

Protective Effect of Silent Mating Type Information Regulation 2 Homolog 1 on TGF- β 1 Pathway via mTOR in Diabetic Nephropathy

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Abstract

Objective: To demonstrate whether the expression of silent mating type information regulation 2 homolog 1 (SIRT1) affects the level of TGF- β 1 and Smad3 in HEK293 cells through regulating mTOR. **Methods:** First, recombinant plasmids DNA (rSIRT1) and siRNA targeting SIRT1 were constructed which were transfected into Human Embryonic Kidney 293 cell (HEK293) cells, respectively. Then, the generation of intracellular ROS in cells was examined by flow cytometry using the oxidation-sensitive probe. Last, the expressions of TGF- β 1, smad3, P53, mTOR, p-mTOR, LC3-I and LC3-II in cells were detected to observe the effect of SIRT1 on TGF- β 1 Pathway by western blot analysis. **Results:** We demonstrated that overexpressing of SIRT1 may decrease TGF- β 1 and Smad3 expression in HEK293 cells through regulating mTOR. In addition, the result is the opposite when SIRT1 was silent in HEK293 cells. **Conclusions:** SIRT1 is closely related to TGF- β 1/Smad3 pathway that correlates with the regulation of mTOR and ROS generation and causes diabetic nephropathy. The available evidence implies that SIRT1 has great potential as a clinical target for the prevention and treatment of renal fibrosis in the development of DN.

Keywords

Silent Mating Type Information Regulation 2 Homolog 1, mTOR, Diabetic Nephropathy, Autophagy, Oxidative Stress

1. Introduction

Diabetic nephropathy (DN) is the leading cause of end stage renal disease (ESRD)

[1]. The treatment of diabetes nephropathy is very limited, such as hypoglycemic drugs, renal replacement therapy, and traditional Chinese medicine used to delay symptoms. Some drugs for the pathogenesis of diabetes nephropathy are under development. Without intervention, the number of individuals with diabetes worldwide will rise to an estimated 629 million 20 years later [2], posing a severe threat to public health [3]. Novel therapeutic approaches to DN are needed. Previous studies reported the protective role of in DN, encouraging the investigation of pathway modulating SIRT1 [4]. It is widely accepted that SIRT1 may regulate multiple cellular functions, including apoptosis, mitochondrial biogenesis, inflammation, autophagy and sodium balance, through the deacetylation of target proteins [5]. The activation of SIRT1 in the kidney may be a new therapeutic target to increase resistance to many causal factors in the development of renal diseases, including DN.

SIRT1, an NAD⁺ dependent deacetylase, plays a pivotal role in modulating autophagy [6]. The activation of SIRT1 protein induction mediated autophagy is an important protective mechanism against oxidative stress mediated diabetic renal fibrosis. In addition, the decline of SIRT1 expression in diabetic-kidney tissue leads to mitochondrial damage, autophagy and oxidative stress by impairing antioxidant capacity and accumulating renal lesions [7]. Targeting various components of autophagy pathway may become a new strategy for clinical treatment of DN. The well-known autophagy regulation pathways include mammalian target of rapamycin (mTOR) [6], which can interact with several proteins to form two different complexes, namely mTORC1 and mTORC2, to regulate autophagy. In recent years, some new findings have been made in this field. SIRT1 maintains a close but inverse relationship with mTOR. For example, the overexpression of SIRT1 was verified to inhibit the expression of mTOR and ameliorate fibrosis and inflammation *in vitro* [8]. In addition, SIRT1 can be beneficial by decreasing mTOR activity to reduce neuronal accumulation of A β . Yet, these pathways are intimately dependent upon one another and may require a careful balance to achieve a desired clinical outcome [9] [10].

In our preliminary study, we investigated that antifibrotic drug exhibited protective effects and inhibited autophagy in DN rats through suppressing TGF- β 1/Smad3 pathway [11]. In a high glucose environment, the autophagic level first increased, and then decreased gradually with time. Recent studies have shown that autophagy and SIRT1 can regulate each other in the procession of DN. Therefore, we hypothesize that there is a close relation between SIRT1 and TGF- β 1/Smad3 pathway in autophagic environment. In this study, we investigated whether the over-expression or silencing of SIRT1 was related to the regulation of the level of TGF- β 1/Smad3 pathway via regulating mTOR activity.

2. Materials and Methods

2.1. Cell Culture and Treatment

We followed the methods of Stepanenko *et al.* 2015 [12]. Human Embryonic

Kidney 293 cell (HEK293) cells are used in this experiment which is kept in the Central Laboratory of Aviation General Hospital. HEK293 cells are a cell line derived from human embryonic kidney cells, which have the characteristics of high transfection efficiency and easy culture. HEK 293 cells are considered to be an *in vitro* model of adrenal cells, rather than typical kidney cells.

We followed the methods of Yan Wang *et al.* 2019. Cells were plated into 96-well plates and incubated for 24 h to allow cell adherence. Then, the culture medium was replaced with DMEM. After 24 h of incubation, cell viability was determined by colorimetry using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Insoluble formazan crystals were dissolved in dimethyl sulfoxide (DMSO) and measured at 490 nm with a Thermo microplate reader (Thermo Fisher Scientific, Waltham, MA, United States).

