PINK1/Park2-Mediated Mitophagy Relieve Non-Alcoholic Fatty Liver Disease

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Summary

Up to now, there's a limited number of studies on the relationship between PINK1/Park2 pathway and mitophagy in NAFLD. To investigate the effect of Park2-mediated mitophagy on non-alcoholic fatty liver disease (NAFLD). Oleic acid was used for the establishment of NAFLD model. Oil red-dyed lipid drops and mitochondrial alternations were observed by transmission electron microscopy. Enzymatic kit was used to test lipid content. The levels of IL-8 and TNF-α were determined by ELISA. Lenti-Park2 and Park2-siRNA were designed to upregulate and downregulate Park2 expression, respectively. The changing expression of PINK and Park2 was detected by RT-qPCR and Western blot. Immunofluorescence staining was applied to measure the amount of LC3. Successful NAFLD modeling was featured by enhanced lipid accumulation, as well as the elevated total cholesterol (TC), triglyceride (TG), TNF-α and IL-8 levels. Mitochondria in NAFLD model were morphologically and functionally damaged. Park2 expression was upregulated by lenti-Park2 and downregulated through Park2-siRNA. The PINK1 expression showed the same trend as Park2 expression. Immunofluorescence staining demonstrated that the when Park2 was overexpressed, more LC3 protein on mitochondrial autophagosome membrane was detected, whereas Park2 knockdown impeded LC3' locating on the membrane. The transmission electron microscopy image exhibited that the extent of damage to the mitochondrial in NAFLD model was revered by enhanced Park2 expression but further exacerbated by reduced Park2 expression. Park2-mediated mitophagy could relive NAFLD and may be a novel therapeutic target for NAFLD treatment.

Keywords: Non-Alcoholic Fatty Liver Disease (NAFLD) • Mitophagy • PINK1/Park2 • Park2 • PINK1

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Introduction

Non-alcoholic fatty liver disease (NAFLD) is characterized by an accumulation of fat in liver that surpasses 5 % of liver weight when patients drink less than 10 g alcohol during a single day [1]. Recent years has witnessed an increasing number of NAFLD cases, which has now affected up to 25.2 % of the general population in the world, prevailing the raised risk of obesity [2]. Nonlethal as it is, NAFLD is identified as leading cause of cardiovascular disease and liver cancer, such as coronary heart disease hypertension and arteriosclerosis [3]. Patients with NAFLD have abnormal lipid accumulation in the liver as the enhanced lipid disposal ultimately fails to deal with the increasing intrahepatic fat deposition [4]. Specifically, excessive accumulation of triglycerides (TG) and total cholesterol (TC) in hepatocytes results from abnormal homeostasis of lipid metabolism in the liver [5]. The initiation and advancement of NAFLD are intricate, influenced by a plethora of risk factors encompassing epigenetic inheritance, environmental stimuli, sleep quality, dietary habits, and stress [6]. The interplay between genetic predisposition and these diverse factors significantly contributes to elucidating the discernible divergence in NAFLD presentation and severity across different individuals. In genetics, the degree of variance in gene

expression leading to alterations in phenotypes is denoted as heritability [7]. Numerous studies have substantiated that NAFLD exhibits a robust heritability component, based on the frequent familial clustering [8]. Furthermore, enhancement of inflammatory response in the liver presented as elevated TNF-α and IL-8 has been implicated in the pathogenesis of NAFLD [9].

The liver necessitates a substantial quantity of energy for metabolizing various substances, with energy generation intricately linked to the function of mitochondria. Hence, mitochondria are commonly referred to as the "powerhouse of cells" [10]. Beyond energy production, mitochondria performed multifaceted roles in normal physiology, including the generation of redox molecules and reactive oxygen species (ROS), and regulation of biosynthetic metabolism and cell signaling, which make them crucial cellular stress sensors [11]. Besides, their quality, quantity, and functional flexibility exert great influence on cellular physiology. However, abnormal mitochondrial functions are link to many human diseases, including age-related disorders, heart failure, neurodegeneration, diabetes, and cancers [12]. More focus have been placed on mitochondria and its role in different diseases in the past decade. For instance, mitochondrial fatty acid oxidation and autophagy activated by adipocytes promote tumor growth in colon cancer [13]. Mitochondria participate in chemoresistance to cisplatin in human ovarian cancer cells [14]. Mitophagy is a mechanism of specific autophagy of mitochondria, through which, excessive or dysfunctional mitochondria can be eliminated. Mitophagy helps to control the number and quality of mitochondria in cells, beneficial to the maintenance of cellular physiology [11]. The impairment of mitochondrial function stands as a significant contributory factor to the onset of NAFLD [15]. Recent research has proposed a correlation between the processes of mitochondrial fusion, fission, and the progression of NAFLD [16]. In NAFLD, there exists an upsurge in mitochondrial fission alongside a reduction in fusion, culminating in a fragmented and dysfunctional mitochondrial network. This phenomenon fosters the accumulation of fat in the liver, as malfunctioning mitochondria generate a diminished amount of ATP, rendering them more susceptible to oxidative stress, ultimately instigating lipid accumulation and inflammation [17].

It has been reported that PINK1/Parkin degrade damaged mitochondria by stimulating mitophagy [18]. The serine/threonine kinase PINK1 and E3 ubiquitin-ligase Parkin were found to be linked to mitophagy in Parkinson's disease at first [19]. With more researches on the association between PINK1/Parking and mitophagy, some underlying mechanisms have also been revealed. For example, PINK1 phosphorylates ubiquitin to activate Parkin E3 ubiquitin ligase activity [20]. Also, PINK1 stabilization or overexpression is sufficient to recruit Parkin to mitochondria and trigger mitophagy [18].

Here in, in this study we aim to reveal the connection between PINK1/Park2 pathway and mitophagy in NAFLD and the underlying mechanisms, hoping to provide a novel insight in to NAFLD therapy.

Materials and Methods

Construction of NAFLD cell model

Hepatocyte SMMC-7721 cells (SC0041, Yuchicell Biological technology, China) were cultured in high glucose DMEM medium (10569010, Gibco, USA) containing 1 % GlutaMAX™ supplement, 1 % sodium pyruvate and 10 % fetal bovine serum (Gibco, USA) at 37 °C in 5 % CO_2. NAFLD cell model was established by incubation with Oleic acid (400 μmol/L) for 24 h. Oleic acid was purchased from Solarbio (SC9320, China).

