, cervical, head-and-neck, colorectal, and prostate cancers, and overexpression of miR-497 sensitizes the resistant ovarian tumor to cisplatin treatment by targeting mTOR and p70S6K1 [227]. Among the three miRs previously reported to target p70S6k1, only miR-128-3p is downregulated in human cardiomyocytes during H/R by Tongxinluo (TXL, a traditional Chinese medicine, widely used to treat cardiovascular and cerebrovascular diseases). Interestingly, TXL restored p70S6K1 but had no effects on miR-145-5p and miR-497-5p [228]. Inhibition of miR-128-3p activated mTOR via increasing the phosphorylation and abundance of p70s6k1.

PI3K/AKT/mTOR pathway has been shown to be suppressed by miR-139 in I/R injury in H9c2 cell line [229]. The overexpression of SOX8, a target of miR-139, alleviates hypoxia-induced cell injury via activation of PI3K/AKT/mTOR and MAPK pathway [229]. Recently, several miR profiling studies revealed that miR-494 was downregulated in human failing hearts as well as ischemic/hypertrophic hearts of animals [230–232]. The cardiac-specific overexpression of miR-494 in mice protected hearts against I/R-triggered injury; conversely, knockdown of endogenous miR-494 by antagomir sensitized hearts to I/R-induced injury [233]. The overexpression of miR-494 suppressed the levels of proapoptotic proteins (PTEN, ROCK1, and CaMKIIδ) after I/R injury, which also induced AKT signaling in concert, a critical survival pathway in the myocardium mediated through mTORC2 activation [233]. Also, the inhibition

of miR-494 using antagomir elevated the level of PTEN while simultaneously suppressing the level of pAKT (S473) after I/R injury [233].

Apart from the intracellular regulation of mTOR pathway by miRs, miRs packed in exosomes can affect cardiac function. Based on the miRNA array data, remote ischemic preconditioning (rIPC) altered the myocardial expression of miR-144 in mice [234]. Initially, it was shown that rIPC increased the myocardial expression of miR-144, whereas I/R injury alone significantly reduced the level of miR-144. Intriguingly, the exosomes isolated from tissue samples of rIPC hearts were rich with the expression of miR-144 upon rIPC. Moreover, intravenous administration of miR-144 via tail vein injection induced early and delayed cardioprotection in Langendorff isolated perfused model of I/R injury [234]. This study also showed that miR-144 directly targeted mTOR as evident with the decreased p-mTOR and increased autophagy signaling upon miR-144 administration. More precisely p-AKT (S473), a marker for mTORC2 activation, was increased in the heart upon miR-144 injection in mice [234]. These finding suggest that miR-144 acts via suppressing mTORC1 while simultaneously activating mTORC2 complex [234]. In silico analysis of miRNA-target mRNA prediction algorithm (TargetScan 6.0) revealed two specific miR-144 binding sites in the mTOR 3′UTR region with perfect Watson–Crick matches at miRNA positions 1–7 and 2–8 [235]. The interaction of miR-144 and mTOR and its clinical significance have been evaluated in human cancer biology. Specifically, the downregulation of miR-144 leads to poor prognosis of cancer patients via activation of the mTOR signaling pathway [235].

2.2. Regulation of mTOR through miRNA in Diabetes and Obesity. Diabetes is a major risk factor for CVD and is characterized by elevated blood glucose, insulin resistance/deficiency, and metabolic abnormalities [236, 237]. Since mTOR is sensitive to nutrient, excessive glucose level in the blood stream activates mTOR [146, 151, 158, 238]. Prolonged activation of mTORC1 induces insulin resistance in adipose tissue through the S6K1-mediated inhibition of insulin signaling that disrupts the recruitment and activation of PI3K via phosphorylation of insulin receptor substrate-1 (IRS-1) [239, 240]. Similar aberrant mechanism in cardiovascular tissues, in conditions like diabetic and obesity, leads to cardiac abnormalities through S6K1-IRS-1 [241] and its effector kinases like MAPK [242], AMPK [241], and glycogen synthase kinase-3β (GSK3β) [148, 243, 244]. Several miRNAs were identified to play a role in diabetes by regulating insulin signaling and glucose metabolism [238, 245]; [246, 247]. Some of the prominent miRs that regulate mTOR pathway are miR-133a, miR-100, miR-221, miR-483-3p, miR-133a, miR-503, miR-214, microRNA-99a, miR-143, miR-126, and miR-181a-5p.

Inhibition of Let-7 family of miR was shown to be beneficial and promoted cardiac function against I/R injury in diabetic rats [248]. I/R injury in diabetic rat significantly increased let-7 miR as well as infarct size, while antagomir let-7-treated diabetic group offered protection against I/R [248]. Moreover, the myocardial expression of IGF1 and GLUT4 as well as p-AKT (S473) were significantly lower with activation of mTOR in diabetic group. Notably, blocking of let-7 expression or treatment with rapamycin effectively increased AKT phosphorylation at S473 residue, while simultaneously blocked mTOR phosphorylation [248]. IGF plays an important role in glucose metabolism and in the development of insulin resistance, which are crucial events in diabetic cardiomyopathy. miR-1 has been shown to directly targeted IGF-1 [249] and regulated PI3-AKT pathway [250]. In support of this notion, it was shown that miR-1 increased during diabetic cardiomyopathy, which led cardiomyocyte apoptosis through targeting Pim-1 (proviral integration site for Moloney murine leukemia virus-1) [251]. Inhibition of miR-1-dependent downregulation of Pim-1 using miR-1 antagomir resulted in the elevation of phosphorylated AKT and abrogation of diabetic-induced cardiac apoptosis [251]. Similarly, miR-320 is also identified to directly target IGF-1 and VEGF and impairs angiogenesis in myocardial microvascular endothelial cells (MMVEC) isolated from Goto-Kakizaki (GK) diabetic rats [252]. Published studies also demonstrated that miR-99a suppressed the expression of IGF-1 and inactivated mTOR in vascular smooth muscle cells (VSMC) [253]. The hyperinsulin-mediated proliferation and migration of VSMC were reversed by overexpression of miR-99 or inhibition of mTOR. Moreover, overexpression of miR-99a reduced AKT and ERK1/2 activity while suppressing p70S6K, a downstream target of mTORC1 [253].

miR-133a is one of the predominantly expressed miRs in the cardiac tissue, which plays a protective role against pathological remodeling by inhibiting cardiac hypertrophy and cardiac fibrosis in diabetes [254, 255]. Studies in the murine model show that diabetes attenuates miR-133a in hearts [256, 257]. Additionally, a diabetic heart failure (DHF) patient population study showed that the attenuation in the level of miR-133a in diabetic hearts was associated with the exacerbation of autophagy and hypertrophy and suppression of mTOR [258]. In contrast, another interesting study conducted to evaluate the cardiac dysfunction in the offspring of maternal diet-induced obesity revealed a role for miR-133a in cardiac hypertrophy [259]. The results showed that the level of miR-133 is significantly increased in ventricular tissue of the Mat-Ob group and cardiac hypertrophy in the offspring [259]. Most notably, AKT1-Ser473 phosphorylation as well as levels of phospho-ERK1/2, phospho-mTOR, and phospho-p38MAPK were significantly elevated in the Mat-Ob group [259], suggesting an active role of mTOR in the development of cardiac hypertrophy upon diet-induced maternal obesity [259].

Elevated levels of fatty acids and glucose observed in obesity and diabetes mellitus (DM) contribute to systematic inflammation [260, 261]. Blood miRNAs signatures in patients with diabetes with/without obesity revealed a significant reduction of circulating miR-100 in obese normoglycemic subjects and subjects with T2D compared to healthy and lean individuals [262]. Visceral adipose miR-100 was also lower in obese patients with T2D compared to those without. Reduced miR-100 levels were associated with adverse metabolic indices, which may lead to the differentiation of fat tissues and subsequent lipid accumulation, potentially contributing

to increased obesity. miR-100 led to the differentiation of adipocytes by modulating its direct target IGFR (insulin growth factor receptor), mTOR, and vLDLR signaling.

A recent study characterized the function of the endothelial-enriched miR-100 during vascular inflammation and atherogenesis [263]. It was reported that miR-100 directly repressed several components of mTORC1-signaling, including mTOR and raptor, which led to the stimulation of endothelial autophagy and diminished activity of the pro-inflammatory transcription factor NF-κB. In a low-density lipoprotein receptor-deficient atherosclerotic mouse model, inhibition of miR-100 enhanced atherosclerotic plaque formation and a higher macrophage content of the plaque, whereas miR-100 mimic attenuated atherogenesis in the aortic root and in the abdominal aorta. Moreover, miR-100 mimic suppressed mTOR and the transcription factor SREBP-2, which subsequently controlled lipid metabolism in hepatocytes. mTOR inhibition with rapamycin showed anti-inflammatory effects through decreasing the expression of E-Selectin, intracellular adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) in response to endothelial cell activation with TNF-α. In addition, rapamycin abolished the effects of miR-100 inhibition with TNF-α on endothelial adhesion molecule protein expression, confirming the essential role of intact mTOR signaling in the anti-inflammatory effects of miR-100 [263].

Vascular remodeling and cardiac hypertrophy is one of the adverse effect of diabetes and results in end-stage heart failure [264, 265]. To address this phenomenon, cardiac hypertrophy was induced by angiotensin II (Ang II) treatment in diabetic OVE26 mice, and the role of miR-221 on autophagy was investigated [266]. The results demonstrated that Ang II treatment increased the phosphorylation of c-Jun, JNK, mTOR, and miR-221, while decreasing the level of p27, a direct target of miR-221 and regulator of p-mTOR [266]. Direct downregulation of p27 by miR-221 led to mTOR activation and diminished cardiac autophagy of diabetic OVE26 and/or Ang II-treated mice, resulting in cardiac hypertrophy [266].

mTOR plays a contrasting role in type I DM, where there is an insufficient insulin secretion due to deficient pancreatic β-cells. In gestational diabetes mellitus (GDM), it was shown that knockdown of miR-503 enhanced insulin secretion of pancreatic β-cells, promoted cell proliferation, and protected cells from apoptosis [267]. mTOR has been identified as a direct target of miR-503, and suppression of miR-503 improves insulin secretion and pancreatic β-cells proliferation [267]. The regulation of mTOR pathway by miR is also evident in renal cortex of type 1 diabetic mice [268]. Elevation of miR-214 under high glucose conditions decreased the levels of its target PTEN and increased AKT activity (p-S473) and led to phosphorylation of its substrates glycogen synthase kinase-3β and phosphorylation of PRAS40. In contrast, antimiR-214 blocked the phosphorylation of both AKT and PRAS40 and attenuated renal cell hypertrophy, suggesting that inactivation of both mTORC1 and C2 is beneficial [268]. Consistent with this finding, studies using placental tissue from women with GDM demonstrated a robust activation of both mTORC1 and C2 as evident with the increased phosphorylation of AKT (S473), (4EBP1), and p70 S6 kinase (S6K) [269]. Data also showed that miR-143 was significantly high using placental tissue and trophoblast cells, and it impaired mitochondrial respiration via targeting hexokinase (HK), a rate-limiting enzyme in glycolysis [269]. Similarly, miR-99a has been shown to be involved in insulin-dependent glucose consumption in human liver cells (HLL7702) via directly targeting mTOR [270]. Cells treated with insulin suppressed the level of miR-99a while increasing glucose consumption and activation of mTOR. In contrast, the overexpression of miR-99a or rapamycin treatment reversed insulin-mediated glucose utilization [270].

2.3. Interaction of mTOR and miRNA in Vascular Remodeling and Hypertrophy. Given the role of mTOR in regulating protein synthesis through S6K [271] and cell cycle control [272, 273], it is well established that mTOR play a key role in cardiac hypertrophy [274–276]. In fact, several reports support this notion as mTOR inhibitors have antihypertrophic property [277–279]. Due to its antiproliferative properties, mTOR inhibitors have also been approved as anticancer drugs [280–282]. Intriguingly, the identification of miRNAs as novel emerging regulators of mTOR signaling has provided new insights into a multitude of biological processes, especially in tissue remodeling and hypertrophy, which has been appreciated by the scientific community in cardiac physiology [103, 169, 185]. Hypertrophic stimuli such as phenylephrine [283], angiotensin II (Ang II) [37, 284, 285], and endothelin-1 [286] are known to activate mTORC1 in the heart and result in robust vascular remodeling leading to heart failure [274]. However, mTORC2 is essential for the preservation of cardiac function and attenuation of pressure overload-induced cardiac hypertrophy [287]. It is increasingly apparent that mTOR [156, 283] and miR [216, 254] have a critical role in the development of cardiac hypertrophy and it is becoming important to understand the mechanism by which these two major regulators communicate with each other.

Cardiomyocyte-specific miR-199a overexpression inhibited autophagy and induced cardiac hypertrophy via targeting glycogen synthase kinase 3β (GSK3β) involving mTOR signaling [39]. The mTOR signaling was activated in miR-199a transgenic hearts [39]. In addition, treatment with rapamycin blocked the activation of p-mTOR and p-S6 in miR-199 transgenic mice and attenuated hypertrophy with induction of autophagy [39]. Data also indicated that miR-761 expression was reduced during Ang II-induced proliferation of VSMCs, and exogenous miR-761 delivery effectively inhibited the Ang II-induced VSMC proliferation. [288]. Experimental evidence showed that miR-761 directly targets mTOR and reduced its abundance [288]. Similarly, miR-99a was shown to negatively regulate hypertrophy through mTOR signaling pathway [185]. Interestingly, mice displayed an increase in mTOR activity starting at first week through 8 weeks following TAC- (transverse aortic constriction-) induced cardiac hypertrophy [185]. Overexpression of miR-99a suppressed mTOR and attenuated cardiac hypertrophy and cell death in TAC mouse model. Overexpression of miR-

99a attenuated cardiac hypertrophy in TAC mice and cellular hypertrophy in cardiomyocytes subjected to Ang II or isoprenaline (ISO) through suppression of expression of mTOR [185].

In contrast, it has been shown that cardiac-specific overexpression of miR-222 induced pathological cardiac remodeling and heart failure in mice [289]. Transgenic mice with cardiac-specific expression of miR-222 (Tg-miR-222 mice) developed severe cardiac fibrosis and apoptosis, which led pathological cardiac remodeling and heart failure. The autophagy was inhibited in the hearts of Tg-miR-222 mice with activation of mTOR, but the expression of p27 was downregulated in the hearts of Tg-miR-222 mice [289]. It was suggested that miR-222 induced autophagy through activation of both mTORC1 and C2 complexes as shown with a substantial increase in both p-mTOR and p-S6 (Ser240/244) in transgenic Tg-miR-222 mice [289]. In the context of these findings, Su et al. also reported a role for p27-mTOR in the development of cardiac hypertrophy [290]. Cardiac-specific overexpression of miR-221, driven by the α-myosin heavy chain, resulted in hypertrophic hearts at 4 weeks of age with increased expression levels of ANP and BNP [290]. Moreover, miR-221 also inhibited autophagy, as demonstrated by downregulation of LC3-1/LC3-II ratio and an increase in p62 expression level [290]. Further, miR-221 overexpression in H9C2 cells and in primary cardiomyocytes showed decreased autophagosome formation as demonstrated with low number of EGFP-LC3 puncta [290]. More importantly, phosphorylation levels of mTOR and its substrates phospho-mTOR (S2448), phospho-4EBP1 (T37/46), and phospho-S6 (S235/236) levels were all significantly increased in Tg-miR-221 hearts at 4 weeks of age compared with those in the nontransgenic controls [290]. Conversely, silencing miR-221 in H9C2 cells and cardiomyocytes decreased the levels of phospho-mTOR, phospho-S6K, and phospho-S6, thereby establishing a link between miR-221 and mTOR signaling in the induction of cardiac hypertrophy [290]. Similarly, miR-365 was shown to promote cardiac hypertrophy through inhibition of autophagy by suppressing S-phase kinase-associated protein 2 (SPK2), an important activator of autophagy [291]. Conceptually, it was demonstrated that Spk2 induces autophagy through inhibition of mTORC1 and reverses adverse effect of cardiac hypertrophy [291]. Notably, Ang II treatment of cardiomyocytes increased the phosphorylation of the mTORC1 downstream effectors S6K and 4EBP1 and decreased the level of Spk2. Inhibition of mTOR activation, using rapamycin, completely abolished the Ang II-mediated inhibition of autophagy via miR-365-Spk2-dependent mechanism [291].

Recent studies have suggested that a long noncoding RNA (lncRNA), myocardial infarction-associated transcript (MIAT), plays a role in vascular remodeling and cardiac hypertrophy [41]. In this study, the authors demonstrated a three way link between MIAT, miR-93, and mTOR network. The upregulation of MIAT was associated with the decrease in miR-93 in Ang II-induced cardiac hypertrophy in rat [41]. Furthermore, it was shown that MIAT positively regulated TLR4 expression by acting as a sponge for miR-93 expression [41]. Overexpression of miR-93 attenuated MIAT-induced increase of TLR4 level in cardiomyocytes and attenuated Ang II-induced cardiac hypertrophy. In contrast, MIAT knockdown or miR-93 overexpression led to a significant inhibition on the protein levels of PI3K, p-AKT, and p-mTOR and blunted Ang II-mediated cardiac hypertrophy [41]. This study also suggested a strong corelation between miR-93, TLR4, and mTOR signaling, since overexpression of TLR4 enhanced the expression of miR-93 and blocked the protection observed with p-mTOR inhibition [41].

High fat diet (HFD) consumption for a prolonged time induces cardiac hypertrophy [292], and mTOR being a nutrition sensor plays an active role in mediating this effect in the heart [293]. Microarray analyses of the heart tissue of mice on HFD for 8 and 20 weeks identified a role for miR-451 in the development of cardiac hypertrophy [103]. Calcium-binding protein 39 (Cab39) is a direct target of miR-451 and an upstream kinase of AMP-activated protein kinase (AMPK). Suppression of miR-451 protected neonatal rat cardiac myocytes against palmitate-induced lipotoxicity through a mechanism that involves Cab39 [103]. In addition, cardiomyocyte-specific miR-451 knockout mice were resistant to HFD-induced cardiac hypertrophy. Protein levels of Cab39 and phosphorylated AMPK were increased, and phosphorylated mTOR and S6 phosphorylation were significantly suppressed in cardiomyocyte of the HFD-fed miR-451 cKO mice compared with control mouse hearts [103]. These findings elucidated an interesting aspect of AMPK-miR-451 and mTOR cross talk in cardiac hypertrophy.

Angiogenesis is an important process that plays a detrimental role in post-MI, and its abnormal regulation leads to cardiac hypertrophy [294]. mTOR and its downstream target AKT have been involved in the control of angiogenesis process during I/R injury [295–298]. Placental growth factor (P1GF), a member of vascular endothelial growth factor (VEGF) family, has been shown to induce cardiac angiogenesis and leads to hypertrophic heart [299]. Cardiac-specific overexpression of P1GF induced cardiac angiogenesis with increased expression of miR-182 at 6 weeks onset of angiogenesis process [299]. The study also found blunting of miR-182 upregulation in PlGF-induced eNOS$^{-/-}$ mice, suggesting that miR-182 acts through NO-independent pathway to regulate angiogenesis [299]. Since NO exerts its function through AKT, it was further shown that mTORC1 was involved in the induction of angiogenesis and cardiac hypertrophy. Suppression of miR-182 using antimiR-182 decreased the phosphorylation of AKT^{Ser473} and p70-S6K^{Thr389}, thus indicating an important regulatory effect of miR-182 on the AKT/mTORC1 pathway [299].

Endothelial cell dysfunction contributes to coronary vascular tone and results in atherosclerosis by affecting various growth factors, such as vascular endothelial growth factor, fibroblast growth factors (FGFs), and platelet-derived growth factors [300, 301]. PI3K/AKT/mTOR pathway plays a role in endothelial function and in the development of atherosclerosis [302]. miR-126 has been shown to play a role in alleviating oxidized low-density lipoprotein (ox-LDL) induced HUVEC injury through suppression of AKT-mTOR pathway [303]. The overexpression of miR-126 reversed ox-LDL-induced cell injury and apoptosis in HUVECs [303]. Conceptually,

treatment of HUVECs with ox-LDL increased the phosphorylation of mTOR through activation of PI3K and AKT, and miR-126 mimics restored the impaired autophagic flux via inhibition of PI3K/AKT/mTOR pathway [303].

A recent study by Bera et al. revealed a significant role of miR-214 in the activation of mTORC1 that contributed to high-glucose-induced mesangial and proximal tubular cell hypertrophy and fibronectin expression [268]. miR-214 expression is increased in the renal cortex of type 1 diabetic mice. High glucose treatment induced the expression of miR-214 and decreased its target, PTEN, in mesangial and proximal tubular epithelial cells [268]. Suppression of PTEN subsequently increased the AKT-dependent mTORC1 activation to induce mesangial and proximal tubular cell hypertrophy and fibronectin expression. Quenching of miR-214 expression inhibited high-glucose-stimulated cell hypertrophy and expression of the matrix protein fibronectin. In contrast, overexpression of miR-214 suppressed PTEN and increased AKT activity similar to high glucose and led to phosphorylation of two mTORC1 inhibitors, PRAS40 and tuberin, which contributes to high-glucose-stimulated mTORC1 activation [268].

Interestingly, a recent study demonstrated that overexpression of lncRNA Plscr4 alleviated pressure overload-induced cardiac hypertrophy in mice and attenuated the increased cell surface area of cultured neonatal mouse cardiomyocytes treated with Ang II [304]. The study identified that Plscr4 elicits the antihypertrophic effects by repressing the prohypertrophy gene miR-214. Mitofusin 2 (Mfn2), which is located at the mitochondrial outer membrane, plays a negative regulator of cardiac hypertrophy by modulating mitochondrial fusion [305, 306]. Mfn2 is a direct target of miR-214 in the hypertrophic heart [307]. The interaction between Plscr4 and miR-214 attenuated the inhibitory effects of miR-214 on Mfn2. The overexpression of Plscr4 rescued the decreased expression of Mfn2 by sponging miR-214 in response to hypertrophic stress and, therefore, resisted mitochondrial dysfunction to alleviate hypertrophic growth [307]. However, the exclusive interplay between lncRNA Plascr4 and mTOR in regulation mediated by miR-214 of cardiac hypertrophy is yet to be identified.

3. Therapeutic Potential of miRNA and mTOR Inhibitors in CVD

Rapamycin, received the FDA approval in 1999, has been successfully used as an effective immunosuppressant post-organ transplantation to prevent allograft rejection [308]. The rapamycin-eluting coronary stent received first FDA approval in 2003 for use in coronary-artery stents to prevent restenosis [309–311]. Rapamycin is also used clinically for some rare forms of cancer (pediatric and adult patients with subependymal giant cell astrocytoma (SEGA), progressive neuroendocrine tumors of pancreatic origin (PNET), and SEGA associated with tuberous sclerosis (TS)) (http://www.cancer.gov/cancertopics/druginfo/fda-everolimus) [312, 313]. Multiple clinical trials of rapamycin are currently underway for several other disease conditions including lymphangioleiomyomatosis (LAM) [314], other metabolism modulating interventions on the elderly (NCT02874924), ALS (amyotrophic lateral sclerosis) (NCT03359538), Sturge-Weber syndrome (SWS) (NCT03047980), and type 1 diabetes (NCT01060605; NCT00014911-both are completed) [315, 316].

Rapalogs, the modified form of rapamycin, are widely considered in clinical trials for its anticancer property. In fact, the National Cancer Institute has registered more than 200 clinical trials involving either rapamycin or modified form of rapamycin both as monotherapy and as combination treatment cancer (NCT01698918; NCT00337376; NCT00930930) [317–320]. Due to the successful outcome of rapamycin in the clinical trials, several drugs analogs of rapamycin with modified chemical structure such as sirolimus, temsirolimus (CCI-779), everolimus (RAD001), and ridaforolimus (AP-23573) are being evaluated for enhanced treatment of several diseases [321–323]. In 2009, everolimus received approval from the FDA for HER2-negative breast cancer (advanced HR+ BC) patients in combination with exemestane after failure of a nonsteroidal aromatase inhibitor (Afinitor: Highlights of Prescribing Information). (http://www.accessdata.fda.gov/drugsatfda_docs/label/2012/022334s016lbl.pdf).

Current mTOR inhibitors available in the market are not complex-specific and can either partially suppress mTORC1 or completely block mTORC1 as well as C2. Therefore, several pharmaceutical companies ventured to develop second generation of mTOR inhibitor that can block both mTORC1 and C2. These inhibitors are designed to completely block the core catalytic activity of mTOR by acting as an ATP-competitive agents to mTOR subunit. On the contrary, diseases like cancer and cell cycle irregularities need specific inhibition of mTORC1 without interfering the activity of mTORC2. Since mTORC1 is vital for basic cellular process, it is indispensable, and its complete inhibition may lead to unwanted side effects. To overcome these obstacles, scientists are also in pursuit of developing inhibitor that target rictor or raptor to silence either mTORC1 or C2. Although mTOR inhibitors are promising drug for cancer treatment and immunosuppressant, an unmet clinical trial is essential for their therapeutic use in cardiovascular diseases. Substantially, evidences from laboratory models and preclinical trials suggest that mTOR inhibition in the heart is beneficial and prevents cell apoptosis [146, 158] and autophagy [324–327]. Interestingly, inhibition of mTOR by rapamycin or other rapalogs are shown to alter the expression pattern of miRs in the cardiovascular system [162, 199, 208, 228]. Especially, alteration of miRs through mTOR inhibition that changes the expression level of PTEN and other downstream targets can offer new treatment strategies [328]. Many miRNA-based therapies for cancer are in clinical trial and have shown efficiency in reducing tumor malignancy [312]. Mimic of miR-34 are currently being tested in phase I clinical trials (NCT01829971) for its anticancer properties [329]. It was demonstrated that low level of miR-34 is an indicator of poor prognosis in osteosarcoma (OS) patients. Sirolimus increases the sensitivity of human OS cells to anticancer drugs *in vitro* by upregulating miR-34b and suppressing its target p21-activated protein kinase 1 (PAK1) and ATP-

binding cassette subfamily B member 1 (ABCB1) [329]. In contrast, the miR-34 family (miR-34a, -34b, and -34c) is upregulated in the heart in response to stress, including myocardial infarction or pressure overload via TAC [330]. Diabetes also increases the expression of miR-34a both in the heart and in circulation [331]. miRNA Therapeutics Inc. is developing an LNA-modified antimiR against miR-34a, which attenuates MI-induced remodeling and dysfunction, and also improves cardiac function and increase angiogenesis with activation of AKT in a model of pressure overload-induced pathological hypertrophy and dysfunction [330]. Mechanistically, miR-34 can directly target protein phosphatase PH domain leucine-rich repeat protein phosphatase (PHLPP2), a negative regulator of the PI3K/AKT/mTOR pathway.

Upregulation of miR-92a was shown to activate PI3K/AKT/mTOR pathway and inhibit cell apoptosis induced by chemotherapy in mantle cell lymphoma (MCL) cells [332]. Downregulation of miR-92a could inhibit the growth of tumors in a xenograft MCL mouse model [332]. Interestingly, pharmaceutical company Miragen developed MRG-110, an inhibitor of miR-92a, to enhance the revascularization process in ischemic heart disease. However, inhibition of angiogenesis is the goal for cancer therapy, and it should be assumed that miR-92a acts differently in cardiovascular system [333].

MGN-1374, a miR-15 inhibitor, is under the developmental stage by miRagen Therapeutics for treating myocardial infarction [334, 335]. Studies conducted in MDA-MB-231 breast cancer cells demonstrated overexpression of miR-15b/16 led to inhibition of cell proliferation causing G1 cell cycle arrest as well as caspase-3-dependent apoptosis by directly suppressing mRNA levels of RPS6KB1 and mTOR [336]. In addition, miR-15 was shown to regulate $CD4^+$ regulatory T cells (Tregs) expression, which is essential for preventing autoimmunity. Overexpression of miR-15b/16 significantly enhanced the induction of Tregs in $Dicer^{-/-}$ $CD4^+$ T cells and suppressed the mTOR expression as evident with the decrease in phosphorylation of its downstream target, ribosomal protein S6 [337].

Cardiac expression of miR-208 was upregulated upon Ang II treatment and induced obesity through upregulation of mTORC1 in Zucker obese (ZO) rats [338]. Whereas, rapamycin treatment attenuated weight gain despite leptin resistance by attenuating the expression of miR-208 and increasing the expression of cardiac mediator complex subunit 13 (MED13), a suppresser of obesity, in ZO rats [338]. In addition, therapeutic inhibition of miR-208 prevents pathological cardiac remodeling, which coincides with a significant improvement in survival and cardiac function during heart disease [339]. MED13 is negatively regulated by a heart-specific miR-208a [340]. In this context, MGN-9103 (a LNA-modified antisense oligonucleotide against a cardiac-specific miR-208LNA) is a novel potential therapeutic candidate developed by miRagen Therapeutics for the treatment of obesity, diabetes, and metabolic syndrome and to improve cardiac function and survival rates during heart failure (http://drugprofiles.informa.com/drug_profiles/18925-mgn-9103). These research findings and clinical trials described above highlight the potential of miRNA-based therapies with an emphasis on mTOR signaling. Although several studies established a clear synergistic effect of miRNA and mTOR in the treatment of cancer, there are scarce reports of clinical trial in cardiovascular field. Nevertheless, conceptual treatments in laboratory models describing mTOR inhibition mediated miR changes and vice versa are encouraging and may lead to novel treatments in cardiovascular diseases in the future.

4. Conclusion

The role of mTOR in controlling the cellular dynamics in cardiovascular system provides confidence to consider mTOR and its related kinases as targets for therapeutic intervention. Most remarkably, changes in epigenetic signature of miRs upon mTOR inhibition can lead to identify novel miRNA-based treatment for cardiovascular diseases. Moreover, antagomir-based treatment options can specifically target individual mTOR complex and eliminate common side effects seen with dual mTOR inhibitors. Further understanding of the interfunctional relationship between mTORC1 and C2 complexes and its association with miRNA is warranted to develop an efficient miRNA-based therapeutics and diagnostics in cardiovascular system.

Conflicts of Interest

The authors declare no conflicts of interest.

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Research Article

Secreted Frizzled-Related Protein-2 Inhibits Doxorubicin-Induced Apoptosis Mediated through the Akt-mTOR Pathway in Soleus Muscle

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Doxorubicin (Dox) is a potent chemotherapeutic drug known for its dose-dependent and serious adverse effects, such as cardiotoxicity and myotoxicity. Dox-induced cardiotoxicity (DIC) and muscle toxicity (DIMT) have been studied; however, the mechanisms of Dox-induced apoptosis in soleus muscle are not well defined. Our data shows that with Dox treatment, there is a significant increase in oxidative stress, apoptosis, proapoptotic protein BAX, pPTEN levels, and wnt3a and β-catenin activity ($p < 0.05$). Moreover, Dox treatment also resulted in decreased antioxidant levels, antiapoptotic BCL2, pAKT, p-mTOR, and endogenous levels of sFRP2 in the soleus muscle tissue ($p < 0.05$). Secreted frizzled-related protein 2 (sFRP2) treatment attenuated the adverse effects of DIMT and apoptosis in the soleus muscle, evidenced by a decrease in oxidative stress, apoptosis, BAX, pPTEN, and wnt3a and β-catenin activity, as well as an increase in antioxidants, BCL2, pAKT, p-MTOR, and sFRP2 levels ($p < 0.05$). This data suggests that Dox-induced oxidative stress and apoptosis is mediated through both the Akt-mTOR and wnt/β-catenin pathways. Moreover, the data also shows that sFRP2 modulates these two pathways by increasing signaling of Akt-mTOR and decreased signaling of the wnt/β-catenin pathway. Therefore, our data suggests that sFRP2 has valuable therapeutic potential in reversing Dox-induced oxidative stress and apoptosis in soleus muscle mediated through the Akt-mTOR pathway.

1. Introduction

Doxorubicin (Dox) is a well-known medication used to treat various types of cancer, including those related to the breast, lung, stomach, and blood [1]. Though it is effective as an antimalignancy agent, there are multiple serious side effects associated with its use, including damage to the heart (cardiotoxicity), skeletal tissue (myotoxicity), hair loss, and arrhythmia [2]. Therefore, the use of doxorubicin has been limited, and various alternative strategies have been planned.