2.2. Cell Transfection

We followed the methods of Hanahan, 1983 [13]. Rat SIRT1 cDNA fragments were then subcloned into pcDNA3.1/V5-His-A vector to form recombinant plasmids DNA (rSIRT1). HEK293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (GIBCO) containing 10% fetal bovine serum (FBS) (Life Technology), 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C with 5% CO₂. One day before transfection, HEK293 cells were seeded in 6-well plates at a concentration of 3.0×10^5 per well. Recombinant plasmid DNA and control vector was transfected into the cells, respectively. Transfection was conducted using FuGENE HD Transfection Reagent (Promega, Madison, USA). Epifluorescence inverted microscopy was employed to detect the transfection efficiency at 24 h, 48 h and 72 h. After 72 hrs of transfection, 80% of 293T cells were able to express GFP with 2 µg DNA. Cells from each group were collected at 72 h for mRNA and protein analyses.

HEK293 cells were transfected with nontargeting small interfering RNA (siRNA) and siRNA targeting SIRT1 (purchased from GenePharma, Shanghai, China) using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's protocol. Cells were then divided into three groups: rSIRT1, rSIRT1 + siRNA and rSIRT1 + Control siRNA. The rSIRT1 cDNA (0.8 µg) alone or combined with siRNA (3.2 µg) was mixed in 50 µL antibiotic-free DMEM to generate transfection complexes. The siRNA targeting human SIRT1 sequences were used: sense, 5'-CCCUGUAAAGCUUUCAGAAAdtdt-3', and antisense, 5'-UUCUGAAAGCUUUCAGGGdtdt-3'. After cotransfection of SIRT1 cDNA and SIRT1 siRNA, HEK293 cells were seeded in 6-well plates at approximately 40% confluence and incubated at 37°C for 24 h.

2.3. ROS Measurement

The generation of intracellular ROS was examined by flow cytometry using the oxidation-sensitive probe, 2,7-dichlorofluorescein diacetate (DCFH-DA) (Ap-plygen Co. Ltd., China). Cells (3.0×10^5) were incubated with 10 mM DCFH-DA

in complete medium for 30 min at 37°C to allow cellular incorporation. The cells were then washed twice softly in 1 mL of PBS. Single cells were then analyzed by fluorescent inverted microscope (OLYMPUS). A total of 10,000 cells were counted per sample.

2.4. Western Blot Analysis

Protein lysates (20 - 50 µg) of cells were separated in 12% sodium dodecyl sulfate gels and then were transferred onto polyvinylidene fluoride membranes. After blocking in 5% BSA, membranes were incubated with specific primary antibodies, including rabbit V5 (1:5000; Cell Signaling Technology, Danvers, MA, United States), rabbit TGF-β1 (1:3000; Cell Signaling Technology, Danvers, MA, United States), rabbit Smad3 (1:1000; Cell Signaling Technology, Danvers, MA, United States), rabbit SIRT1 (1:1000; Cell Signaling Technology, Danvers, MA, United States), rabbit P53 (1:2000; Cell Signaling Technology, Danvers, MA, United States), rabbit mTOR (1:1000; Cell Signaling Technology, Danvers, MA, United States), rabbit p-mTOR (1:1000; Cell Signaling Technology, Danvers, MA, United States), rabbit LC3 antibody (1:2000; Sigma-Aldrich, St. Louis, MO, United States) and mouse β-actin antibody (1:3000; Santa Cruz Biotechnology, Santa Cruz, CA, United States) overnight at 4°C. After washing with TBST, the membranes were incubated with horseradish peroxidase-conjugated IgG. Blots were developed using ECL Detection Kit (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) and then detected using a ChemiDoc XRS system (Bio-Rad, Hercules, CA, United States). Protein bands were quantified using densitometry with Image J (NIH, Bethesda, MD, United States).

2.5. Statistical Analyses

Quantitative data were presented as the mean ± standard error. Comparisons among groups were performed using one-way analysis of variance, and GraphPad Prism software version 6.0 (GraphPad Prism, San Diego, CA, United States) was used for the analysis. $P < 0.05$ and < 0.01 were considered statistically significant.

3. Results

3.1. Recombinant SIRT1 Expressed Successfully in HEK293 Cells

After 72 h transfection of recombinant SIRT1 in HEK293 cells, there was a significant elevation of SIRT1 protein in SIRT1 overexpression group (rSIRT1) while control vector group had no fusion protein using V5 antibody (**Figure 1**). The nucleus is stained blue by DAPI, and the result shows that SIRT1 is mainly distributed in the nucleus when SIRT1 staining and nuclear staining has overlapped.