Oil red O staining

The oil red O (O8010; Solarbio, China) isopropanol saturated solution was mixed with distilled water in a ratio of 3:2 to prepare the dyeing solution. The treated cells subjected to PBS washing for 3 times were fixed with 4 % paraformaldehyde for 30 min at room temperature. For lipid qualification, the absorbance values were measured at 490 nm after staining the cells with the prepared solution for 30 min.

Detection of total cholesterol (TC) and triglyceride (TG) level

To evaluate the degree of lipid accumulation in NAFLD models, TC and TG were detected by enzymatic kits (TC: E1026-105, TG: E1025-105, Beijing Applygen Technologies, China) based on the manufacturer's instruction. BCA protein assay kit (P1511; Applygen Technologies, China) was bought for the measurement of protein concentration. The lipid level/protein concentration was calculated to reflect lipid accumulation.

Detection of IL-8 and TNF-α by ELISA

The supernatant obtained in the process of cell

culture was centrifuged and transferred into clean tubes for subsequent detection. The levels of IL-8 and TNF-α were measured by specific ELISA kit (IL-8: ab214030; TNF-α: ab181421; Abcam, China).

Transmission electron microscopy

SMMC-7721 cells were fixed with glutaraldehyde and osmic acid, and observed by transmission electron microscopy (H-7500; Hitachi, Tokyo, Japan). The number of mitophagosomes and mitophagosome lysosomes per unit cross-sectional area of cells were detected and analyzed by image analyzer.

Transfection

To upregulate Park2 expression, cells were incubated with lentivirus oePark2 consisting of vector plasmid pLVX-Puro (Clonetech, USA) and Park2 gene fragment in fresh medium containing polybrene at 37 °C for 4 h. Then, the polybrene concentration was diluted by addition of an equal volume of fresh medium. After 24-h incubation, cells were transferred to a fresh medium without lentivirus and then cultured for 72 h.

To knockdown Park2 expression, Park2-siRNA (5'-UCCA GCUCAAGGAGGU GGUUGCUAA-3') was designed according to Park2 gene sequence. The negative control siRNA (5'-UUCUCCGAACGUCACGU-3') was obtained from Thermo Fisher (USA). After cultured in the medium without antibodies, cells were transfected with Park2-siRNA for 72 h using LipofectamineTM2000 kit (11668030; ThermoFisher, USA).

After transfection, SMMC-7721 cells were divided into control group (normal cells), NAFLD group (model group), vector group (lenti-Park2 vector), oe-Park2 group (lenti-Park2), si-NC group (negative control siRNA) and si-Park2 group (Park2-siRNA).

RT-qPCR

Total RNA was extracted from cell lines using the RNA Clean and Concentrator Kit (K1069, APExBIO) following the manufacturer's instruction. Reverse transcription was performed using RT SuperMix for qPCR (K1074, APExBIO). qRT-PCR was carried out using GoTaq® qPCR Master Mix (A6002, Promega, USA) on a 7500 Fast Real-Time PCR System (351106, Applied Biosystems, Singapore). Amplification was repeated 40 times as the following process：Hot start reaction was performed at 95 °C for 10 minutes, followed by 15-second denaturation at 95 °C, 35-second annealing at 55 °C, 40 seconds extension at 72 °C. GAPDH was used as a loading control. The primers were as follows: PINK1, forward 5'-CATGGCTTTGGATGGAGAGT-3' and reverse 5'-TGGGAGTTTGCTCTTCAAGG-3'; PARK2, forward 5'- ATCGCAACAAATAGTCGG-3' and reverse 5'-GGCAGGGAGTAGCCAAGT-3; GAPDH, forward 5'-TGAAGCAGGCATCTGAGGG-3' and reverse 5'-CGAAGGTGGAAGAGTGGGAG-3'. The relative expression level of mRNA was calculated using 2-ΔΔCt method.

Western blot

Cells washed twice by using PBS were lysed with RIPA buffer (ThermoFisher, USA). BCA protein assay kit (P1511-1) purchased from Applygen Technologies Inc. (China) was utilized to measure the protein concentration in lysates according to the manufacturer's instructions. Subsequently, the protein was separated by 8-12 % SDS-polyacrylamide gels and transferred to PVDF membrane (KGP114-1; Titeca, Jiangsu, China). To remove the western transfer buffer from the PVDF membranes, TBST (Tris Buffered Saline with Tween 20) (T1082; Solarbio, Beijing, China) were adopted to wash the membrane twice. TBST on the membrane was digested by a pipette. Then, the membranes were blocked using blocking buffer (W3010; Solarbio, Beijing, China) for 2 h and subjected to an overnight immunoblotting with primary antibodies at 4 °C. Later, the membrane was washed with TBST for three times and incubated with corresponding second antibodies (Beyotime, China) at room temperature for 1 h following by TBST washing again. Antibodies against PINK1 (ab216144), Park2 (ab77924) and GAPDH (ab8245) were purchased from Abcam (Shanghai, China). ECL detection reagents bought from Beyotime (P0018M, Shanghai, China) was used to detect protein signals. Image J software was adopted to measure the gray value of protein bands.

Immunofluorescence

The cells were fixed and blocked with normal serum after incubated with Mitotracker solution (C1049-50μg, Beyotime, China) at 37 °C for 30 min. Subsequently, monoclonal antibody against LC3 (14600-1-AP, Proteintech, USA) was used for cell incubation overnight at 4 °C and discard the next day. When PBST washing was done for 3 times, cells were cultured with secondary antibody Alexa Fluor 555-labeled Donkey Anti-Rabbit IgG (H+L) (A0453, Beyotime, China) at 37 °C in the dark for 30 min and then mounted with fluorescence decay resistant medium. To detect mitophagy, the number of LC3 dots colocated

with mitochondria in each cell was systematically analyzed by IN Cell Analyzer living cell fluorescence microscopic image system.

Statistical analysis

The data were analyze using GraphPad Prism 8. Each group was analyzed three times. The experimental results are presented as mean ± standard deviation (SD). Comparisons between 2 data sets were analyzed with T-test. One-way ANOVA with Bonferroni post hoc test was used for analysis of multiple data sets.

Results

Alternations of mitochondria in successful NAFLD model

NAFLD models were established to explore the differences between mitochondria in normal hepatocytes and NAFLD cells. As shown in Fig. 1A, noticeable accumulation of lipid droplets in NAFLD cells was observed by electronic telescope after red oil O staining, yet was not found in normal liver cells. At the same time, significantly elevated lipid content of TC and TG suggested lipid metabolism disorders in NAFLD cells (p<0.001, Fig. 1B-D). Meanwhile, the concentration of TNF-α and IL-8 in NAFLD cells was remarkably higher than that in normal SMMC-7721 cells (p<0.001, Fig. 1E). These data indicated the successful establishment of NAFLD cell model. Then, changes of mitochondria in NAFLD cells were shown clearly by transmission electron microscopy imaging. Fig. 1F showed the accumulation of damaged mitochondria in cells with NAFLD, which exhibited crest rupture and a swollen morphology. Besides, the number of mitochondrial autophagosomes was obviously reduced. Overall, suppressed mitophagy was provoked in cells with NAFLD.