Dox has been found to induce both acute and late-onset dysfunction of the heart, eventually leading to heart failure and potentially death [3, 4]. Dox-induced muscle toxicity (DIMT) can also cause dose-dependent muscle dysfunction; however, DIMT is associated with adverse changes to skeletal muscle tissue, leading to effects such as fatigue, atrophy, and eventually muscle cell death [5–8]. Significant loss in muscle can result in decreased response to treatment, worsening of prognosis, and a reduction in quality of life [5, 7]. Although the mechanism of Dox-induced cardiotoxicity has been studied extensively, the exact mechanism responsible for DIMT in soleus muscle has yet to be fully understood.

Dox treatment causes cardiac toxicity and involves multiple mechanisms, such as induced oxidative stress, inflammation, necrosis, apoptosis, and fibrosis [1, 2]. However, it remains unknown whether Dox toxicity in skeletal muscle involves these mechanisms or if it adopts a different pathway. Therefore, we proposed an investigation of oxidative stress-

induced apoptosis in soleus muscle. Furthermore, these studies were extended to investigate mechanisms of apoptosis mediated by the Akt-mTOR pathway.

Moreover, the wnt/β-catenin pathway was traditionally viewed to serve a role in development [9]. However, the heart is now known to activate various pathways such as the wnt/β-catenin signaling pathway under states of stress [10]. During cardiac remodeling, inhibition of the wnt-signaling pathway at the soluble frizzled receptor level has been shown to be beneficial in repairing the damaged tissue [10]. This pathway has also been shown to have involvement in skeletal muscle remodeling [11]. A recent study showed a shift in skeletal muscle fiber type of both the quadriceps and soleus muscles in the dilated cardiomyopathy (DCM) mouse model, leading to skeletal myopathy [11]. Induction of tissue damage and repair also depends on specific activation of wnts. For example, wnt3a activation has been shown to induce apoptosis in the heart [12]. Therefore, we also designed the study to investigate if Dox-induced apoptosis of the skeletal muscle involves the wnt3a pathway.

Secreted frizzled-related proteins (sFRP) are considered to be antagonists of the wnt-signaling pathway [9, 13]. Therefore, these proteins can be used to inhibit the wnt signaling pathway and have been shown to be beneficial in disease states [12, 14–16]. sFRP2 is a part of the sFRP family and has been previously shown to reduce fibrosis and improve the left ventricular functionality of the heart in the rat myocardial infarction model [15]. Furthermore, sFRP2 has been shown to be a significant paracrine factor for stem cells, aiding in repair of the myocardium [16]. This suggests that if wnt signaling is involved in DIMT, this potentially could be a treatment option that is clinically significant in muscle tissue in addition to the heart.

The significant side effects of Dox give rise to an urgent need to understand the molecular mechanisms of the disease state in order to generate new treatment options and improve patients' quality of life. To the best of our knowledge, there are no studies on the role of sFRP2 in doxorubicin-induced toxicity of the soleus muscle mediated through the Akt-mTOR and wnt3a/β-catenin pathways. Therefore, this study was designed to investigate if oxidative stress-induced apoptosis is mediated through the Akt-mTOR and wnt3a/β-catenin pathways in the soleus muscle as well as to understand whether this process can be inhibited by sFRP2.

2. Materials and Methods

2.1. Study Groups. C57BL/6 mice were divided into three treatment groups: control (saline), Dox, and Dox + sFRP2, with $n = 8$ in each group. The Institutional Animal Care and Use Committee of the University of Central Florida approved the animal protocol used in this study.

2.2. Doxorubicin and sFRP2 Treatment. C57BL/6 male and female mice of eight to ten weeks of age were administered a dose of 4 mg/kg doxorubicin (Fisher Scientific, cat. number BP 2516-50) one time every other day (M, W, and F) via intraperitoneal (IP) injection, resulting in a cumulative dose of 12 mg/kg. Recombinant mouse sFRP2 (Sino Biological Inc., cat. number 50028-M08H) was reconstituted according to the manufacturer's instructions and injected via the tail vein at day one (D1) and day six (D6) after the final Dox injection at a dose of 40 μg/kg, for a cumulative dose of 80 μg/kg.

2.3. Tissue Harvest and Paraffinization. Mice were sacrificed, and bilateral soleus muscle was harvested at day 14 (D14). The left soleus was kept in RNA later, and the right soleus was kept in 10% paraformaldehyde (PFA) for storage. Paraffin blocks were made of the samples, and blocks were sectioned (5 μm) and placed on ColorFrost Plus microscope slides (Fisher Scientific, cat. number 12-550-17).

2.4. Catalase, MnSOD, and Lipid Peroxide Assays. Catalase activity was measured as previously reported [3] using a colorimetric assay kit (Abcam, cat. number ab83464). The colorimetric assay was measured at 570 nm, and these were adjusted for total protein concentration.

Manganese-containing mitochondrial superoxide dismutase (MnSOD) levels were analyzed as previously reported [3] using an assay kit (Applied Bioanalytical Labs, cat. number SOD-560). The manufacturer's protocol was followed, and the absorbance of the sample was measured at 560 nm, using a plate reader.

Lipid peroxides were analyzed following the LPO-CC assay kit per the manufacturer's instructions (Kamiya Biomedical Co., cat. number CC-004) and as previously reported by this lab [3]. This sample was analyzed at 675 nm with a Bio-Rad plate reader.

2.5. Dihydroethidium Staining. Dihydroethidium (DHE) (Invitrogen cat. number D23107) staining was performed as previously reported [17]. The samples were deparaffinized and incubated with DHE (1 μm/mL) for 15–25 minutes at room temperature in the dark. The samples were then washed with phosphate-buffered saline (PBS) and counterstained with DAPI in order to determine total nuclei count. Confocal microscopy was used for representative imaging.

2.6. Myosin Staining. Myosin staining was performed to show the soleus muscle tissue. Before staining, the sections were deparaffinized and rehydrated using sequential decreasing alcohol concentration. They were then blocked in 10% normal goat serum (NGS) for one hour and decanted thereafter. Antimyosin (raised in rabbit, Sigma-Aldrich, cat. number M7523) was added in 10% NGS at a 1 : 50 concentration. This was incubated overnight at 4°C. The sections were then washed with 1x PBS, and Alexa Fluor 488 goat anti-rabbit (Invitrogen, cat. number A11008) was added in 1x PBS for 1 hour at room temperature. Finally, the sections were washed with 1x PBS.

2.7. TUNEL Staining. TUNEL staining was performed to determine the percentage of apoptotic nuclei (TMR Red, Roche, cat. number 12156792910). Slides prepared with soleus muscle tissue sections were deparaffinized and permeabilized with proteinase K (25 μg/mL in 100 mM Tris-HCl), as previously described [17]. The sections were

counterstained and mounted with Antifade Mounting Medium with 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, cat. number H-1200) to show the total nuclei. The percentage of apoptotic cells was quantified by dividing the number of TUNEL-positive cells by the total nuclei. Confocal microscopy was used to obtain representative images of the muscle tissue.

2.8. Caspase-3 Immunohistochemistry and ELISA. Caspase-3 staining utilizing anti-caspase-3 (Rabbit, Abcam, cat. number ab13847) was performed to corroborate apoptosis' involvement in DIMT. This stain was carried out following standard staining procedures, as published previously [2]. In brief, the samples were blocked with NGS and washed with 1x PBS, and the primary antibody, anti-caspase-3 (1 : 50), was diluted in 10% NGS and incubated at 4°C overnight. The slides were washed with 1x PBS, and the secondary antibody, Alexa Fluor 568 goat anti-rabbit, was added and allowed to incubate for one hour at room temperature. The slides were washed one more time and finally were mounted and stained with DAPI.

Caspase-3 activity was analyzed using an ELISA kit from BioVision according to the manufacturer's instructions and as previously reported [17]. Soleus muscle was washed with 1x PBS and homogenized in cell lysis buffer. The sample was then centrifuged to isolate the supernatant. Then, the sample was analyzed to determine protein concentration at 405 nm using a plate reader.

2.9. pPTEN, pAKT, Wnt3A, and β-Catenin ELISA Analyses. pPTEN was analyzed using the PathScan Phospho-PTEN (Ser380) Sandwich ELISA kit (Cell Signaling Technology, cat. number 7285), as previously reported [3]. In brief, the soleus muscle tissue was homogenized. Then, protein concentration was estimated using the Bradford Assay, and the tissue was incubated for 2 hours at 37°C. Next, a colorimetric assay was performed following the manufacturer's instructions. Once complete, stop solution was added, and the samples were read at 450 nm using the ELISA plate reader. Data was expressed in arbitrary units (AU).

pAKT activity was measured using a commercially available kit (Exalpha Biologicals Inc., cat. number X1844K), as previously reported [3]. In brief, soleus muscle tissue was homogenized, and protein estimation was performed. The sample was incubated for 2 hours, and a colorimetric assay was performed per the manufacturer's instructions. Using a Bio-Rad plate reader, samples were analyzed at 450 nm. Data was expressed in AU.

A WNT3A ELISA kit (USCN Life Sciences Inc., cat. number E83155Hu) was used to determine WNT3A activity per the manufacturer's instructions. In brief, the kit's detection reagent A was added to the samples and they were incubated at 37°C for 1 hour. The solution was washed, and then detection reagent B was added and incubated for another 30 minutes at 37°C. The samples were washed again, and the substrate solution was added to the samples and incubated for 10–15 minutes at 37°C. Finally, stop solution was added, and the samples were analyzed at 450 nm using a plate reader.

β-Catenin was analyzed using a commercially available ELISA kit (Enzo Life Sciences, cat. number ADI-900-135) following the manufacturer's instructions. In brief, the samples were added to the assay buffer and allowed to sit at room temperature for 1 hour on a shaker. The primary antibody was added to each well and incubated for another hour on the shaker. The plate was washed 5 times, and 100 μL of blue conjugate was added. The plate was washed 5 more times, and 100 μL of soluble substrate was added and incubated for another 30 minutes at room temperature on the shaker. Finally, stop solution was added, and the samples were analyzed using a plate reader at 450 nm.

2.10. Western Blot Analysis of p-mTOR, sFRP2, BCL2, and BAX. sFRP2 presence was confirmed with Western blot analysis, using a standard Western blotting procedure. In brief, sFRP2 antibody (Abcam, cat. number ab111874) at a 1 : 250 concentration was analyzed with β-actin (1 : 1000) as a loading control. The secondary antibody was anti-rabbit at a concentration of 1 : 1000 for both.

Analysis of p-mTOR over total mTOR was performed using Western blot, following standard technique. Primary antibodies, p-mTOR and mTOR, were used at concentrations 1 : 1000 and 1 : 750, respectively (Cell Signaling, cat. number 2971L and 2972S). The secondary antibody for both was HRP-conjugated anti-rabbit, at concentrations of 1 : 1000 and 1 : 750, respectively.

Western blot analysis was performed to analyze BCL2, an antiapoptotic protein, and BAX, a proapoptotic protein, following a standard Western blotting procedure (Cell Signaling Technology, cat. numbers SC-492 and 2772, resp.). The primary antibodies were BCL2 and BAX, both at a 1 : 100 concentration. The secondary antibody was HRP-conjugated anti-rabbit for both BCL2 and BAX, at a concentration of 1 : 1000. Quantitative densitometry analysis of Western blotting was performed using ImageJ software (NIH).

2.11. Statistical Analysis. The data is expressed as mean ± SE. Statistical significance was determined when $p < 0.05$, using one-way ANOVA and Tukey's test.

3. Results

3.1. Effects of sFRP2 on Oxidative Stress (Lipid Peroxidases) and Antioxidants (MnSOD and Catalase). Figure 1(a) shows quantitative ELISA analysis of an oxidative stress marker, lipid peroxidase. Dox treatment shows a significant increase of lipid peroxidases; however, this increase was significantly decreased by sFRP2 treatment (Figure 1(a), $p < 0.05$). Furthermore, we performed ELISAs to detect the levels of antioxidants, MnSOD and catalase. Following Dox treatment, there was a decrease in antioxidants significantly, whereas sFRP2 treatment significantly increased MnSOD and catalase (Figures 1(b) and 1(c), $p < 0.05$). This data suggests that sFRP2 treatment improves antioxidant levels in Dox-treated soleus muscle (Figures 1(b) and 1(c), $p < 0.05$).

3.2. Effects of sFRP2 Treatment on Oxidative Stress Marker DHE. Figure 2(a) shows staining for total nuclei in blue with DAPI (A, D, and G), DHE stain in red to determine

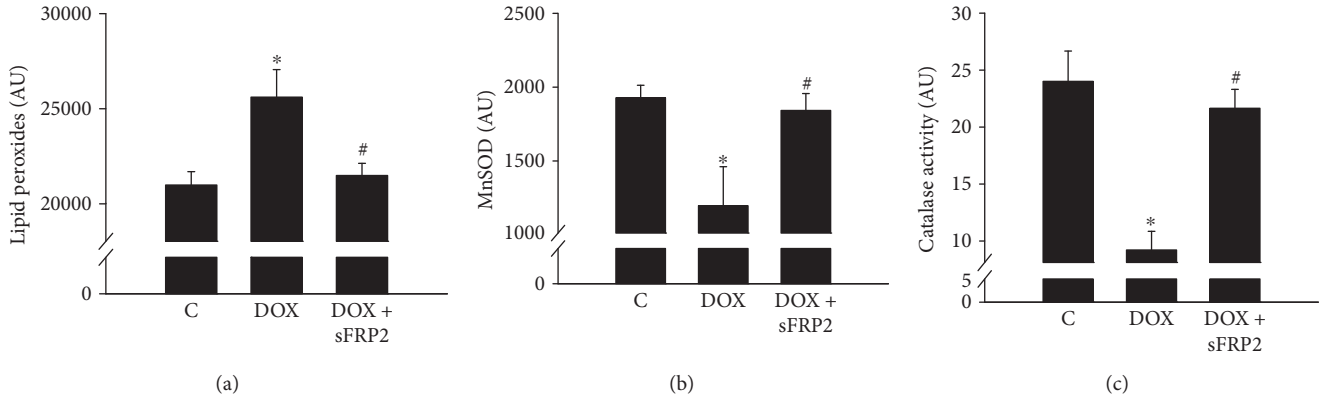

Figure 1: Effect of sFRP2 treatment on lipid peroxides, superoxide dismutase, and catalase activity. Figure 1 shows quantitative data from the ELISA kits for lipid peroxides (a) to determine oxidative injury to the muscle, MnSOD (b) to determine the presence of the antioxidant superoxide dismutase, and (c) to determine the presence of the antioxidant, catalase. Units represented in arbitrary units. $^{*}p < 0.05$ compared to control, and $^{\#}p < 0.05$ compared to the Dox group. $n = 4$-5 for lipid peroxides, $n = 5$-6 for MnSOD, and $n = 6$ for catalase activity.

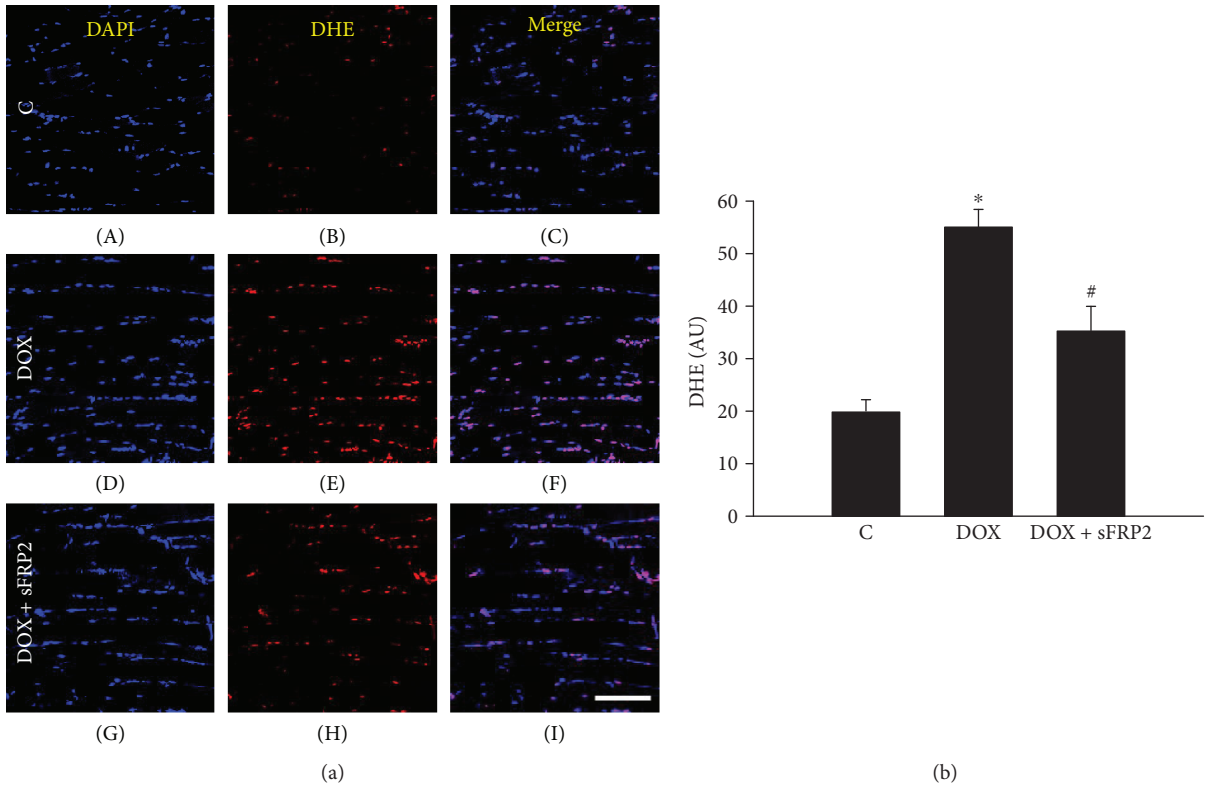

Figure 2: Significant decrease in DHE-positive cells post-sFRP2 treatment. (a) shows DAPI staining to determine the total number of nuclei in (A, D, and G), DHE staining to measure oxidative stress levels in (B, E, and H), and the merged photomicrographs (C, F, and I). (b) shows the quantitative immunohistochemistry data for the DHE staining. Units represented in arbitrary units. $^{*}p < 0.05$ compared to control, and $^{\#}p < 0.05$ compared to the Dox group. Scale for A is 100 μm. $n = 4$-5.

superoxide levels (B, E, and H), and the merged images (C, F, and I). Quantitative analysis of DHE-positive cells shows that with treatment of Dox, superoxide levels significantly increased (Figure 2(b), $p < 0.05$). This significant increase was attenuated with sFRP2 treatment, further suggesting that sFRP2 treatment inhibits increased oxidative stress (Figure 2(b), $p < 0.05$), in a similar fashion observed with lipid peroxidase in Figure 1(a).

3.3. Effects of sFRP2 on Apoptosis and Caspase-3 Activity. Figure 3(a) shows detection of apoptosis by TUNEL staining. The muscle tissue is stained for myosin in green in A, E, and

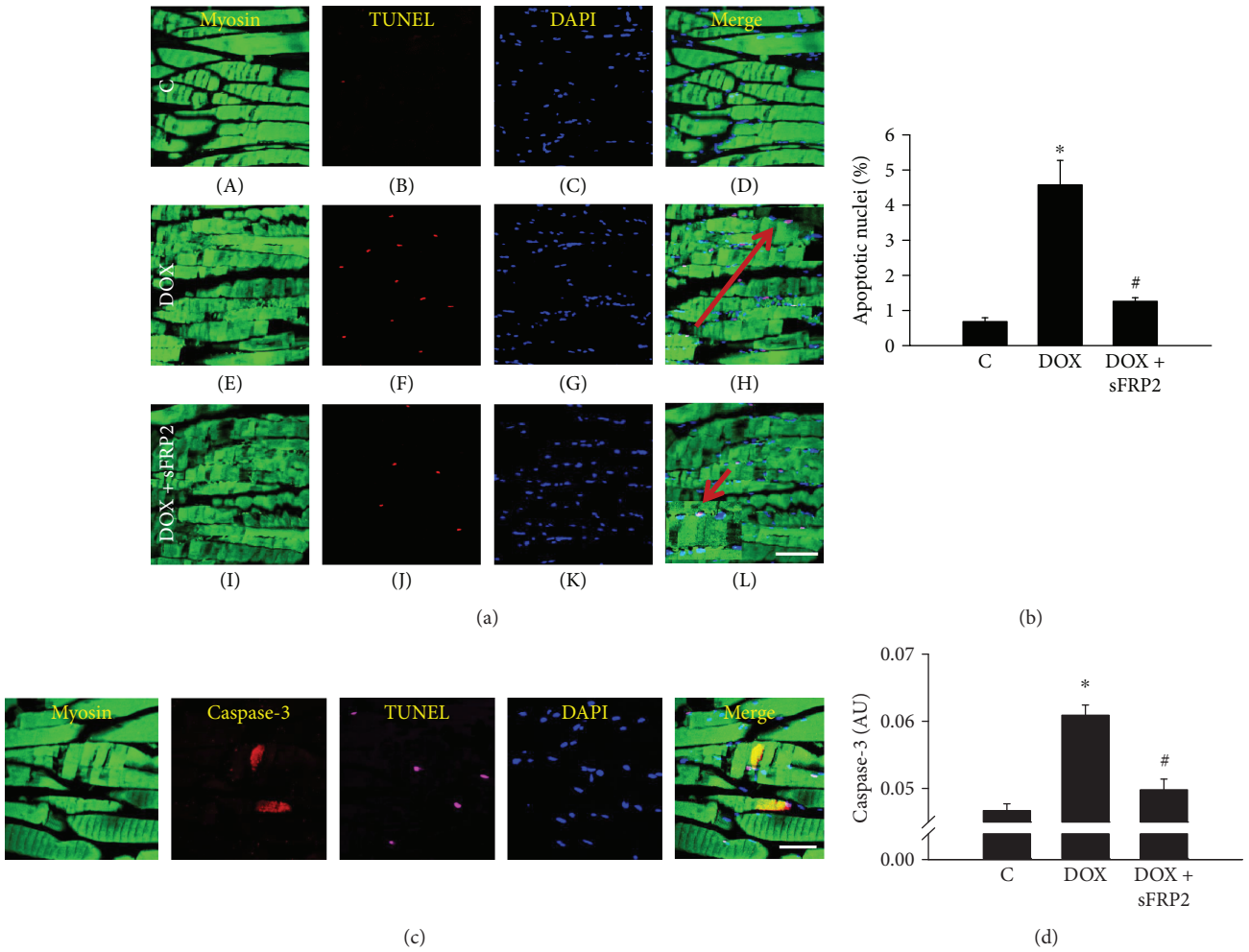

Figure 3: sFRP2 treatment decreases caspase-3 activity and inhibits apoptosis. (a) shows representative imaging of soleus muscle. The muscle has been stained with antimyosin (A, E and I), TUNEL to confirm apoptosis (B, F, and J), and DAPI to determine total nuclei (C, G, and K), and the merged images of all staining can be seen (with enlargements, denoted by a red arrow) in (D, H, and L). (b) shows a graph of the quantitative data from immunohistochemistry for the percentage of apoptotic nuclei. (c) shows a stain of the soleus muscle using antimyosin, caspase-3, TUNEL, and DAPI, from left to right. (d) shows the quantitative results from an ELISA kit for caspase-3 activity, a key mediator in apoptosis. Units represented in percentage of apoptotic nuclei in (b) and in arbitrary units for caspase-3 activity in (d). $^{*}p < 0.05$ compared to control, and $^{\#}p < 0.05$ compared to the Dox group. Scale for (a) is 100 μm. Scale for (c) is 50 μm. $n = 5$-6 for apoptotic nucleus percentage, and $n = 7$-8 for caspase-3 activity.

I; the apoptotic nuclei are stained in red as seen in B, F, and J; total nuclei are stained in C, G, and K; and the merged images are seen in D, H, and L (Figure 3(a)).

Quantitative analysis of the apoptotic nuclei was obtained. Figure 3(b) shows a graph of the percentage of apoptotic nuclei. Our data shows a significant increase in the number of apoptotic nuclei in the Dox treatment group compared to the control; however, this increase was significantly decreased following treatment with sFRP2 ($p < 0.05$).

An additional staining was performed to determine the presence of caspase-3 in apoptotic muscle, as seen in Figure 3(c). From left to right, myosin, caspase-3, TUNEL, DAPI, and merged images show that the TUNEL-positive soleus muscle is also positive with caspase-3, suggesting that Dox-induced apoptosis does occur in the soleus muscle. Moreover, we performed a caspase-3 ELISA to quantify apoptosis in these soleus muscle cells (Figure 3(d)). Figure 3(d) shows a significant increase in caspase-3 activity following treatment with Dox; however, this increase in caspase-3 activity was attenuated with sFRP2 treatment ($p < 0.05$). Noticeably, our TUNEL staining corresponds with the additional method of caspase-3 activity ELISA analysis, suggesting that apoptosis is occurring in soleus muscle and that this apoptosis is attenuated by sFRP2.

3.4. Effects of sFRP2 on Proapoptotic Protein BAX and Antiapoptotic BCL2. Figure 4(a) shows the Western blot on BAX and BCL2, with β-actin as the loading control. The quantitative Western blot analysis shows that Dox induces an increase in proapoptotic protein BAX and a decrease in antiapoptotic protein BCL2 ($p < 0.05$). The BAX levels significantly decreased after sFRP2 treatment, and BCL2 level

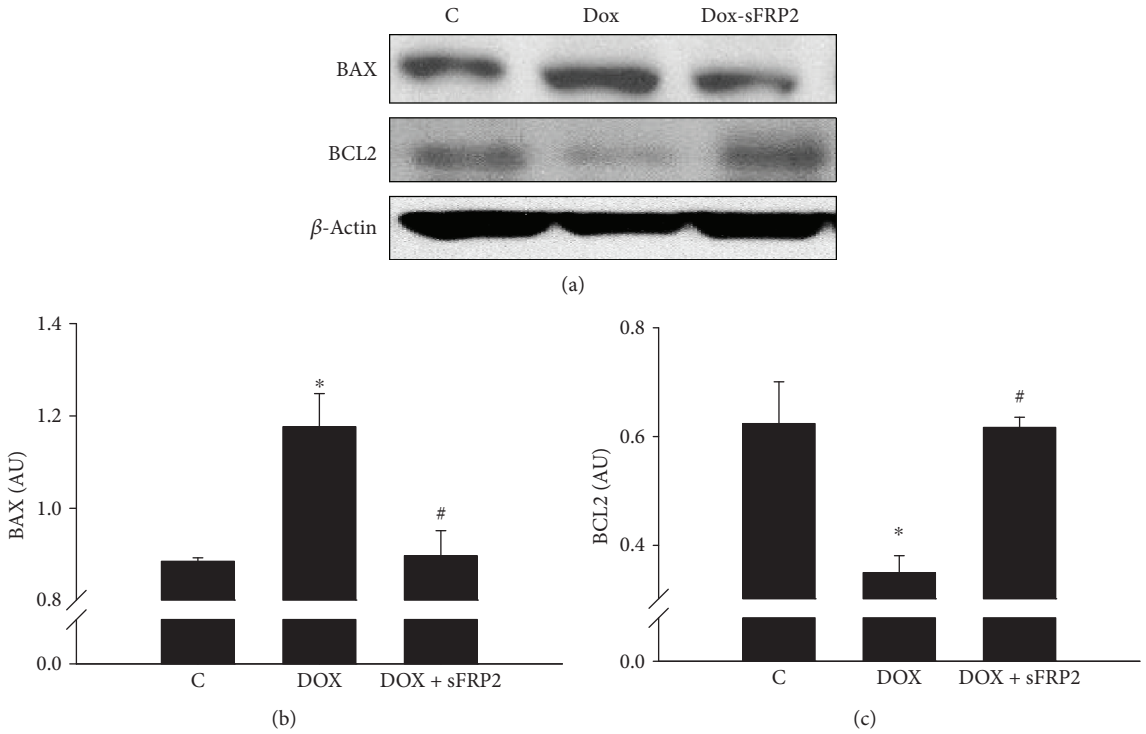

Figure 4: sFRP2 treatment post-administration of Dox decreases proapoptotic protein BAX and increases antiapoptotic protein BCL2. Figure 4 shows a Western blot analysis of BAX (proapoptotic), BCL2 (antiapoptotic), and β-actin as the control. Qualitative data can be seen in (a), and quantitative data in the form of graphs can be seen below for BAX in (b) and BCL2 in (c). Units represented in arbitrary units. $^{*}p < 0.05$ compared to control, and $^{\#}p < 0.05$ compared to the Dox group. $n = 3$ for both BAX and BCL2.

significantly increased compared to the Dox group (Figures 4(b) and 4(c), resp., $p < 0.05$), suggesting that sFRP2 inhibits apoptosis in the soleus muscle induced by Dox.

3.5. sFRP2 Treatment on PTEN, AKT, and mTOR. Figure 5 shows the quantitative data for ELISAs performed on pPTEN and pAKT, whereas Western blot analysis was performed to detect p-mTOR. When treated with Dox, the pPTEN levels significantly increased; however, this increase was mitigated by the addition of sFRP2 (Figure 5(a), $p < 0.05$). In contrast, when treated with Dox, the pAKT levels significantly decreased, and this decrease was then attenuated by the addition of sFRP2 (Figure 5(b), $p < 0.05$). The Western blot in Figure 5(c) shows a significant decrease in p-mTOR, whereas total mTOR was used as a loading control. Densitometry quantification shows a significant decrease in p-mTOR with Dox treatment; however, this decrease in p-mTOR was significantly increased following sFRP2 treatment ($p < 0.05$). This data suggests involvement of the Akt-mTOR pathway in Dox-induced apoptosis in the soleus muscle.

3.6. Effects of sFRP2 Treatment on Wnt3a and β-Catenin. Figure 6 shows quantitative ELISA analysis of wnt3a and β-catenin. Our data shows a significant increase in both wnt3a and β-catenin with Dox treatment; however, this increase was reduced with sFRP2 treatment (Figures 6(a) and 6(b), resp., $p < 0.05$). This is indicative of the wnt3a/β-catenin pathway's involvement in Dox-induced cytotoxicity of the soleus muscle and that sFRP2 attenuates the wnt3a/β-catenin pathway.

3.7. Determining Levels of sFRP2 in Soleus Muscle. Figure 7(a) shows Western blot analysis of sFRP2's presence in the soleus muscle tissue, with and without Dox treatment, with β-actin as the loading control. Our data shows a decrease in sFRP2 in the Dox treatment group compared to the control (Figure 7(b), $p < 0.05$). This decrease in sFRP2 was attenuated following administration of sFRP2 (Figure 7(b), $p < 0.05$).

We have also developed a flow chart to demonstrate that the wnt3a and Akt-mTOR pathways are involved in Dox-induced apoptosis of the soleus muscle.