3.2. Over-Expression of SIRT1 Reduced TGF-β1/Smad3 Pathway via Regulating mTOR in HEK293 Cells

The phosphorylation of multiple key molecules within the TGF-β1/Smad3 pathway

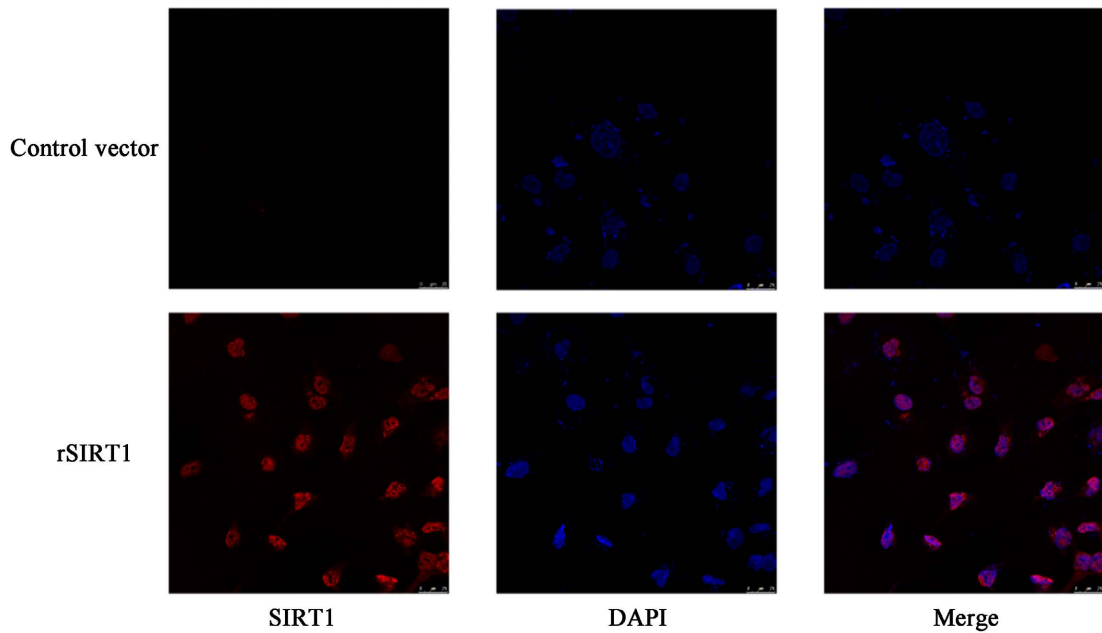


Figure 1. Recombinant SIRT1 expressed successfully in HEK293 Cells. Immunofluorescence staining showed that fusion protein expressed in rSIRT1 group via V5 antibody, and SIRT1 is mainly distributed in the nucleus.

plays an essential role in TGF- β 1 modulation. Phosphorylated pSmad3 is a molecular marker of TGF- β /activin activity [14]. TGF- β 1 treatment markedly increased the phosphorylation of mammalian target of rapamycin (mTOR) and its downstream targets p70S6K and 4EBP1. Blocking TGF- β receptor I with SB431542 completely blunted the phosphorylation of mTOR [15].

Recombinant SIRT1 was expressed successfully in the cells, which significantly increased SIRT1 expression and decreased the P53 expression, p-mTOR/TOR ratio, TGF- β 1 expression, Smad3 expression, LC3-II expression and the ratio of LC3-II to LC3-I (**Figure 2**), strongly indicating an inhibition in TGF- β 1/Smad3 pathway via alleviating mTOR phosphorylation. These SIRT1-dependent mechanisms of TGF- β 1/Smad3 pathway regulation provide significant protection against DN and require further research for effective therapy.

3.3. SIRT1 Reduced ROS Production in rSIRT1 Group

Apart from performing transfection of recombinant SIRT1 studies on HEK293 cells, studies of ROS production were also performed using HEK293 cells *in vitro* tests. The intrinsic levels of ROS produced by the cells were indirectly measured by flow cytometry using DCFH-DA. The results indicate a higher level of ROS in control vector.

Consistent with the above results, overexpression of SIRT1 reduced the generation of ROS while alleviated TGF- β 1/Smad3 pathway. The emerging role of TGF- β 1/Smad3 pathway during DN has not explicitly been summarized, and the present review aims to fill this gap in the literature [16]. TGF- β 1 increases translation of Nox4 through the Smad-mTORC1 axis, which is independent of transcriptional regulation. Activation of this pathway plays a crucial role in ROS