PINK1 expression in SMMC-7721 cells induced by Park2

SMMC-7721 cells were respectively subjected to transfection with lenti-vector, lenti-Park2, si-NC and Park2-siRNA to investigate the relationship between PINK1 and Park2. The expression level of PINK1 and Park2 was tested by RT-qPCR and Western blot. Lenti-vector and si-NC groups exerted no significant change of PINK1 expression compared to corresponding control groups. Importantly, the expression of Park2 was significantly raised in lenti-Park2-transfected cells (p<0.01, p<0.001) and significantly reduced when Park2 was knocked down by Park2-siRNA (p<0.001, Fig. 2). Noticeably, PINK1 expression in NAFLD group presented a significant lower level (p<0.001), whereas significantly increased after Park2 overexpression (p<0.001) (Fig. 3A-C). In addition, the downregulated expression of Park2 contributed to dropped expression of PINK1 compared to that in si-NC group (p<0.01, p<0.001, Fig. 3D-F). Taken together, upregulated Park2 expression markedly promoted PINK1 expression while downregulated Park2 expression inhibited PINK expression.

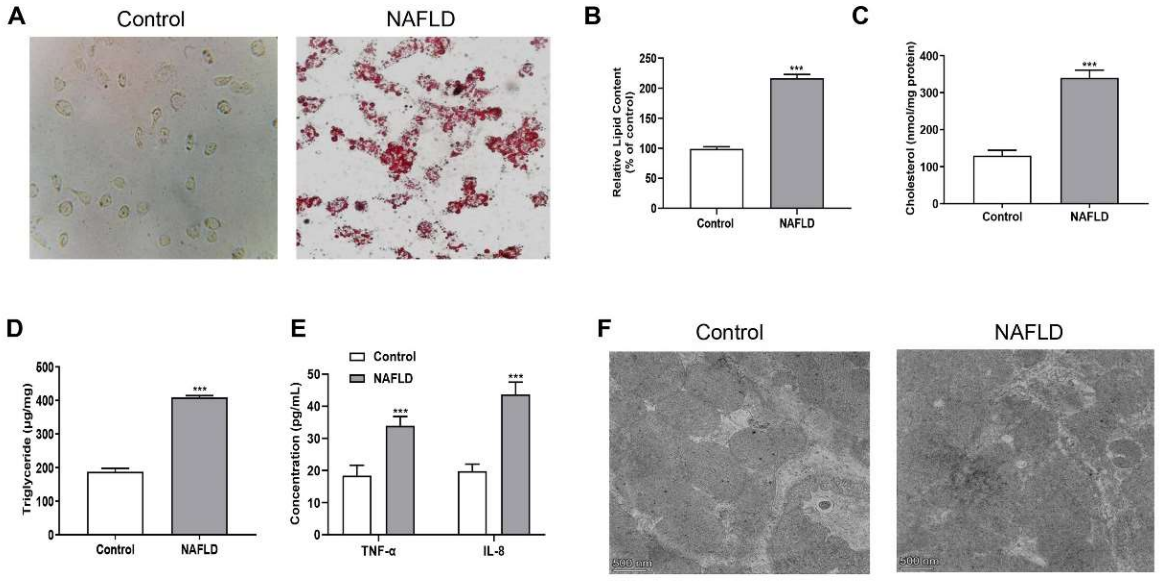

Fig. 1. The successful establishment of NAFLD cell model. (**A**) Lipid accumulation, (**B**) lipid content and level of (**C**) total cholesterol, (**D**) triglyceride, (**E**) TNF-α and IL-8 level in NAFLD model cells. (**F**) The changed phenotype of mitochondria. $^{***}p$<0.001 compared to control group.

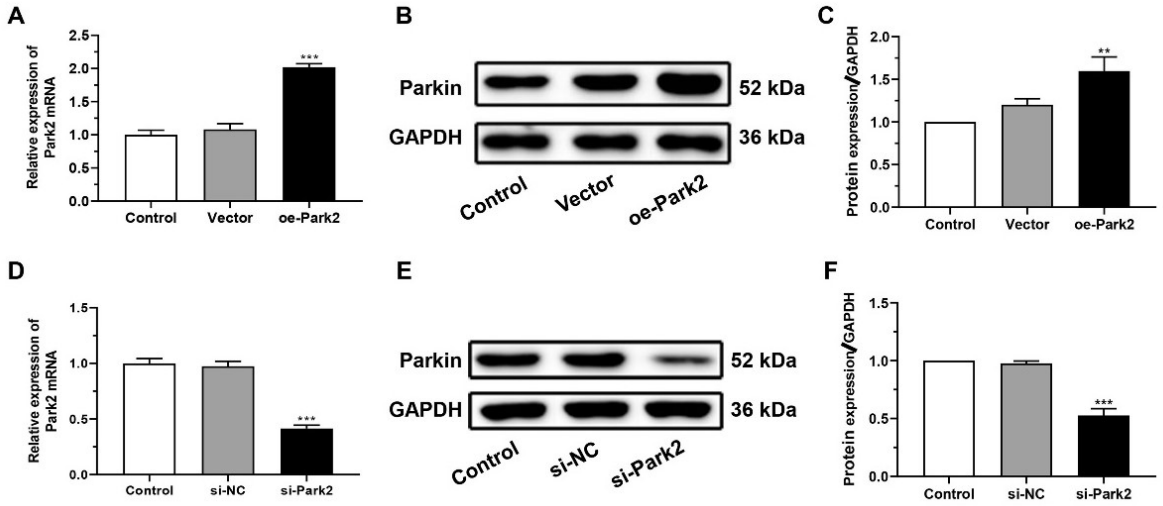

Fig. 2. Park2 transfection efficiency. (**A**) Relative expression of Park2 mRNA evaluated by RT-qPCR when cells were transfected with lenti-Park2. (**B**) Western blot result and (**C**) its quantitative analysis of Park2 protein level after cells were subjected to lenti-Park2 transfection. GAPDH functioned as loading control. $^{**}p<0.01$, $^{***}p<0.001$, compared to the Vector group. (**D**) RT-qPCR demonstrated relative expression of Park2 mRNA in respond to Park2-siRNA transfection. (**E**) Western blot result and (**F**) its quantitative analysis of Park2 protein level when Park2 was knocked down. GAPDH served as loading control. $^{***}p<0.001$, compared to the siNC group.

