Effect of The Autophagy Mechanism of TSCs Induced by Oxidative Stress Under Beclin1-mTOR Interaction

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Research Article

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Abstract

Background

Tendinopathy is a chronic injury disease caused by repeated traction. It is characterized by exercise-related pain, increased local tendon sensitivity, and imaging changes in the tendon. Rotator cuff injury is one of the typical tendinopathy. Tendon-derived stem cells (TDSC) play a vital role in the development of tendinopathy. Our previous studies have found that reactive oxygen species increase after rotator cuff injury and the oxidative stress response is strengthened, but whether oxidative stress induces TDSC autophagy to promote tendon bone healing is not clear.

Methods

First, we collected the injured and normal tendon tissues of patients with rotator cuff injury, detected the levels of reactive oxygen species (ROS) and superoxide anion (SOD) in the tissues, detected Beclin1, mTOR gene expression by qPCR, and WB (Western blotting). Beclin1, p-mTOR/mTOR protein expression. Then, we extracted human tendon stem cells (TDSC) from tendon tissue, infected TDSC with recombinant lentivirus pLKO.1-shBeclin1, and verified the expression of Beclin1 by qPCR and WB. Finally, H2O2 and 3-MA were used to intervene TSCs. CCK8 was used to detect the proliferation ability of H2O2 on human TSCs; autophagy staining (MDC), autophagy-lysosome staining (Lyso-Tracker Red) and transmission electron microscopy were used to observe autophagy. Immunofluorescence staining detects the expression of autophagy factor LC3A/B; DCFH-DA detects cellular reactive oxygen species ROS level, Annexin V/PI detects cell apoptosis; WB detects Beclin1, mTOR, p-mTOR (Ser2448), LC3A/B, cleaved caspase-3 protein expression.

Results

In this study, it was found that the expression levels of ROS and Beclin1 in the damaged rotator cuff tissue were higher, while the expression levels of SOD and mTOR were lower. After the recombinant lentivirus pLKO.1-shBeclin1 was infected with TDSC, the expression of Beclin1 decreased. After treating TDSCs with H2O2 and 3-MA, it was found that H2O2 caused an increase in reactive oxygen species ROS content, autophagy levels, and LC3A/B expression in TDSCs, and an increase in Beclin1, mTOR, LC3A/B, and cleaved caspase-3 protein expression. Lead to a decrease in the level of apoptosis.

Conclusion

Under the mutual regulation of Beclin1-mTOR, oxidative stress induces the occurrence of autophagy in TSCs, and autophagy may protect TSCs from oxidative stress by reducing the accumulation of ROS.
Introduction

The function of the motor system is inseparable from the close cooperation of bone, skeletal muscle and tendon. Tendons connect muscles and bones, and are prone to damage due to overuse or aging [1]. Tendon is a unique dense fibrous connective tissue that connects muscle and bone tissue, and can transmit force from muscle to bone tissue to maintain posture or produce movement. According to the adjacent anatomical relationship with muscles and bone tissues, tendons can be divided into three parts: Enthis (also called bone-tendon junction), middle part, and muscle insertion end (Myotendinous). The tendon is mainly composed of a small number of cells and a large amount of extracellular matrix. The cells in normal tendon tissue include tendon cells and tendon stem cells (TSCs). The extracellular matrix mainly includes collagen, proteoglycan, glycoprotein and water. Tendinopathy is a chronic traumatic disease caused by repeated traction. It is characterized by exercise-related pain, increased sensitivity of the local Achilles tendon, and imaging changes in the tendon. In the past, tendinopathy was a simple inflammatory response, so it was called “tendinitis”. However, recent histopathological studies showed that its pathological process is tendinosis, and its specific The pathophysiological changes still need to be elucidated in detail.

Oxidative stress refers to the fact that the reactive oxygen species (ROS) generated by internal and external sources exceeds the antioxidant capacity of cells, thereby affecting the cell signal transduction system, or causing damage to biological macromolecules such as proteins and nucleic acids or organelles.. ROS mainly includes O2-, H2O2, HOCl and singlet oxygen. The oxidative stress effect is positively correlated with the increase of ROS. Normal physiological activities such as aerobic respiration of cells, a small amount of ROS produced can be used as cell signaling molecules to regulate cell survival, proliferation, and differentiation. However, the continuous production of excessive ROS stimulated by external risk factors will cause severe oxidative stress in cells, leading to cell apoptosis, necrosis, autophagy and other outcomes. In the process of degenerative changes of the tendon, the dysfunction of mitochondria will lead to the excessive production of reactive oxygen species (ROS) [4], and the oxidative stress caused by the excessive production of reactive oxygen will play a role in the degeneration of the tendon. A vital role, causing continuous loss of tendon function [5]. Therefore, the physiological dysfunction of the tendon caused by aging may damage the antioxidant defense function and increase the sensitivity of the tendon to oxidative stress, which will be more obvious in the elderly [6]. Therefore, oxidative stress is inevitable in tendon degeneration, and the theory of tendon damage caused by oxidative stress has attracted more and more attention.