4. Discussion

Doxorubicin is a chemotherapeutic drug known to induce cardiotoxicity and myotoxicity as major side effects [2, 18, 19]. A previous study has reported that when treated with Dox, the skeletal muscle gives rise to increased muscle fatigue and reduced blood flow, interferes with actin-myosin interaction and contractile alterations, and results in overall lower functionality [19]. The aforementioned adverse effects warrant study for potential therapeutics to attenuate muscle toxicity induced by doxorubicin. The major new and important information in the present work, following Dox toxicity in the soleus muscle and protective effects of sFRP2 treatment, includes (1) a decrease in oxidative stress markers, lipid

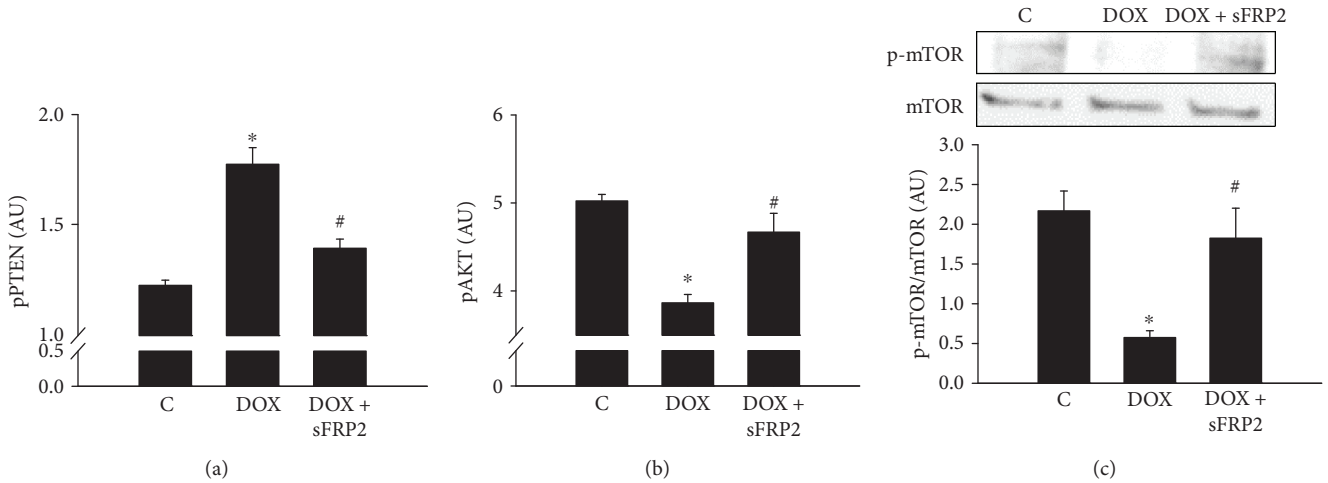

Figure 5: sFRP2 treatment decreases pPTEN levels and increases pAKT and p-mTOR/mTOR. Figure 5 shows the quantitative results of the ELISA kits for pPTEN (a) and pAKT (b). (c) shows analysis of p-mTOR over total mTOR activity using Western blot. Units represented in arbitrary units. $^{*}p < 0.05$ compared to control, and $^{\#}p < 0.05$ compared to the Dox group. $n = 7$ for pPTEN and $n = 6$-7 for pAKT. $n = 4$-5 for p-mTOR/mTOR analysis.

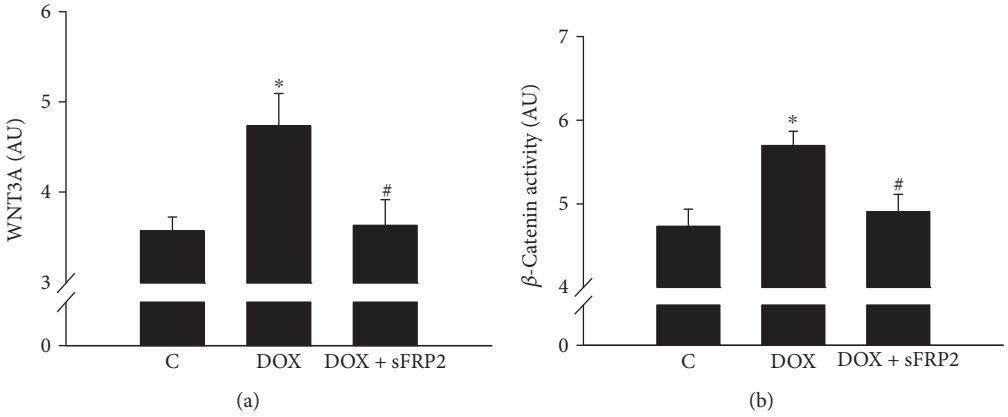

Figure 6: Wnt3a and β-catenin decrease with sFRP2 administration. Figure 6 shows graphs for the quantitative data from the ELISA assay kits for wnt3a to determine the involvement of the wnt signaling pathway and β-catenin to determine levels of this signaling molecule (a and b, resp.). Units represented in arbitrary units. $^{*}p < 0.05$ compared to control, and $^{\#}p < 0.05$ compared to the Dox group. $n = 6$-7 for wnt3a, and $n = 6$ for β-catenin activity.

peroxide, and DHE; (2) an increase in antioxidant levels of MnSOD and catalase; (3) a decrease in soleus muscle cell apoptosis; (4) a decrease in proapoptotic protein BAX and an increase in antiapoptotic protein BCL2; (5) a decrease in the negative regulation of PTEN and an increase in cell survival proteins pAKT and p-mTOR; (6) effects on the wnt3a/β-catenin pathway by decreasing wnt3a and β-catenin activity; (7) and, finally, an improvement of the decrease seen in endogenous sFPR2 in Dox-treated animals. The findings that sFRP2 inhibits oxidative stress and improves antioxidant levels in the soleus muscle are consistent with other published studies on decreased oxidative stress and apoptosis in skeletal muscle following exercise, as reported by Smuder et al. [20, 21]. Moreover, the role of increased oxidative stress and decreased antioxidant reserve following muscle injury in Duchenne muscular dystrophy (DMD) has also been reported, which is in agreement on the alteration of oxidative and antioxidant defense following Dox toxicity in muscle [22–25]. It has been shown in muscle atrophy and muscle inactivity studies that there is an increase in reactive oxygen species (ROS) and a decrease in antioxidants [26–29]. In contrast, recent data in a denervation study shows that antioxidant genes increase immediately as a result of increased ROS, suggesting that muscular atrophy and weakness is independent of the oxidative stress pathway [28]. Moreover, they further confirmed that the process is not mediated through oxidative stress, as antioxidants trolox and resveratrol were shown not to have an effect on oxidative stress-induced atrophy and muscle weakness [28]. Our data differed with this denervation study with respect to antioxidants and lipid peroxidase determination. For example, in their study,

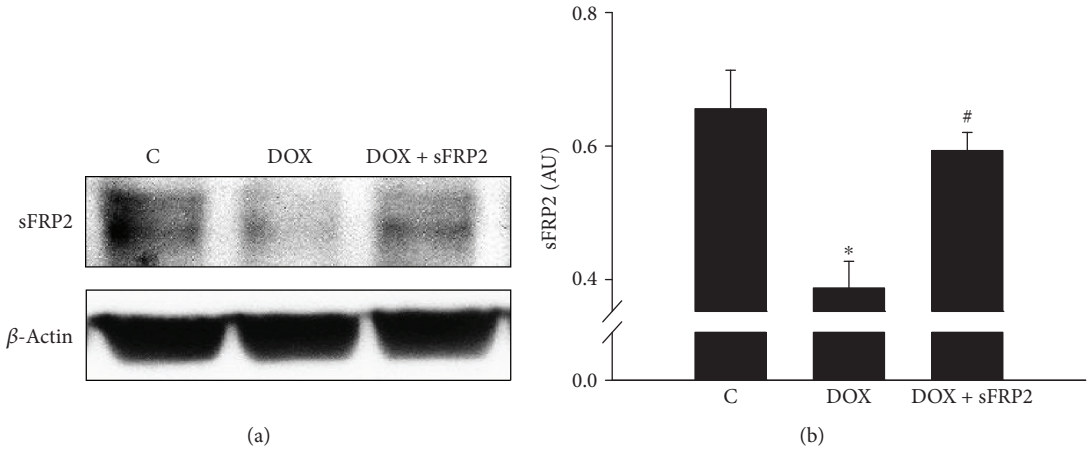

Figure 7: Endogenous presence of sFRP2 in soleus muscle. (a) shows a qualitative Western blot of sFPR2 with β-actin as the loading control. To the right of this Western blot picture in (b) is the quantitative results from the sFRP2 densitometry analysis using ImageJ software (NIH), confirming its presence in the soleus muscle. Units represented in arbitrary units. $^{*}p < 0.05$ compared to control, and $^{\#}p < 0.05$ compared to the Dox group. $n = 4$-5 for sFRP2.

lipid peroxidase levels were not assessed related to oxidative stress and antioxidant levels were not changed [28], whereas our data in the DIMT model shows a significant increase in lipid peroxide levels as well as a decrease in the antioxidants in the Dox-treated group. Therefore, it is anticipated that the role of oxidative stress in muscle injury is mediated through two independent pathways: one mediated through increased oxidative stress and decreased antioxidant reserves as reported in the current study, and another through nonoxidative stress in the denervation study, as published previously [28]. Overall, our data is also consistent with Dox-induced cardiotoxicity and in various other muscle conditions such as atrophy that involve changes in oxidative stress and antioxidants [2–4, 15, 16, 18, 27, 30]. Moreover, we suggest that sFRP2, which has never been reported before, could be a potential target to decreasing oxidative stress and increasing antioxidant reserves following Dox treatment.

Apoptosis is a programmed cell death mediated by mitochondrial proteins, caspase-3 and -9, which has been reported in various skeletal muscle diseases such as exercise-induced muscle damage, dystrophinopathies, inflammatory myopathies, ischemic atrophy, and spinal muscular atrophy [31–36]. The presence of apoptosis is confirmed by TUNEL, BAX, and BCL2 staining, and this apoptosis significantly contributes to the development and progression of these disease states [32]. Moreover, a recent study shows that Dox induces apoptosis in rat soleus muscle [20, 21]. Data from another recent study shows that TNF-α-induced apoptosis in C2C12 myoblast cells involves a decrease in the BCL2 to BAX ratio, which is consistent with our data [37]. The current study corroborates the previously published muscle studies on Dox-induced muscle apoptosis. The presence of apoptosis in the current study is also confirmed by TUNEL staining, caspase-3 activity, proapoptotic BAX, and antiapoptotic BCL2, which is in agreement with other studies published on muscle apoptosis [32]. Moreover, the presence of apoptosis in muscle cells was further confirmed for caspase-3 and TUNEL occurring in single muscle cells, as confirmed with muscle-specific protein, myosin, and nuclear stain, DAPI (Figure 3). Therefore, this data confirms that apoptosis is present in injured soleus muscle cells following Dox treatment.

Next, we confirmed whether apoptosis in the soleus muscle is mediated through the Akt-mTOR pathway and/or the wnt3a/β-catenin pathway. The Akt-mTOR pathway plays a major role in cell processes such as cell proliferation, survival, growth, and death [3, 16, 30, 38, 39]. An *in vivo* study published in Nature Cell Biology suggests that the Akt-mTOR pathway plays a major role in skeletal muscle hypertrophy, where an increase in activity of this pathway resulted in decreased muscle atrophy [38]. Additionally, the role of Akt-mTOR pathway activation has also been shown to play a role in decreasing DMD-associated fibrosis and inflammation [39]. Additionally, a previous study has shown that the PI3K/Akt/mTOR pathway is involved in Dox-induced apoptosis in the heart and the process is inhibited by transplantation of embryonic stem cells [3]. However, the role of the Akt-mTOR pathway in Dox-induced apoptosis in soleus muscle is not well defined. Therefore, in the present study, as per the best of our knowledge, we are the first to report that pAKT and p-mTOR decrease significantly compared to the control following Dox treatment, whereas sFRP2 treatment significantly ($p < 0.05$) increases Akt and mTOR, suggesting involvement of the Akt-mTOR pathway in DIMT of the soleus muscle.

PTEN, an endogenous inhibitor of the Akt pathway, has been shown to be modulated in different muscle and cardiac diseases [3, 40]. A significant increase in PTEN and apoptosis was observed in insulin-resistant skeletal muscle cells following insulin stimulation [40]. This increase in PTEN was suppressed when cells were treated with metformin, a common drug for diabetes, suggesting that PTEN regulates apoptosis in this insulin-resistant muscle model [40]. In agreement with these studies, PTEN in the current study was significantly increased ($p < 0.05$) in Dox-treated mice along with apoptosis, whereas PTEN and apoptotic levels were significantly attenuated following administration of sFRP2 ($p < 0.05$). This data suggests that the PTEN pathway

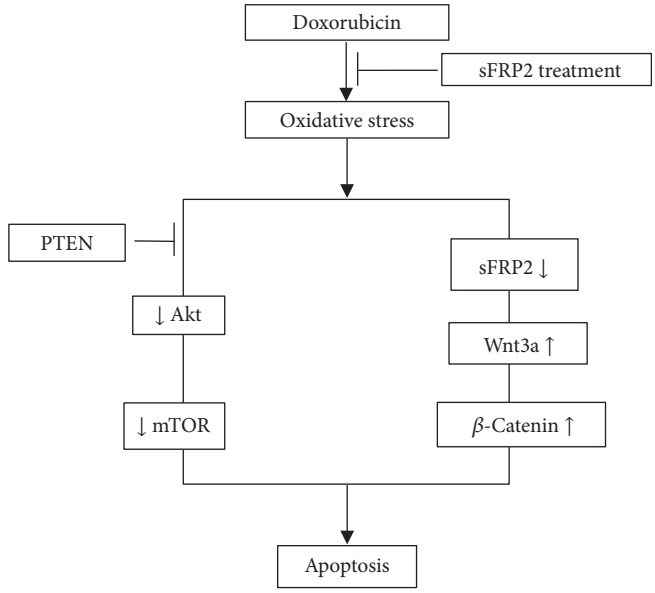

Figure 8: sFRP2 treatment inhibits oxidative stress induced by doxorubicin. Figure 8 is a flow chart that describes the effects of sFRP2 treatment on the two pathways involved in doxorubicin-induced apoptosis: Akt/mTOR and Wnt/β-catenin.

is involved in regulation of apoptosis mediated through the Akt-mTOR pathway in soleus muscle.

The wnt family consists of 19 members with discrete types of cellular functions, such as stem cell differentiation, myogenesis, cell survival, muscle fibrosis, and apoptosis, in organ development [41–44]. The function of wnts depends on their isoform, type of injury, and organ development [44]. Wnt3a treatment increases deposition of connective tissue in the muscle [43, 44]. Moreover, mouse embryos that were deficient in wnt1 and wnt3a demonstrate abnormal growth and a reduction in expression of muscle protein, Myf5 [44, 45]. Additionally, wnt3a has been published in previous studies of myocardial infarction-induced apoptosis, where increased levels of wnt3a were observed after the infarct was generated, whereas sFRP2 administration attenuated this wnt3a activity [15]. Noticeably, there is no published report on wnt3a and sFRP2 in DIMT. Therefore, our data on the increase in apoptosis as well as wnt3a and β-catenin activity is in agreement with the published myocardial infarction model and provides novel information in Dox-induced muscle toxicity. Moreover, the current study shows that an increase in wnt activity was significantly attenuated following treatment with sFPR2, which is also in agreement with studies published in the heart, and in some forms of cancer such as medulloblastoma [15, 46].

Next, we examined whether Dox treatment decreases exogenous levels of sFRP2 in soleus muscle, which may play a role in the increased oxidative stress and apoptosis seen in DIMT. Our data in Figure 7 shows that Dox treatment significantly decreases sFRP2 in soleus muscle, whereas treatment with sFRP2 injection brings back levels of this protein close to control values ($p < 0.05$). This suggests that baseline levels of sFRP2 are present in healthy skeletal muscle and play a protective role against various muscle disorders.

There are published studies that show significant decrease in muscle mass in early and late stages of cancer progression that ultimately leads to a decrease in muscle function [47–50]. It has been observed that this process can be mediated through inflammation from presence of a tumor [47, 48]. Interestingly, a recent study by Yu et al. report that the compound ghrelin was shown to inhibit Dox-induced apoptosis in the gastrocnemius muscle, suggesting a therapeutic role in the associated cancer cachexia [51]. Noticeably, we are suggesting in this study that sFRP2 inhibits apoptosis in soleus muscle, which could be a part of induced muscle cachexia as Yu et al. also report apoptosis in the gastrocnemius muscle. Based on our data on the decrease of apoptosis in Dox-induced muscle cachexia, we anticipate that sFRP2 could be a potential therapeutic target for cancer cachexia that is formed due to tumor formation.

In conclusion, our data suggests that Dox induces oxidative stress and apoptosis and that the process is mediated through the Akt-mTOR and wnt3a/β-catenin pathways. We also presented a flow chart in Figure 8 to depict involvement of two pathways in Dox-induced muscle toxicity and apoptosis: Akt-mTOR and wnt3a/β-catenin. Interestingly, the link of wnt7a binding to frizzled protein-7 that activates the PI3K-Akt-mTOR pathway has been reported [44, 52]. However, in the current study we do not provide a link between wnt3a and the Akt-mTOR pathway in the regulation of apoptosis, which we propose as a future study by us or others. Finally, further studies in large animals are needed to confirm these findings, so that sFRP2 can be potentially used in the clinical setting.

Data Availability

The data generated in the present study is available upon request.

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgments

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Research Article

Implication of the PI3K/Akt/mTOR Pathway in the Process of Incompetent Valves in Patients with Chronic Venous Insufficiency and the Relationship with Aging

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Chronic venous insufficiency (CVI) is a multifactorial disease, commonly caused by valvular incompetence (clinically diagnosed by venous reflux) and venous hypertension. The incidence of these factors clearly increases with patient age, and aging is one of the risk factors involved. The activity of the PI3K/Akt/mTOR pathway is considered fundamental in vascular pathologies, and understanding its involvement would help in the development of possible therapeutic targets. This is an observational, analytical, and prospective cohort study that reviewed 110 patients with CVI scheduled to undergo stratified saphenectomy. They were distributed according to the presence (R = 81) or absence (NR = 29) of valvular incompetence (venous reflux) diagnosed clinically. Each of the groups was further divided according to age, with a cutoff point of 50 years (NR < 50 = 13, NR ≥ 50 = 16, R < 50 = 32, and R ≥ 50 = 49). The involvement of the PI3K/Akt/mTOR pathway, as well as that of HIF-1α and HIF-2α and of CD4+, CD8+, and CD19+ cells and mastocytes, was assessed. Saphenous vein tissue samples obtained during surgery were processed for RT-qPCR and immunohistochemistry. Patients with venous reflux showed a significant increase in mRNA and protein expression levels for PI3K/mTOR and HIF-1α/HIF-2α. The number of mast cells was significantly elevated in the R group. In distribution by age, PI3K/Akt/mTOR and HIF-1α were significantly higher in R < 50 patients. Furthermore, these patients had a significant increase in the number of CD4+, CD8+, and CD19+ cells and mastocytes in the saphenous vein wall. These findings provide a basis for the possible existence of changes in PI3K/Akt/mTOR pathway expression in young patients, with potential accelerated asynchronous aging that is enhanced by CVI.

1. Introduction

Venous pathology develops when venous pressure increases and blood return is disrupted. The peripheral venous system functions as a conduit that returns blood to the heart. Proper functioning of this system depends on the venous valves and the muscular pump [1]. Chronic venous insufficiency (CVI) is a multifactorial disease, commonly caused by valvular incompetence (clinically visible due to the presence of venous reflux) and venous hypertension [2]. The incidence of varicose veins per year according to the Framingham study is 2.6% in women and 1.9% in men [3]. The incidence clearly increases with patient age, family history of CVI, and history of multiple pregnancies [4]. Aging is considered one of the risk factors involved in this pathology [5]. Pocock et al. [6] reported that cellular events have a special importance in the pathophysiology of CVI and are a starting point for the response and progression of this pathology.

PI3K/Akt/mTOR pathway activity is reported by numerous researchers to be fundamental in vascular pathologies [7, 8]. Classically, PI3K activity has been considered a hallmark of the oncogenic process [9]. Parallel to research by cancer biologists, research in other fields has uncovered disturbing and often unpredictable roles of PI3K in normal cell function and disease [10, 11]. Many of these functions affect cellular homeostasis by influencing cellular dynamics [12]. PI3K induces the phosphorylation of secondary proteins, triggering the recruitment of cytoplasmic proteins [13]. This event activates the cellular PI3K/Akt/mTOR signaling pathway, which is an important intracellular signaling pathway in cell cycle regulation. Therefore, it is directly related to cellular quiescence, proliferation, cancer, and longevity [14, 15]. Balancing the appropriate amount of proliferation versus differentiation is challenging and motivates the need for research to determine this balance for use in the development of different therapies [16].

Varicose veins develop through a series of gradual stages; it is possible to consider the development of venous wall insufficiency as an aging process. Generally, this condition proceeds according to the age of the individual, especially after the fifth decade of life [17]. With aging comes changes in the structure of the venous wall induced by alterations in the connective tissue [18]. This situation is related to loss or poor organization of elastic fibers and muscles of the middle layer, which facilitates venous dilation [17–19]. CVI has been demonstrated in young patients and can be considered a premature and accelerated asynchronous aging process [6, 18, 20]. These young CVI patients may possess a specific genetic background, but no studies on valvular incompetence have been reported. Therefore, in young people with CVI or with certain genetic susceptibility, valvular incompetence becomes an important risk factor. Considering previous research, the PI3K/Akt/mTOR pathway can affect cellular function. Therefore, the aim of this study is to demonstrate how the pathological environment created in the process of valvular incompetence (venous reflux) development in patients with CVI is produced by a set of cellular-level events modulated by the PI3K/Akt/mTOR pathway.

2. Patients and Methods

2.1. Design of the Study. This is an observational, analytical, and prospective cohort study that reviewed patients with CVI scheduled to undergo stratified saphenectomy and divided them according to age (cutoff point of 50 years). The research was developed through a collaboration between the Service of Angiology and Vascular Surgery of the Ruber International Hospital and the Department of Medicine and Medical Specialties of the University of Alcalá. The study cohort was selected according to the following criteria. Inclusion criteria are as follows: women and men diagnosed with CVI, with and without venous reflux in the great saphenous vein; BMI ≤ 25; informed consent signed; and commitment to have a follow-up during the pre- and postoperative periods plus tissue sample collection. Exclusion criteria are as follows: patients with venous malformations or arterial insufficiency, patients who did not provide their clinical history, patients with pathology affecting the cardiovascular system (infectious diseases, diabetes, dyslipidemia, and hypertension), patients with toxic habits, and patients who doubted they could complete the full follow-up.

Each patient underwent exploration with the aid of an M-Turbo Eco-Doppler (SonoSite) transducer at 7.5 MHz. The examination of the lower limbs was performed in a standing position with the leg that was explored maintained in external rotation and supported by the contralateral leg; the study included the great saphenous axis from the inguinal region to the ankle and femoral vein. A study of the small saphenous vein and popliteal vein was also performed standing, with the back to the examiner and the body weight resting on the examined leg. A distal compression maneuver was performed. In this study, Valsalva maneuvers were performed, which when producing a proximal circulatory stop will allow exploration of venous insufficiency proximal to the detection point, as well as the identification of leakage points (it evaluates the absence of reflux in the femoral-iliac and saphenous-femoral union). The distal compression and decompression maneuver was also performed to assess the direction of the truncal venous flow, although it was not a physiological maneuver. Pathological reflux was considered when this was greater than or equal to 0.5 sec.

The present study was conducted in accordance with the basic ethical principles (autonomy, beneficence, nonmaleficence, and distributive justice), and its development followed Good Clinical Practice standards and the principles set forth in the last Declaration of Helsinki (2013) and the Convention of Oviedo (1997). The patients were duly informed, and each was asked to provide written informed consent. The project was approved by the Clinical Research Ethics Committee of the Ruber International Hospital.

2.2. Samples. Once saphenectomy was performed, the entirety of the great saphenous vein was extracted. These fragments were introduced into two different sterile tubes, one containing MEM (minimum essential medium) with 1% antibiotic/antimycotic (both from Thermo Fisher Scientific, Waltham, MA, USA) and another containing RNAlater®

Table 1: Primary antibodies that were used and their dilutions.

Antigen	Species	Clone	Dilution	Provider	Protocol specifications
Akt	Rabbit	Polyclonal	1 : 1000	Abcam (ab8805)	—
CD4	Rabbit	Monoclonal	1 : 50	Abcam (ab133616)	EDTA pH = 9 before incubation with blocking solution
CD8	Rabbit	Polyclonal	1 : 25	Abcam (ab4055)	EDTA pH = 9 before incubation with blocking solution
CD19	Mouse	Monoclonal	1 : 200	Abcam (ab31947)	—
HIF-1α	Mouse	Monoclonal	1 : 800	Abcam (ab16066)	EDTA pH = 9 before incubation with blocking solution
HIF-2α	Mouse	Monoclonal	1 : 2000	Abcam (ab8365)	EDTA pH = 9 before incubation with blocking solution
PI3K	Mouse	Monoclonal	1 : 500	Abcam (ab86714)	—
mTOR	Rabbit	Polyclonal	1 : 500	Abcam (ab1093)	—

Table 2: Secondary antibodies that were used and their dilutions.

Antigen	Species	Clone	Dilution	Provider
IgG (mouse)	Goat	Polyclonal	1/300	Sigma
IgG (rabbit)	Mouse	RG-96	1/1000	Sigma

Solution (Ambion, Austin, TX, USA). All samples are transferred refrigerated to the Department of Medicine and Medical Specialties (Faculty of Medicine and Health Sciences, University of Alcalá) for processing. In all cases, the transfer was made within four hours after the sample was taken.

2.3. Structural Studies. The samples were processed in a Telstar AV 30/70 Müller class II laminar flow hood 220 V 50 MHz (Telstar SA Group, Terrassa, Spain), thus allowing an environment of sterility. Samples conserved in RNAlater remained in 1 mL of the same solution at −80°C until further processing for analysis of gene expression. The samples conserved in MEM were destined for histological studies of venous tissue. The samples were washed/hydrated several times with MEM without antibiotic to remove blood cells and then cut into fragments that were kept in different fixatives: F13 (60% ethanol, 20% methanol, 7% polyethylene glycol, and 13% distilled H_2O). After the necessary fixing time for each fixative solution, the samples were dehydrated. At the end of the inclusion, paraffin blocks were made using molds. Once the paraffin solidified, an HM 350 S rotation microtome (Thermo Fisher Scientific) was used to obtain 5 μm thick sections on glass slides impregnated with 10% poly-L-lysine solution. Once dry, the sections were deparaffinized for 30 minutes in xylol (PanReac AppliChem, Barcelona, Spain) and then rehydrated by passing through solutions with decreasing alcohol concentrations. After rehydration, the sample sections were subjected to different staining (toluidine blue) and immunohistological processes.

Toluidine blue staining was conducted as follows: (1) staining with toluidine blue in 0.03% aqueous solution for 15 minutes, (2) washing with running water for 10 minutes, (3) dehydration in 96% alcohol for 5 minutes, (4) dehydration in 100% alcohol for 5 minutes, (5) clarification in xylol for 5 minutes, and (6) assembly with Cytoseal™ balsam. Toluidine blue is often used to identify mast cells, by virtue of the heparin present in their granules.

2.4. Immunohistochemical Studies. Antigen-antibody detection was achieved using the ABC (avidin-biotin complex) method with chromogenic peroxidase or alkaline phosphatase according to the following protocol: (1) washing the samples with PBS 1x, three passes of 5 minutes. (2) Blocking of nonspecific binding sites with BSA (bovine serum albumin) at 3% in PBS for 30 minutes at room temperature. (3) Incubation with the primary antibody (Table 1) diluted in 3% BSA and PBS overnight at 4°C. (4) Washing with PBS, three passes of 5 minutes each. (5) Incubation with the secondary antibody bound to biotin (Table 2) and diluted in PBS for 1 hour and 30 minutes at room temperature. (6) Washing with PBS, three passes of 5 minutes. (7) Incubation with the avidin-peroxidase ExtrAvidin®-Peroxidase (Sigma-Aldrich, St. Louis, MO, USA) for 1 hour at room temperature. Dilution 1/200 in PBS. In the case that the conjugate was avidin-alkaline phosphatase (ExtrAvidin-Alkaline Phosphatase, Sigma-Aldrich), it was used for 60 minutes at room temperature. Dilution 1/200 in PBS. (8) Washing in PBS, three passes of 5 minutes each. (9) (A) Development by incubation with the chromogenic substrate diaminobenzidine (Kit DAB, SK-4100) (Vector, Burlingame, CA, USA). The preparation of the chromogenic substrate is carried out immediately before development (5 mL of distilled water, 2 drops of buffer, 4 drops of DAB, and 2 drops of hydrogen peroxide). This technique results in labeling with a brown color. (B) Development with the alkaline chromogenic substrate for 15 minutes (controlling the appearance of marking under the microscope). The chromogenic substrate preparation was performed immediately before development by adding 10 mL of PBS (10 mg of α-naphthol AS-BI phosphate, 10 mg of Fast Red, and 100 μL of 0.1 M levamisole). (10) Washing with distilled water to stop the development reaction, with three 5-minute passes. (11) Contrasting the nuclei by staining with Carazzi's hematoxylin for 5–15 minutes. (12) Washing in running water for 10 minutes. (13) Mounting in aqueous medium with Plasdone. In all immunohistochemical studies, sections of the same tissue were used as a negative control, in which the incubation with the primary antibody was replaced by incubation in blocking solution.

2.5. Genetic Expression. Through real-time polymerase chain reaction (qPCR), the amount of cDNA in each sample of the gene of interest was quantified (Table 3). The results were normalized using the constitutive expression gene of

Table 3: Primers used for RT-qPCR: sequences and binding temperatures (Temp).

Gene	Sequence fwd ($5' \rightarrow 3'$)	Sequence rev ($5' \rightarrow 3'$)	Temp (°C)
GAPDH	GGA AGG TGA AGG TCG GAG TCA	GTC ATT GAT GGC AAC AAT ATC CAC T	60
Akt	TGT CTC GTG AGC GCG TGT TTT	CCG TTA TCT TGA TGT GCC CGT C	60
HIF-1α	ACG TGT TAT CTG TCG CTT TGA G	ATC GTC TGG CTG CTG TAA TAA TG	59
HIF-2α	ACC CAG TAC CAG GAC TAC AGC	GGC ACG TTC ACC TCA CAG TC	61
PI3K	CTT GCC TCC ATT CAC CAC CTC T	GCC TCT AAT CTT CTC CCT CTC CTT C	60
mTOR	ATC CAG ACC CTG ACC CAA AC	TCC ACC CAC TTC CTC ATC TC	60

GAPDH (Table 3). Primer-specific primers were designed for all genes studied using the Primer-BLAST [21] and Auto-Dimer [22] online applications. The extraction of RNA was carried out capped by the guanidine-phenol-chloroform isothiocyanate method of Chomczynski and Sacchi [23]. The qPCR was performed on a StepOnePlus™ System (Applied Biosystems-Life Technologies), using the relative standard curve method. To this end, 5 μL of each sample, previously diluted 1/20 in nuclease-free water, was mixed with 10 μL of iQ™ SYBR® Green Supermix (Bio-Rad Laboratories), 1 μL forward primer, 1 μL Ml of reverse primer, and 3 μL of DNase and RNase-free water in a MicroAmp® 96-well plate (Applied Biosystems-Life Technologies), for a total reaction volume of 20 μL. Fluorescence detection is performed at the end of each repeat (amplification) cycle and at each step of the dissociation curve. The data obtained from each gene are interpolated in a standard curve made by serial dilutions of a mixture of the study samples which is included in each plate. All tests are performed in duplicate.

2.6. Statistical Analysis and Evaluation of Expression. For statistical analysis, the GraphPad Prism® 6.0 program was used to apply the Mann–Whitney *U* test. The data are expressed as the mean ± standard deviation of the population. Significance was established at $^{*}p < 0.05$, $^{**}p < 0.005$, and $^{***}p < 0.001$. In the case that the study variables were not qualitative, a Pearson chi-square test or Fisher's exact test was used when applicable. In the case of inequality, possible confounding factors were defined for which the final analysis of the main efficacy variable was adjusted.

For each of the patients in the established groups, five sections and 10 fields per section were randomly selected and examined. The patients were described as positive when the average of the analysis of the labeled sample for each study subject was greater than or equal to 5% of the total, following the anatomopathological protocol of Ortega et al. [24]. Infiltrated cells were counted under a microscope (1000x) in 10 aleatory areas of 0.5 mm^2 per patient. All values are expressed as means ± SE. Sample observation was carried out using a Zeiss Axiophot optical microscope (Carl Zeiss, Germany) equipped with an AxioCam HRc digital camera (Carl Zeiss, Germany).

3. Results

3.1. Clinical and Demographic Characteristics. For the present study, a total of 110 patients were contacted. These patients were classified according to the absence of venous reflux (NR; $n = 29$) (51.51 ± 14.04) or the presence of reflux (R; $n = 81$) (50.09 ± 15.91). The classification according to age was established as follows: patients under 50 years of age with an absence of venous reflux (NR < 50; $n = 13$) (38.53 ± 6.21), patients greater than or equal to 50 years with an absence of venous reflux (NR ≥ 50; $n = 16$) (62.06 ± 8.54), patients younger than 50 years with the presence of venous reflux (R < 50; $n = 32$) (35.09 ± 7.31), and patients greater or equal to 50 years with the presence of venous reflux (R ≥ 50; $n = 49$) (59.98 ± 11.81).