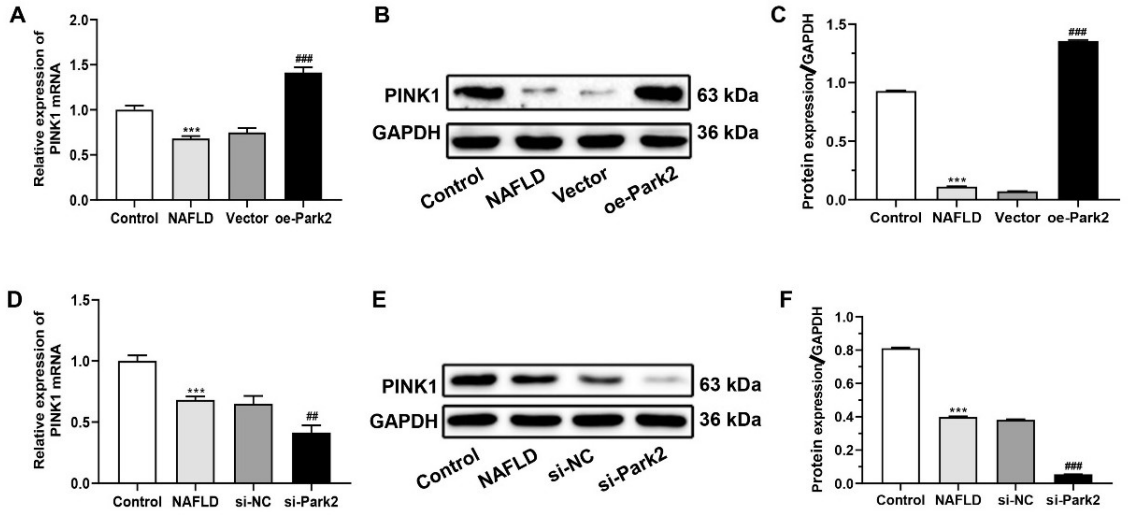

Fig. 3. PINK1 expression in respond to changed Park2 expression. (**A**) Relative expression of PINK1 mRNA when Park2 was overexpressed. (**B**) Western blot result and (**C**) its quantitative analysis of PINK1 protein level when Park2 was overexpressed. GAPDH served as loading control. $^{***}p<0.001$, compared to the Control group; $^{###}p<0.001$, compared to the Vector group. (**D**) RT-qPCR demonstrated relative expression of PINK1 mRNA in respond to Park2-siRNA transfection. (**E**) Western blot result and (**F**) its quantitative analysis of PINK1 protein level when Park2 was downregulated. GAPDH served as loading control. $^{***}p<0.001$, compared to the Control group; $^{##}p<0.01$, $^{###}p<0.001$, compared to the si-NC group.

The effect of Park2 on mitophagy in SMMC-7721 cells induced by Oleic acid

Since LC3 is identified the mammalian protein localized in the autophagosome membrane, reflecting the extent of mitophagy [21]. Immunofluorescence staining was performed to detect LC3 level in this study. As revealed in Fig. 4, the relative fluorescence intensity of LC3 in NAFLD cells was markedly reduced ($p<0.001$, Fig. 4&5), suggesting the blocked mitophagy as corroborated before in this study. Also, the corresponding quantitative analysis results showed that the amount of LC3 was significantly increased in oe-Park2 group ($p<0.001$, Fig. 4), yet significantly decreased in si-Park2 group ($p<0.01$, Fig. 5). Overexpressed Park2 significantly enhanced the co-localization of LC3 (green dots) with Mitotracker-stained mitochondria (red dots) ($p<0.01$, Fig. 4), whereas the limited co-localization of green and red dots was demonstrated when Park2 had lower expression ($p<0.05$, Fig. 5).

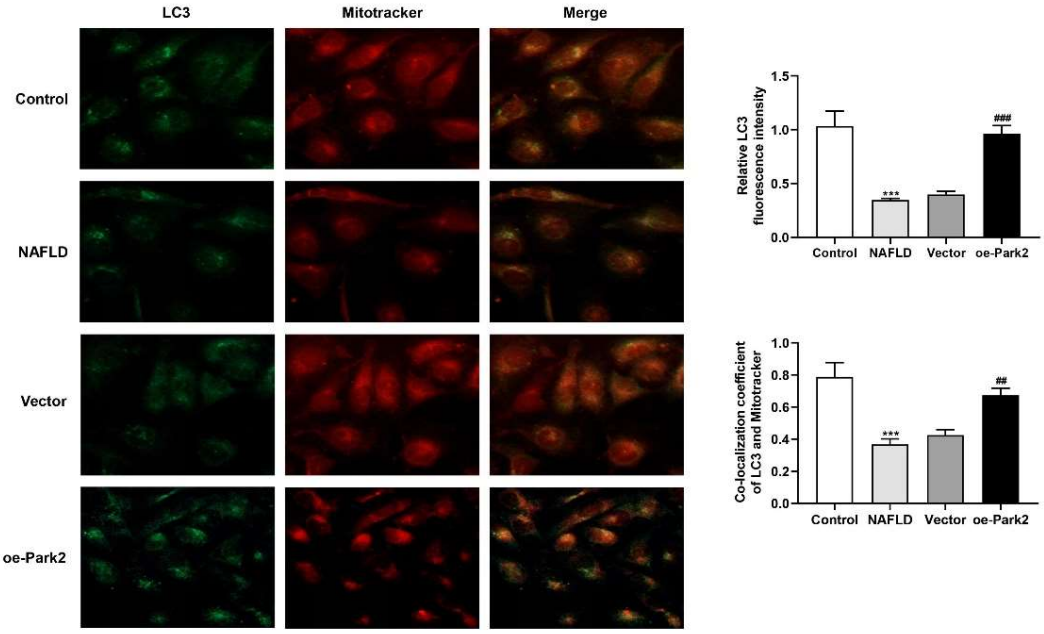

Fig. 4. Colocalization of LC3 and Mitotracker-stained mitophagosomes when Park2 was upregulated. LC3 was stained green and mitophagosomes was stained red. $^{***}p<0.001$, compared to the Control group; $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$, compared to the Vector group.