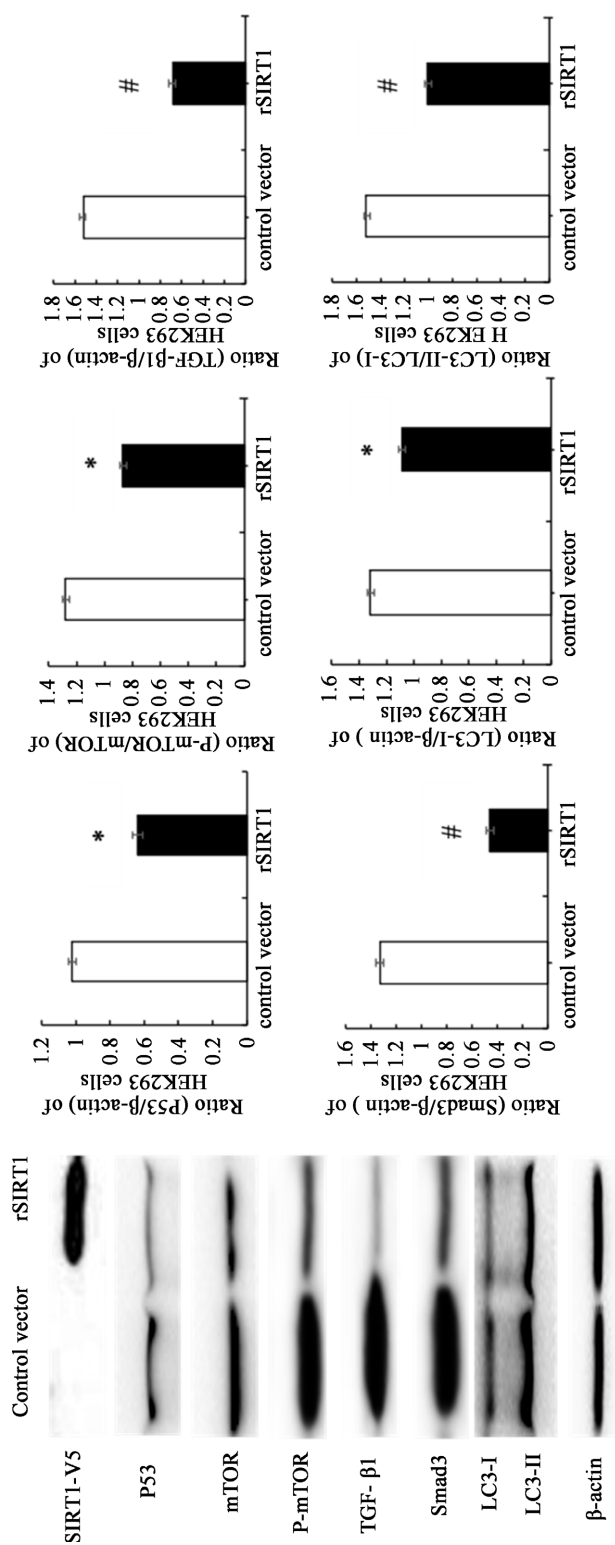


Figure 2. Overexpression of SIRT1 reduced TGF-β1 by decreasing mTOR in HEK293 cells. Overexpression of SIRT1 induced downregulation of the P53 expression, p-mTOR/TOR ratio, TGF-β1 expression, Smad3 expression, LC3-II expression and the ratio of LC3-II to LC3-I determined by western blot assay and semi-quantitative analysis; data are expressed as the mean ± SEM of three independent experiments performed in triplicate. **P* < 0.05, #*P* < 0.01 vs. control vector group.

generation and mitochondrial dysfunction, leading to podocyte apoptosis [17].

In our study, we first summarize the effect of SIRT1 on TGF- β 1/Smad3 pathway closely related to DN through regulating P53, mTOR, and LC3-II expression. In addition, these SIRT1-dependent factors can mediate intracellular production of ROS (Figure 3).

3.4. siRNA Targeting SIRT1 Expressed Successfully in HEK293 Cells

After cotransfection of SIRT1 cDNA and SIRT1 siRNA, there was no SIRT1 protein in rSIRT1 + siRNA group while rSIRT1 and rSIRT1 + Control siRNA groups had obvious expression of fusion protein using V5 antibody (Figure 4). The nucleus is stained blue by DAPI, and the result shows that siRNA blocked the expression of SIRT1 in the HEK293 cells successfully.