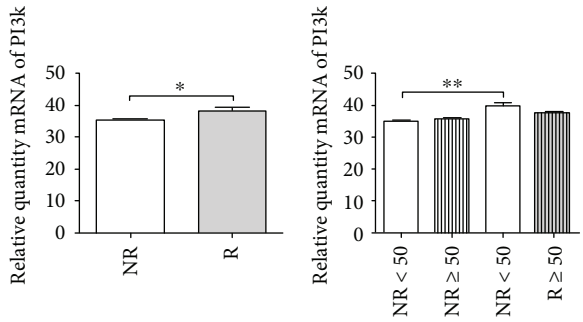
There were no significant differences in the hemogram or in the general biochemistry (data not shown). This nonsignificant relationship was maintained when the absence or presence of venous reflux was considered, as well as when age was considered. No significant differences were found in the clinical history of the patients when studying demographic factors (data not shown).

3.2. Expression of the PI3K/Akt/mTOR Pathway. The expression of the PI3K/Akt/mTOR pathway was revealed using protein and relative quantity mRNA detection techniques.

3.2.1. PI3K. The relative quantity mRNA of PI3K showed a significant increase in patients with R in comparison with the NR group ($^{*}p < 0.05$). In distribution by age, statistically significant differences were established between NR < 50 and R < 50 patients ($^{**}p < 0.005$) (Figure 1(a)).

The percentage of patients with positive protein expression based on immunohistochemical studies of PI3K expression was higher (75.31%) in the group of R individuals. Notably, NR ≥ 50 patients and R < 50 patients exhibited greater expression than the rest of the individuals, with 75.00% and 93.75%, respectively (Figure 1(b)). PI3K expression was distributed in the vein wall and was present throughout the NR < 50 and NR ≥ 50 patients (Figure 1(c), A and B).

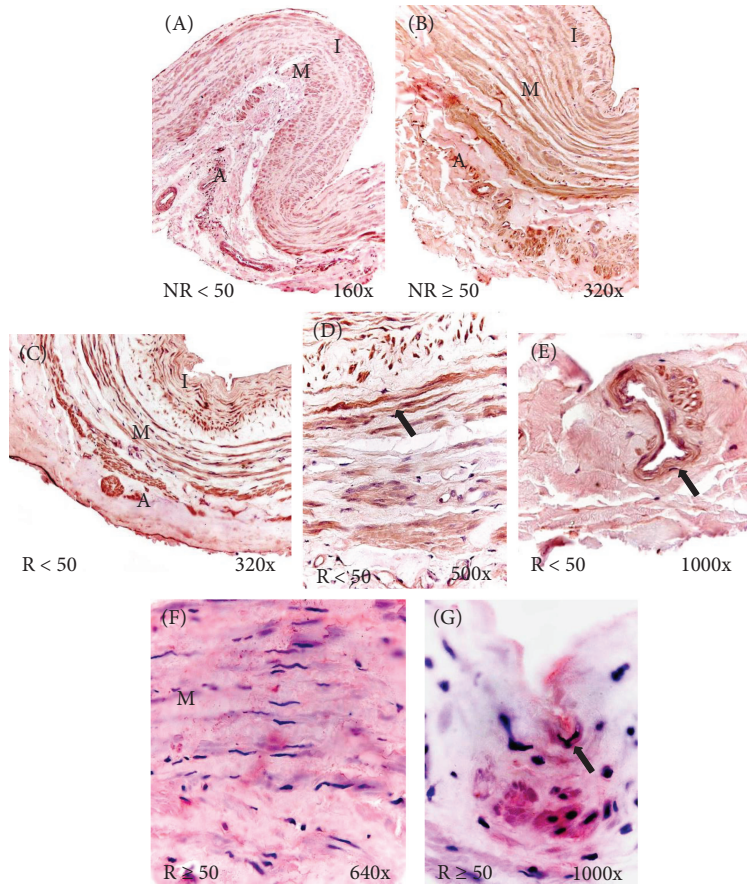
Patients with venous reflux exhibited differential expression depending on their age. In the R < 50 patients, PI3K expression was observed throughout the entire vein wall (Figure 1(c), C) and was most intense in the smooth muscle bundles of the tunica media (Figure 1(c), D arrow) and in the venula of the tunica adventitia (Figure 1(c), E arrow). In the case of the R ≥ 50 patients, lower PI3K expression intensity was observed. The expression in this group of patients presented as small heterogeneous accumulations along the wall of the vein (Figure 1(c), F), which were



(a)

% positive expression for PI3K (n)			
NR	65.52 (19)	NR < 50	53.85 (7)
		NR ≥ 50	75.00 (12)
R	75.31 (61)	R < 50	93.75 (30)
		R ≥ 50	63,26 (31)

(b)



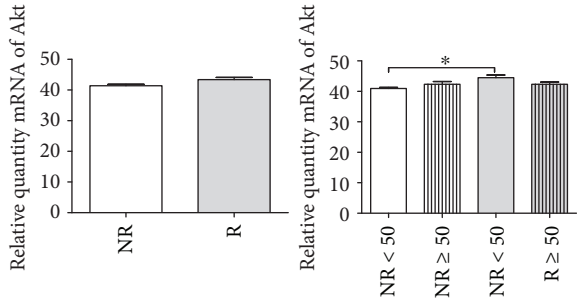
(c)

Figure 1: (a) Significant levels of mRNA for PI3K quantified by RT-qPCR in R and R < 50 patients. Results were normalized to that of the reference gene GAPDH and are provided in arbitrary units. NR = no reflux; R = reflux. $^{*}p < 0.05$ and $^{**}p < 0.005$. (b) Distribution of the percentage of patients with positive protein expression for PI3K in NR and R patients and by age. n = number of patients. (c) A-B: Histological images for PI3K protein expression in the different tunicae of venous wall in NR < 50 (160x) and NR ≥ 50 patients (320x). C-D: Images of PI3K expression of R > 50 throughout the vein wall (C, 320x) smooth muscle bundles (arrow) in the tunica media (D, 500x) and in the venula (arrow) of the tunica adventitia (E, 1000x). F-G: PI3K protein expression images in R ≥ 50 patients along the wall of the vein (F, 640x) and in the insertion areas of the venous valves (G, 1000x). The red coloration indicates the specific precipitate that correlates with the expression of the said protein. A = tunica adventitia; M = tunica media; I = tunica intima.

especially intense in the insertion areas of the venous valves (Figure 1(c), G arrow).

3.2.2. Akt. mRNA expression did not reveal any significant differences between the study groups (NR versus R). The distribution by age showed a significant increase in R < 50 patients ($^{*}p < 0.05$) (Figure 2(a)).

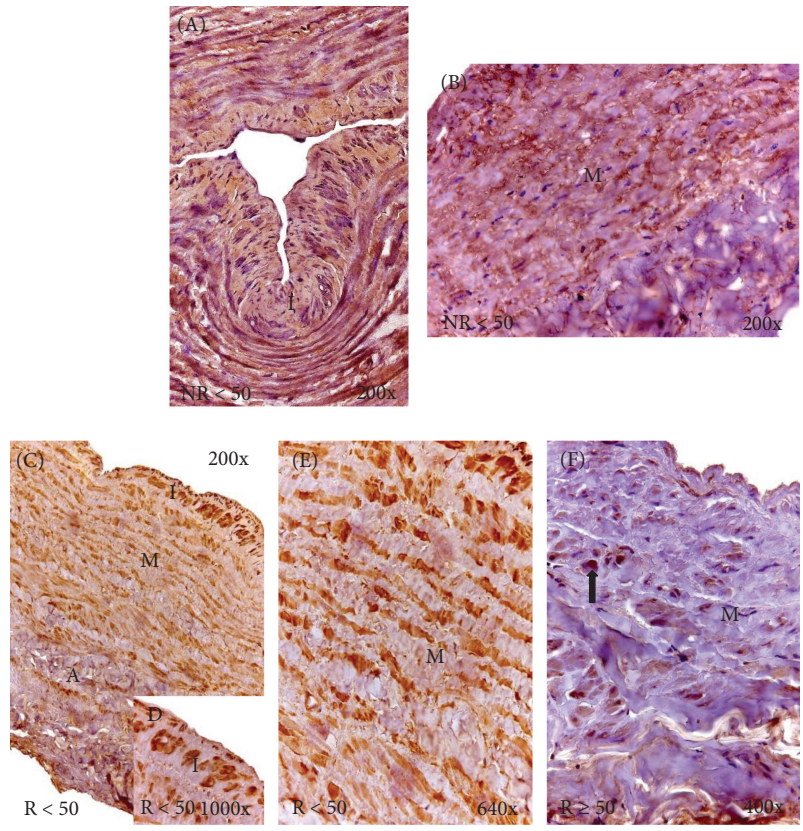
The percentage of Akt protein expression was not different between NR (79.31%) and R (75.31%) individuals. Remarkably, R < 50 patients had the highest percentage of protein expression at 96.88% (Figure 2(b)). In the NR < 50 patients, Akt expression was distributed by the three tunicae of the venous wall (Figure 2(c), A). In the case of the NR ≥ 50 patients, the percentage of individuals with



(a)

% positive expression for Akt (n)			
		NR < 50	76.92 (10)
NR	79.31 (23)		
		NR ≥ 50	81.25 (13)
		R < 50	96.88 (31)
R	75.31 (61)		
		R ≥ 50	61.22 (30)

(b)



(c)

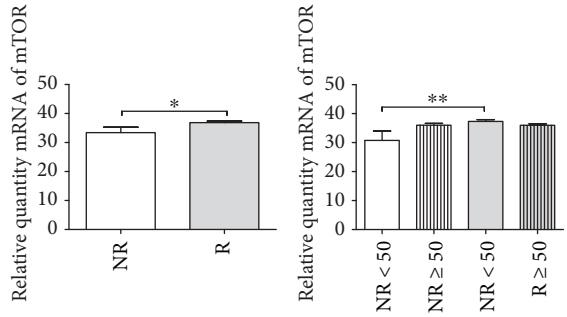
Figure 2: (a) Significant levels of mRNA for Akt quantified by RT-qPCR. In R < 50 patients, results were normalized to that of the reference gene GAPDH and are provided in arbitrary units. NR = no reflux; R = reflux. $^{*}p < 0.05$. (b) Distribution of the percentage of patients with positive protein expression for Akt in NR and R patients and by age; n = number of patients. (c) A–C: Histological images for Akt protein expression in the different tunicae of venous wall in NR < 50 (200x), NR ≥ 50 (200x), and R < 50 patients (200x). D-E: Detail of expression for Akt at greater magnification in R < 50 for tunica intima (640x) and tunica media (640x). F: R ≥ 50 patients show a heterogeneously Akt protein expression as small accumulations (arrow) in the tunica media (400x). A = tunica adventitia; M = tunica media; I = tunica intima. The brown coloration indicates the specific precipitate that correlates with the expression of the said protein.

positive expression was greater than that of young patients; however, the distribution was more heterogeneous. Akt was observed in large accumulations along the wall of the vein (Figure 2(c), B).

Microscopic observation revealed that the R < 50 patients had high Akt expression intensity throughout the entire venous wall (Figure 2(c), C). This expression was visualized acutely in the tunica intima (Figure 2(c), D) and measured in the smooth muscle bundles (Figure 2(c), E). The R ≥ 50 patients had low Akt expression. Akt was observed heterogeneously as small accumulations in the tunica media within the smooth muscle fibers (Figure 2(c), F).

3.2.3. mTOR. A relative amount mRNA of mTOR was higher in the R patients versus the NR group, establishing a statistically significant difference ($^{*}p < 0.05$). In the case of the distribution by age, the R < 50 patients showed a significant increase in comparison with the NR<50 group ($^{**}p < 0.005$) (Figure 3(a)).

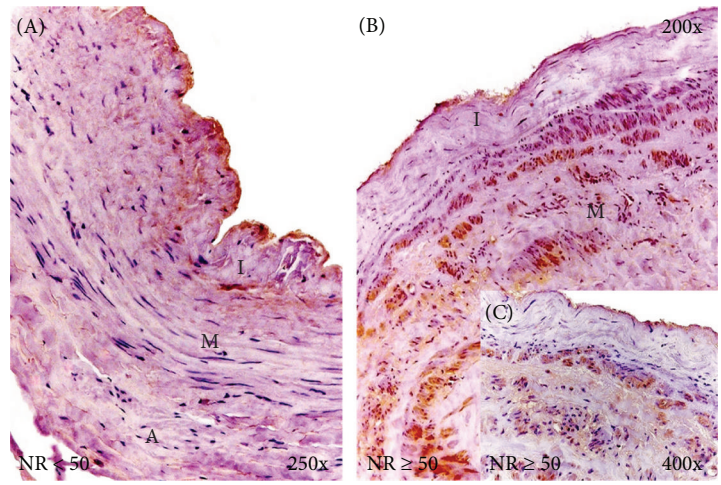
The percentage of positive mTOR expression was higher in patients with venous reflux (R), with 62.07% in the NR and



(a)

% positive expression for mTOR (n)			
NR	62.07 (18)	NR < 50	61.54 (8)
		NR ≥ 50	62.50 (10)
R	75.31 (61)	R < 50	100 (32)
		R ≥ 50	63.26 (31)

(b)



(c)

Figure 3: (a) Significant levels of mRNA for mTOR quantified by RT-qPCR in R and R < 50 patients. Results were normalized to that of the reference gene GAPDH and are provided in arbitrary units. NR = no reflux; R=reflux. $^{*}p < 0.05$ and $^{**}p < 0.005$. (b) Distribution of the percentage of patients with positive protein expression for mTOR in NR and R patients and by age; n = number of patients. (c) A: Histological images for protein expression mTOR in the different tunicae of venous wall in NR < 50 patients (350x). B, C: mTOR protein expression images in NR ≥ 50 patients (200x–400x). The brown coloration indicates the specific precipitate that correlates with the expression of the said protein. A = tunica adventitia; M = tunica media; I = tunica intima.

77.78% in the R patients. Consideration of the age factor showed that the percentage was more elevated in the R < 50 patients, being 100% in these patients and 63.27% in the R ≥ 50 patients. In the case of the NR patients, the expression percentages were similar: 61.54 in NR < 50 and 62.50 in NR ≥ 50 (Figure 3(b)).

The microscopic observation showed that mTOR was distributed differently in the different study groups. The NR < 50 patients exhibited reduced mTOR expression, which was limited to the environment of the tunica media and appeared as small heterogeneous accumulations along the wall of the vein (Figure 3(c), A). In the case of the NR ≥ 50 patients, mTOR was present in the tunica media and slightly in the tunica intima (Figure 3(c), B). This expression was irregular, with intense small points in the smooth muscle bundles of the vein wall (Figure 3(c), C).

The R < 50 patients had high mTOR protein expression that extended throughout the entire wall of the vein (Figure 4(a)). Importantly, mTOR was differentially distributed in the smooth muscular bundles of the tunica media, with intense labeling observed (Figure 4(b)). In these patients, venula in the tunica adventitia were visualized with a high expression for mTOR protein (Figure 4(c), arrow). The R ≥ 50 patient samples exhibited lower mTOR protein expression intensity. The protein extended along the tunica media and was mildly expressed in the tunica adventitia, with low expression in the endothelium (Figure 4(d)). The smooth muscular bundles appeared labeled (Figure 4(e), arrow) and blood capillary (Figure 4(e), arrowhead), and this expression was heterogeneous.

3.3. Expression of HIF-1α and HIF-2α. The study of the hypoxic component was performed by relative quantity mRNA and protein detection of hypoxia-inducible factors (HIFs) based on the expression of the subunits 1 alpha (HIF-1α) and 2 alpha (HIF-2α).

3.3.1. HIF-1α. A relative amount of mRNA of HIF-1α was higher in the R group versus the R patients, establishing a statistically significant difference ($^{*}p < 0.05$). The distribution of amount by age showed statistically significant differences between the NR < 50 and R < 50 patients ($^{*}p < 0.05$) (Figure 5(a)).

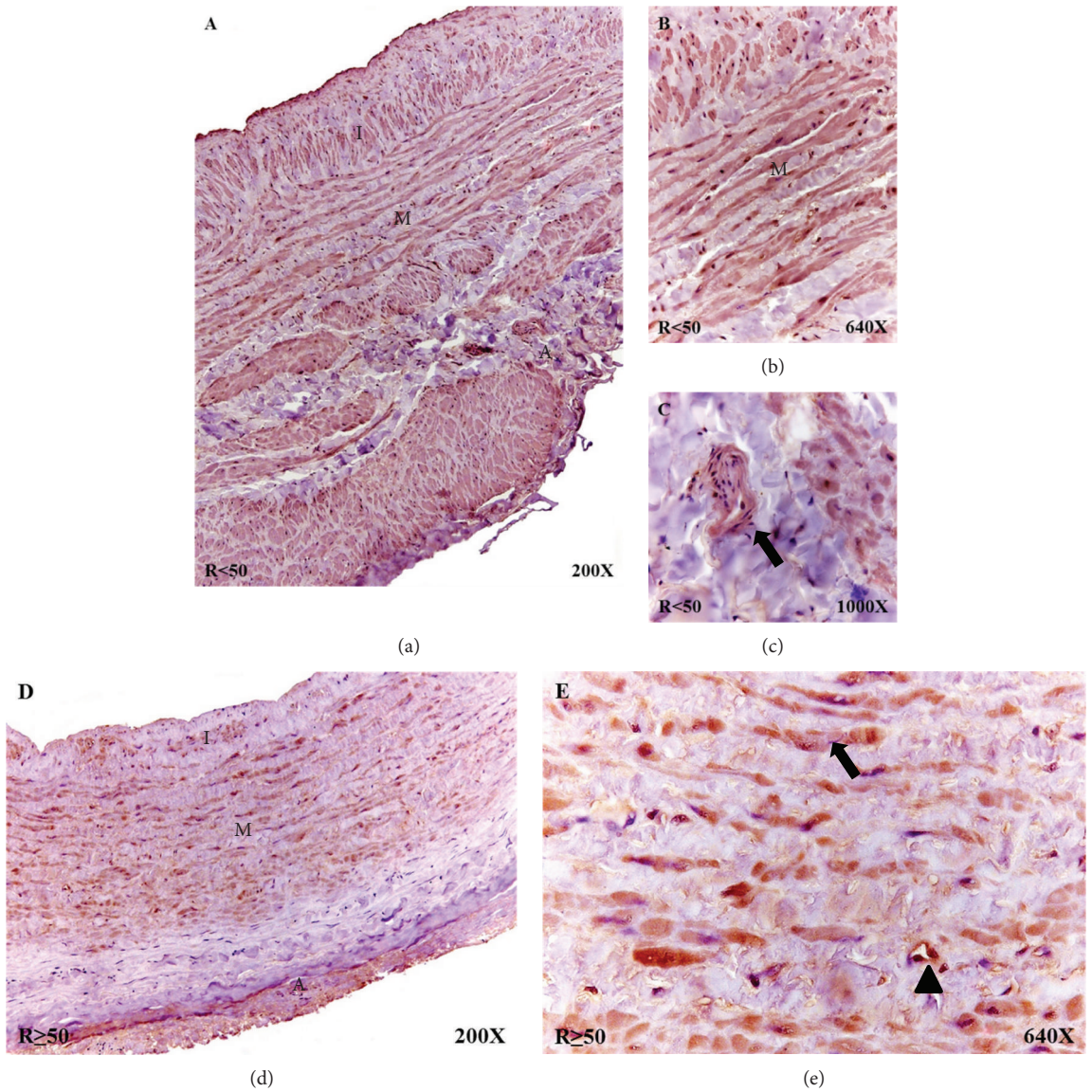


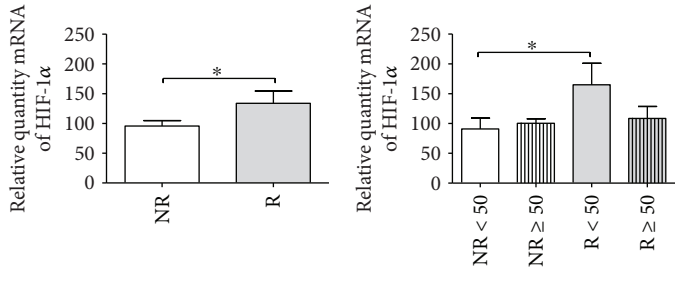
Figure 4: (a) Histological images for protein expression of mTOR in the different tunicae of the venous wall in R < 50 patients (200x). (b-c) Detail of expression for mTOR at greater magnification in media tunica (640x) and venula (arrow) in adventitia tunica (1000x). (d-e) Protein expression images of mTOR in R ≥ 50 patients, with detail in smooth muscle bundles (arrow) and blood capillary (arrowhead) in the tunica media (640x). The brown coloration indicates the specific precipitate that correlates with the expression of the said protein. A = tunica adventitia; M = tunica media; I = tunica intima.

When studying HIF-1α protein expression, differences were observed in the percentage of expression in patients with positive expression. Overall, the R patients presented an expression percentage of 76.54%, but in the R < 50 group, the expression percentage was 93.75%, which was greater than that of patients without reflux (Figure 5(b)). The HIF-1α protein expression intensity was similar in all study groups, and a uniform distribution along the vein wall was observed (Figure 5(c)).

3.3.2. HIF-2α. A relative quantity of mRNA of HIF-2α was higher in the R patients versus the NR group, establishing a statistically significant difference ($^{*}p < 0.05$). In the case of distribution by age, mRNA expression did not reveal any significant differences between the study groups (Figure 6(a)).

The study of HIF-2α protein expression showed that the percentage of patients reactive for this factor was similar in the NR (58.62%) and R (60.49%) patients. The highest percentage of positive HIF-2α expression was observed in the R < 50 patients (71.88%) (Figure 6(b)).

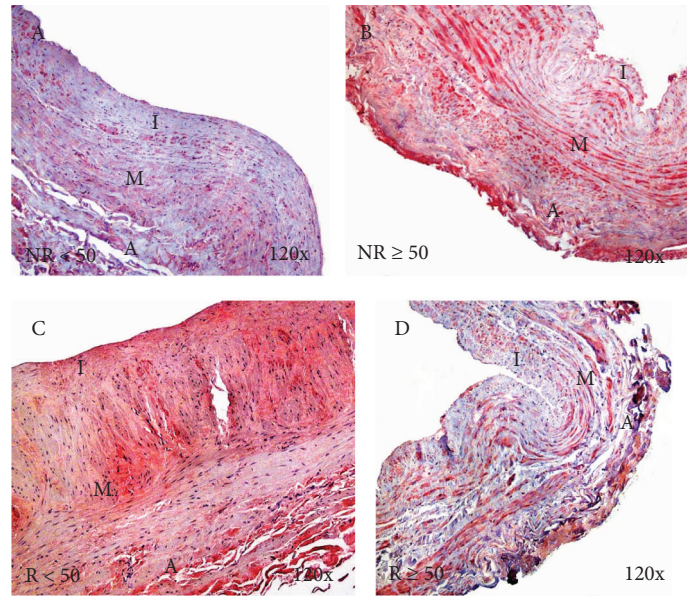
Protein expression was present in the nuclei of smooth muscle fibers (Figure 6(c), A–C); in the R < 50 patients, there was slight expression in the vessels of the tunica adventitia (Figure 6(c), arrow). The rest of the patients presented with diffuse protein labeling similar to that previously described for muscle fibers.



(a)

% positive expression for HIf-1α (n)			
NR	65.52 (19)	NR < 50	61.54 (8)
		NR ≥ 50	68.75 (11)
R	76.54 (62)	R < 50	93.75 (30)
		R ≥ 50	65.31 (32)

(b)



(c)

Figure 5: (a) Significant levels of mRNA for HIF-1α quantified by RT-qPCR in R and R < 50 patients. Results were normalized to that of the reference gene GAPDH and are provided in arbitrary units. NR = no reflux; R = reflux. $^*p < 0.05$. (b) Distribution of the percentage of patients with positive protein expression in HIF-1α NR and R patients and by age; n = number of patients. (c) A–D: Histological images for HIF-1α protein expression in the different tunicae of the venous wall in NR < 50, NR ≥ 50, R > 50, and R ≥ 50 patients (120x). Red coloration indicates the specific precipitate that correlates with the expression of said protein. A = tunica adventitia; M = tunica media; I = tunica intima.

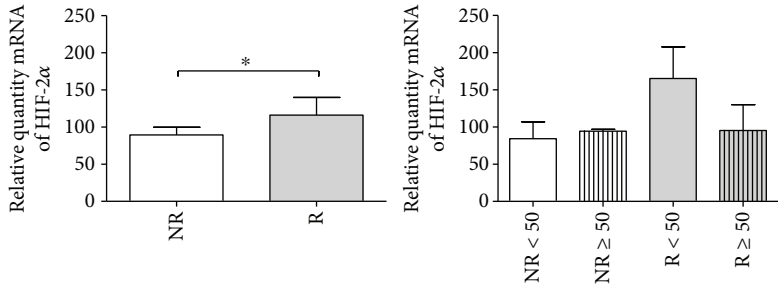
3.4. Expression of CD4+, CD8+, and CD19+ and Mast Cells

3.4.1. CD4+ Cells. In terms of quantification between the NR and R patient groups, no significant differences were observed. By considering age, we found significant differences. The highest number of CD4+ cells was observed in the NR ≥ 50 and R < 50 patients, which presented significant differences in comparison with the rest of the study groups (Figure 7(a)). CD4+ cells were present along the entire wall of the vein in all the study groups (Figure 7(b)).

3.4.2. CD8+ Cells. Quantification of the number of CD8+ cells did not show significant differences between the NR and R patients. Including age in the analysis did not reveal differences between the different patient groups but showed a slight tendency toward an increase similar to that observed in the CD4+ cells (Figure 7(a)). The CD8+ cells were visualized by immunodetection techniques and appeared in the tunica intima and adventitia of the vein (Figure 7(b)).

3.4.3. CD19+ Cells. The presence of B lymphocytes was evidenced by immunodetection of CD19+ cells, and an increasing tendency in the R patients with respect to the NR patients was found. In terms of age, statistically significant differences were established between the NR < 50 and R < 50 patients ($^*p < 0.05$) and between the R < 50 and R ≥ 50 patients ($^*p < 0.05$). The data obtained show that the highest elevation of CD19+ cells occurred in the R < 50 patients (Figure 7(a)). CD19+ cells were visualized on the endothelium and tunica adventitia of the vein wall (Figure 7(b)).

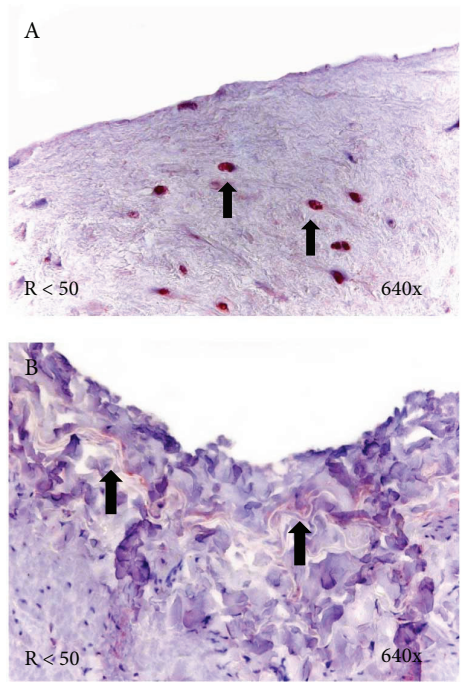
3.4.4. Mast Cells. The presence of mast cells in the vein wall was detected with toluidine blue. The results revealed a significant increase in the number of mastocytes in the R patients with respect to the NR patients ($^*p < 0.05$). Statistically significant differences were observed between the NR < 50 and R < 50 patients ($^*p < 0.05$) (Figure 7(a)). Toluidine blue allowed us to identify these cells because the stain lends them a purple tone due to the mast cell



(a)

% positive expression for HIf-2α (n)			
NR	58.62 (17)	NR < 50	53.85 (7)
		NR ≥ 50	62.50 (10)
R	60.49 (49)	R < 50	71.88 (23)
		R ≥ 50	53.06 (26)

(b)



(c)

Figure 6: (a) Significant levels of mRNA for HIF-2α quantified by RT-qPCR in R. Results were normalized to that of the reference gene GAPDH and are provided in arbitrary units. NR = no reflux; R = reflux. $^*p < 0.05$. (b) Distribution of the percentage of patients with positive protein expression in HIF-2α NR and R patients and by age; n = number of patients. (c) A: Protein expression images of HIF-2α in cell nucleus (arrow) of R > 50 patients (640x). B: Protein expression images of HIF-2α in capillary (arrow) of adventitia tunica in R > 50 patients (640x). Red coloration indicates the specific precipitate that correlates with the expression of the said protein.

cytoplasmic granules, which are rich in anionic substances, such as histamine and heparin. When performing the microscopy study, mast cells were observed near blood capillaries in the tunica media and adventitia, as well as in the vasa vasorum of the vein wall (Figure 7(b)).

4. Discussion

Despite scientific and technological advances, the failure of the venous wall responsible for CVI still has no clear etiology. Therefore, multiple factors have been proposed to contribute to venous wall failure and to potentially determine the failure either by distension of the wall, valvular failure, or valvular agenesis in some key places [25, 26]. The measure of venous reflux is clinically valuable, and one of the tests aids in estimating the degree of venous involvement in the lower limbs. However, to date, there are no clear data on the correlation between reflux and damage to the venous wall, thus limiting the implementation of specific corrective measures.

Previous works have shown that of the multiple noxae that affect the wall and venous functioning, the very process of life (aging) is one contributor to venous failure [18]. CVI with or without reflux will produce a dilatation of the venous wall that initially leads to alterations in the structure of the compensatory wall in the form of hypertrophic areas, and after failure, the wall will return to being fibrosclerotic [27]. These alterations, which are induced, promoted, and maintained by the phenomena of inflammation and ischemia, lead to cytoarchitectonic remodeling of the venous wall, causing in turn a manifest functional incompetence known as venous reflux. Although aging and CVI develop in parallel, the aging process can be accelerated in CVI, coinciding with the secondary remodeling induced by valvular incompetence [27–29]. CVI induces changes in the return of venous flow, which can increase venous filling and subsequently induce an increase in intraluminal pressure. This would produce an increase in venous stasis and relative hypoxia, with a consequent associated increase in oxidative metabolism and reactive oxygen species. All this would contribute to venous wall remodeling and damage [30].