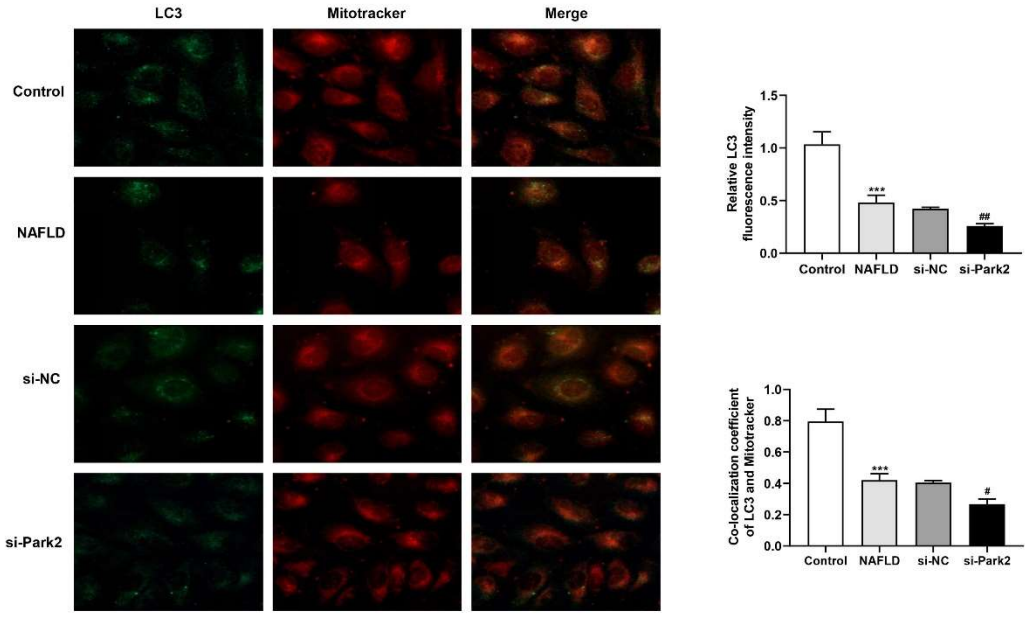

Fig. 5. Colocalization of LC3 and Mitotracker-stained mitophagosomes when Park2 was downregulated. LC3 was stained green and mitophagosomes was stained red. $^{***}p<0.001$, compared to the Control group; $^{\#}p<0.05$, $^{\#\#}p<0.01$, compared to the si-NC group.

PINK1/Park2-mediated mitophagy mitigates Oleic acid-induced NAFLD cells

It was previously confirmed in this study that mitochondria in NAFLD cells had difficulty to form autophagosomes with its torn membrane, crest rupture and swollen morphology, which was remarkably rescued by the overexpressed Park2 expression whereas developed to a more serious damage after Park2 knockdown (Fig. 6A). Importantly, oleic acid-caused lipid accumulation in oe-Park2 group was alleviated, accompanied by an obvious decrease in relative lipid content ($p<0.01$, Fig. 6B-C). On the contrary, the noticeable enhancement of lipid accumulation and escalated level of lipid content in respond to downregulated Park2 expression was demonstrated in Fig. 6B&D ($p<0.001$). Therefore, PINK1/Park2-regulated mitophagy aided to relieve NAFLD.

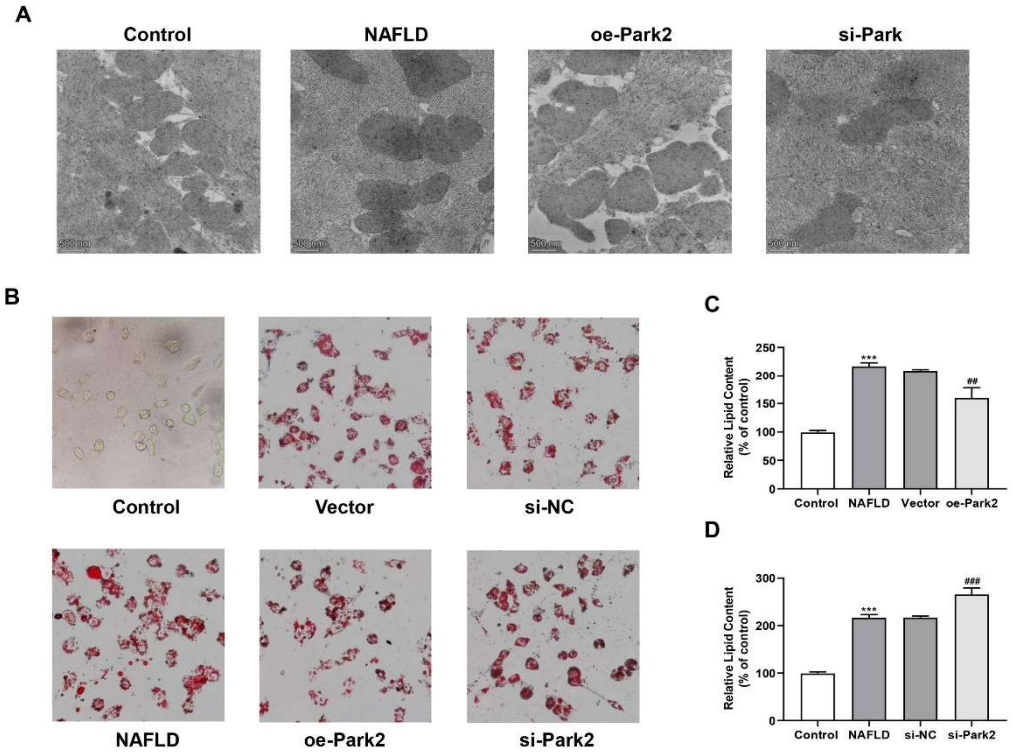

Fig. 6. PINK1/Park2-mediated mitophagy mitigates NAFLD. (**A**) Impaired mitochondria was rescued in oe-Park group while subjected to a more serious damage in si-NC group. (**B**) Lipid accumulation in NAFLD cells in respond to overexpressed and reduced Park2 expression. (**C**) Quantitative analysis of lipid content in cells transfected with lenti-Park2. $^{***}p<0.001$, compared to the Control group; $^{\#\#}p<0.01$, compared to the Vector group. (**D**) Quantitative analysis of lipid content in cells transfected with si-Park2. $^{***}p<0.001$, compared to the Control group; $^{\#\#\#}p<0.001$, compared to the si-NC group.