3.5. SIRT1 siRNA Increasing mTOR Expression via Regulating TGF- β 1 Pathway in HEK293 Cells

To determine whether TGF- β 1 pathway was associated with SIRT1 induction

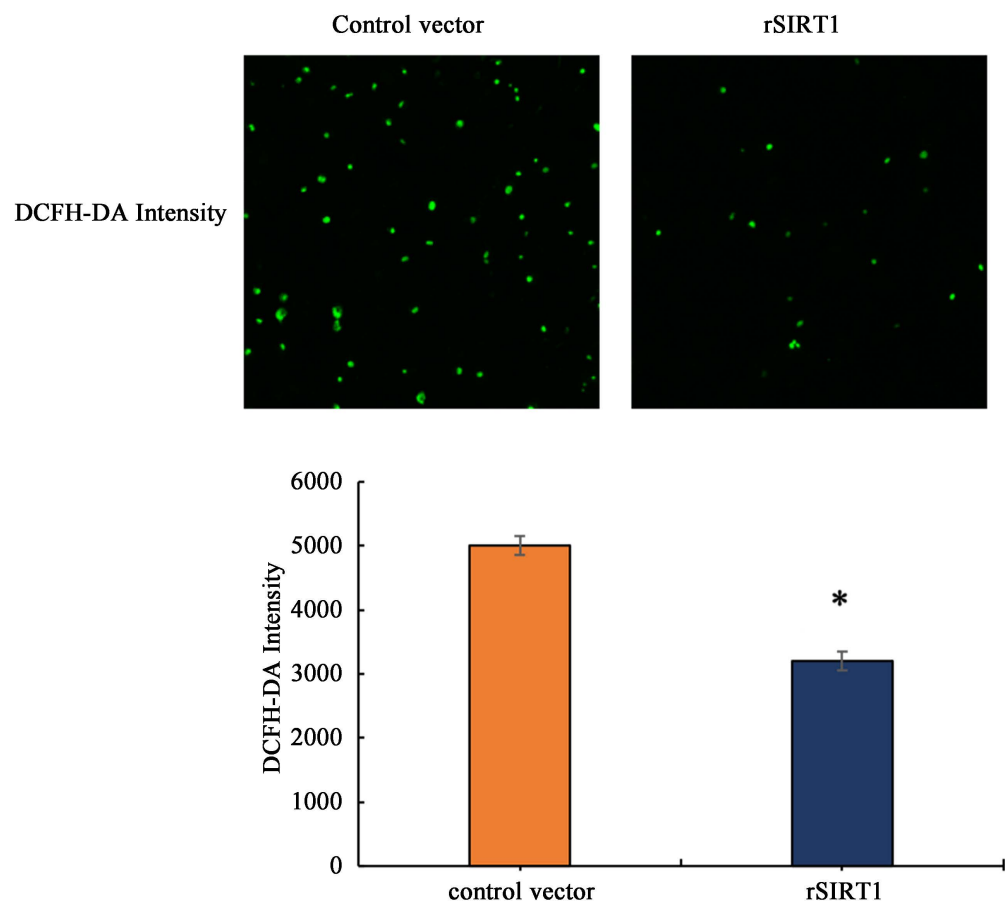


Figure 3. Overexpression of SIRT1 reduced ROS generation in HEK293 cells. Compared with the control vector, ROS was significantly reduced in the rSIRT1 group which had more SIRT1 expression; data are expressed as the mean \pm SEM of three independent experiments performed in triplicate. * $P < 0.05$, vs. control vector group.