One of the events observed by our research group is the gene and protein expression of different components of the PI3K/Akt/mTOR cellular transduction pathway. Our results have shown that patients with venous reflux, especially young individuals, have a significant increase in vein activation. Yuan et al. [31] noted that PI3K is essential in the regulation of embryonic vasculogenesis, and alterations in this pathway can have severe consequences. The implication of these molecules in different pathologies is well tested [32–34], and they are described as triggers for numerous pathophysiological processes. Among these processes, we must highlight hypertension. Another of the points of interest noted by numerous authors is that inflammation processes can trigger the activation of these pathways with severe consequences in the tissue itself [35]. Castel et al. [36] and Castillo et al. [37] reported that mutations in this pathway can have consequences in

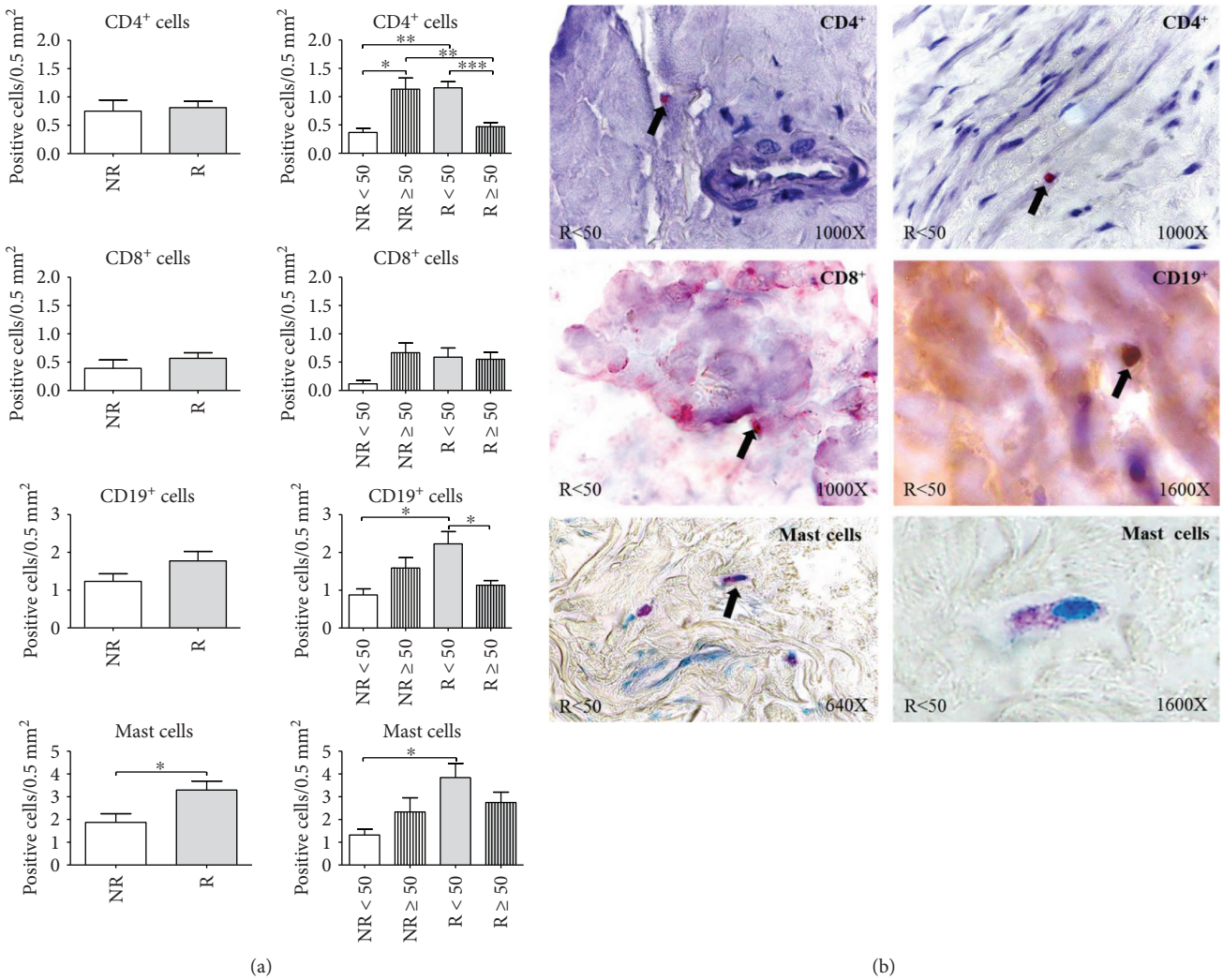


Figure. 7: (a) Positive cell quantification of CD4+, CD8+, CD19+, and mast cells in the three tunicae of the vein wall for patients without reflux (NR) and with reflux (R), as well as by the ages of the same. $^{*}p < 0.05$; $^{**}p < 0.005$; $^{***}p < 0.001$. (b) Histological images showing the specific detection of immune cells CD4+, CD8+, and CD19+ and mast cells in R < 50 patients.

the vascular system, at both the cytoarchitectural and the physiological levels. Therefore, in light of what was previously stated, the PI3K/Akt/mTOR pathway, as observed by numerous authors, may have an essential role in the remodeling processes in human veins with CVI, especially in those with valvular incompetence.

At another point, hypoxia caused by venous hypertension sets in motion molecular pathways involved in the cellular response to the lack of oxygen, such as PI3K/Akt/mTOR and consequently the HIF transcription factors. Research in the field of these factors suggests that HIF-2α stabilization requires less severe hypoxia than HIF-1α, but the mechanisms involved are still unknown. Therefore, it is thought that HIF-2α should be a first line of response in the face of moderate or less severe decreases in O_2 [38]. In general, we observed the presence of HIF-1α in all the patients studied, which suggests a significant level of hypoxia in the venous walls studied. This hypoxia appeared in the young population with venous reflux at a higher proportion. Related to this event is the mTOR gene and protein expression that has a similar pattern, unlike other PI3K/Akt/mTOR pathway components. In general, our results coincide with those of authors who observed a significant increase in the expression of the HIF pathway in patients with varicose pathology. There has been talk about deregulation of this pathway, which produces an increase in angiogenic factors [39–42]. Lee et al. [39, 40] described a significant increase in HIF-1α in the muscle layers of diseased vessels. They observed that this expression was related to the increased Bcl-2 in the vessel endothelium, which would lead to apoptosis inhibition and therefore to an increase in the dilation of the wall of the human vein.

Interestingly, in our study, we observed that reflux becomes a point of inflection for adaptation relative to the age profile of the patients studied, especially in relation to mTOR expression. All these findings are compatible with the process of the hypertrophy-atrophy sequence that an insufficient venous wall undergoes during this process, which is aggravated in venous reflux due to valve incompetence, as shown by Buján et al. [27].

The presence of CD4+, CD8+, and CD19+ cells suggests the existence of an inflammatory process and stress in relation to the above. A variety of studies have shown an increase in degranulation and extravasation of leukocytes in patients with venous hypertension. Saharay et al. [43] showed via flow cytometry an alteration in the number of neutrophils and monocytes in patients with CVI with venous hypertension. T lymphocytes have been demonstrated to be at least partially involved in the inflammatory process observed in venous disease. By analyzing the blood obtained from varicose veins of patients with CVI, Ojdana et al. [44] were able to demonstrate that the levels of CD4+ T lymphocytes measured from varicose veins were significantly elevated compared with the total CD4+ cell levels. The increase in the level of these cells, as well as other subpopulations of T cells measured by this group, indicate that T cells may also partially participate in the pathogenesis of cardiovascular disease. The increase in the number of CD19+ cells in patients with venous reflux could be related to changes in the inflammatory status produced by the increase in CD4+ cells. Zhang et al. [45] proposed that the presence of CD19+ cells was related to PI3K/Akt/mTOR pathway activation, with systemic consequences.

In relation to the above, it should be noted that mast cells can perform a wide variety of functions that induce the inflammatory cascade via mediators derived from their granules in endocytosis. Pascual et al. [18] showed significantly greater mast cell infiltration in varicose vein walls than in healthy controls. Chymase from mast cells is an MMP activator and stimulates the release of TGF-β, which plays an integral role in vascular remodeling. Mast cells also secrete tryptase, which can degrade elastin, collagen, proteoglycans, and fibronectin, consequently affecting extracellular matrix remodeling of the vessel wall.

Current research suggests that the PI3K/Akt/mTOR pathway plays an important role in cellular metabolism regulation and in the functions of the immune system, two subjects that are closely related to cell and organ functionality [16, 46]. All this makes us consider the importance of this pathway in the homeostasis of the venous wall and in its activation capacity in pathological processes. Therefore, these facts provide a basis for the possible existence of changes in the PI3K/Akt/mTOR pathway expression in young CVI patients, with a possible accelerated asynchronous aging. In these individuals, the presence of gene mutations or epigenetic changes, as expressed by some authors [47], may play a role. Our results show that older patients with venous reflux have less expression of this pathway. We think that this expression decrease could be explained in relation to cellular senescence, autophagy, and/or apoptosis. In our studied population, patients did not have an oncogenic background. Buján et al. [27] observed that older patients with varicose veins had more TUNEL-positive cells. This fact could be related to that these patients have less activity of PI3K/Akt/mTOR, because they have lost the ability to react to the aggression and they have damage that makes them enter apoptosis. In contrast, young patients with venous reflux have greater expression, which could be related according to some authors [12, 48, 49] with possible cellular senescence and autophagy process. Young patients with venous reflux have a greater ability to react to the cell damage that occurs.

Our results help demonstrate the notion that among several possible mechanisms of activation, venous reflux in CVI is to a considerable degree an inflammatory disease induced by blood pressure. The venous pressure elevation (change in the type of pressure) and the displacement of the shear stress generate an abnormal biomechanical environment in the venules, in their walls, and in the valves, which can initiate early activation of enzymatic activity and in turn set in motion a cascade of cellular transduction, leading to cytoarchitectonic changes to compensate for these alterations.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Miguel A. Ortega and Ángel Asúnsolo contributed equally to this work.

Acknowledgments

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Research Article

Mitofusin2 Induces Cell Autophagy of Pancreatic Cancer through Inhibiting the PI3K/Akt/mTOR Signaling Pathway

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Aim. Pancreatic cancer is one of the most quickly fatal cancers around the world. Burgeoning researches have begun to prove that mitochondria play a crucial role in cancer treatment. Mitofusin2 (Mfn2) plays an indispensable role in mitochondrial fusion and adjusting function. However, the role and underlying mechanisms of Mfn2 on cell autophagy of pancreatic cancer is still unclear. Our aim was to explore the effect of Mfn2 on multiple biological functions involving cell autophagy in pancreatic cancer. *Methods*. Pancreatic cancer cell line, Aspc-1, was treated with Ad-Mfn2 overexpression. Western blotting, caspase-3 activity measurement, and CCK-8 and reactive oxygen species (ROS) assay were used to examine the effects of Mfn2 on pancreatic cancer autophagy, apoptosis, cell proliferation, oxidative stress, and PI3K/Akt/mTOR signaling. The expression of tissue Mfn2 was detected by immunohistochemical staining. Survival analysis of Mfn2 was evaluated by OncoLnc. *Results*. Mfn2 improved the expression of LC3-II and Bax and downregulated the expression of P62 and Bcl-2 in pancreatic cancer cells. Meanwhile, Mfn2 also significantly inhibited the expression of p-PI3K, p-Akt, and p-mTOR proteins in pancreatic cancer cells. In addition, Mfn2 inhibited pancreatic cancer cell proliferation and ROS production. Assessment of Kaplan-Meier curves showed that $Mfn2^{-}$ pancreatic cancer has a worse prognosis than $Mfn2^{+}$ pancreatic cancer has. *Conclusions*. Our finding suggests that Mfn2 induces cell autophagy of pancreatic cancer through inhibiting the PI3K/Akt/mTOR signaling pathway. Meanwhile, Mfn2 also influences multiple biological functions of pancreatic cancer cells. Mfn2 may act as a therapeutic target in pancreatic cancer treatment.

1. Introduction

Accompanied with nearly 100% of 5-year mortality rate, pancreatic cancer is one of the most quickly fatal cancers around the world [1]. Although in recent year we have some amazing improvements in the development of surgery, radiation therapy, and chemotherapy, pancreatic cancer still has a desperate prognosis, mainly because of its aggressive biological behavior and late breaking out of symptoms for clinical diagnosis [2]. Traits like that bring a mass of difficulties for therapeutic interventions of pancreatic cancer treatment. One of the main problems for clinical treatment of pancreatic cancer is that we still do not fully understand the pathogenesis and development of this disease. Thus, a deep-going exploration of the malignant essence of pancreatic cancer is urgently needed for the development of novel therapies.

Mitochondria play a significant role in the intermediates needed for macromolecule biosynthesis and the production of ATP [3]. Mitochondria also take part in the activation of signaling pathways. Current evidence suggests that biosynthesis, signaling, and mitochondrial bioenergetics are needed for tumorigenesis [4]. Burgeoning researches have begun to prove that mitochondria play a crucial role in cancer treatment [5]. In the meantime, more and more evidence shows that tumor suppressors and key oncogenes modify the mitochondrial dynamics through significant signaling pathways and that function and mitochondrial mass variables in different tumors and individuals [6, 7].

Mitofusin2 (Mfn2) is a mitochondrial outer membrane protein that plays an indispensable role in mitochondrial fusion, adjusting function and mitochondrial morphology [8]. Research reported that ER stress up-adjusted Mfn2,

and genetic ablation of Mfn2 increased cell death during ER stress [9]. In skeletal muscle, Mfn2 regulates the optimal biological properties by maintaining mitochondrial quality control and efficient mitochondrial metabolism [10]. With the knockdown of Mfn2 in Hela cells and a human smooth muscle cell line, impaired autophagic degradation, reduced ATP production, inhibited cell glycolysis and mitochondrial oxygen consumption rate, and suppressed cell proliferation were observed [11].

In recent years, Mfn2 has also shed new light on the area of tumor research. Several studies have found the antitumor effect of Mfn2 in different malignancies, including gastric cancers, breast cancer, hepatocellular carcinoma and urinary bladder cancer [12–14]. The latest study showed that in pancreatic cancer, overexpressed Mfn2 makes cells under apoptotic stress with cleaved caspases. But the cell cycle was not significantly changed with the overexpression of Mfn2. Tumor cells' migration and invasion abilities were inhibited [15]. It is indicated that the overexpression of Mfn2 may become an effective treatment strategy in pancreatic cancer. However, the role and underlying mechanisms of Mfn2 on autophagy of pancreatic cancer cells is still unclear.

In this research, we used adenovirus to deliver Mfn2 to pancreatic cancer cells, so that we can assess the effect of Mfn2 on autophagy. Besides, we uncovered the mechanism of Mfn2-induced autophagy of pancreatic cancer cells. At the same time, we further deeply explore some potential biological mechanisms of Mfn2 by a bioinformatics analyzing.

2. Material and Methods

2.1. Cell Culture and Passaging. The Aspc-1 cell line was a gift from the cell laboratory of the Beijing Chao Yang Hospital, Capital Medical University. Cryopreserved Aspc-1 cells were thawed and then cultured at 37°C and 5% CO_2, in proper volume of 10% fetal bovine serum (FBS) in Dulbecco's modified Eagle's medium (DMEM) bought from Gibco (USA). Cells grown to logarithmic growth phase were trypsinized and then passaged.

2.2. Adenovirus. Adenovirus encoding the Mfn2 open reading frame (Ad-Mfn2) and control adenovirus were constructed by JI KAI Gene Technology Co. Ltd. (Beijing, China). Aspc-1 cells were cultured for 24 h for synchronization and then incubated with adenovirus at a multiplicity of infection (MOI) of 100 pfu per cell at 37°C for 4 h.

2.3. Cell Viability Analysis. Cell viability assay was tested by Cell Counting Kit-8 (CCK-8, Beyotime, China) following the instructions. Cell viability was calculated as follows:

$$\text{Cell viability} = \frac{\text{A 450 nm mean value of infected cells}}{\text{A 450 nm mean value of uninfected cells}} \times 100\%. \quad (1)$$

2.4. Caspase-3 Activity Measurement. The activity of caspase-3 was tested using the caspase-3 Activity Assay Kit (C1115, Beyotime, China). The absorbance (A405) was measured using an ELISA reader (BioTek, USA).

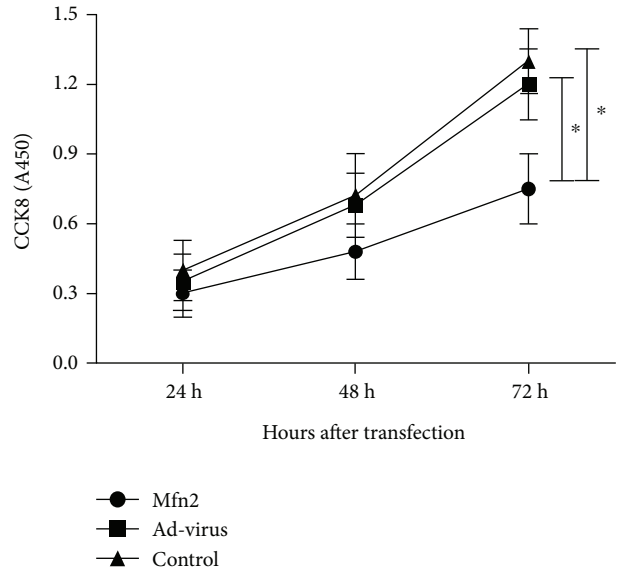

Figure 1: CCK-8 assay was used to verify Aspc-1 cell proliferation ($^*p < 0.05$; $N = 3$).

2.5. Reactive Oxygen Species (ROS) Measurement. Changes in intracellular ROS levels were detected by the oxidative conversion of cell-permeable 2′, 7′-dichlorofluorescein diacetate (DCFH-DA) to fluorescent dichlorofluorescein (DCF). Aspc-1 cells were incubated with Ad-Mfn2 or control media. DCF fluorescence was measured using the FACScan flow cytometer (Becton Dickinson).

2.6. Western Blotting Analysis. Western blotting was performed, following that described above [16]. The primary antibody and secondary antibody are listed in Supplementary Table S1 online. Protein bands were visualized using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA, USA). Average intensity analysis was used to quantify the protein expression. And the average intensities of each standard protein band were quantified using Photoshop CS5 (Adobe Systems Incorporated), and these results were normalized using GAPDH. The results were column-plotted by GraphPad Prism 7 software.

2.7. Immunohistochemical Stains. Immunohistochemistry (IHC) staining was tested as described in previous research [17]. Sections were incubated with mouse monoclonal to Mfn2 (ab56889, Abcam, USA) primary antibodies over one night at 4°C. Then, sections were incubated using the horseradish peroxidase conjugate antibody, while the chromogen used was 2% 3,3′-diaminobenzidine (DAB). As for the histological and immunohistochemical assessment, to analyze Mfn2 expression, according to the number of positive cells, the immunohistochemical results were categorized as follows: +++, positive (>70%); ++, positive (50–70%); +, positive (30–50%); ±, weakly positive (10–30%); and −, negative

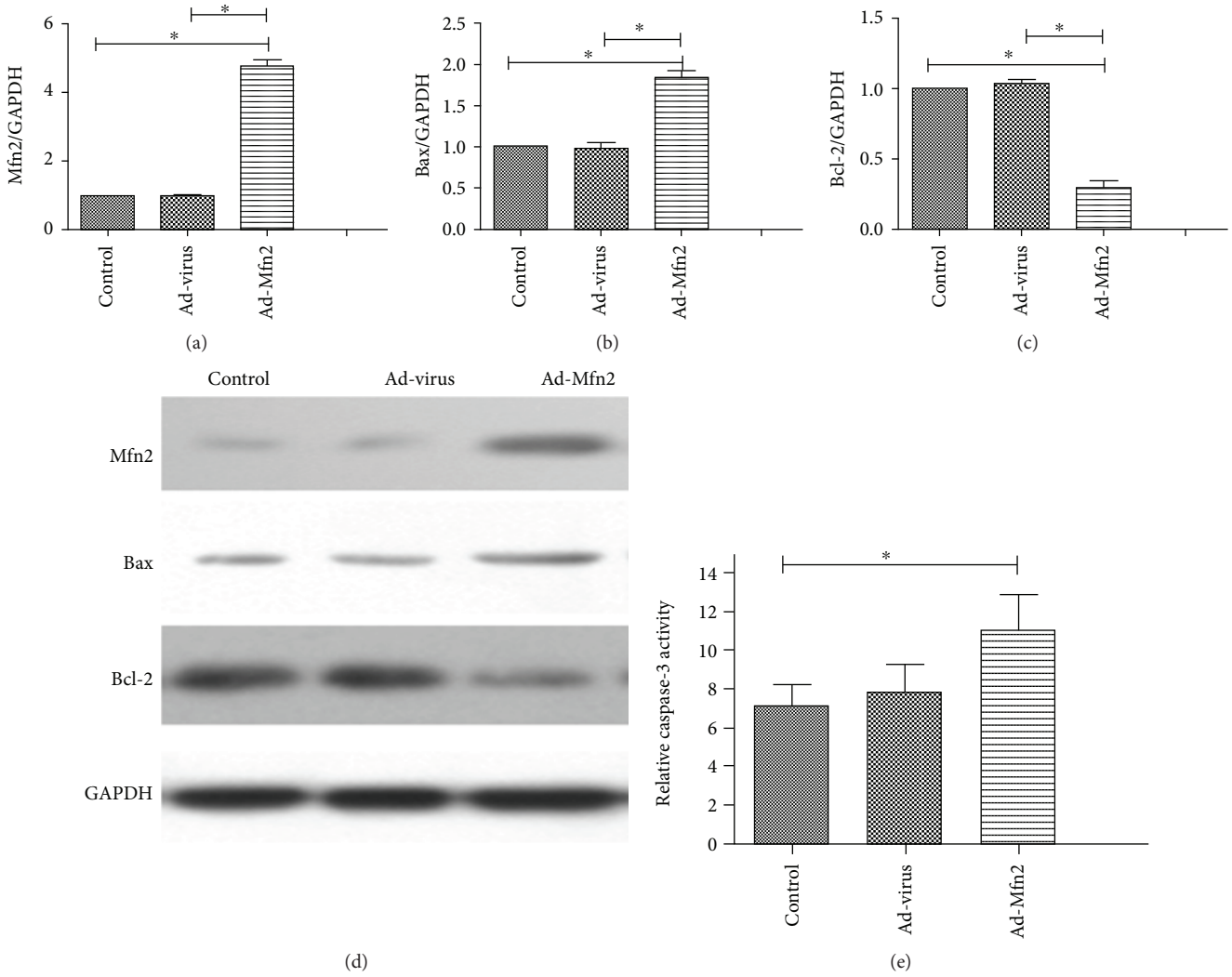

Figure 2: (a) Quantification of Western blots for Mfn2 expressions in the Ad-Mfn2 group compared to control group ($^*p < 0.05$; $N = 3$). (b) Quantification of Western blots for Bax expressions in the Ad-Mfn2 group compared to the control group. ($^*P < 0.05$; $N = 3$). (c) Quantification of Western blots for Bcl-2 expressions in the Ad-Mfn2 group compared to the control group ($^*P < 0.05$; $N = 3$). (d) Western blotting was used to detect the expression of Bcl-2 and Bax to verify cell apoptosis in pancreatic cancer cells. (e) Caspase-3 activity measurement ($^*P < 0.05$; $N = 3$).

(<10%). The expression of Mfn2 was assessed blindly and independently by two investigators.

2.8. PPI Network Construction and Identification. The Cytoscape app can build a composite gene-gene functional interaction network. The edges are annotated with the results derived from publication or public database [18]. Potential Mfn2-regulated genes were obtained through the GeneMANIA Cytoscape app.

2.9. Functional Enrichment Analysis of Mfn2-Regulated Genes. FunRich (Functional Enrichment analysis tool) from ExoCarta (http://www.exocarta.org/) was used to perform analysis. FunRich is an independent software instrument used mainly for interaction network analysis and functional enrichment of proteins and genes. The cut-off standard was $p < 0.01$.

Gene Ontology (GO) mainly contains molecular function (MF), biological process (BP), and cellular component (CC) [19]. The Database for Annotation, Visualization and Integrated Discovery (DAVID, http://david.abcc.ncifcrf.gov/) is a functional annotation tool to understand biological meaning [20]. The Kyoto Encyclopedia of Genes and Genomes (KEGG) [21] provides information about how molecules or genes are networked. The GO-BP, GO-CC, and GO-MF terms were screened with a p value of <0.05. Significant enriched KEGG pathways were identified with a p value of <0.05.

2.10. Survival Analysis of Mfn2 in Human Pancreatic Cancer. OncoLnc (http://www.oncolnc.org) is an instrument for interactively discovering survival correlations. OncoLnc has 8647 patient survival data collected from 21 cancer researches by The Cancer Genome Atlas (TCGA). The total survival of

pancreatic cancer patients was analyzed by a Kaplan-Meier plot. The pancreatic cancer patients were separated into two groups on the basis of high or low expression for a particular gene.

2.11. Statistical Analysis. Statistical analyses were used by SPSS 16.0 (SPSS, Chicago, IL, USA). p values < 0.05 were considered statistically significant. A one-way ANOVA or two-tailed Student t-test was performed for intergroup comparison of variance.

3. Results

3.1. Mfn2 Suppressed Pancreatic Cancer Cell Proliferation. To further test the effects of Mfn2 on pancreatic cancer cell proliferation, CCK-8 assay was carried out. Compared to the control group, proliferation of Aspc-1 cells was inhibited by Mfn2 overexpression (Figure 1).

3.2. Mfn2 Triggers Cell Apoptosis in Pancreatic Cancer. For assessing cell apoptosis in pancreatic cancer cells with Mfn2 overexpression, the expression of Bcl-2 and Bax was measured using Western blotting analysis. Caspase-3 activity was also performed.

The Bax levels were significantly increased in Ad-Mfn2 groups. In addition, Mfn2 significantly reduced Bcl-2 levels of Aspc-1 compared with the control group. Compared with controls, the increased caspase-3 activity was also observed in Ad-Mfn2 groups (Figure 2).

3.3. Effect of Mfn2 on Reactive Oxygen Species (ROS) in Pancreatic Cancer. ROS levels were performed by flow cytometry in the DCFH-DA fluorescent probe. There was a significant decrease in ROS-positive cells in the Ad-Mfn2 group compared to the control. In the control group, the average rate of DCF-positive cells was $93.12 \pm 2.28\%$, while $71.79 \pm 2.42\%$ in the Ad-Mfn2 group contributed to intracellular ROS production ($P = 0.003$) (Figure 3).

3.4. Mfn2 Enhances Cell Autophagy in Pancreatic Cancer. Western blotting analyses showed that the expression of LC3-II/LC3-I was increased in the Mfn2 overexpression group. There was also a decreased expression for P62 in the Ad-Mfn2 group (Figure 4).

3.5. Mfn2 Enhances Cell Autophagy through Inhibiting the PI3K/AKT/mTOR Signaling Pathway. Next, Western blotting analyses showed that Mfn2 significantly decreased the expression of phosphorylated-PI3K (p-PI3K), phosphorylated-Akt (p-Akt), and phosphorylated-mTOR (p-mTOR) (Figure 5). The expression of p-PI3K, p-Akt, and p-mTOR was significantly decreased in the Mfn2 overexpression group. As an activator of the PI3K/AKT signaling pathway, IGF-1 was given to perform a rescue experiment. After adding IGF-1 in the Ad-Mfn2 group, the expression of p-PI3K, p-Akt, and p-mTOR was significantly increased compared with that of the Mfn2 overexpression group. We consider that Mfn2 induces pancreatic cancer cell autophagy by inhibiting the PI3K/AKT/mTOR signaling pathway.

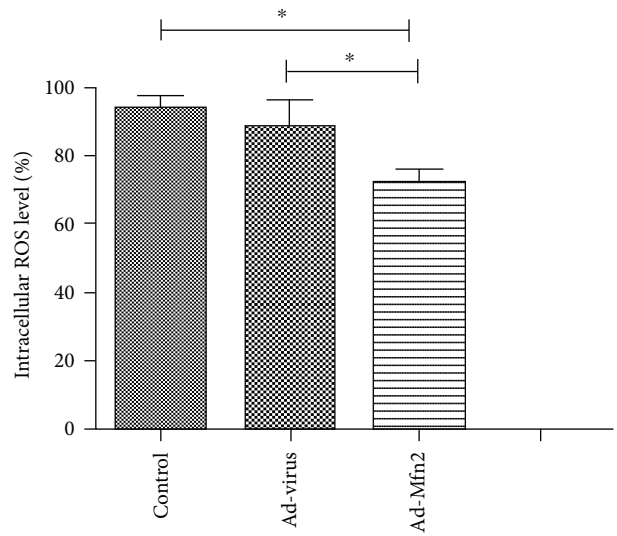

Figure 3: ROS levels were tested using flow cytometry by the DCFH-DA fluorescent probe. There were significant differences between the Mfn2 overexpression group and the control group for ROS level of pancreatic cancer ($^*P < 0.05$; $N = 3$).

3.6. Association between Mfn2 Expression and Clinic Pathological Factors. The IHC results showed that the Mfn2-positive protein is mainly located in the cytoplasm and is dyed into yellow or yellow granules in the cytoplasm (Figure 6). Mfn2 is expressed both in normal pancreas and in pancreatic cancer tissues. The relationship between Mfn2 expression and clinic pathological factors, including gender, age, differentiation grade, and TNM stage, is shown in Table 1.

3.7. Relationship between Mfn2 Immunosubtype and Survival in Pancreatic Cancer. Assessment of Kaplan-Meier curves showed that $Mfn2^-$ pancreatic cancer has a worse prognosis than $Mfn2^+$ pancreatic cancer has. Patients with an Mfn2-positive expression had a significantly longer survival time than those with an Mfn2-negative expression ($P = 0.0346$, log-rank test) (Figure 7).

Based on the data we observed above, the Mfn2 immunophenotype is closely relevant to the malignant behavior of pancreatic cancer. $Mfn2^-$ pancreatic cancer is an aggressive subtype, alongside $Mfn2^+$ pancreatic cancer, a less aggressive subtype.

3.8. PPI Network Construction. Based on data from GeneMANIA, the PPI network comprised of Mfn2 regulatory genes which were constructed by Cytoscape software (Supplementary Figure S1 Online 1). The network consisted of 20 nodes and 112 links. 112 links included physical interactions (67.64%), coexpression (13.50%), prediction (6.35%), pathway (4.35%), colocalization (6.17%), genetic interactions (1.40%), and shared protein domains (0.59%). The full list of Mfn2 regulatory genes is shown in Supplemental Table S2.

3.9. GO Analysis of Mfn2 Regulatory Genes. Following GO analyses for Mfn2 regulatory genes, significant GO terms

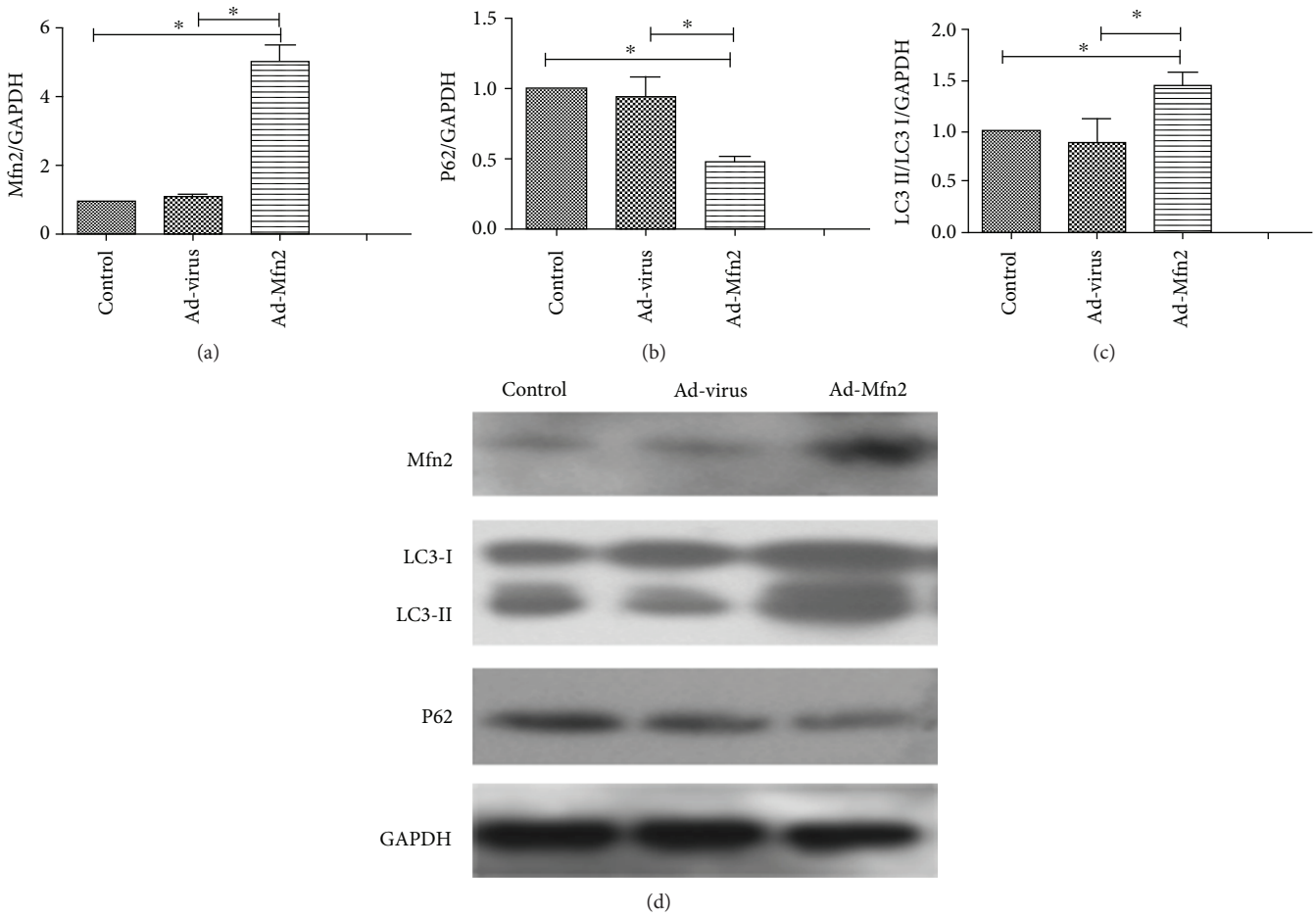

Figure 4: (a) Quantification of Western blots for Mfn2 expressions in the Ad-Mfn2 group compared to the control group ($^*P < 0.05$; $N = 3$). (b) Quantification of Western blots for P62 expressions in the Ad-Mfn2 group compared to the control group ($^*P < 0.05$; $N = 3$). (c) Quantification of Western blots for LC3 II/LC3 I expressions in the Ad-Mfn2 group compared to the control group ($^*P < 0.05$; $N = 3$). (d) Western blotting was used to detect the expression of LC3 and P62 to verify cell autophagy in pancreatic cancer cells.

including cellular component, biological process, and molecular function were collected. Mitochondrion organization and biogenesis was the most significant enrichment of the biological process ($p < 0.01$); mitochondrion was the highest enrichment of the cellular component ($p < 0.001$); and GTPase activity was the highest enrichment of molecular function ($P = 0.03$), as shown in Supplementary Figures S2, S3, and S4 online).