Discussion

At present, lifestyle modification that focus on weight reduction by healthy diet and regular exercise is still the main treatment for NAFLD patients. When doing sports may not be practical for some patient, bariatric surgery can be a good alternative for them. Up to now, no drug is approved for NAFLD [22], but many researchers never give up finding appropriate targets for drug development. For example, Yu *et al.* [23] have proved that liraglutide ameliorates non-alcoholic steatohepatitis by inhibiting NLRP3 inflammasome and pyroptosis activation via mitophagy. What's more, a study of Zhang *et al.* [24] has revealed that impaired mitophagy triggers NLRP3 inflammasome activation during the progression from nonalcoholic fatty liver to nonalcoholic steatohepatitis. In this study, we found that PINK1/Park2-mediated mitophagy alleviated NAFLD.

The pervasive consensus holds that cytokines assume a pivotal role as mediators in the processes of fibrosis, inflammation, and cirrhosis in NAFLD [25]. Prior investigations have documented several inflammatory mediators, such as TNF-α and IL-8, playing a central role in the genesis and advancement of NAFLD [26]. TNF-α, as the foremost proinflammatory cytokine liberated during the body's immune response, serves to recruit an array of inflammatory agents and initiates the progression of NAFLD [27]. TNF-α exerts a potent inhibitory impact on lipoprotein lipase, which can curtail the breakdown of peripheral adipose tissue, foster the synthesis of TG in hepatocytes, and provoke the accrual of lipids in the liver [28]. In patients with NAFLD, notable escalations in IL-8 levels were observed, inciting the intrahepatic neutrophil infiltration and contributing to hepatocyte injury through the activation and chemotaxis of neutrophils [29]. Moreover, IL-8 has the capacity to activate hepatic macrophages and further the progression of liver fibrosis and cirrhosis among individuals afflicted with NAFLD [30]. Here, we assessed the content of TNF-α and IL-8, and the results involved that the level of inflammation in NAFLD cell model substantial increase, suggesting that the successful establishment of NAFLD cell model, with the inflammatory cytokines TNF-α and IL-8 undeniably implicated in the pathogenesis of NAFLD.

Mitophagy stands as a pivotal component for maintaining intracellular homeostasis by effectively eliminating damaged mitochondria, thereby serving as a key mechanism of mitochondrial quality control [31].

The clearance of mitochondria through mitophagy has been widely considered as a protective mechanism in NAFLD [32]. More recently, mounting evidence has substantiated the correlation between mitophagy and liver fibrosis, with PINK1 standing as one of the most extensively studied molecules linked to mitophagy [33]. Bueno *et al.* [34] illustrated that the absence of PINK1 deficiency led to defective mitophagy, consequently fostering lung fibrosis in aging mice. Additionally, Qiu *et al.* [35] proposed that the inhibition of PINK1-mediated mitophagy could alleviate PM2.5-induced liver fibrosis. Moreover, Xu *et al.* indicated the suppression of PINK1-mediated mitophagy in mice experiencing liver fibrosis associated with NAFLD [36]. Consequently, PINK1-mediated mitophagy appears to wield a crucial role in NAFLD.

PINK1 and Parkin, two gene products mutated in familial Parkinsonism, serve as pivotal components of mitophagy, operating in synergy to facilitate the degradation of dysfunctional mitochondria. Parkin's E3 ubiquitin ligase is recruited and activated on the surface of damaged mitochondria where the prior accumulation of PINK1 happens [37]. Subsequently, ubiquitin chains on mitochondrial outer membrane proteins are built by Parkin, helping to recruit autophagy receptors that initiate mitophagy. Surprisingly, Michael Lazarou [38] have found that PINK1 recruit two primary autophagy receptors NDP52 and optineurin to mitochondria to provoke mitophagy directly without the involvement of Parkin, which means that PINK1-induced mitophagy can be independent from Parkin. For example, Piquereau *et al.* [39] confirmed that PARK2-independent mitophagy and upregulation of macroautophagy partly compensate for autophagic clearance of damaged mitochondria in Park2-deficient mice. The coexist Parkin-dependent and independent mechanism underlying mitophagy aroused our curiosity about Parkin's interference in PINK1-mediated mitophagy. In this study, we measured PINK1 expression in respond to the changed expression level of Park2. The same trend of the expression level of the two genes demonstrated that Park2 could affect the PINK expression in Oleic acid-induced cells.

LC3 is identified to be the first mammalian protein that accumulates on the membrane of autophagosomes thus it is regard as a suitable marker for autophagosomes [21]. According to existing researches, the amount of LC3 is closely linked to the extent of autophagosome formation. For instance, Padman *et al.* [40] have proved that the absence of LC3s result in the slower rate of autophagosome formation. Furthermore, even when Parkin is knocked down, PINK1 can recruit some autophagy receptors to adjacent mitochondria, which would interact with LC3 to induce low-level mitophagy [38]. In the current study, immunofluo-rescence staining was performed to determine the amount of LC-3 and the extent of autophagosome. The colocation of LC3 and autophagosomes was limited when Park2 was downregulated by Park-2 si-RNA whereas more LC3 and autophagosome located at the same spots when Park2 was overexpressed. Taken together, we believe that in Oleic acid-induced cells Park2 could enhance the function of PINK1 that accelerate the formation of autophagosomes presented as the accumulation of LC3 on the autophagosome membrane.

It is reported that mitophagy is beneficial to the repression of cell senescence [41], renal ischemia/ reperfusion injury [42] and glioma progression [43]. Also Park2-mediated mitophagy is confirmed to play a protective role in many diseases, For instance, Drp1-regulated PARK2-dependent mitophagy protects against renal fibrosis in unilateral ureteral obstruction [44]. Interestingly, overactive mitophagy triggered off under certain stressful status may function as a disease contributor. According to Yi *et al.* [45] found that excessive mitophagy mediated by PINK1/Park2 is involved in the pathogenesis of polycystic ovary syndrome. To verify the effect of Park2-mediated mitophagy in NAFLD, we checked Oleic acid-stimulated NAFLD model in respond to the presence and absence of Park2. We found that the morbid states of NAFLD in cells were reversed by upregulated Park2 expression while exacerbated by downregulated Park2 expression, suggesting the protective role of PINK1/Park2-mediated mitophagy on Oleic acid-induced NAFLD.