Autophagy is a dynamic catabolic process [13], which is closely related to cell damage, repair, and proliferation, and plays an important role in cell stress and environmental adaptation. The initiation of autophagy is composed of a series of autophagy-related proteins, such as Atg1, Atg5 and Beclin1. Autophagy is activated by various internal and external cellular stresses, such as starvation, reactive oxygen species (ROS) accumulation and hypoxia, and plays a vital role in maintaining cell homeostasis under these stress conditions. There is evidence that autophagy plays a key role in the self-renewal and stemness control of stem cells [7, 8]. There are also reports that autophagy can promote self-renewal and
maintain the dryness of mesenchymal stem cells during hypoxia, starvation or radiation [12–15]. The mammalian specific target of rapamycin (mTOR) is an important molecular complex that regulates energy metabolism and protein synthesis in mammals. It has a regulatory effect on mammalian bone development, bone formation, bone remodeling, and blood vessel formation. It is clearly affirmed [9] that local osteogenesis and angiogenesis have always been regarded as the important basis of tendon bone healing process. Beclin1 is a key regulator of autophagy and an indispensable condition for the formation of autophagosomes. It can mediate the localization of autophagy proteins in phagocytic vesicles and regulate the formation and maturation of mammalian autophagosomes. mTOR and Beclin1 are important factors in the regulation of autophagy. The role of mTOR cell signaling channels in the network function of Beclin1 regulating autophagy and the mutual feedback regulation mechanism between them are also one of the hot topics today. Based on these results, we explored the effect of oxidative stress-induced autophagy of TSCs on tendon bone healing under the interactive regulation of Beclin1-mTOR.

**Materials And Method**

**Reagents and antibodies**

DMEM high sugar medium (Gibco, #11965092), Penicillin-Streptomycin (Gibco, #15070063), fetal bovine serum (FBS, Gibco, #10099133), Tissue ROS detection kit (Beibo Biological, #BB-470532), cell reactive oxygen detection kit (Biyuntian, #S0033S), cell apoptosis detection kit (KGI Bio, #KGA108-1), CCK8 reagent (Dongren Chemical, #CK04), superoxide Substance detection kit (Biyuntian, #S0060), autophagy staining detection kit (Solebao, #G0170-100T), Lyso-Tracker Red(Solebao, #L8010-50μl); Goat Anti-Rabbit IgG H&L (HRP) (Jackson, #111-035-003), Beclin-1 (D40C5) Rabbit mAb (CST, #3495T), mTOR (7C10) Rabbit mAb (CST, #2983T), Phospho-mTOR (Ser2448) (D9C2) Rabbit (CST, #5536T), LC3A/B (D3U4C XP® Rabbit mAb (CST, #12741T), Cleaved Caspase-3 (Asp175) Antibody (CST, #9661T), 3-MA (MCE, #HY-19312), Endonuclease SgrAI (NEB, #R0603S), Endonuclease EcoRI (NEB, #R3101V).

**Detection of clinical specimens**

A total of 10 normal rotator cuff tissues (5 males and 5 females) and 10 injured rotator cuff tissues (5 males and 5 females) of clinical rotator cuff injuries were taken, and divide them into male control group and male injury rotator cuff group, the female control group and female injury rotator cuff group, and arthroscopic rotator cuff repairs were performed. The protocol for collecting normal and injured tendon tissues during the operation and obtaining tendon samples has been approved by the Research Ethics Committee of the Third Affiliated Hospital of Guangzhou University of Chinese Medicine (Guangzhou, China), and an informed consent form has been signed. This study was registered with the China Clinical Trial Registry, registration number ChiCTR-2000033948. There were no differences in clinical characteristics between the groups of patients, including age, body mass index, gender, diabetes, hypertension, and peripheral arterial disease. All treated tendon tissues are frozen using liquid nitrogen and then ground. The ROS detection kit (Bebo, BB-470532) was used to detect the level of ROS in the
tissue, and the tissue superoxide anion detection kit (GENME, GMS10096.2 v.A) was used to detect the level of superoxide anion (SOD) in the tissue. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, USA). qPCR detection of Beclin1 and mTOR expression levels. WB detects Beclin1, p-mTOR/mTOR protein expression. The primers used are shown in Table 1, and the expression of Beclin1, mTOR, and GAPDH are used as endogenous controls. All experiments were repeated three times, and the data was calculated using the $2^{-\Delta\Delta Ct}$ method.