3.10. Functional Enrichment Analysis for TFs. The TFs for coexpressed DEGs were significantly enriched in CTCF, OTX1, ELF1, and PITX1 (all $p < 0.01$). CTCF is 11.8% for all transcription factor enrichment analysis. All TFs for coexpressed DEGs are shown in Supplementary Figure S5 online.

3.11. KEGG Enrichment Pathways of Mfn2 Regulatory Genes. Following KEGG enrichment analysis for Mfn2 regulatory genes, significant KEGG terms were collected. The pathways enriched were mainly related to viral carcinogenesis by DAIVD ($p < 0.012$) (BAK1, BAX, and UBR4).

4. Discussion

Recently, Mfn2 has become a rising star in tumor research [12–14]. In this study, we first proposed that Mfn2 can increase cell autophagy by the PI3K/Akt/mTOR signaling pathway in pancreatic cancer. Mfn2 is considered to perform antiproliferative and proapoptotic functions in pancreatic cancer. Meanwhile, Mfn2 is associated with a good survival rate in pancreatic cancer. Above all, it is indicated that Mfn2 can be a potential clinical therapeutic target in pancreatic cancer.

Autophagy has a complex role in the development of pancreatic cancer, promoting growth of established tumors but suppressing early stages of tumorigenesis. The exact pathways that control the dual roles of autophagy in the pathogenesis of pancreatic cancer, and whether autophagy is efficient or defective, remain to be elucidated [22]. Important mechanisms that could link aberrant autophagy to inflammation in pancreatitis and pancreatic cancer include accumulation of p62 and mitochondrial dysfunction, resulting in increased levels of ROS [23]. In this study, Mfn2 was discovered increasing the cell autophagy by the PI3K/Akt/mTOR signaling pathway in pancreatic cancer.

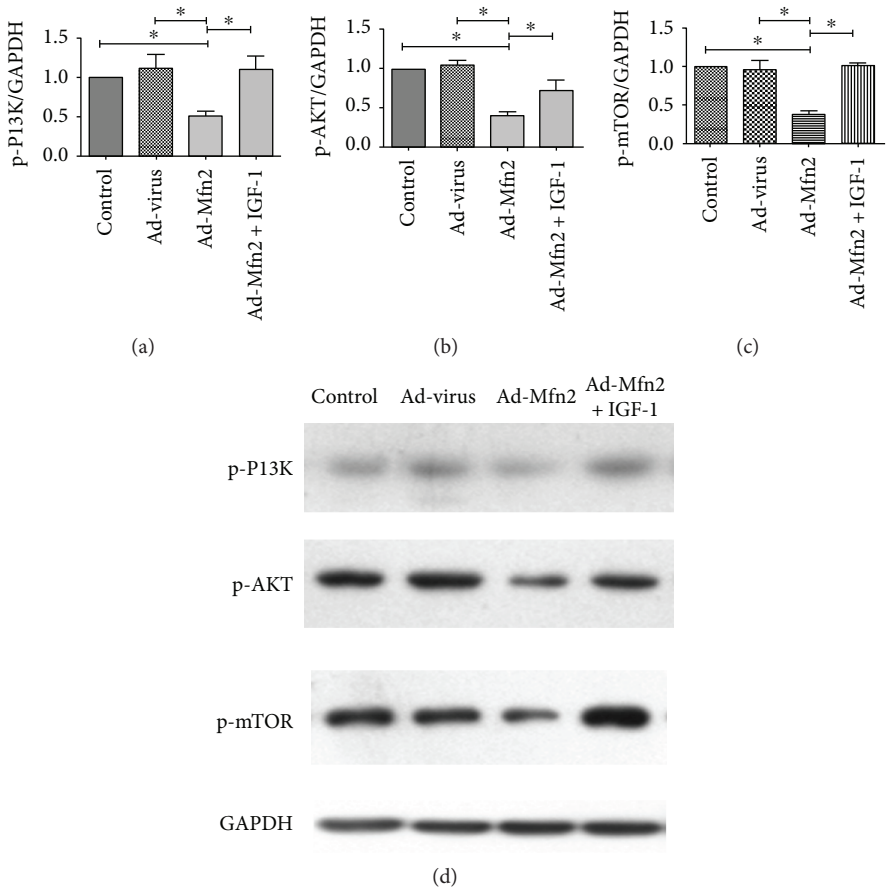

Figure 5: Mfn2 promotes cell autophagy by inhibiting the PI3K/AKT/mTOR signaling pathway. (a) Quantification of Western blots for p-PI3K expressions in the Ad-Mfn2 group compared to the control group and Ad-Mfn2$^+$ IGF-1 group ($^*P < 0.05$; $N = 3$). (b) Quantification of Western blots for p-AKT expressions in the Ad-Mfn2 group compared to the control group and Ad-Mfn2$^+$ IGF-1 group ($^*P < 0.05$; $N = 3$). (c) Quantification of Western blots for p-mTOR expressions in the Ad-Mfn2 group compared to the control group and Ad-Mfn2$^+$ IGF-1 group ($^*P < 0.05$; $N = 3$). (d) Western blotting was used to detect the expression of p-PI3K, p-Akt, and p-mTOR in pancreatic cancer cells.

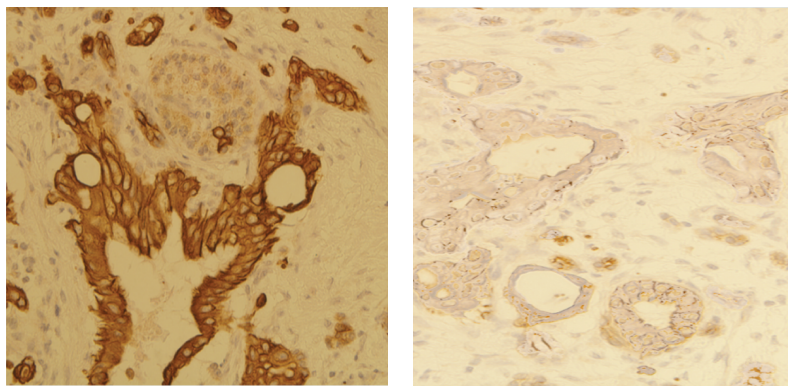

Figure 6: Pancreatic cancer is shown in a pancreas surgical resection specimen cell block with cytoplasmic brown staining with Mfn2 (immunohistochemistry; original magnification, ×400). (b) These cells were almost entirely ASPH$^+$ staining. (b) ASPH$^-$ staining cells can be found in this case.

The autophagy declines the ROS production of pancreatic cancer. The effect of autophagy in pancreatic cancer is also the potential mechanism of proapoptotic and antiproliferative functions of Mfn2 in pancreatic cancer.

Mitochondria are highly dynamic organelles, which respond to cellular stress by changes in interconnectedness, overall mass, and subcellular localization [24, 25]. The change in overall mitochondrial mass reflects the balance

Table 1: The relationship between Mfn2 expression and clinic pathological factors.

Number	Age	Sex	TNM	Grade	Stage	Mfn2
1	66	F	T2N0M0	1	I	+++
2	66	F	T2N0M0	1	I	++
3	54	F	T3N0M0	2	II	+
4	54	F	T3N0M0	1	II	+
5	44	M	T3N0M0	2	II	+
6	44	M	T3N0M0	2	II	++
7	59	M	T2N0M0	3	I	+
8	59	M	T2N0M0	–	I	+++
9	63	F	T2N0M0	3	I	+++
10	63	F	T2N0M0	3	I	++
11	53	F	T3N0M0	3	II	+
12	53	F	T3N0M0	3	II	++

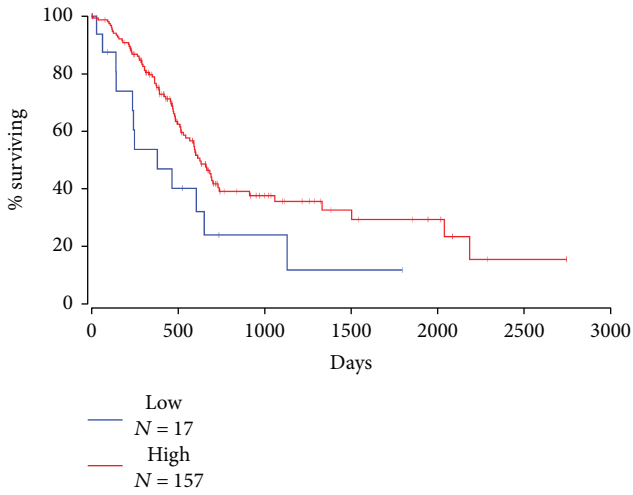

Figure 7: Overall survival curves of patients with pancreatic cancer. The Mfn2 expression curve was calculated according to the Kaplan-Meier method. The red line is the higher expression group ($n = 157$), and the blue line is the lower expression or without group ($n = 17$).

between the rates of mitophagy and mitochondrial biogenesis [26, 27]. Mfn2 plays an important role in the mitochondrial dynamic fission. In this study, Mfn2 is associated with a good survival rate in pancreatic cancer. This study also indicated that mitochondrial dynamics may be a key aspect of treating cancer.

In this study, we also used bioinformatics methods to analyze potential regulatory genes of Mfn2, aiming to provide valuable information for further biological mechanism elucidation of Mfn2 and provide the groundwork for therapeutic target identification for pancreatic cancer. The pathways enriched were mainly related to viral carcinogenesis by DAIVD, which indicated that Mfn2 is closely linked to the pathogenesis of cancer. This study also has some limitations. Further studies concerning the in vivo effect of Mfn2 for the pancreatic cancer is still required.

5. Conclusions

Our finding suggests that Mfn2 induces cell autophagy of pancreatic cancer through inhibiting the PI3K/Akt/mTOR signaling pathway. Meanwhile, Mfn2 also influences multiple biological functions of pancreatic cancer cells. Mfn2 can act as a therapeutic target in pancreatic cancer treatment.

Data Availability

All data used to support the findings of this study are included within the article and supplementary information files.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Supplementary Materials

Supplementary 1. Supplementary Figure S1: (online 1) PPI network of the Mfn2-regulated gene. The lines represent the protein-protein interaction relationships that correspond to the genes.

Supplementary 2. Supplementary Figure S2: (online) following GO analyses for Mfn2 regulatory genes, significant GO terms for the biological process were collected.

Supplementary 3. Supplementary Figure S3: (online) following GO analyses for Mfn2 regulatory genes, significant GO terms for the cellular component were collected.

Supplementary 4. Supplementary Figure S4: (online) following GO analyses for Mfn2 regulatory genes, significant GO terms for the molecular function were collected.

Supplementary 5. Supplementary Figure S5: (online) functional enrichment analysis of transcription factors (TFs). The transcription factor (TF) enrichment analysis for the Mfn2-regulated gene.

Supplementary 6. Supplementary Table S1: (online) antibodies and conditions used for Western blotting analyses.

Supplementary 7. Supplementary Table S2: Mfn2 regulatory genes obtained by GeneMANIA.

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Review Article

mTOR Inhibitor Therapy and Metabolic Consequences: Where Do We Stand?

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mTOR (mechanistic target of rapamycin) protein kinase acts as a central integrator of nutrient signaling pathways. Besides the immunosuppressive role after solid organ transplantations or in the treatment of some cancers, another promising role of mTOR inhibitor as an antiaging therapeutic has emerged in the recent years. Acute or intermittent rapamycin treatment has some resemblance to calorie restriction in metabolic effects such as an increased insulin sensitivity. However, the chronic inhibition of mTOR by macrolide rapamycin or other rapalogs has been associated with glucose intolerance and insulin resistance and may even provoke type II diabetes. These metabolic adverse effects limit the use of mTOR inhibitors. Metformin is a widely used drug for the treatment of type 2 diabetes which activates AMP-activated protein kinase (AMPK), acting as calorie restriction mimetic. In addition to the glucose-lowering effect resulting from the decreased hepatic glucose production and increased glucose utilization, metformin induces fatty acid oxidations. Here, we review the recent advances in our understanding of the metabolic consequences regarding glucose metabolism induced by mTOR inhibitors and compare them to the metabolic profile provoked by metformin use. We further suggest metformin use concurrent with rapalogs in order to pharmacologically address the impaired glucose metabolism and prevent the development of new-onset diabetes mellitus after solid organ transplantations induced by the chronic rapalog treatment.

1. Introduction

The mammalian target of rapamycin (mTOR) is a cytoplasmic serine/threonine protein kinase that belongs to the phosphoinositide 3-kinase, PI3K-related kinase family, which operates as a central regulator of cell metabolism, growth, proliferation, and survival. It is activated by nutrients (glucose, amino acids, and lipids), growth factors, insulin, and inflammatory cytokines [1, 2]. The mTOR has a unique intracellular signaling position, integrating all those factors, and is a critical regulator of the immune response because it plays a central role in sensing nutrient availability, cytokine/growth factor signaling, and costimulatory factors. Except from the inhibition of interleukin-2-induced T-cell proliferation, mTOR inhibitors induce the development of Treg cells, suppress dendritic cell proliferation and maturation, and play so many complex roles in immune cell cross-talks, including the promotion of proinflammatory cytokine production in some circumstances [3–6].

The increasing use of mTOR inhibitors in recent years, as immunosuppressants both in solid organ transplantation and in the treatment of certain tumors, such as the advanced renal cell carcinoma, also has confronted us with the development of the unwanted effects of this therapy. The development of the adverse effects is primarily a consequence of pleiotropy, a central role for mTOR in a variety of signaling pathways regulating metabolism, growth, and senescence. Among the most common undesirable effects of mTOR inhibitor therapy is metabolic syndrome that implies hyperglycemia with de novo diabetes mellitus (DM) and dyslipidemia.

The retrospective analysis of data from the US Renal Data System ($N = 20{,}124$ renal transplant patients) has shown that sirolimus was independently associated with an increased risk of new-onset DM [7]. The patients treated with everolimus may develop new-onset diabetes mellitus in up to 32% of cases as a result of hyperglycemia and insulin resistance [8]. The prevalence of hyperlipidemia is significantly higher and occurs in as many as 75% of the patients who are treated with mTOR inhibitors [9, 10].

However, the already known facts that the increased mTOR activity is associated with insulin resistance [11–13] and that the caloric restriction and short-term treatment with rapamycin have led to an increase in insulin sensitivity and glucose uptake [14, 15] suggest a contradictory or dual role of mTOR and mTOR inhibitors. In this review, we will highlight and compare the mechanisms of mTOR inhibitor therapy to the mechanisms of the excessive activation of mTOR leading to metabolic abnormalities. In addition, we will discuss potential therapeutic strategies to mitigate these abnormalities.

2. mTOR Signaling Pathways and Pharmacological Inhibition

mTOR is composed of two distinct multiprotein complexes with different cellular functions named mTORC1 and mTORC2 [16]. mTORC1 complex contains five components: mTOR, which is the catalytic subunit; regulatory-associated protein of mTOR (Raptor); mammalian lethal with Sec13 protein8 (mLST8); proline-rich Akt substrate 40 kDa (PRAS40); and DEP domain containing mTOR-interacting protein (Deptor) [17]. Raptor and mLST8 positively regulate mTOR's activity and functions, whereas PRAS40 and Deptor are the negative regulators of the mTORC1 [18–21].

The main inhibitor of mTORC1 is tuberous sclerosis complex 1 (TSC1) and TSC2. Growth factors, nutrients, cytokines, hormones such as insulin, and cellular energy level activate several pathways such as PI3K-Akt and RAS-mitogen-activated protein kinase (MAPK), leading to the inhibition of the TSC1-TSC2 complex [1, 22]. As a consequence, the uninhibited, that is, activated mTORC1, further through S6 kinase 1 (S6K1), 4E-binding protein-1 (4EBP1), cyclin-dependent kinases (CDKs), and the hypoxia-inducible factor 1α (HIF1α), promotes energy metabolism, protein synthesis and lipogenesis, proliferation, and growth [22]. Actually, the activated mTORC1 via an interaction between Raptor and a TOR signaling (TOS) motif in S6K and 4EBP1 phosphorylates S6K1 and 4EBP1 [23, 24]. The phosphorylated S6K1 then phosphorylates S6 (40S ribosomal protein S6), thereby enhancing the translation of mRNAs. The role of 4EBP1 is to inhibit the initiation of protein translation. It binds and inactivates the eukaryotic translation initiation factor 4E (eIF4E) [25]. When 4EBP1 is phosphorylated by mTORC1, it dissociates from eIF4E, enabling the increased translation of mRNAs and G1-to-S phase transition [25, 26]. mTORC1 also promotes growth by negatively regulating autophagy, which is the central degradative process in cells, but it is beyond the scope of this article [27].

The PI3K/Akt and mTOR signaling are closely interconnected. The binding of growth factors to insulin-like growth factor receptor (IGFR), platelet-derived growth factor receptor (PDGFR), or epidermal growth factor receptor (EGFR) generates downstream signal, which activates the PI3K/Akt pathway. When insulin binds to its cell surface receptor, the recruitment of insulin receptor substrate 1 (IRS) is promoted with the activation of PI3K and the production of phosphatidylinositol (3,4,5)-trisphosphate (PIP3) [2] (Figure 1). PIP3 binds to Akt and then engages this kinase to the cell membrane, to be activated by phosphorylation by PDK1 [28]. Activated Akt phosphorylates several downstream substrates, including TSC1/TSC2 complex, thereby activating mTORC1 and downstream effectors of mTORC1 [29, 30]. The upstream IRS pathway is negatively regulated by the mTOR-S6K1 pathway through a direct phosphorylation on specific residues [31, 32]. This increased degradation of IRS1, caused by hyperphosphorylation on serine/threonine residues, can lead to insulin resistance associated with the mTOR overactivation.

Compared to mTORC1, much less is known about the upstream activators of the mTORC2 pathway. mTORC2 responds to the growth factors such as insulin, via direct associations to ribosome in a PI3K-dependent fashion [33]. mTORC2 directly activates Akt by phosphorylating its hydrophobic motif (Ser473) and SGK1, a kinase controlling ion transport and growth [1, 34]. The loss of mTORC2 does not prevent phosphorylation of some Akt targets such as TSC2 but completely abolishes the activity of SGK1 [34, 35]. Thus, PI3K/Akt, in addition to the activation of mTORC2 by promoting its association with ribosomes, also controls the mTORC1 activation through the Akt-dependent TSC1/TSC2 inhibition [36]. Except from Akt and SGK1, PKC-α is another kinase activated by mTORC2, which regulates cell shape by affecting the actin cytoskeleton [37].

Originally, it was thought that acute treatment with rapamycin in contrast to the mTORC1 inhibition does not perturb mTORC2 signaling, but recent data confirm that there is a cell-type specificity to the rapamycin sensitivity of mTORC2 assembly [38]. Anyway, although mTORC2 is less responsive to rapamycin and rapalogs, a prolonged exposure to these compounds leads to a suppressed mTORC2 assembly, with a consequent inhibition of Akt signaling [39]. At the same time, rapalogs therapy results in a reduced or modified efficacy, due to the existence of numerous negative feedback loops in the mTOR pathway. The direct phosphorylation of IRS1 by the mTOR-S6K1 pathway, which promotes IRS1 degradation and PI3K/Akt downregulation, has already been mentioned [31, 32, 40]. That is why rapalogs lead to a decrease in negative feedback of the mTOR-S6K1 pathway on IRS pathway, thereby increasing the growth factor and Akt signaling with a decreased apoptotic potential. This is one of the reasons for insufficient antitumor activity of the mTOR inhibitors.

However, regardless of the association of mTOR overactivation and insulin resistance, rapalogs may also cause insulin resistance and hyperglycemia. In order to explain this phenomenon, it is necessary to look at the effects of mTOR

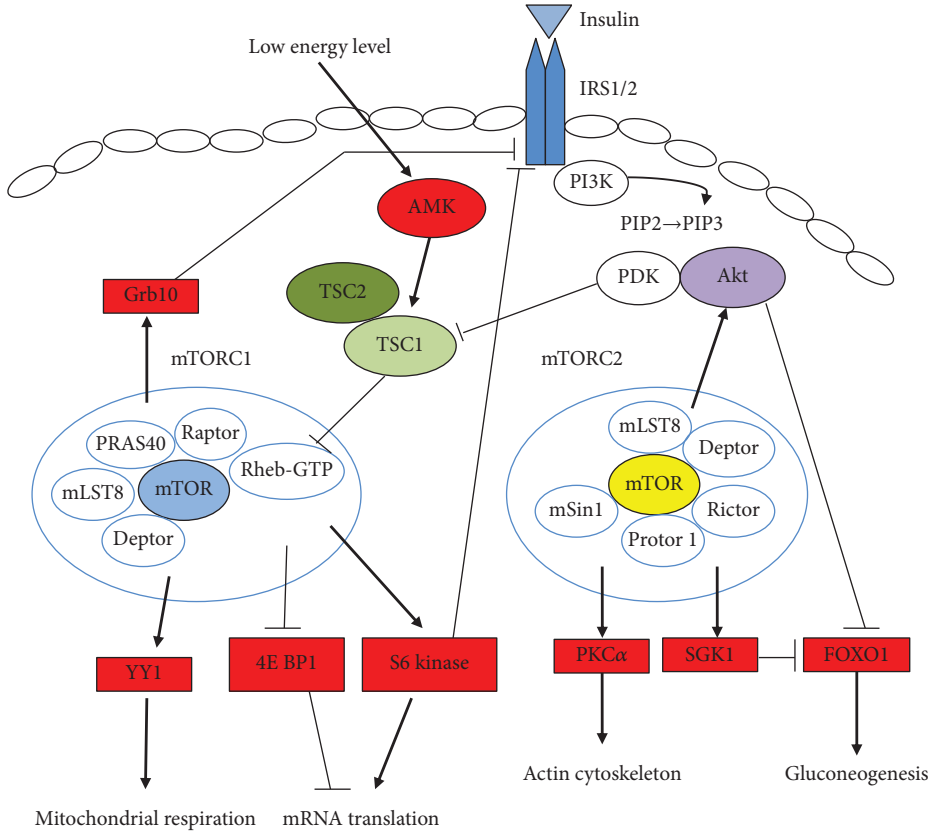

Figure 1: mTOR signaling pathways. IRS 1/2: insulin receptor substrate protein-1/2; PI3K: phosphoinositide 3-kinase; AKT: protein kinase B; Grb 10: growth factor receptor-bound protein 10; AMPK: adenosine monophosphate-activated protein kinase; TSC1: tuberous sclerosis complex 1; TSC2: tuberous sclerosis complex 2; mTORC1: mTOR complex 1; mTORC2: mTOR complex 2; PDK: phosphoinositide-dependent protein kinase 1.

inhibition in several organs, in the first place including the pancreas and the liver.

3. Metabolic Consequences of Overactivated mTOR

The postprandial increase of glucose and insulin activates mTOR and consequently protein kinase B (Akt) through mTORC2. The activation of Akt leads to glucose uptake by an increased GLUT4 translocation to the membrane in adipocytes [41]. The GSK-3 phosphorylation and deactivation by Akt decrease the rate of phosphorylation of glycogen synthase and increase the glycogen synthase activity and the accumulation of glycogen, most importantly in the liver and muscles [42]. Additionally, Akt controls glucose homeostasis by phosphorylating and inhibiting FOXO1, a transcription factor that regulates gluconeogenesis [43]. In addition, mTORC2 promotes glycogen synthesis and decreases gluconeogenesis in the liver [44].

As we have already mentioned, it is important to emphasize that both nutrients and insulin activate mTOR, but the overactivated mTOR further causes insulin resistance by at least two mechanisms [13, 32, 45]. S6K1 activated by mTORC1 causes the phosphorylation and degradation of insulin receptor substrate 1/2, thereby impairing insulin signaling. By affecting the growth factor receptor-bound protein 10, mTORC1 may also cause insulin resistance. The deletion of S6K1 is sufficient to improve insulin sensitivity in mice and in fat-fed rodents, while the activated mTOR pathway leads to an impaired insulin signaling and insulin resistance [46, 47]. In humans, the infusion of amino acids activates the mTOR/S6K1 pathway and consequently causes insulin resistance in skeletal muscles [45].

Thus, the overactivation of mTOR in the liver, muscles, adipose tissues, and pancreas leads to insulin resistance. Initially, mTORC1 stimulates β-cell functions causing an increased insulin secretion and the expansion and hypertrophy of β cells. The mTORC2-Akt axis positively affects β-cell mass by promoting proliferation and survival [27]. In further course of the chronic mTOR stimulation, mTOR renders β-cells resistant to IGF-1 and insulin, fostering cell death [48, 49]. It means that the overactivated mTORC1 in pancreas β-cells causes an increased insulin secretion to compensate for insulin resistance, but eventually, it leads to β-cell failure.

The mTOR activity affects lipid metabolism, too. Signaling promotes lipogenesis in the liver. Through sterol regulatory element-binding protein (SREBP), mTOR promotes lipogenesis in the liver [50]. The insulin-stimulated mTORC1 enhances lipogenesis and lipid storage, while it inhibits lipolysis, β-oxidation, and ketogenesis. The activated mTORC1

has an impact on three lipases: adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), and lipoprotein lipase (LPL) [51]. In adipocytes, ATGL catalyzes the lipolysis of triacylglycerol to diacylglycerol, and then HSL converts diacylglycerol to monoacylglycerol. mTORC1 reduces the HSL activity and decreases the activity of extracellular LPL, which is important for lipoprotein uptake in tissues. The mTORC1 activation reduces ketone body production by inhibiting PPAR-α activity in the liver [27].

By coordinating various levels of the gene expression, mTORC1controls mitochondrial mass and functions. The loss of mTORC1 in the muscle of mice reduces oxidative function and muscle mass leading to an early death [52]. The loss of mTORC1 or rapamycin treatment reduces peroxisome proliferator-activated receptor coactivator 1-alpha (PGC-1α) expression and inhibits the complex of PGC-1α with the transcription factor yin-yang 1 YY1 [53]. Rapamycin decreases the gene expression of PGC-1alpha, oestrogen-related receptor alpha, and nuclear respiratory factors, which are mitochondrial transcriptional regulators, resulting in a decrease in mitochondrial gene expression and oxygen consumption. YY1 regulates mitochondrial gene expression and is a common target of mTOR and PGC-1alpha. The inhibition of mTOR results in a failure of YY1 to interact and has coactivated by PGC-1alpha, thereby depressing mitochondrial oxidative function [53].

Ultimately, insulin resistance due to elevated mTOR activity, characterized by increased hepatic gluconeogenesis, reduced glucose uptake by muscles, and pancreatic β-cell apoptosis, leads to type II diabetes. Taking into consideration that insulin resistance and associated complications such as retinopathy, neuropathy, and nephropathy can precede the diagnosis of type II diabetes raises the question of the possibility for the prevention of diabetic complications using pharmacological inhibition of the mTOR pathway.

4. Glucose Intolerance Induced by mTOR Inhibitors

It is obvious that mTOR has multiple roles in metabolism and, when overactivated by nutrient overload and obesity, participates in causing glucose intolerance and insulin resistance. Calorie restriction, which means a reduction in caloric intake, while maintaining adequate nutrition, improves glucose tolerance and insulin sensitivity and extends lifespan [54, 55]. Given the assumption that rapamycin is a starvation mimetic, its role has been suggested in reversing insulin resistance. The acute treatment with rapamycin (single injection) increases insulin sensitivity and glucose uptake [14, 56]. In healthy volunteers, a single dose of rapamycin as a pretreatment abrogates nutrient-induced insulin resistance [57]. In contrast to the results of acute or intermittent rapamycin treatment, the chronic treatment with rapamycin impairs glucose homeostasis. Paradoxically, the chronic rapamycin treatment leads to glucose intolerance in both animals and humans [7, 58, 59]. Although chronic rapamycin treatment reduces fat content, it also promotes insulin resistance, glucose intolerance, and gluconeogenesis in the liver. Despite the improved insulin signaling in the liver of rapamycin-treated rats, which came out from the blockade of the mTOR/S6K1 negative feedback loop, the induction of gluconeogenic pathway in the liver potentiates glucose intolerance [59, 60]. Although white adipose tissue and skeletal muscles take up glucose normally in response to continuous insulin stimulation during the chronic rapamycin treatment, hepatic insulin resistance is a major contributor to the impaired glucose homeostasis [59]. It has been shown that the insulin-mediated suppression of hepatic gluconeogenesis is directly mediated by rapamycin-induced mTORC2 disruption [59]. Except from the mTORC2 inhibition, the chronic rapamycin treatment contributes to insulin resistance, due to inability to activate fatty acid β-oxidation and ketogenesis, leading to an imbalance in lipid metabolism [61]. Additionally, a prolonged rapamycin treatment leads to a decreased β-cell viability and decreased insulin secretion, probably via the inhibition of mTORC2 [62, 63]. This increased β-cell toxicity induced by the chronic mTOR inhibitor treatment might be a bridge leading to the development of new onset of diabetes mellitus after solid organ transplantations, imposing the need of the development of strategies to avoid this adverse effect.

5. The Role of Metformin in the Reversal of Insulin Resistance Induced by mTOR Inhibitors

The clinical significance of insulin resistance is associated with coronary artery disease and ischemic stroke [64, 65]. Metformin, a widely prescribed antidiabetes drug, is a biguanide and represents the first line of the treatment for type II diabetes mellitus [66]. It not only decreases hyperglycemia primarily by lowering hepatic gluconeogenesis but also increases insulin sensitivity and lowers blood lipid level [67]. However, in addition to the treatment of type II diabetes mellitus, metformin has shown its beneficial effect in aging-related diseases such as cancer and cardiovascular diseases [68–70]. In all of these aging-related conditions, metformin has achieved effects similar to the effects of rapamycin therapy. Several epidemiological studies have confirmed that the treatment of diabetes type II with metformin was associated with a reduced cancer incidence and cancer-related death [68, 71–73]. Different animal experimental models have as well shown varying anticancer and prolongevity effects depending on dosage, sex, and age at the onset of metformin treatment [74–76].

The molecular mechanisms of metformin are only partially understood. The multiple mechanisms of action have been studied, suggesting inhibition of the mitochondrial respiratory chain (complex I) as the primary mode of action. [77, 78]. As a result, a decrease in cellular energy status with an increased cellular AMP : ATP ratio activates AMP-activated protein kinase (AMPK), which inhibits mTORC1 signaling in the liver, the primary site of metformin action, with different downstream effects [78–80]. At a lower dosage, metformin requires AMPK and the TSC to inhibit mTORC1, whereas at higher dosage, this effect is AMPK and TSC independent [80]. Anyway, metformin decreases a hepatic protein synthesis through a mechanism implicating inhibitory

effect on mTORC1. By inducing the phosphorylation of GLUT4 enhancer factor, metformin enhances the peripheral glucose uptake, thereby increasing insulin sensitivity. Additionally, metformin decreases an insulin-induced suppression of fatty acid oxidation [81]. The effect appears to be attributable to a stimulation of AMPK and the reduction of malonyl-CoA content in the muscles.