In summary, this study proved that PINK1/Park2-mediated mitophagy aided to ameliorate NAFLD. This finding may provide a promising target for the treatment of NAFLD.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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References

1. Mir HM, Stepanova M, Afendy H, Cable R, Younossi ZM. Association of sleep disorders with nonalcoholic fatty liver disease (NAFLD): A Population-based Study. J Clin Exp Hepatol 2013;3:181-185. https://doi.org/10.1016/j.jceh.2013.06.004
2. Younossi ZM, Golabi P, de Avila L, Paik JM, Srishord M, Fukui N, Qiu Y, Burns L, Afendy A, Nader F. The global epidemiology of NAFLD and NASH in patients with type 2 diabetes: A systematic review and meta-analysis. J Hepatol 2019;71:793-801. https://doi.org/10.1016/j.jhep.2019.06.021
3. Chacko KR, Reinus J. Extrahepatic complications of nonalcoholic fatty liver disease. Clin Liver Dis 2016;20:387-401. https://doi.org/10.1016/j.cld.2015.10.004
4. Saigo Y, Sasase T, Tohma M, Uno K, Shinozaki Y, Maekawa T, Sano R, Miyajima K, Ohta T. High-cholesterol diet in combination with hydroxypropyl-beta-cyclodextrin induces NASH-like disorders in the liver of rats. Physiol Res 2023;72:371-382. https://doi.org/10.33549/physiolres.934981
5. Hu Y, Xu J, Chen Q, Liu M, Wang S, Yu H, Zhang Y, Wang T. Regulation effects of total flavonoids in Morus alba L. on hepatic cholesterol disorders in orotic acid induced NAFLD rats. BMC Complement Med Ther 2020;20:257. https://doi.org/10.1186/s12906-020-03052-w
6. Younossi Z, Anstee QM, Marietti M, Hardy T, Henry L, Eslam M, George J, Bugianesi E. Global burden of NAFLD and NASH: trends, predictions, risk factors and prevention. Nat Rev Gastroenterol Hepatol 2018;15:11-20. https://doi.org/10.1038/nrgastro.2017.109
7. Sharma D, Mandal P. NAFLD: genetics and its clinical implications. Clin Res Hepatol Gastroenterol 2022;46:102003. https://doi.org/10.1016/j.clinre.2022.102003
8. Huang G, Wallace DF, Powell EE, Rahman T, Clark PJ, Subramaniam VN. Gene variants implicated in steatotic liver disease: opportunities for diagnostics and therapeutics. Biomedicines 2023;11. https://doi.org/10.3390/biomedicines11102809
9. Zhang J, Xie H, Yao J, Jin W, Pan H, Pan Z, Xie D, Xie D. TRIM59 promotes steatosis and ferroptosis in non-alcoholic fatty liver disease via enhancing GPX4 ubiquitination. Hum Cell 2023;36:209-222. https://doi.org/10.1007/s13577-022-00820-3
10. Ng MYW, Wai T, Simonsen A. Quality control of the mitochondrion. Developmental cell 2021;56:881-905. https://doi.org/10.1016/j.devcel.2021.02.009
11. Vyas S, Zaganjor E, Haigis MC. Mitochondria and cancer. Cell 2016;166:555-566. https://doi.org/10.1016/j.cell.2016.07.002
12. Kang D, Hamasaki N. Alterations of mitochondrial DNA in common diseases and disease states: aging, neurodegeneration, heart failure, diabetes, and cancer. Curr Med Chem 2005;12:429-441. https://doi.org/10.2174/0929867053363081
13. Wen YA, Xing X, Harris JW, Zaytseva YY, Mitov MI, Napier DL, Weiss HL, Mark Evers B, Gao T. Adipocytes activate mitochondrial fatty acid oxidation and autophagy to promote tumor growth in colon cancer. Cell Death Dis 2017;8:e2593. https://doi.org/10.1038/cddis.2017.21
14. Zampieri LX, Grasso D, Bouzin C, Brusa D, Rossignol R, Sonveaux P. Mitochondria participate in chemoresistance to cisplatin in human ovarian cancer cells. Mol Cancer Res 2020;18:1379-1391. https://doi.org/10.1158/1541-7786.MCR-19-1145
15. Alshehri AS, El-Kott AF, El-Kenawy AE, Khalifa HS, AlRamlawy AM. Cadmium chloride induces non-alcoholic fatty liver disease in rats by stimulating miR-34a/SIRT1/FXR/p53 axis. Science of The Total Environment 2021;784:147182. https://doi.org/10.1016/j.scitotenv.2021.147182
16. Li R, Toan S, Zhou H. Role of mitochondrial quality control in the pathogenesis of nonalcoholic fatty liver disease. Aging (Albany NY) 2020;12:6467. https://doi.org/10.18632/aging.102972
17. Ramanathan R, Ali AH, Ibdah JA. Mitochondrial dysfunction plays central role in nonalcoholic fatty liver disease. Int J Mol Sci 2022;23. https://doi.org/10.3390/ijms23137280
18. Bingol B, Sheng M. Mechanisms of mitophagy: PINK1, Parkin, USP30 and beyond. Free Radic Biol Med 2016;100:210-222. https://doi.org/10.1016/j.freeradbiomed.2016.04.015