**TABLE 1 Primers used for quantitative real-time polymerase chain reaction**

| Gene       | primers 5’-3’                         | Amplicon size bp |
|------------|---------------------------------------|-----------------|
| h-GAPDH    | Forward: CAAGAGCACAAGAGGAAGAGAG       | 102             |
|            | Reverse: CTACATGGCAACTGTGAGGAG        |                 |
| h-Beclin1  | Forward: TCCATGCTCTGGCCAATAAG         | 111             |
|            | Reverse: ACGGCAGCTCTTAGATTTG          |                 |
| h-mTOR     | Forward: GGTGCTGGAGAACATGGATTAG       | 91              |
|            | Reverse: ACCAGTGAGGTCTTGGGATA         |                 |

**Beclin1 shRNA lentivirus packaging and verification**

According to the shRNA design rules, the target interference sequence of Beclin1 shRNA was designed: sh-Beclin1 F: 5’-CCGGCGGACAGTTTGGCACAATCAACTCGAGTTGTGCCAAACTGTCCGTTTTTG-3’; Sh-Beclin1 R: 5’-AATTCAAAAACGGACAGTTTGGCACAATCAACTCGAGTTGTGAGCAGGGAAACTGACCG-3’, and EcoRⅡ and SgrAⅡ restriction sites were added to the shRNA end. The designed shRNA sequence was synthesized by Guangzhou Aiji Biosciences. Surgical repair of the remaining human tendon tissue, the third generation of vigorously growing human tendon stem cells were digested and counted, and the human tendon stem cells were infected with the recombinant lentivirus pLKO.1-shBeclin1. 72 hours after the recombinant pLKO.1-shBeclin1 was infected with TSCs, divided into blank group, NC-shRNA group, Beclin1 shRNA lentivirus group, the cells were collected and total RNA was extracted. The changes in Beclin1 mRNA were detected by qPCR. The RNA extraction and qPCR detection methods were the same as before; the cells were collected and the protein was extracted. WB was used to detect the changes in Beclin1 expression, protein extraction and The WB detection method is the same as before. All experiments were repeated three times.

**The effect of H2O2 on the proliferation of human TSCs cells**

Collect human TSCs cells in logarithmic growth phase, count them, resuspend the cells in DMEM high glucose complete medium, adjust the cell concentration to 1×105 cells/ml, inoculate the 96-well plate, add 0.1 ml cell suspension to each well, and keep at 37 °C, Incubate overnight under 5% CO2 conditions.
Discard the old culture medium in the culture well, add 0.1 ml of DMEM high glucose complete medium containing 0, 0.05, 0.1, 0.2, 0.4 mM H2O2, and continue the culture at 37 °C, 5% CO2. After culturing for 24, 48, 72 hours, discard the medium in the well, add 0.1ml DMEM high glucose complete medium containing 10% CCK8, incubate for 2~3 hours at 37°C, 5% CO2, and measure OD450 on the microplate reader. And draw the cell growth curve. CCK-8 detects the effect of H2O2 on the proliferation of human TSCs. All experiments were repeated three times.

**The mechanism of H2O2 on autophagy of human TSCs**

Collect human TSCs cells in logarithmic growth phase, count them, resuspend the cells in DMEM high glucose complete medium, adjust the cell concentration to 1×10^5 cells/ml, inoculate the 6-well plate, add 2 ml cell suspension to each well at 37 °C , Incubate overnight under 5% CO2 conditions. Discard the old medium in the wells, and process them in groups as follows: blank group: add 2 ml of fresh DMEM high sugar complete medium; H2O2 intervention group: add 2 ml of DMEM high sugar complete medium containing 0.05 mM H2O2; 3-MA pretreatment Treatment group: Add 2 ml of DMEM high glycosyl complete medium containing 5 mM 3-MA for 30 min, discard the medium, and add 2 ml of DMEM high glycosyl complete medium containing 0.05 mM H2O2. Autophagy staining (MDC), autophagy-lysosomal staining (Lyso-Tracker Red) and transmission electron microscopy were used to observe autophagy, immunofluorescence staining to detect the expression of autophagy factor LC3A/B; DCFH-DA to detect cellular reactive oxygen species ROS level, Annexin V/PI detection of cell apoptosis; WB detection of Beclin1, mTOR, p-mTOR (Ser2448), LC3A/B, cleaved caspase-3 protein expression. The protein extraction and WB detection methods are the same as before. All experiments were repeated three times.