All these metabolic effects are almost identical to the effects of hunger, that is, dietary restriction. It has been shown that metformin-treated mice had a transcriptional profile resembling mice subjecting to dietary restriction [82]. Although both rapamycin and dietary restriction inhibit lipogenesis and activate lipolysis with consequent increased serum levels of nonesterified fatty acids, in contrast to dietary restriction, rapamycin does not activate β-oxidation [61]. Considering that the two main characteristics of metabolic disorder caused by rapamycin are the stimulation of gluconeogenesis in the liver and the decrease of β-oxidation, metformin is imposed as a potential solution. Since decreased fatty acid oxidation is associated with the development of insulin resistance, the metformin-induced fatty acid oxidation might contribute to the increase of insulin sensitivity. The addition of metformin to chronic rapamycin treatment may provide a therapeutic approach to treat insulin resistance and dyslipidemia. Most of the literature discusses the combined use of metformin and rapamycin for the purpose of treating aging and aging-related diseases. Another option suggested for prevention, that is, treat metabolic disorder caused by rapamycin, is an intermittent application of rapamycin, taking into account the fact that after the cessation of rapamycin therapy, insulin resistance and glucose intolerance are reversible. It is clear that this approach cannot be used in patients treated with immunosuppressive therapy to prevent transplant rejection or in patients who take mTOR inhibitors as an anticancer treatment, where therapy with mTOR inhibitors must be continuous.

The so-far conducted study summarized rapamycin effects on mTORC1 and mTORC2, pointing to the fact that a long-term treatment with rapamycin in addition to mTORC1 also disrupts mTORC2, thereby causing β-cell toxicity and insulin resistance [62, 83]. This effect of mTORC2 inhibition was confirmed in vivo in multiple tissues, including the liver, white adipose tissue, and skeletal muscle [59]. Given the assumption that the immunosuppressive effects of rapamycin are mediated predominantly via mTORC1, one may suppose that the mTORC1-specific inhibitors would achieve the same immunosuppressive effects as rapamycin, but without any mTORC2-mediated toxicity. This assumption might be operating when mTOR inhibitors are used as antiaging therapeutics because mTORC1 inhibition would achieve the desired effects by avoiding metabolic disorders caused by mTORC2 inhibition [84].

Would it be so if the mTORC1-specific inhibitors were used as immunosuppressive drugs? It seems that important immunosuppressive effects of mTOR inhibitor therapy are mediated by the inhibition of both mTORC1 and mTORC2. In addition to the inhibition of T-cell proliferation and blockade of dendritic cell maturation, one of the hallmarks of the immunoregulatory properties of mTOR inhibitors is the development of Tregs whose differentiation and expansion are suppressed by mTORC2 activity [85, 86]. This means that the specific mTORC1 inhibition in the cells belonging to the immune system without mTORC2 disruption may lead to an insufficient immunosuppression.

There are some indications that other rapalogs, such as everolimus and temsirolimus, achieve a lower degree of mTORC2 inhibition and thus a lower degree of insulin resistance, but this still needs to be confirmed in other studies [87].

Anyway, the necessity of the constant use of mTOR inhibitors after solid organ transplantations, such as kidney transplantation, prevents the regimen of intermittent application of rapamycin or the use of rapamycin in smaller doses. In an attempt to solve this problem, that is, to prevent insulin resistance and new-onset diabetes after a kidney transplantation, the combined therapy of rapamycin and metformin has been suggested [88, 89]. By inducing AMPK at clinically relevant doses, metformin inhibits mTORC1, helping to reduce the dose of rapalogs and associated adverse metabolic effects. If the patients with kidney transplants have GFR > 60 ml/min, metformin may be prescribed for the treatment of preexisting type 2 diabetes mellitus or new-onset diabetes mellitus [90].

6. Conclusion

We are trying to show that metformin use is also possible in order to prevent the onset of diabetes mellitus after a kidney transplantation. So far, no studies have been carried out to investigate the role of metformin in the prevention of new-onset diabetes mellitus after a transplantation. The future research can result in clinical guidelines, which will allow us to better counteract rapalog-mediated adverse effects.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Exendin-4 and Liraglutide Attenuate Glucose Toxicity-Induced Cardiac Injury through mTOR/ULK1-Dependent Autophagy

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Mitochondrial injury and defective autophagy are common in diabetic cardiomyopathy. Recent evidence supports benefits of glucagon-like peptide-1 (GLP-1) agonists exendin-4 (Exe) and liraglutide (LIRA) against diabetic cardiomyopathy. This study was designed to examine the effect of Exe and LIRA on glucose-induced cardiomyocyte and mitochondrial injury, oxidative stress, apoptosis, and autophagy change. Cardiomyocytes isolated from adult mice and H9c2 myoblast cells were exposed to high glucose (HG, 33 mM) with or without Exe or LIRA. Cardiac contractile properties were assessed including peak shortening, maximal velocity of shortening/relengthening ($\pm dL/dt$), time to PS, and time-to-90% relengthening (TR_{90}). Superoxide levels, apoptotic proteins such as cleaved caspase-3, Bax, and Bcl-2, and autophagy proteins including Atg5, p62, Beclin-1, LC3B, and mTOR/ULK1 were evaluated using Western blot. Mitochondrial membrane potential (MMP) changes were assessed using JC-1, and autophagosomes were determined using GFP-LC3. Cardiomyocyte exposure to HG exhibited prolonged TR_{90} associated with significantly decreased PS and $\pm dL/dt$, the effects of which were partly restored by GLP-1 agonists, the effects of which were negated by the mTOR activator 3BDO. H9c2 cell exposure to HG showed increased intracellular ROS, apoptosis, MMP loss, dampened autophagy, and elevated p-mTOR and p-ULK1, the effects of which were nullified by the GLP-1 agonists. These results suggested that GLP-1 agonists rescued glucose toxicity likely through induction of mTOR-dependent autophagy.

1. Introduction

Diabetes mellitus is becoming a major health threat as the International Diabetes Federation (IDF) predicts a startling number of 642 million patients with diabetes by the year of 2040 [1]. This chronic metabolic disease can steadily trigger a cascade of long-term severe complications such as cardiovascular diseases, peripheral vascular diseases, and central nervous system and kidney diseases [2–5]. Among these comorbidities, diabetic cardiomyopathy occurs independent of macro- and micro-coronary artery diseases and other cardiovascular diseases and imposes a high risk for cardiovascular morbidity and mortality [6]. The major pathological manifestations of diabetic cardiomyopathy include hypertrophy, ventricular dilatation, and compromised contractile function, which may be attributed to apoptosis and interstitial fibrosis, leading to ventricular remodeling [7, 8]. Previous studies from our lab and others have depicted a number of pathophysiological factors for the onset and development of diabetic cardiomyopathy including glucose and lipid toxicity, inflammation, oxidative stress, mitochondrial injury, interstitial fibrosis, apoptosis, and dysregulated autophagy [9–12]. Nevertheless, the precise molecular mechanism behind diabetic cardiomyopathy remains obscure.

Autophagy denotes a cellular degradation process for long-lived or damaged proteins and cytoplasmic organelles, through which these cytoplasmic proteins are degraded and recycled by lysosomes [13]. Autophagy plays a pivotal role

for cardiac homeostasis in both physiological and pathological conditions [14]. Constitutive autophagy helps to sustain a balance between the synthesis, degradation, and subsequent recycling of cellular elements. Recent studies have indicated that levels of autophagy may be suppressed in diabetes, leading to the development of diabetic cardiomyopathy [15]. To this end, there is a growing interest in the administration of an autophagy inducer as a therapeutic approach in diabetes although many of the autophagy inducers suffer from pitfalls such as toxicity and undesired off-target effects.

Glucagon-like peptide-1 (GLP-1), synthesized and secreted from L-cells of the small intestine, is a 30-amino acid peptide with potent biological effects. The GLP-1 receptor is a G protein-coupled receptor broadly expressed in peripheral tissues including islet cells, kidney, lung, brain, and gastrointestinal tract [16]. Many peripheral tissues including the heart possess a GLP-1 receptor reminiscent of those found in pancreatic cells [16]. Clinical and experimental evidence has shown the utility of GLP-1 at the time of reperfusion in reducing myocardial infarct size, mitigating ischemic-reperfusion injury, and improving cardiac functions [17, 18]. Moreover, recent date suggested that GLP-1, its analogues, and receptor agonists are capable of benefiting diabetes, diabetic retinopathy, nephropathy, and peripheral neuropathy [19–21]. More interestingly, the GLP-1 receptor agonist exendin-4 (Exe) offers protective effects against type 2 diabetes-induced brain injury via autophagy induction [22]. However, whether GLP-1 receptor agonists play any role in diabetic cardiomyopathy remains unknown. Therefore, this study was designed to evaluate the effect of GLP-1 agonists Exe and liraglutide (LIRA) on high glucose-induced cardiomyocyte contractile dysfunction, mitochondrial injury, oxidative stress, apoptosis, and changes in autophagy.

2. Materials and Methods

2.1. Cardiomyocyte Isolation and Mechanics. All animal procedures used here were approved by the Animal Care and Use Committee at the University of Wyoming (Laramie, WY). In brief, hearts were removed rapidly from adult wild-type mice sedated with ketamine (80 mg/kg, ip) and xylazine (12 mg/kg, ip) and perfused with Krebs-Henseleit bicarbonate (KHB) solution consisting of (in mM) 118 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 10 HEPES, and 11.1 glucose. Hearts were digested with Liberase Blendzyme™ (Roche Diagnostics, Indianapolis, IN) for 15 min. After removal and mincing of the left ventricle, Ca^{2+} was added back to a final concentration of 1.25 mM. Cardiomyocytes with no spontaneous contractions and clear edges were used for shortening. The IonOptix SoftEdge system (IonOptix, Milton, MA) was employed to assess the mechanical properties of isolated myocytes. Myocytes were mounted on the stage of an Olympus IX70 microscope in contractile buffer containing (in mM) 131 NaCl, 4 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose, and 10 HEPES. Myocytes were stimulated at 0.5 Hz with cell shortening and relengthening evaluated using the following indices: peak shortening (PS), time to peak shortening (TPS), time to 90% relengthening (TR_{90}), and maximal velocities of shortening/relengthening ($\pm dL/dt$) [11]. To evaluate the effect of the GLP-1 agonist on glucose toxicity-induced changes in cardiac function, cells were cultured for 4 hours in a KHB solution containing 33 mM or 5.5 mM glucose (designated as high glucose and normal glucose) with or without Exe (10 nM) or LIRA (100 nM) prior to the assessment of cardiomyocyte mechanical properties.

2.2. Cell Culture and Treatment. Rat cardiomyocyte-derived cell line H9c2 was purchased from the American Type Culture Collection (ATCC, CRL-1446™) and was cultured in DMEM supplemented with 10% FBS, 1% penicillin, and streptomycin at 37°C in a humidified atmosphere (5% CO_2 and 95% air). When cells reached confluence at 70%–80%, they were exposed to normal glucose (NG, 5.5 mM) or high glucose (HG, 33 mM) for 48 hours in the absence or presence of Exe (10 nM) or LIRA (100 nM). To assess whether high glucose affects autophagy, H9c2 cells were exposed with high glucose medium in the absence or presence of Exe (10 nM) or LIRA (100 nM) in the presence or absence of the autophagy inducer rapamycin (100 nM) or the autophagy inhibitor 3-methyladenine (3-MA, 10 mM) [23]. To assess the role of mTOR in Exe- and LIRA-induced response, if any, against high glucose-induced cardiomyocyte mechanical dysfunction, murine cardiomyocytes were exposed with high glucose medium in the absence or presence of Exe (10 nM) or LIRA (100 nM) for 4 hours with or without the mTOR activator [3-benzyl-5-((2-nitrophenoxy) methyl)-dihydrofuran-2(3H)-one (3BDO),120 μM] [24] prior to assessment of mechanical function.

2.3. Intracellular Reactive Oxygen Species (ROS) Measurement. Intracellular superoxide anions were measured using the dihydroethidium (DHE) fluorescence probe. The cells were incubated in a light-impermeable chamber at 37°C for 30 min after application of 10 μM DHE (Life Technologies, USA) and then were cultured with 5 μg/ml DAPI (Sigma, USA) for 5 min. The images of H9c2 cardiomyocytes were captured and analyzed immediately under a fluorescence microscope (Olympus BX51, Japan). Production of reactive oxygen species (ROS) in cultured cells was assessed by 5-(6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H2DCFDA) molecular probe staining. In brief, H9c2 cells were loaded with 1 μM H_2DCFDA at 37°C for 30 min. The cells were rinsed with PBS, and the fluorescence intensity was then detected by a fluorescent microplate reader (Molecular Devices, Sunnyvale, CA) at an excitation wavelength of 480 nm and an emission wavelength of 530 nm [25].

2.4. Measurement of MitoSOX. The mito-ROS level was assessed using the MitoSOX™ Red mitochondrial superoxide indicator (Life Technologies, USA) according to the manufacturer's instructions. Briefly, after treatment, the cells were washed three times with PBS and incubated with 5 μM MitoSOX for 30 min in the dark. The level of mito-ROS was detected by a fluorescent microplate reader (Molecular Devices, Sunnyvale, CA) at 485 nm for excitation and 590 nm for emission [26].

2.5. Measurement of Mitochondrial Membrane Potential (MMP, $\Delta\Psi m$). Changes of MMP was measured using the fluorescent dye, 5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide (JC-1 fluorochrome, Sigma, USA) as described [27]. Briefly, after the treatment of the cells, 5 μM JC-1 staining was added to the cells and incubated in the dark for 30 min at 37°C. PBS buffer was used to wash H9c2 cells for three times and subsequently examined under a fluorescent microplate reader (Spectra Max Gemini XS) at 530 nm (monomer form of JC-1, green) and at 590 nm (aggregate form of JC-1, red). The mitochondrial uncoupler carbonyl cyanide m-chlorophenyl hydrazone (CCCP, 10 μM) was used as the positive control [28]. In addition, cells labeled with JC-1 were observed using a fluorescence microscope (Olympus BX51, Japan).

2.6. LC3B-GFP-Adenovirus Infection in H9c2. H9c2 cells were assessed by autophagy using GFP fluorescence [29]. An adenovirus containing a GFP-LC3 construct was provided by Dr. Cindy Miranti from the University of Arizona (Phoenix, AZ) and was propagated using the HEK293 cell line. Cells were transfected with GFP-LC3 adenovirus for 8 hours and then refreshed with normal medium. After 48 hours, cells were visualized for autophagy using fluorescence microscopy and were treated with either normal or high glucose in the absence or presence of GLP-1 agonists or the autophagy inhibitor 3-MA. Rapamycin was used as the positive control. For autophagy evaluation, cells were captured under a fluorescence microscope (Olympus BX51) and the percentage of GFP-LC3-positive cells showing numerous GFP-LC3 puncta (>10 dots/cell) was achieved as described previously [29]. Approximately 300–400 cells in each group were calculated in at least three independent experiments.

2.7. Western Blot Analysis. Cardiomyocytes were homogenized in a lysis buffer containing RIPA (Cell Signaling Technology, Danvers, MA), 1% NaF, 1% Na_3VO_4, and 1% protease inhibitor cocktail. Supernatants were separated after centrifugation at 12,000g for 15 min at 4°C. The protein levels of supernatant were quantified using the BSA protein assay. Equal amounts (30 mg protein/lane) of proteins were separated on 10% or 12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked and incubated overnight at 4°C with the following antibodies: p-mTOR (Ser^{2448}), mTOR, p-ULK1 (Ser^{757}), ULK1, Bcl-2, Bax, cleaved caspase-3, Atg5, P62, Beclin-1, LC3B, and GAPDH (Cell Signaling). Membranes were incubated for 1 hour at 37°C with a horseradish peroxidase-conjugated secondary antibody. Blots were assessed by the luminescence method. The Quantity One software (Bio-Rad, version 4.4.0, ChemiDoc XRS) was used for analysis quantification of immunoblots [29].

2.8. Statistical Analysis. Data were mean ± SEM. All statistical analyses were subjected to one-way ANOVA, and a p value less than 0.05 was considered to be significant.

3. Results

3.1. Effect of Exendin-4 and Liraglutide on Cardiomyocyte Shortening. Short-term exposure (4 hours) of high glucose (33 mM) did not affect the resting cell length in murine cardiomyocytes. However, high glucose incubation suppressed peak cell shortening (PS), maximal velocity of shortening/relengthening (+dL/dt and −dL/dt), and prolonged time-to-90% relengthening (TR_{90}) without affecting time-to-peak shortening (TPS). Although Exe and LIRA themselves did not affect these cardiomyocyte mechanical functions, they effectively rescued against glucose toxicity-induced changes in PS, ±dL/dt, and TR90 without affecting TPS and resting cell length (Figure 1).

3.2. Exendin-4 and Liraglutide Attenuated High Glucose-Induced ROS/O_2^- Production and Downregulation of Nrf2. ROS plays an essential role in glucose toxicity and diabetic cardiomyopathy [30, 31]. Here we examined levels of ROS and O_2^- using fluorescence techniques. As shown in Figure 2(a), O_2^- production assessed using DHE fluorescence was significantly elevated in the high glucose group compared with the normal glucose group. Although GLP-1 agonists did not affect O_2^- levels in normal glucose groups, they attenuated high glucose-induced elevation in O_2^- levels. To further verify these results, H9c2 cells were stained with the specific oxidation-sensitive fluorescent dye DCF; high glucose significantly enhanced the relative fluorescent intensity of DCF, corresponding to an increased ROS level, the effects of which were attenuated by either Exe or LIRA. Neither GLP-1 agonist had any effect on DCF fluorescence intensity themselves (Figure 2(b)).

Earlier evidence suggested a pivotal role for mitochondrial ROS in the development of diabetic cardiomyopathy [31]. As demonstrated in Figure 2(c), mitochondrial O_2^- evaluated using MitoSOX Red revealed that high glucose facilitated the generation of mitochondrial O_2^-, the effect of which was partly reversed by Exe and LIRA. Neither GLP-1 agonist exerted any notable effect on mitochondrial O_2^- themselves. We went on to examine the changes of the cytosolic antioxidant Nrf2 in H9c2 cells. As shown in Figure 2(d), high glucose overtly downregulated the Nrf2 expression, the effects of which were alleviated by either Exe or LIRA, with little effect from the GLP-1 agonists themselves.

3.3. Exendin-4 and Liraglutide Inhibited High Glucose-Induced Apoptosis. To determine the role of GLP-1 activation on glucose-induced apoptosis, apoptotic-related proteins including cleaved caspase-3, Bax, and Bcl-2 were evaluated. As shown in Figure 3, high glucose incubation significantly upregulated the levels of cleaved caspase-3 and Bax-to-Bcl-2 ratio, the effects of which were abrogated by Exe and LIRA with little effect from either GLP-1 agonist itself.

3.4. Exendin-4 and Liraglutide Ameliorated High Glucose-Induced Loss in MMP. JC-1 staining was performed to determine the stability of MMP in H9c2 cells. Our results revealed that high glucose significantly decreased MMP, as evidenced by the decreased aggregate-to-monomer ratio.

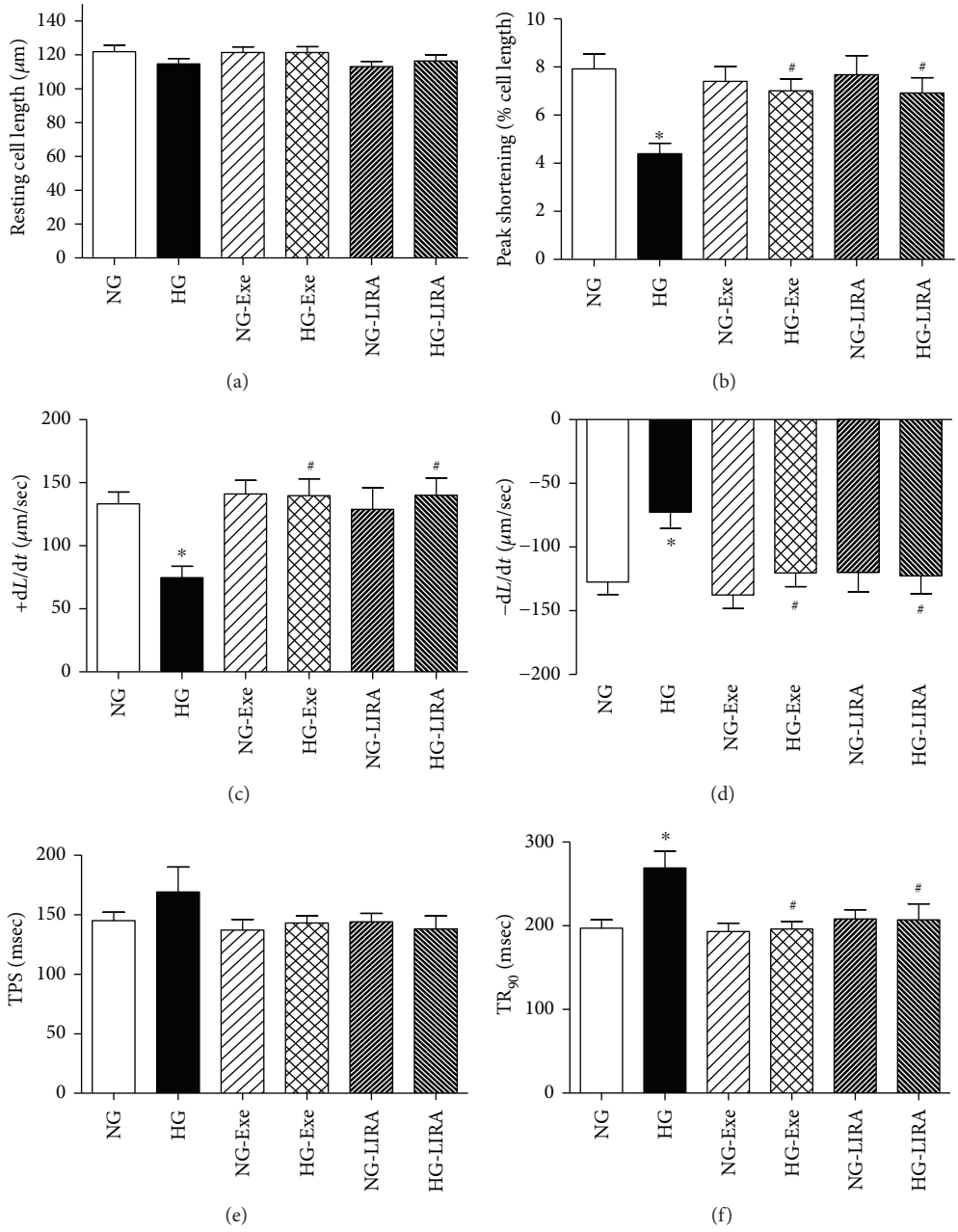


Figure 1: Effect of exendin-4 (Exe) and liraglutide (LIRA) on cardiomyocyte shortening in ventricular myocytes isolated from adult mouse hearts: (a) resting cell length; (b) peak shortening (PS); (c) maximal velocity of shortening (+dL/dt); (d) maximal velocity of relengthening (−dL/dt); (e) time to PS (TPS); (f) time-to-90% relengthening (TR_{90}). Mean ± SEM, $n = 63 – 66$ cells per group, $^{*}p < 0.05$ versus the NG group; $^{\#}p < 0.05$ versus the HG group.

Although neither Exe nor LIRA affected MMP levels in the normal glucose group, they effectively nullified the high glucose-induced drop in MMP (Figure 4).

3.5. Exendin-4 and Liraglutide Protected against Glucose Toxicity-Induced Loss in Autophagy. Defective autophagy was reported in the setting of diabetes or hyperglycemia and may contribute to the pathogenesis of diabetic cardiomyopathy [11, 30, 32]. To discern the role of autophagy in the GLP-1 activation-induced beneficial role against glucose toxicity, levels of autophagy-related proteins were examined using Western blot analysis. As shown in Figure 5, high glucose challenge (for 48 hours) significantly decreased the levels of autophagy protein markers including Beclin-1, Atg5, LC3II, LC3II-to-LC3I ratio, and p62, the effects of which were negated by Exe and LIRA. Neither GLP-1 agonist produced any notable effect on autophagy protein markers in a normal glucose environment. Next, fluorescence microscopy was employed to visualize autophagosome formation. LC3-II accumulates due to increased autophagosome formation or impaired autophagosome-lysosome fusion. As shown in Figure 6, H9c2 cells cultured in a high-glucose medium

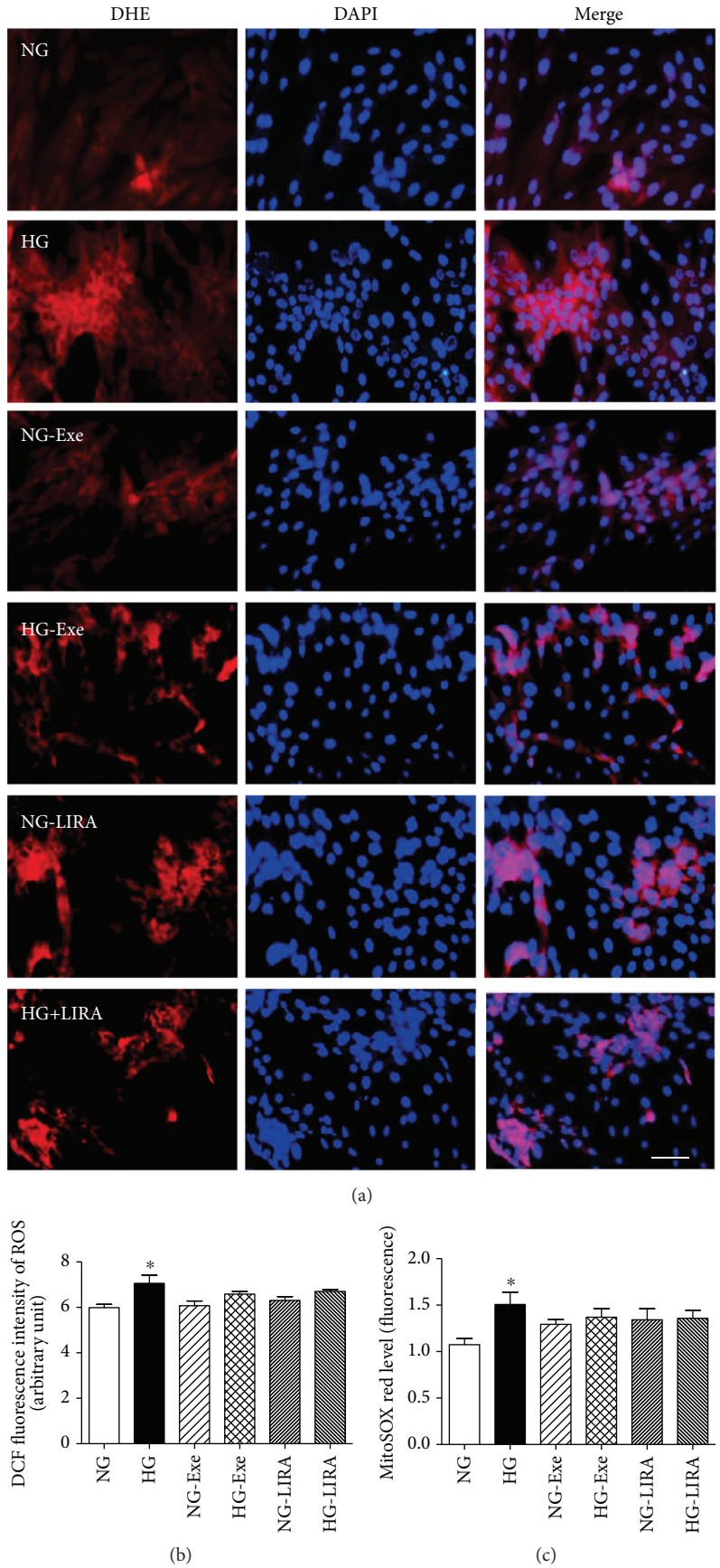


Figure 2: Continued.

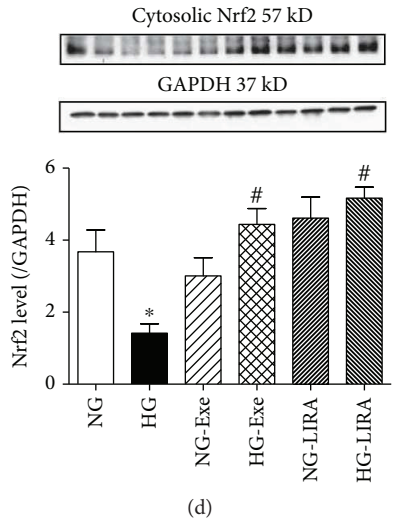


Figure 2: Effect of exendin-4 (Exe) and liraglutide (LIRA) on accumulation of ROS and mitochondrial O_2^- as well as antioxidant Nrf2: (a) representative images of DCF staining depicting the effect of Exe and LIRA on high glucose-induced ROS production in H9c2 cells, scale bar = 50 μm; (b) pooled data of DCF quantification, $n = 7$; (c) quantification of MitoSOX red intensity, $n = 7$; (d) levels of Nrf2 normalized to GAPDH. Inset: representative gel blots of Nrf2 and GAPDH using specific antibodies, $n = 4$, independent cell cultures per group, mean ± SEM, $^*p < 0.05$ versus the NG group and $^\#p < 0.05$ versus the HG group.

exhibited a decrease in the number of punctate GFP-LC3 structures. Although neither GLP-1 agonist had any effect on GFP-LC3 puncta formation, they effectively rescued against a glucose toxicity-induced decrease in the number of punctate GFP-LC3. Furthermore, class III PtdIns3K inhibitor 3-MA, an autophagy inhibitor, ablated Exe- or LIRA-induced restoration of LC3II accumulation. These data strongly indicated that Exe and LIRA protects against high glucose-induced loss in autophagy.

3.6. Exendin-4 and Liraglutide Promoted mTOR/ULK1-Dependent Signaling. To determine whether Exe and LIRA activates autophagy through the classical mTOR/ULK1-dependent pathway, mTOR and ULK1 signaling was examined using Western blot. As demonstrated in Figure 7, high glucose incubation significantly increased phosphorylation of both mTOR and ULK1 in murine cardiomyocytes, the effects of which were negated by Exe and LIRA. Neither GLP-1 agonist exhibited any effect on phosphorylation of mTOR and ULK1. Neither GLP-1 agonist nor glucose challenge overly affected the pan protein expression of mTOR and ULK1. These results demonstrated that mTOR/ULK1 signaling is likely involved in GLP-1 activation-offered restoration of autophagy in the face of high glucose challenge.

3.7. Role of mTOR in Exendin-4- and Liraglutide-Induced Cardiomyocyte Mechanical Responses against High Glucose. To evaluate whether mTOR plays a permissive role in GLP-1 agonists Exe- and LIRA-induced beneficial response against high glucose challenge, adult murine cardiomyocytes were incubated with normal- (5.5 mM) or high-glucose (33 mM) medium in the absence or presence of GLP-1 agonists and the mTOR activator 3BDO (120 μM) for 4 hours prior to the assessment of cardiomyocyte function. As shown in the Figure 8, the mTOR activator negated Exe- and LIRA-induced protection against high glucose-induced cardiomyocyte mechanical dysfunction (decreased PS, $+dL/dt$, and $-dL/dt$ as well as prolonged TR_{90}, with unchanged resting cell length and TPS). mTOR activation itself did not affect any cardiomyocyte mechanical property in cells incubated in normal- or high-glucose medium. These results favored a permissive role for mTOR in GLP-1 agonist-offered beneficial response against glucose toxicity.

4. Discussion

The salient findings from our study suggested that the GLP-1 agonists Exe and LIRA protect against short-term high-glucose incubation-induced impairment in cardiac contractile function, ROS/O_2^- production, apoptosis, and mitochondrial injury. Glucose toxicity-induced cardiomyopathy is believed to contribute to ventricular dysfunction and the onset of heart failure in diabetes [33–36]. While treatment of diabetic cardiomyopathy remains challenging, our work suggested that GLP-1, an incretin hormone, may serve as an alternative avenue for cardiovascular complications in diabetes. Our data revealed a likely role for autophagy in GLP-1 activation-offered beneficial effects.