19. Sliter DA, Martinez J, Hao L, Chen X, Sun N, Fischer TD, Burman JL, Li Y, Zhang Z, Narendra DP, Cai H, Borsche M, Klein C, Youle RJ. Parkin and PINK1 mitigate STING-induced inflammation. Nature 2018;561:258-262. https://doi.org/10.1038/s41586-018-0448-9
20. Kane LA, Lazarou M, Fogel AI, Li Y, Yamano K, Sarraf SA, Banerjee S, Youle RJ. PINK1 phosphorylates ubiquitin to activate Parkin E3 ubiquitin ligase activity. J Cell Biol 2014;205:143-153. https://doi.org/10.1083/jcb.201402104
21. Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, Kominami E, Ohsumi Y, Yoshimori T. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. EMBO J 2000;19:5720-5728. https://doi.org/10.1093/emboj/19.21.5720
22. Friedman SL, Neuschwander-Tetri BA, Rinella M, Sanyal AJ. Mechanisms of NAFLD development and therapeutic strategies. Nat Med 2018;24:908-922. https://doi.org/10.1038/s41591-018-0104-9
23. Yu X, Hao M, Liu Y, Ma X, Lin W, Xu Q, Zhou H, Shao N, Kuang H. Liraglutide ameliorates non-alcoholic steatohepatitis by inhibiting NLRP3 inflammasome and pyroptosis activation via mitophagy. Eur J Pharmacol 2019;864:172715. https://doi.org/10.1016/j.ejphar.2019.172715
24. Zhang NP, Liu XJ, Xie L, Shen XZ, Wu J. Impaired mitophagy triggers NLRP3 inflammasome activation during the progression from nonalcoholic fatty liver to nonalcoholic steatohepatitis. Lab Invest 2019;99:749-763. https://doi.org/10.1038/s41374-018-0177-6
25. Zhang T-s, Qin H-l, Wang T, Li H-t, Li H, Xia S-h, Xiang X-h. Global publication trends and research hotspots of nonalcoholic fatty liver disease: a bibliometric analysis and systematic review. Springerplus 2015;4:1-9. https://doi.org/10.1186/s40064-015-1542-1
26. Duan Y, Pan X, Luo J, Xiao X, Li J, Bestman PL, Luo M. Association of inflammatory cytokines with non-alcoholic fatty liver disease. Front Immunol 2022;13:880298. https://doi.org/10.3389/fimmu.2022.880298
27. Stojsavljević S, Palčić MG, Jukić LV, Duvnjak LS, Duvnjak M. Adipokines and proinflammatory cytokines, the key mediators in the pathogenesis of nonalcoholic fatty liver disease. World J Gastroenterol 2014;20:18070. https://doi.org/10.3748/wjg.v20.i48.18070
28. Méndez-Sánchez N, Valencia-Rodríguez A, Coronel-Castillo C, Vera-Barajas A, Contreras-Carmona J, Ponciano-Rodríguez G, Zamora-Valdés D. The cellular pathways of liver fibrosis in non-alcoholic steatohepatitis. Annals of Translational Medicine 2020;8. https://doi.org/10.21037/atm.2020.02.184
29. Nassir F, Ibdah JA. Role of mitochondria in nonalcoholic fatty liver disease. International journal of molecular sciences 2014;15:8713-8742. https://doi.org/10.3390/ijms15058713
30. Zimmermann HW, Seidler S, Gassler N, Nattermann J, Luedde T, Trautwein C, Tacke F. Interleukin-8 is activated in patients with chronic liver diseases and associated with hepatic macrophage accumulation in human liver fibrosis. PloS one 2011;6:e21381. https://doi.org/10.1371/journal.pone.0021381
31. Shen S, Wang J, Lin LM. Downregulation of long non-coding RNA AIRN promotes mitophagy in alcoholic fatty hepatocytes by promoting ubiquitination of mTOR. Physiol Res 2021;70:245-253. https://doi.org/10.33549/physiolres.934549
32. Ma X, McKeen T, Zhang J, Ding WX. Role and mechanisms of mitophagy in liver diseases. Cells 2020;9. https://doi.org/10.3390/cells9040837
33. Ding Q, Xie XL, Wang MM, Yin J, Tian JM, Jiang XY, Zhang D, Han J, Bai Y, Cui ZJ, Jiang HQ. The role of the apoptosis-related protein BCL-B in the regulation of mitophagy in hepatic stellate cells during the regression of liver fibrosis. Exp Mol Med 2019;51:1-13. https://doi.org/10.1038/s12276-018-0199-6
34. Bueno M, Lai YC, Romero Y, Brands J, St Croix CM, Kamga C, Corey C, Herazo-Maya JD, Sembrat J, Lee JS, Duncan SR, Rojas M, Shiva S, Chu CT, Mora AL. PINK1 deficiency impairs mitochondrial homeostasis and promotes lung fibrosis. J Clin Invest 2015;125:521-538. https://doi.org/10.1172/JCI74942
35. Qiu YN, Wang GH, Zhou F, Hao JJ, Tian L, Guan LF, Geng XK, Ding YC, Wu HW, Zhang KZ. PM2.5 induces liver fibrosis via triggering ROS-mediated mitophagy. Ecotoxicol Environ Saf 2019;167:178-187. https://doi.org/10.1016/j.ecoenv.2018.08.050
36. Xu ZX, Li JZ, Li Q, Xu MY, Li HY. CircRNA608-microRNA222-PINK1 axis regulates the mitophagy of hepatic stellate cells in NASH related fibrosis. Biochem Biophys Res Commun 2022;610:35-42. https://doi.org/10.1016/j.bbrc.2022.04.008

37. Nguyen TN, Padman BS, Lazarou M. Deciphering the Molecular Signals of PINK1/Parkin Mitophagy. Trends Cell Biol 2016;26:733-744. https://doi.org/10.1016/j.tcb.2016.05.008
38. Lazarou M, Sliter DA, Kane LA, Sarraf SA, Wang C, Burman JL, Sideris DP, Fogel AI, Youle RJ. The ubiquitin kinase PINK1 recruits autophagy receptors to induce mitophagy. Nature 2015;524:309-314. https://doi.org/10.1038/nature14893
39. Piquereau J, Godin R, Deschenes S, Bessi VL, Mofarrahi M, Hussain SN, Burelle Y. Protective role of PARK2/Parkin in sepsis-induced cardiac contractile and mitochondrial dysfunction. Autophagy 2013;9:1837-1851. https://doi.org/10.4161/auto.26502
40. Padman BS, Nguyen TN, Lazarou M. Autophagosome formation and cargo sequestration in the absence of LC3/GABARAPs. Autophagy 2017;13:772-774. https://doi.org/10.1080/15548627.2017.1281492
41. Bakula D, Scheibye-Knudsen M. MitophAging: Mitophagy in Aging and Disease. Front Cell Dev Biol 2020;8:239. https://doi.org/10.3389/fcell.2020.00239
42. Fu ZJ, Wang ZY, Xu L, Chen XH, Li XX, Liao WT, Ma HK, Jiang MD, Xu TT, Xu J, Shen Y, Song B, Gao PJ, Han WQ, Zhang W. HIF-1alpha-BNIP3-mediated mitophagy in tubular cells protects against renal ischemia/reperfusion injury. Redox Biol 2020;36:101671. https://doi.org/10.1016/j.redox.2020.101671
43. Qu F, Wang P, Zhang K, Shi Y, Li Y, Li C, Lu J, Liu Q, Wang X. Manipulation of Mitophagy by "All-in-One" nanosensitizer augments sonodynamic glioma therapy. Autophagy 2020;16:1413-1435. https://doi.org/10.1080/15548627.2019.1687210
44. Li S, Lin Q, Shao X, Zhu X, Wu J, Wu B, Zhang M, Zhou W, Zhou Y, Jin H, Zhang Z, Qi C, Shen J, Mou S, Gu L, Ni Z. Drp1-regulated PARK2-dependent mitophagy protects against renal fibrosis in unilateral ureteral obstruction. Free Radic Biol Med 2020;152:632-649. https://doi.org/10.1016/j.freeradbiomed.2019.12.005
45. Yi S, Zheng B, Zhu Y, Cai Y, Sun H, Zhou J. Melatonin ameliorates excessive PINK1/Parkin-mediated mitophagy by enhancing SIRT1 expression in granulosa cells of PCOS. Am J Physiol Endocrinol Metab 2020;319:E91-E101. https://doi.org/10.1152/ajpendo.00006.2020