**Statistical Analysis**

SPSS 20.0 software was used for statistical analysis. Mean±SD was used to represent measurement data. All data were tested for normality and homogeneity of variance. The comparison between groups was tested by t, and the non-parametric test was used when the analysis of variance was not satisfied. The detection level was α= 0.05, P<0.05, the difference is statistically significant.

**Result**

**The expression levels of reactive oxygen species ROS, superoxide anion, Beclin1 and mTOR in normal and injured tendon tissues.**

The expression levels of ROS and Beclin1 in the damaged rotator cuff tissue were higher, while the expression levels of SOD and mTOR were lower (Fig. 1). It shows that the excessive production of reactive oxygen species after tendon injury stimulates the oxidative stress response, and there is a mutual feedback regulation mechanism between Beclin1 and mTOR cell signal transduction in the process of regulating autophagy.
Beclin1 shRNA lentivirus packaging and verification, recombinant lentivirus infection of TSCs cells, verification of Beclin1 expression level.

The decrease of Beclin 1 shRNA lentivirus expression level (Fig. 2), indicates that the autophagy regulation ability of TDSCs after Beclin 1 silences, which in turn initiates cell apoptosis.

The effect of H2O2 on the proliferation ability of human TSCs cells. H2O2 caused an increase in ROS in TSCs, which in turn led to a decrease in cell proliferation (Fig. 3). It shows that activating oxidative stress will damage the proliferation ability of TSCs cells.

The mechanism of H2O2 on autophagy of human TSCs, observe the level of cellular ROS, autophagy and apoptosis, and detect the expression of Beclin1, mTOR, LC3A/B, caspase-3 and other proteins.

TDSCs were pretreated with H2O2 and 3-MA, and it was found that H2O2 increased the content of reactive oxygen species, autophagy and LC3A/B expression in TDSCs, and increased Beclin1, mTOR, LC3A/B, and cleaved caspase-3 protein expression. Lead to a decrease in the level of apoptosis (Fig. 4). It shows that under the mutual regulation of Beclin1-mTOR, oxidative stress activates autophagy, increases the lysis of LC3A/B and the expression of Beclin-1, mTOR, and caspase-3 proteins, reduces the accumulation of ROS and the apoptosis of TDSCs, to protect TDSCs Protect from damage induced by oxidative stress.

Discussion

Tendinopathy is a chronic activity limitation syndrome characterized by pain, swelling, or dysfunction associated with activity. Tendon and ligament injuries are common in occupational and sports environments. The causes of tendinopathy are multifaceted and are currently unclear. Sports or occupations have received much attention as risk factors. Tendon injury caused by overuse or aging is currently a huge challenge in clinical treatment, because the self-repair of damaged tendon tissue is very slow and incomplete. The molecular mechanism of tendon differentiation is largely uncertain, which hinders the development of new therapies for tendon repair. It is well known that mechanical overload induces cellular stress in tendon cells, such as oxidative stress and endoplasmic reticulum stress, which leads to tendinitis and tendon degeneration [16, 17]. In the process of degenerative changes of the tendon, the dysfunction of mitochondria will lead to an excessive increase of reactive oxygen species (ROS) [4], and the oxidative stress caused by the excessive production of reactive oxygen will play a role in the degeneration of the tendon. A vital role, causing continuous loss of tendon function [5]. Both the mammalian target of rapamycin (mTOR) and Beclin1 mediate the occurrence of autophagy, are important factors in the regulation of autophagy, and form a Beclin1-mTOR interactive regulatory network, which regulates autophagy in Beclin1. The role of mTOR cell signal transduction channels in function and the mutual feedback regulation mechanism between them. In this study, we first detected the ROS and SOD levels of normal rotator cuff and damaged rotator cuff tissues collected in the clinic, and found that the expression level of ROS in the damaged rotator cuff tissue was higher, while the expression level of SOD was lower, indicating The excessive production of reactive oxygen species after
tendon injury stimulates the oxidative stress response. Furthermore, we detected the expression levels of Beclin1 and mTOR, and found that Beclin1 expression levels were higher in the damaged rotator cuff tissues, while mTOR expression levels were lower, indicating that Beclin1 has a mutual feedback regulation between the process of regulating autophagy and mTOR cell signal transduction mechanism.