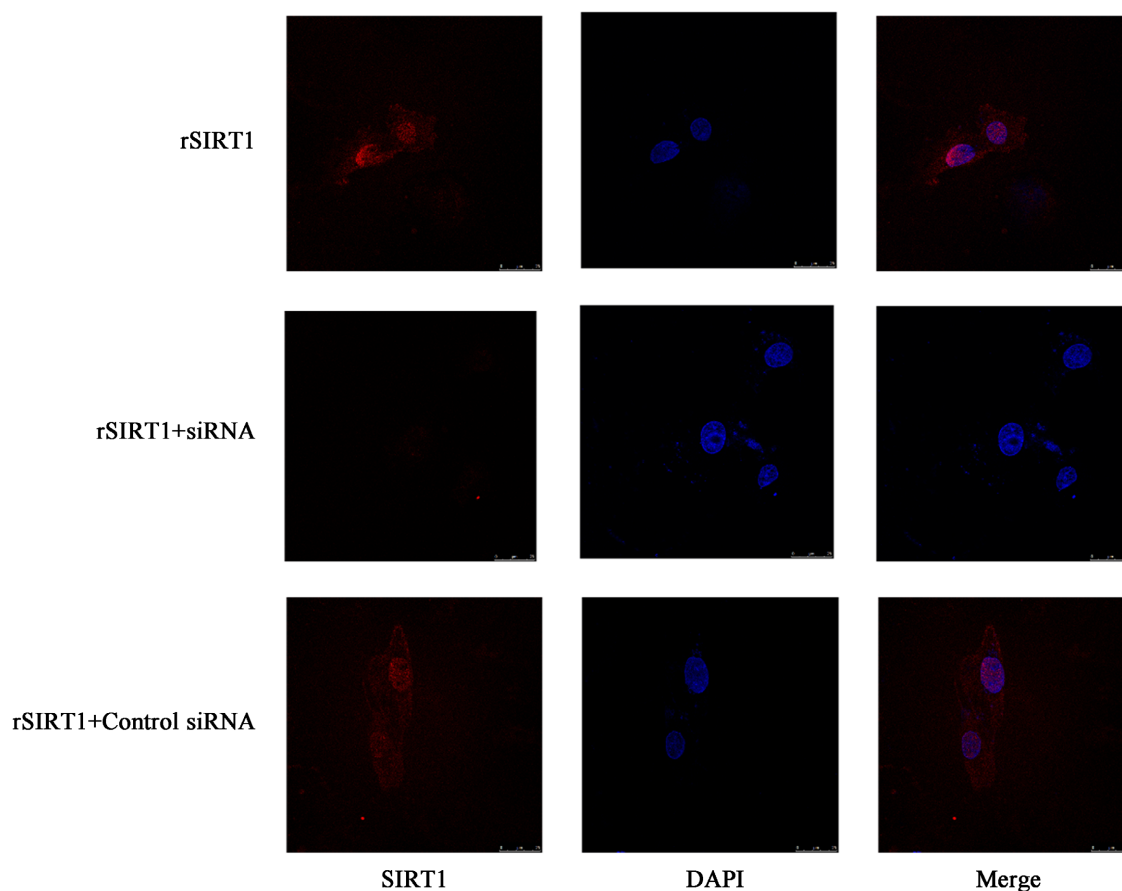


Figure 4. Immunofluorescence staining showed that recombinant SIRT1 expressed successfully in rSIRT1 and rSIRT1 + Control siRNA group in HEK293T Cells as well as recombinant SIRT1 was knockdown in rSIRT1 + siRNA group via V5 antibody. Nuclei were stained with DAPI, and fluorescent images and intensity data were collected and assessed using a high-content screening system, bar = 25 μ m.

and mTOR activation, we used specific SIRT1 siRNA to knock down SIRT1 expression in HEK293 cells. As shown in **Figure 5**, the upregulation of the P53 expression, p-mTOR/TOR ratio, TGF- β 1 expression, Smad3 expression, LC3-II expression and the ratio of LC3-II to LC3-I were notably observed following siRNA-mediated SIRT1 knockdown, which is consistent with the result of SIRT1 overexpression. These findings indicate the involvement of SIRT1 in the TGF- β 1 pathway via regulating mTOR expression in HEK293 cells. Thus, we concluded that SIRT1 alleviated fibrosis process induced by TGF- β 1, and it may be related to the regulation of mTOR expression.

3.6. SIRT1 siRNA Increasing ROS Generation via Regulating TGF- β 1 Pathway in HEK293 Cells

To further ensure the relationships between SIRT1 and TGF- β 1 pathway, we adopted DCFH-DA to determine the generation of ROS. Interestingly, the results of the optical density analysis showed that the generation of ROS in rSIRT1 + siRNA group had an obvious elevation, which was consistent with the results of SIRT1 overexpression test (**Figure 6**). These results demonstrated that SIRT1