Data from our study suggested that short-term high-glucose challenge compromised cardiac function, promoted accumulation of ROS and O_2^-, and triggered apoptosis and loss of MMP, in a manner somewhat similar to our earlier reports [35, 36]. Oxidative stress and apoptosis are considered the main contributing factors in the pathogenesis of diabetic heart anomalies [37]. Our observation of DHE, DCF, and MitoSOX Red staining supported the earlier notion of

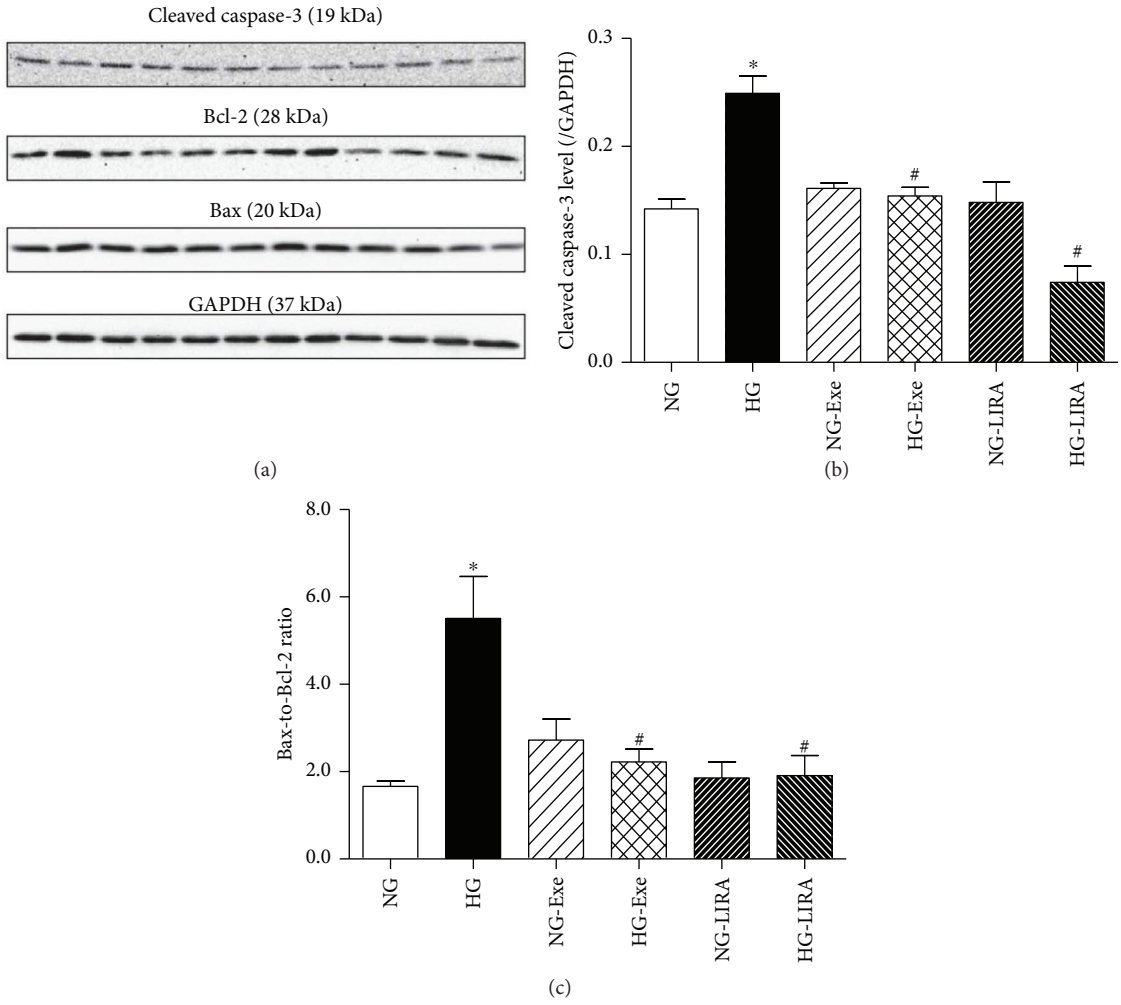


Figure 3: Effect of exendin-4 (Exe) and liraglutide (LIRA) on glucose toxicity-induced elevation of apoptotic proteins: (a) representative gel bands of cleaved caspase-3, Bax, Bcl-2, and GAPDH (loading control) using specific antibodies; (b) quantitative analysis of cleaved caspase-3; (c) quantitative analysis of the Bax-to-Bcl-2 ratio. Mean ± SEM, $n = 4 – 6$ cultures per group, $^{*}p < 0.05$ versus the NG group and $^{\#}p < 0.05$ versus the HG group.

oxidative stress upon high glucose challenge. Mitochondria usually yield energy through oxidative phosphorylation by way of the electron transport chain although incomplete reduction of O_2 could result in O_2^- production. Our MitoSOX measurement indicated much higher MitoSOX fluorescence in high glucose-challenged H9c2 cells, the effect of which was attenuated by GLP-1 activation. Our further examination noted overtly decreased levels of Nrf2, an oxidative stress-activated transcription factor to maintain MMP and ATP production [38], upon high glucose challenge. It is likely that lack of Nrf2 reduces adaptation and intrinsic resistance to defend against glucose toxicity-induced oxidative stress and apoptosis (as shown by elevation of caspase-3 and the Bax-to-Bcl-2 ratio). Bcl-2 is an antiapoptotic protein to suppress mitochondrial apoptosis via antagonism of Bax oligomerization and later cytochrome c release [39]. On the other hand, high glucose incubation promoted levels of caspase-3, a terminal proapoptotic effector. These data collectively suggested that Exe and LIRA offer their cardioprotective effects possibly through inhibition of oxidative stress and mitochondria-dependent apoptosis. Involvement of mitochondrial injury in GLP-1 agonist-offered protection against glucose toxicity received further supports by MMP and is in line with the notion of disturbed preservation of MMP by mitochondrial respiratory chain in diabetes [40, 41]. It is plausible to speculate that sustained MMP in the face of Exe and LIRA treatment contributes to the inhibition of ROS generation.

Reduced autophagy leads to the buildup of damaged organelles including mitochondria, which subsequently releases proapoptotic factors and ROS, prompting cardiac dysfunction and the development of diabetic cardiomyopathy [42]. In our study, we noted suppressed autophagy in glucose-challenged cardiomyocytes, the effect of which was restored by GLP-1 agonists Exe and LIRA. This is supported by several experimental data. (1) Autophagosome formation (GFP-LC3 puncta) was drastically dampened by high glucose, the effect of which was reversed by GLP-1 agonists. Interestingly, the GLP-1 activation-elicited autophagosome formation against high glucose was cancelled off by the

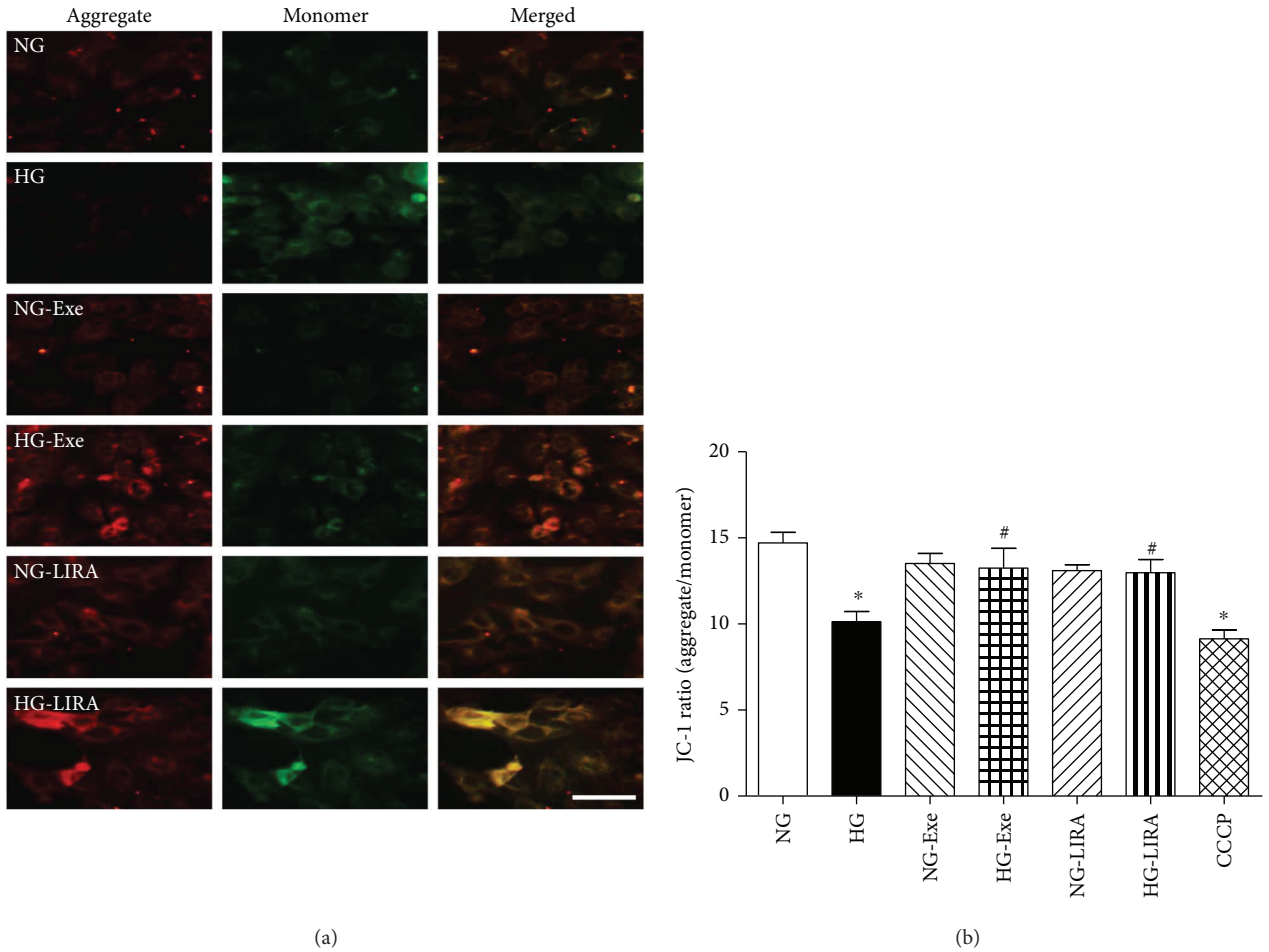


Figure 4: Effect of exendin-4 (Exe) and liraglutide (LIRA) on glucose toxicity-induced loss of mitochondrial membrane potential (MMP) assessed using JC-1: (a) representative fluorescent images of JC-1 staining depicting aggregates (red fluorescence), monomer (green fluorescence), and merged (yellow fluorescence), scale bar = 50 μm; (b) pooled data depicting quantitative analysis of the JC-1 ratio. Mean ± SEM, $n = 10$ images per group, $^{*}p < 0.05$ versus the NG group and $^{\#}p < 0.05$ versus the HG group.

autophagy inhibitor 3-MA. (2) Western blot data revealed decreased Atg5, Beclin-1, LC3II levels, and LC3II-to-LC3I ratio in the high glucose group, the effect of which was reversed by Exe and LIRA. Endogenous Atg5 and Atg12 are manifested as the Atg12-Atg5 conjugate, vital for autophagy [43]. On the other hand, Beclin-1, a core complex of the class III PI3K, helps to recruit autophagy-related proteins onto the isolation membrane in the autophagy process [44]. LC3, commonly referred to as MAP1LC3, plays a pivotal role in autophagosome formation via conversion of LC3I localized in the cytosol to the autophagosome-bound LC3II. Although the increased p62 levels seem to be somewhat paradoxical to reduced autophagy with high glucose challenge, it is possible that suppressed autophagy or autophagosome formation may end up delivering fewer autophagosomes to be degraded in lysosomes, therefore yielding few autophagolysosomes. With the induction of autophagy by GLP-1 activation, p62 levels were restored as observed in our study.

Our study showed that the inactivation of the mTOR/ULK1-dependent pathway may serve as a key mechanism for the cardiac protective role of Exe and LIRA. This was further supported by the fact that the mTOR activator 3BDO effectively nullified GLP-1 agonist-offered beneficial response against glucose toxicity, favoring a permissive role for mTOR in GLP-1 analogue-offered protection against cardiomyopathy. mTOR serves as the most important negative regulator of autophagy through ULK1 [45]. In our hands, high glucose incubation promoted phosphorylation of mTOR at Ser^{2448}, in line with an earlier report [45]. ULK1 serves as a downstream signaling molecule for mTOR where mTOR phosphorylates ULK1 at Ser^{757} to suppress ULK1 activation and subsequently autophagy induction [46]. Our data revealed that GLP-1 analogues effectively reversed high glucose-induced overactivation or phosphorylation of mTOR and ULK1, favoring autophagy induction. These findings support a likely role for the mTOR/ULK1 signaling cascade in the regulation of GLP-1 agonist-elicited autophagic responses in the face of glucose toxicity.

In summary, findings from our study provided convincing evidence that Exe and LIRA rescue against high glucose-induced cardiac contractile function, oxidative stress, mitochondrial injury, and apoptosis possibly via regulation of mTOR and autophagy. Exe and LIRA may offer their beneficial effect through inhibited phosphorylation of

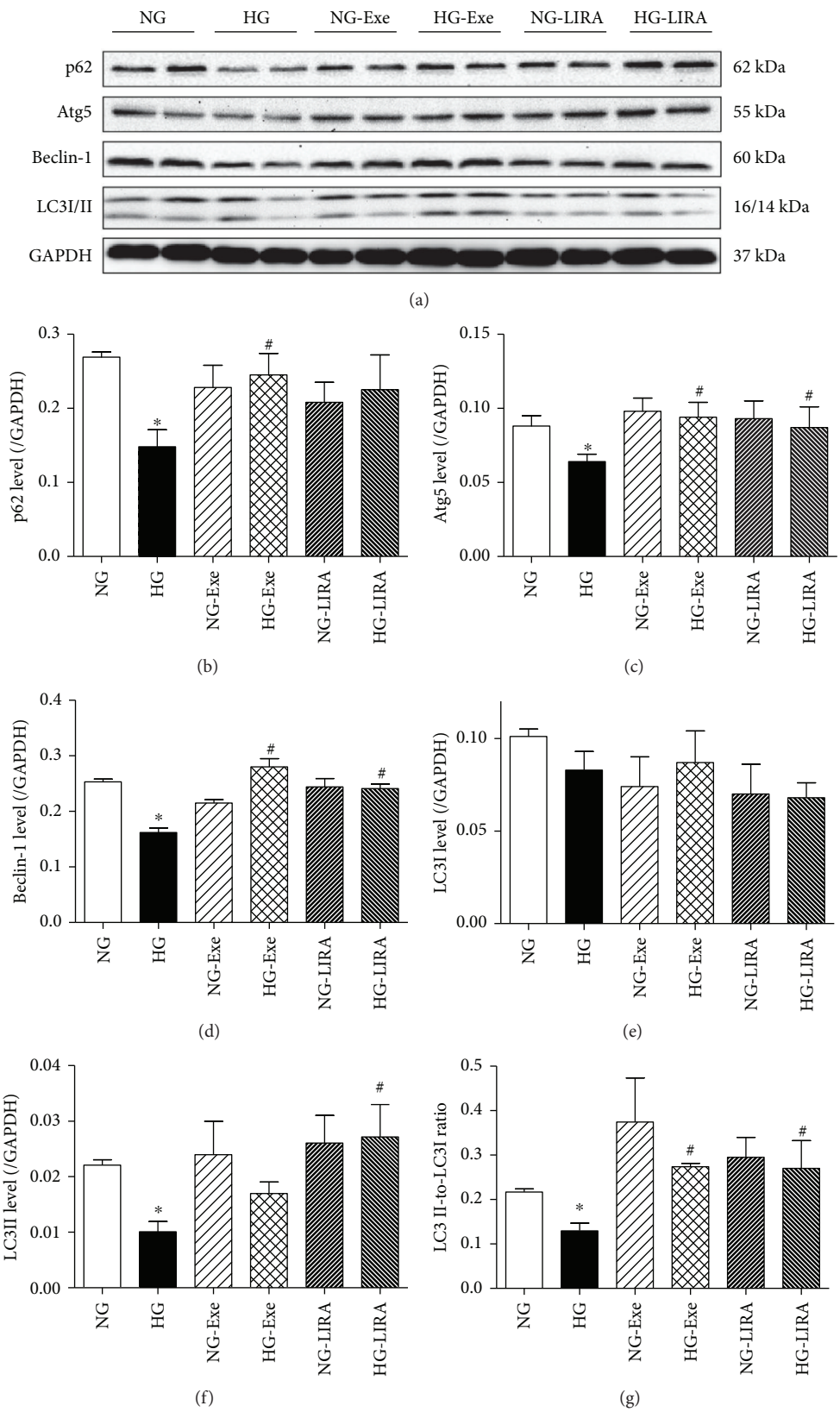
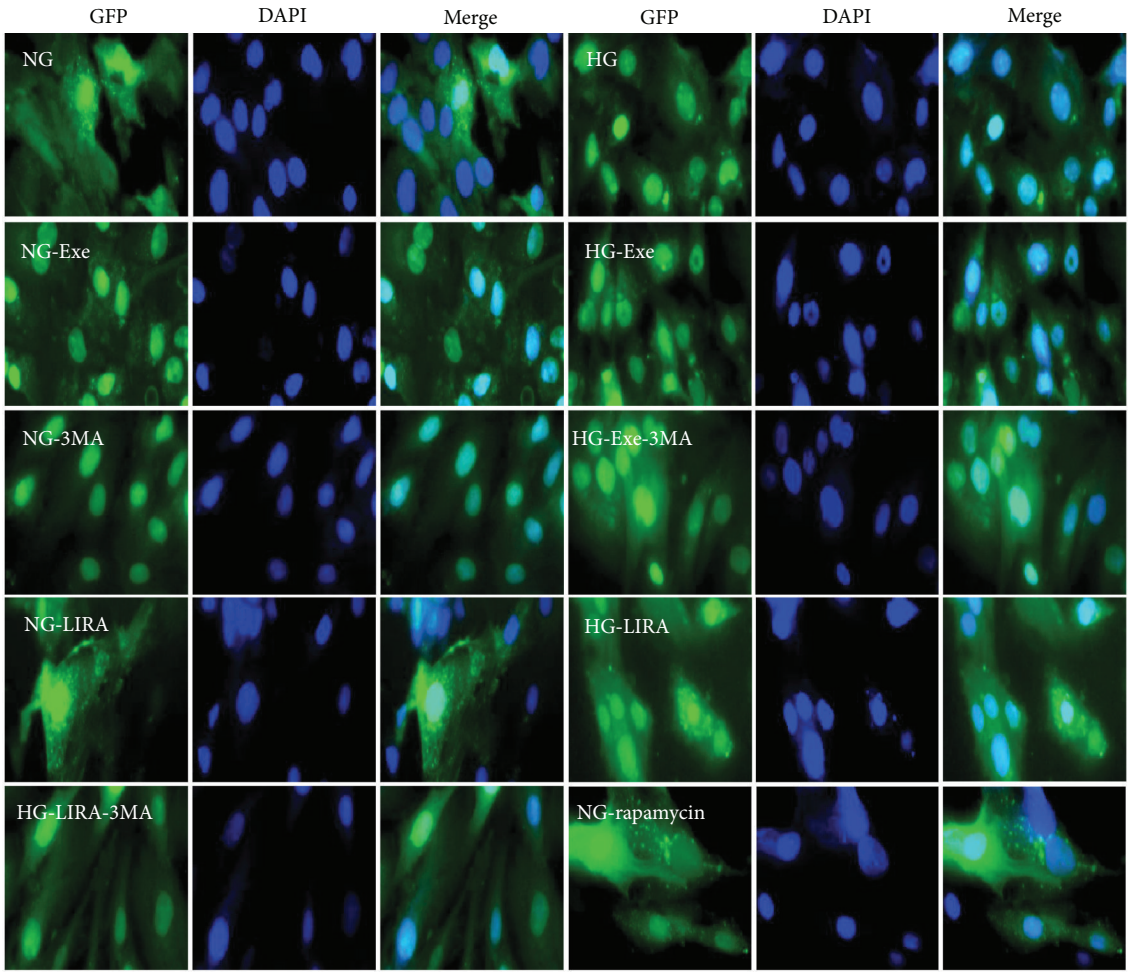
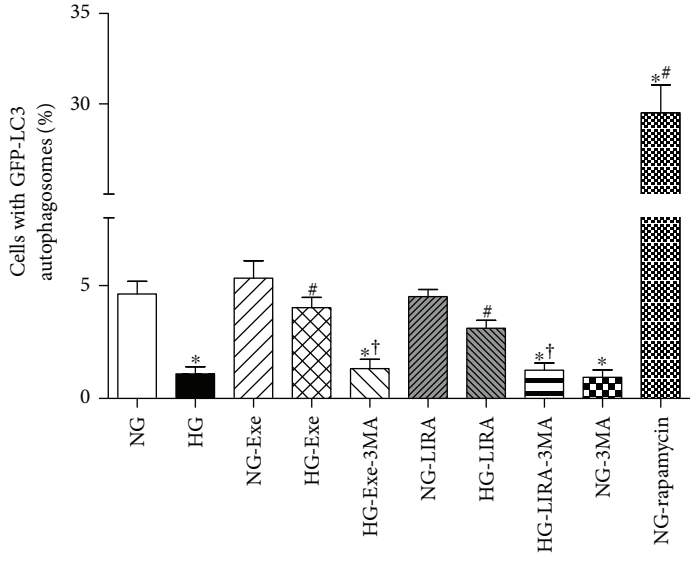


Figure 5: Effect of exendin-4 (Exe) and liraglutide (LIRA) on glucose toxicity-induced loss of autophagy: (a) representative pictures of p62, Beclin-1, Atg5, and LC3I/II using GAPDH as the loading control; (b) p62 levels; (c) Atg5 levels; (d) Beclin-1 levels; (e) LC3I levels; (f) LC3II levels; (g) LC3II-to-LC3I ratio. Mean ± SEM, $n = 3 – 6$ cultures per group, $^{*}p < 0.05$ versus the NG group and $^{\#}p < 0.05$ versus the HG group.



(a)



(b)

Figure 6: Effect of exendin-4 (Exe) and liraglutide (LIRA) on glucose toxicity-induced loss of GFP-LC3 puncta; (a) representative images of GFP-LC3, DAPI staining and merged images of GFP-LC3, and DAPI staining in H9c2 cells cultured in normal-glucose or high-glucose medium treated with or without Exe, LIRA, the autophagy inhibitor 3MA, or the autophagy inducer rapamycin; (b) quantitative analysis of cells with GFP-LC3 positive puncta. Mean ± SEM, $n = 10 - 14$ fields of independent cultures per group, $^{*}p < 0.05$ versus the NG group, $^{\#}p < 0.05$ versus the HG group and $^{\dagger}p < 0.05$ versus Exe or LIRA-treated HG group.

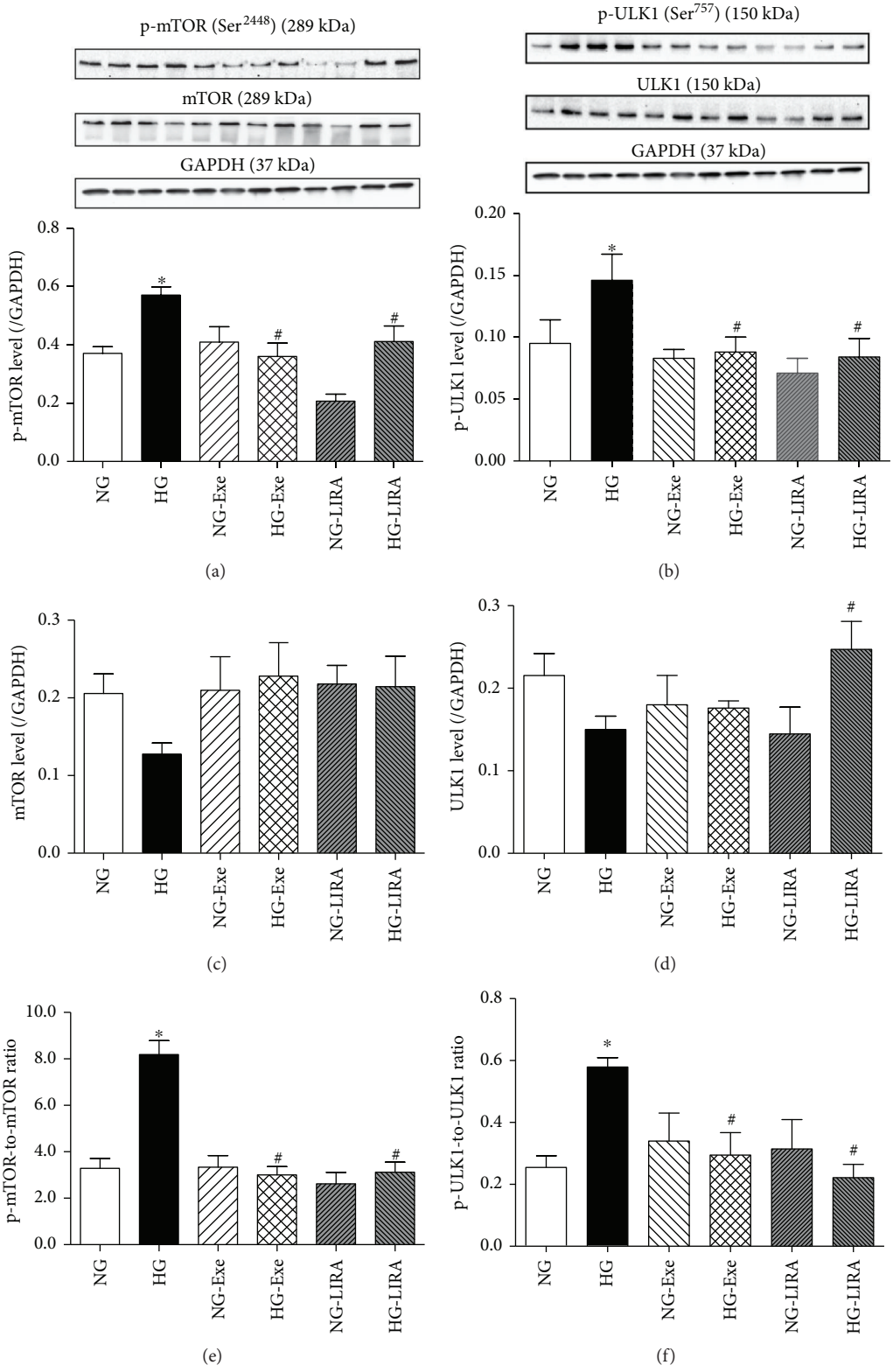


Figure 7: Effect of exendin-4 (Exe) and liraglutide (LIRA) on glucose toxicity-induced changes in the phosphorylation of mTOR and ULK1 in murine cardiomyocytes: (a) p-mTOR levels; (b) p-ULK1 levels; (c) mTOR levels; (d) ULK1 levels; (e) p-mTOR-to-mTOR ratio; (f) p-ULK1-to-ULK1 ratio. Insets: Representative pictures of mTOR, p-mTOR, ULK1, and p-ULK1 using GAPDH as the loading control. Mean ± SEM, $n = 4 – 6$ per group, $^{*}p < 0.05$ versus the NG group and $^{\#}p < 0.05$ versus the HG group.

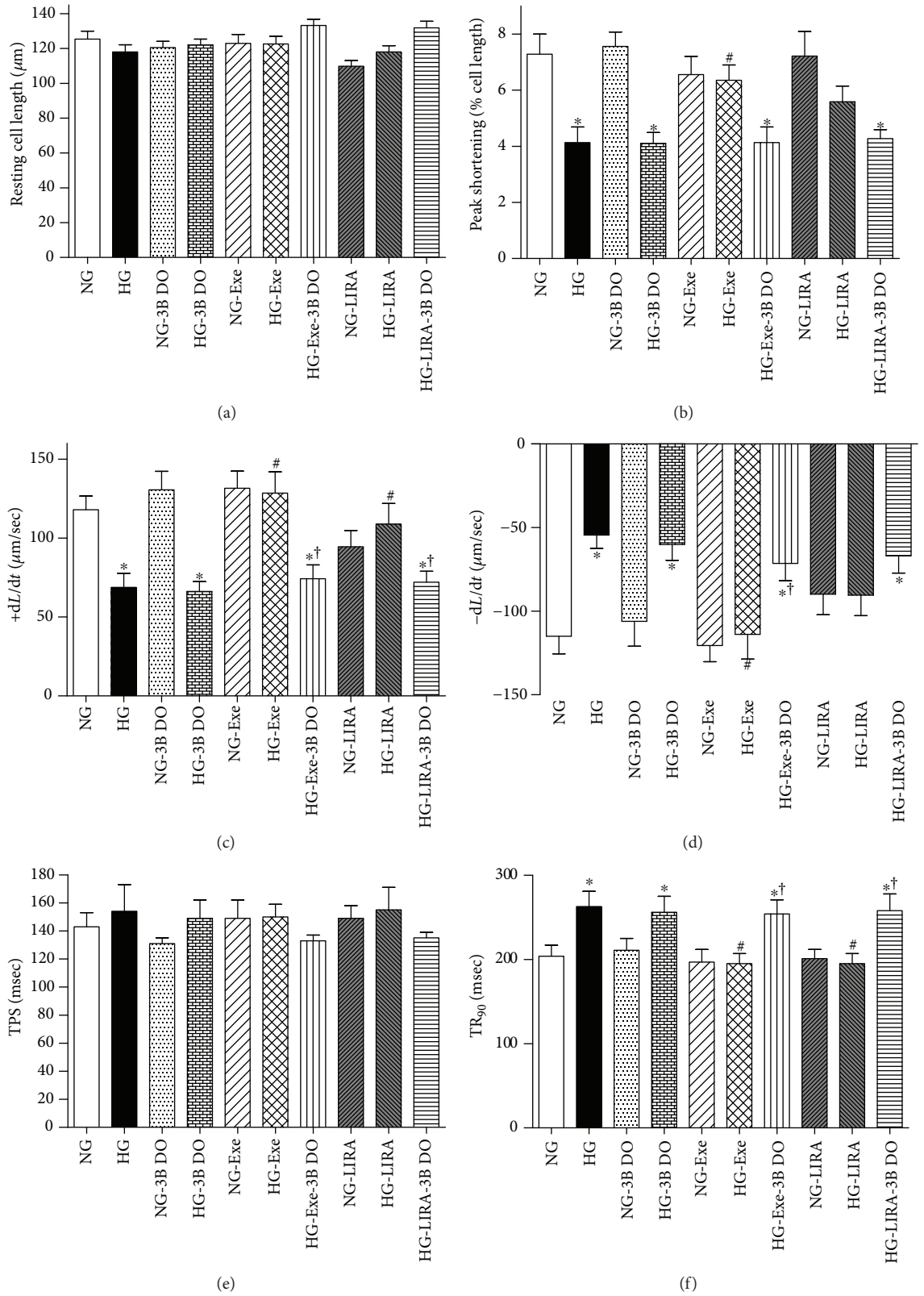


Figure 8: Effect of the mTOR activator 3-benzyl-5-((2-nitrophenoxy) methyl)-dihydrofuran-2(3H)-one (3BDO, 120 μM) on exendin-4- (Exe-) and liraglutide- (LIRA-) induced response against high glucose- (HG-) induced cardiomyocyte mechanical dysfunction in ventricular myocytes isolated from adult mouse hearts: (a) resting cell length; (b) peak shortening (PS); (c) maximal velocity of shortening (+dL/dt); (d) maximal velocity of relengthening (−dL/dt); (e) time-to-PS (TPS); (f) time-to-90% relengthening (TR_{90}). Mean ± SEM, $n = 39$ cells per group, $^{*}p < 0.05$ versus the NG group, $^{\#}p < 0.05$ versus the HG group, and $^{\dagger}p < 0.05$ versus the corresponding GLP-1 agonist-treated group.

mTOR and, subsequently, ULK1 (at Ser^{757} residue). These outcomes should shed some light towards a better understanding of the utility of GLP-1 agonists in diabetes- or hyperglycemia-induced cardiac anomalies, which merit further investigation in a more clinically relevant setting.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

None of the authors declares any conflict of interest in this work.

Authors' Contributions

Wei Yu and Wenliang Zha contributed equally to this work.

Acknowledgments

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