With the latest advances in stem cell biology, research on the use of stem cells to repair tendon and cartilage damage is increasingly emerging, most of which use mesenchymal stem cells or embryonic stem cells. TSCs have high self-renewal ability, multi-differentiation potential and low immunogenicity. Therefore, these cells may provide a new cell source for tendon regeneration. Traditionally, a tendon is believed to be composed only of tendon cells (the resident cells of the tendon). However, tendon cells are usually quiescent, non-dividing cells. Current theories indicate that a small number of resident stem cells maintain the maintenance and repair of adult tissues [8]. Recently, tendon-derived stem cells (TSCs) from human, mouse and rat tendon tissues have been identified [9, 10]. Unlike static tendon cells, TSCs have the potential for multiple differentiation and self-renewal, and are the core of tendinopathy’s occurrence, development and healing. The microenvironment in which they are located is critical to the process of tendinopathy injury and repair. The viability and tendon differentiation of TSCs are closely related to the maintenance of the tendon microenvironment and the development of tendinopathy [8]. It has been reported in the literature that there is a common molecular regulatory network between bone, muscle and tendon [27]. mTOR protein is the central controller of cell growth and mesenchymal stem cell differentiation [28, 29]. It plays an important role in regulating the function of the musculoskeletal system [30, 31]. Beclin1 can mediate the localization of autophagy proteins in phagocytic vesicles, and regulate the formation and maturation of mammalian autophagosomes [26]. It is a key regulator of autophagy and an indispensable condition for the formation of autophagosomes. It can be cleaved by a variety of Caspases. After cleaved Beclin1 loses its original autophagy regulation ability and initiates the apoptosis program instead. Many autophagy regulatory proteins directly or indirectly bind with different domains or amino acids of Beclin 1 to form protein complexes, which in turn regulate the level of autophagy [27–29].

In this study, we extracted TSCs and recombined pLKO.1-shBeclin1 to infect TSCs for 72 hours, and found that Beclin 1 shRNA lentivirus expression decreased, indicating that Beclin 1 silenced the TSCs autophagy regulation ability, which in turn initiates cell apoptosis.

The recent study showed that TSCs cultured under hypoxic conditions showed increased cell proliferation and stem cell marker expression in vitro, and formed more tendon-like structures in the matrix derived from tendons in vivo. Considering that hypoxia is a recognized autophagy inducer, we believe that autophagy may also play a role in the maintenance of TSCs under hypoxic conditions. Reactive oxygen species (ROS) produced by cell metabolism is the active form of oxygen [17]. A moderate level of ROS promotes cell proliferation and differentiation, while excessive ROS induces apoptosis or autophagy due to oxidative damage to lipids, proteins and DNA [18]. A large amount of evidence shows that ROS mediates many signaling pathways and plays a trigger role in autophagy and apoptosis [32]. Autophagy is another key factor that controls cell fate. Autophagy plays a dual role, causing cell death or promoting cell survival [28]. Autophagy can also promote cell growth by protecting cells from apoptosis [29]. The loss of cell self-renewal ability and pluripotency is the main obstacle based on stem cell
Considering that the TSCs implanted in the injury site will be exposed to various cellular stresses, we therefore studied the effect of oxidative stress in vitro on TSCs. We found that TSCs treated with H2O2 showed increased ROS accumulation and decreased colony formation and proliferation, and weakened the differentiation ability. Therefore, maintaining TSCs under stress conditions such as increased oxidative stress may be a challenge to be overcome in TSC-based treatments. According to reports, autophagy is a mechanism for stem cells to maintain self-renewal and dryness under various external injuries. mTOR and Beclin1 are important factors in autophagy regulation and form a Beclin1-mTOR mutual regulatory network. Therefore, we studied the mechanism of H2O2 on TSCs autophagy. TSCs were pretreated with H2O2 and 3-MA, and it was found that H2O2 caused an increase in reactive oxygen species ROS in TSCs and an increase in Beclin1, mTOR, LC3A/B, and cleaved caspase-3 protein expression, leading to a decrease in cell apoptosis. These protective effects are accompanied by activated autophagy activity, such as increased LC3 lysis, and increased expression of Beclin-1, mTOR, and caspase-3. In addition, the autophagy inhibitory effect of Beclin-1 silenced by 3-MA or shRNA will reduce the protective effect of rapamycin on H2O2 treated TSCs. These data indicate that autophagy helps maintain the self-renewal ability, dryness and differentiation ability of TSCs under oxidative stress. Considering that autophagy usually acts as an antioxidant by scavenging oxidized cellular components, we tested the level of ROS in TSCs. It was found that after H2O2 treatment, the level of intracellular ROS in TSCs increased, while apoptosis decreased. These results indicate that under the mutual regulation of Beclin1-mTOR, TSCs can be protected from damage induced by oxidative stress by inducing autophagy, thereby reducing the accumulation of ROS. Lavagnino et al. [17] stated that hypoxic environment will reduce the tolerance of the Achilles tendon to repeated exercise and increase the risk of chronic tendinopathy. Therefore, any factors that may affect the proliferation, apoptosis, differentiation and microenvironment of TSCs can indirectly change the prognosis of tendinopathy injury and repair.