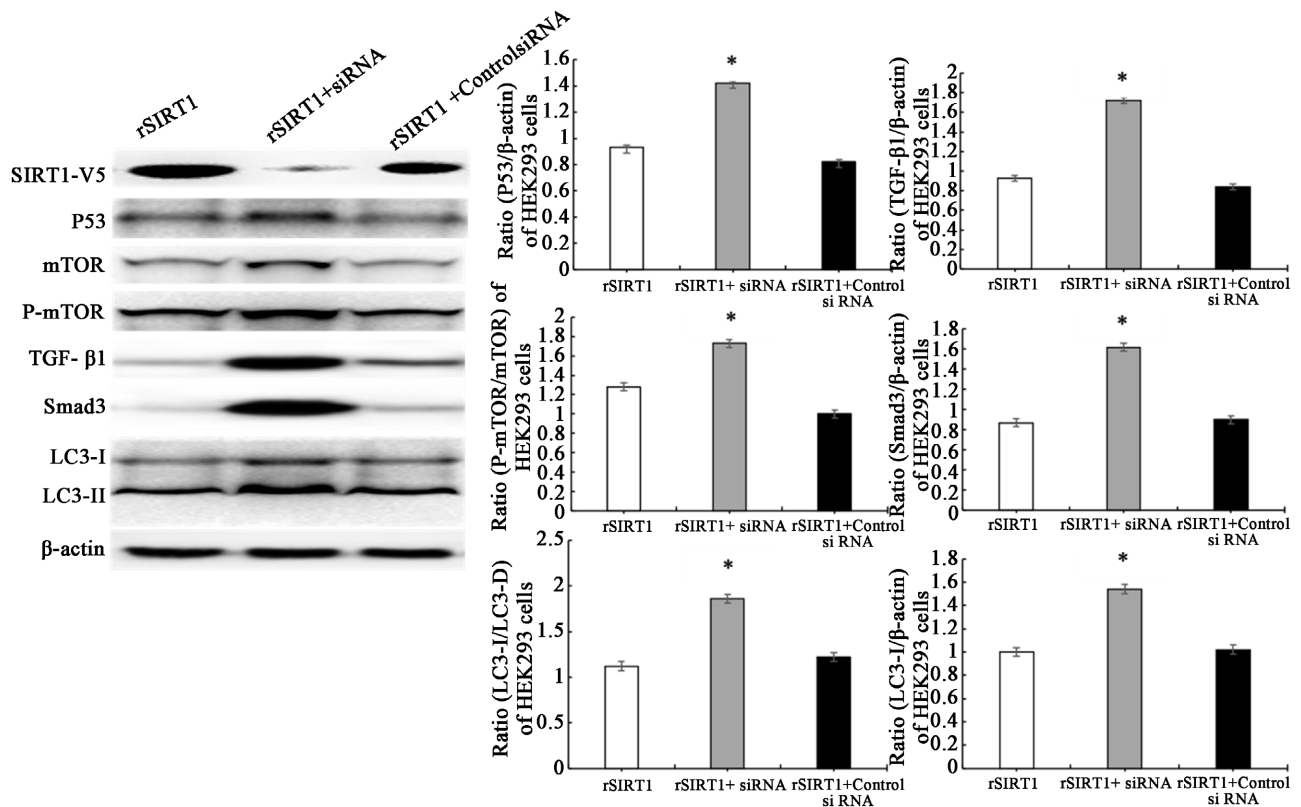


Figure 5. SIRT1 siRNA reinforced TGF- β 1 expression by increasing mTOR in HEK293 cells. SIRT1 siRNA induced upregulation of the P53 expression, p-mTOR/TOR ratio, TGF- β 1 expression, Smad3 expression, LC3-II expression and the ratio of LC3-II to LC3-I determined by western blot assay and semi-quantitative analysis; data are expressed as the mean \pm SEM of three independent experiments performed in triplicate. * $P < 0.05$, vs. rSIRT1 + Control siRNA group.

not only regulated TGF- β 1 pathway but also inhibited oxidative stress in HEK293 cells.

4. Discussion

Many studies have reported that the pathophysiology of DN is very complex, involving many molecules and abnormal cellular activities. Given the respective pivotal roles of NF- κ B, mTOR and TGF- β 1 in inflammation, oxidative stress, and fibrosis during DN [18], at the cellular level, SIRT1 may play important roles in several biological processes, including energetic homeostasis, apoptosis, mitochondrial biogenesis and autophagy through modulation of P53 [19] [20]. Interest in the role of SIRT1 in the pathophysiology of various renal diseases has grown recently. In the present study, we examined the effect of SIRT1 on TGF- β 1/Smad3 pathway in HEK293 cells via regulating mTOR expression and the relationship between SIRT1-dependent factors and ROS.

Various mechanisms have been suggested that SIRT1 regulates cellular homeostasis via inhibition of the mammalian target of rapamycin (mTOR) [21]. The protective effect of SIRT1 in DN by regulating TGF- β 1 pathway was verified in HEK293 cells. Overexpression of SIRT1 has been shown to directly inhibit TGF- β 1 and Smad3 expression, thereby demonstrating the inhibitory effects of