In short, the results of this study show that under the mutual regulation of Beclin1-mTOR, oxidative stress can induce autophagy, and autophagy may protect TSCs from oxidative stress-induced damage by reducing the accumulation of ROS. However, whether TSCs autophagy induced by oxidative stress can promote tendon-bone healing in vivo needs further research.

Abbreviations

TSCs
Tendon stem cells; ROS: Reactive oxygen species; SOD: Superoxide anion; mTOR: Mammalian target of Rapamycin. qRT-PCR: Quantitative polymerase chain reaction. MDC: Monodansylcadaverine. Lyso-Tracker Red: Autophagy Lysosome Red Fluorescent Probe.

Declarations

Acknowledgements

Not applicable.
Authors' contributions

Hewei Wei conceived and designed the experiments; Zhijun Liu, Shaojin Liu, and Weipeng Zhen performed the experiments; Zhijun Liu and Shaojin Liu performed data analysis; Zhihao Liao and Sheng Chen contributed to sample collection; Zhijun Liu and Shaojin Liu wrote the paper; Hewei Wei assisted with writing and proofreading. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analysed during this study are included in this published article.

Ethics approval and consent to participate

This study was approved by the Internal Review and the Ethics Boards of Guangzhou University of Chinese Medicine and The Third affiliated hospital, Guangzhou University of Chinese Medicine. Informed written consent was obtained from all study subjects. This study was registered with the China Clinical Trial Registry, registration number ChiCTR-2000033948.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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**Figures**
Figure 1

Detection of tissue specimens. (a-b) Differential expression of ROE and SOD in the tendon tissue of the normal control group and the injury group (P<0.05); (c-d) qRT-PCR and WB to detect the expression levels of Beclin1 and mTOR in the tendon tissue of the normal control group and the injury group (P <0.05).
Figure 2

Packaging and verification of Beclin1 shRNA lentivirus. (a) Recombinant plasmid pLKO.1-Beclin1-shRNA sequencing results; (b) Recombinant lentivirus infection of TSCs cells; (c-d) qRT-PCR and WB detection of Beclin1 mRNA and protein to verify recombinant lentivirus infection of TSCs.
Figure 3

The effect of H2O2 on the proliferation of human TSCs. CCK8 was used to detect the proliferation ability of H2O2 on human TSCs cells, and culture it for 24, 48, 72h in DMEM high glucose medium with 0, 50, 100, 200, 400 μM H2O2.
Figure 4

The mechanism of H2O2 on autophagy of human TSCs. H2O2 and 3-MA treat TDSCs. (a) DCFH-DA was used to detect the reactive oxygen species (ROS) levels of TSCs in each group; (b-d) Immunofluorescence staining (MDC), lysosome staining (Lyso-Tracker Red), and transmission electron microscope were used to observe the autophagy levels of TSCs in each group; (e) Immunofluorescence staining to detect the expression level of LC3A/B in TSCs cells of each group, and contrast with DAPI and Merge; (f) WB to detect Beclin1, mTOR, p-mTOR (Ser2448), LC3A/B in TSCs cells of each group, The expression of cleaved caspase-3 protein; (g) Annexin V/PI was used to detect the apoptosis level of TSCs in each group.