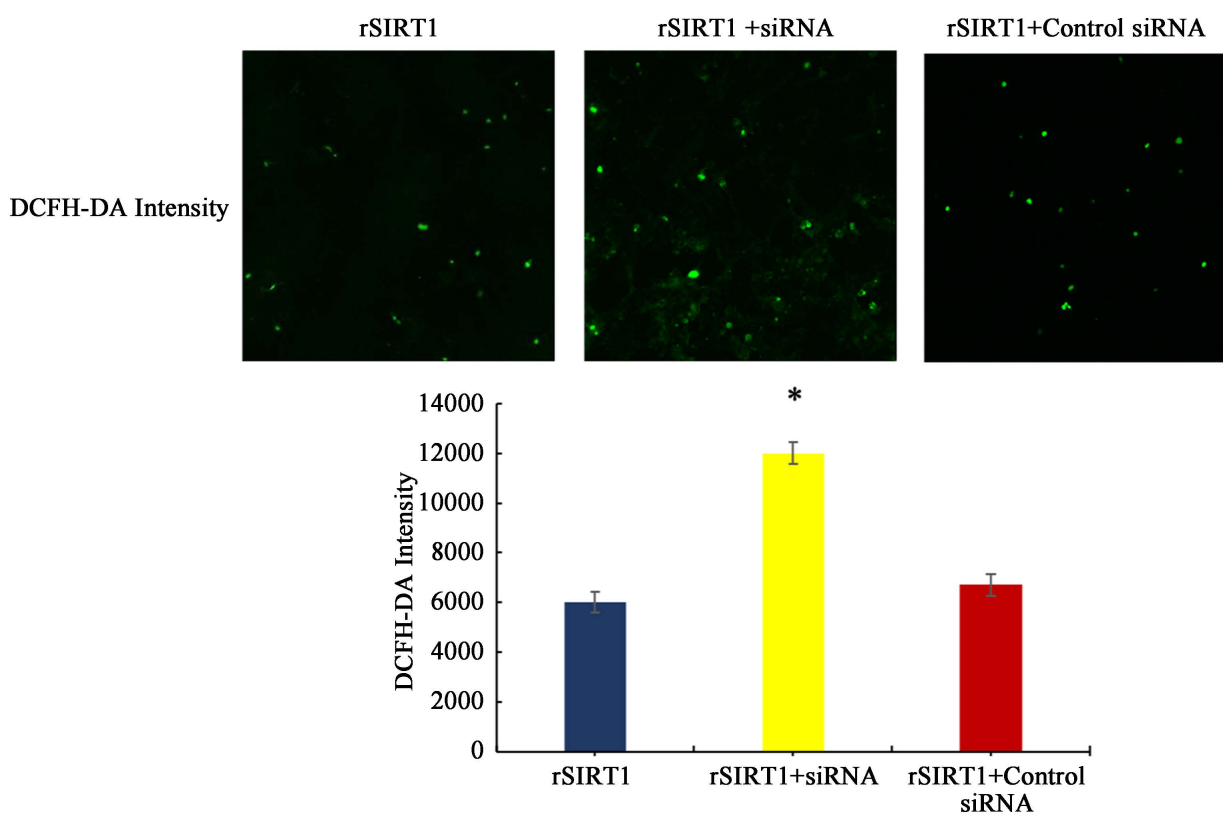


Figure 6. SIRT1 siRNA Increasing ROS generation in HEK293 cells. Compared with the control vector, ROS was significantly reduced in the rSIRT1 group which had more SIRT1 expression; data are expressed as the mean \pm SEM of three independent experiments performed in triplicate. * $P < 0.05$, vs. rSIRT1 + Control siRNA group.

SIRT1 on DN pathogenesis. This phenomenon may be associated with reduced activation of transcription factors, such as P53, phosphorylation of mTOR, LC3B-II levels, LC3B-II/LC3B-I ratio, thus triggering oxidative stress to regulate energy homeostasis and metabolic stress. Furthermore, ROS generation was reduced in SIRT1 overexpression group in the present study.

Kume *et al.* demonstrated SIRT1 and its target proteins may play an important role in renoprotection of aging kidneys, which is accomplished through stimulation of autophagy. Pharmacologically induced SIRT1 activation significantly reduced tubulointerstitial fibrosis and improved renal function through enhancement of Nrf2/HO-1 signaling and AMPK/PGC-1 α signaling [22]. To the best of our knowledge, many cytokines/chemokines/growth factors contribute to the development of fibrosis; however, TGF- β is considered to be the most potent and ubiquitous profibrogenic cytokine. TGF- β mRNA and/or protein expression is increased in almost all fibrotic diseases involved in different organ systems and in experimental fibrosis models [23] [24] [25]. However, TGF- β and smad2/3 have also been identified as targets of SIRT1. Although SIRT1 acts as the key transcriptional modulator of cell survival via regulation of p53, nuclear factor- κ B (NF- κ B) p65, signal transducer and activator of transcription 3 (STAT3) [26], crosstalk between SIRT1 and TGF- β 1/Smad3 pathway was still unclear.

Mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase

and component of the mTOR complex, which is a key modulator of cellular proliferation, autophagy, and lipid metabolism. It is reported that decreases in SIRT1 expression alleviate suppression of the mTOR pathway, which promotes HG-induced renal cell autophagy dysfunction and senescence [27]. It is also well known that high glucose-stimulated ROS production *in vitro*, resulted in increased TGF- β 1 expression as well as an increase in the Akt and mTOR phosphorylation ratio, resulting in epithelial mesenchymal transition (EMT) in the development and progression of DN [28].

Consistent with observations in SIRT1 up-regulation, SIRT1 deletion caused SIRT1-mediated factors such as P53, phosphorylation of mTOR, LC3B-II levels, LC3B-II/LC3B-I ratio increase. Moreover, oxidative stress, produced by ROS generation is a common feature of mitochondrial dysfunction. ROS is associated with oxidative stress induced by TGF- β 1, aldosterone, albumin, or oxidized low-density lipoprotein, which is consistent with earlier studies [29]. Interestingly, after SIRT1 expression was abolished by gene regulation, ROS generation was observed to increase significantly in our study, which indicated that SIRT1 deletion aggravated oxidative stress in HEK293 cells. These results suggest that the upregulation and downregulation of SIRT1 in regulating TGF- β 1/Smad3 pathway and ROS production *in vitro* serves a significant role in the pathogenesis of DN.

SIRT1 plays critical roles in cellular homeostasis, and numerous published studies have revealed that SIRT1 participate in various acute and chronic kidney diseases through the regulation of oxidative stress, apoptosis, inflammation, fibrosis, cell survival. According to the findings of the present study, our studies demonstrate the powerful effect of SIRT1 on the improvement of DN. However, the underlying mechanisms of SIRT1 affecting the progress of DN remain unclear. For more convincing evidence of the beneficial effect of SIRT1 against TGF- β 1/Smad3 pathway via mTOR phosphorylation, further studies are needed to be explored in a future study using podocytes and mesangial cells. In addition, SIRT1-mediated molecular factors, such as AMP-activated protein kinase (AMPK) and nuclear factor erythroid 2-related factor 2 (Nrf2), which may underpin its protective effects, are also required.

5. Conclusion

In conclusion, the present study demonstrated that SIRT1 is closely related to TGF- β 1/Smad3 pathway and this situation correlates with the regulation of mTOR phosphorylation and inhibition of ROS generation. These findings may improve our current understanding of the protective effect of SIRT1 in the development of DN and provide an experimental basis for the clinical application of SIRT1 agonists in DN.

Authors' Contributions

JDC and WYC designed the experiments. GYT, YPC and FR performed the experiments. GYT analyzed, interpreted the data and drafted the manuscript.

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Data Availability Statement

The data that support the findings of this study are available in the methods and/or supplementary material of this article.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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