2020 년 2 월

석사학위 논문

Role of Parkin-mediated mitophagy in Hepatic Stellate Cell Activation

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이 논문을 약학 석사학위신청 논문으로 제출함

2019 년 10 월

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2019 년 11 월

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ABBREVIATIONS

HSCs	Hepatic stellate cells
TGF-β	Transforming growth factor-beta
PAI-1	Plasminogen activator inhibitor-1
CCl_4	Carbon tetracholoride
BDL	Bile buct ligation
ActD	Actinomycin-D
CQ	Chloroquine
MG132	Z-Leu-Leu-Leu-al
α-SMA	alpha-smooth muscle actin
COX Ⅳ	Cytochrome c oxidase Ⅳ
HSP 90	Heat shock protein 90
mtROS	Mitochondrial reactive oxygen species
MMP	Mitochondrial membrane potential
Rho-123	Rhodamin-123
CCCP	Carbonyl cyanide *m*-chlorophenyl hydrazone
PINK1	PTEN-induced kinase 1

국 문 초 록

간성상세포 활성화에 따른 Parkin 매개 미토파지 역할연구

정 은 희

지 도 교 수: 기 성 환

약학과

조선대학교 대학원

간성상세포 (hepatic stellate cell)는 간을 이루고 있는 비실질세포의 하나로, 휴지 상태(quiescent state)로 존재하다가 다양한 자극에 의해 활성화(activated state) 되면 콜라겐, fibronectin 과 같은 세포 외 기질(ECM)을 분비시킨다. ECM의 축적은 간섬유화, 간경변증을 유발하며 간기능 부전에 이르게 한다. 이러한 간섬유화 발병 및 진행 과정에 간성상세포의 활성화는 중요한 영향을 미치는 것으로 알려져 있다. 최근 간성상세포 활성화 과정에서 미토콘드리아의 β-oxidation 매개 가능성에 관한 연구가 진행되었다. 미토콘드리아는 지방산의 β-oxidation 을 통해 에너지를 합성하는 이화 기능을 담당하는 세포소기관이다. 이러한 미토콘드리아는 미토파지 (mitophagy)라고 하는 기전에 의해 자가포식작용(autophagy)을 통해

손상되거나 불필요한 미토콘드리아를 선택적으로 분해하는 과정- 손상된 미토콘드리아들을 자가포식포(autophagosome) 및 리소좀 (lysosome)에 융합시켜 제거하여 미토콘드리아 기능이 정상적으로 유지될 수 있도록 한다-을 통해 항상성이 유지되고 있다. Parkin 은 이러한 미토파지를 조절하는 핵심 분자로, 미토콘드리아의 기능 저하로 인해 미토콘드리아의 막전위가 상실되면 serine-threonine kinase 의 하나인 PINK1 이 안정화되고 이는 다시 Parkin 을 미토콘드리아 막으로 이동시켜 E3 ligase 활성을 증가시킴으로써 미토파지를 유도하는 것으로 알려져 있다. 간에서 미토파지의 변화는 지방간, 간암, 약물 및 허혈성-재관류로 인한 간손상 등을 유발하여 간기능 저하를 유발하는 것으로 알려져 있다. 하지만 간성상세포의 활성화 과정에서 미토파지의 역할과 조절 기전에 관한 연구는 전무한 상태이다.

본 연구에서는 간성상세포 활성화 과정에서 미토파지의 역할과 이의 핵심 매개 분자인 Parkin 의 발현 변화 및 역할에 관해 탐구하였다. Parkin 은 실질세포인 간세포에 비해 간섬유화 유발 세포인 간성상세포에 더 많이 분포하고 있음을 발견하였다. 또한 두 가지 간섬유화 유발 동물 모델 (사염화탄소 투여, 담관 결찰 동물 모델) 간조직 샘플에서 각 대조군에서보다 그 발현이 증가되어 있음을 관찰하였다. 나아가, 간섬유화 유발 동물 및 간섬유화가 동반된 사람 간 조직에서 Parkin 의 발현이 증가하였고 이는 간성상세포 활성화 지표인 desmin 과 일치함을 발견하였다. 이와 더불어 TGF-β (transforming growth factor-β)를 처리한 LX-2 세포와 사염화탄소를 투여한 마우스로부터 분리한 일차성상세포에서 Parkin 의 발현이 증가되어 있음을 관찰할 수 있었다. 이러한 TGF-β 매개 Parkin 의 발현 증가는 전사 만 아니라 전사 이후 단계에서도 조절됨을 발견하였다. 또한, TGF-β 처리는 미토콘드리아의 기능에는 변화를 주지 못하였지만, 자가포식작용을 활성화시켰으며 이러한 자가포식작용활성화는 미토파지저해제인 Mdivi-1 처치를 통해 억제되었으

며, 실제로 미토콘드리아 분획에서의 자가포식작용이 증가되었으며 Parkin 이 미토콘드리아로 이동됨을 증명하였다. TGF-β에 의해 유도된 이러한 결과들을 종합해 볼 때, 본 연구에서는 간성상세포에서 TGF-β에 의해 유도된 Parkin 이 미토콘드리아 기능에 직접적인 영향을 주진 않지만 미토파지를 통한 미토콘드리아 항상성 유지에 기여함으로써 간성상세포 활성화를 촉진시킴을 규명하였다. 이러한 결과는 간성상세포 활성화를 조절하는 Parkin 이 간섬유화 치료 및 예방을 위한 신규 표적으로 활용될 수 있음을 제시한다.

I. INTRODUCTION

Liver fibrosis and cirrhosis is global burden that is associated with various complications and high mortality. Liver fibrosis is a major disorder caused by chronic HCV infection, alcohol abuse, cholestatic liver diseases, nonalcoholic steatohepatitis (NASH) and autoimmune hepatitis [1]. Upon repeated injury, the liver undergoes the wound-healing response leading to accumulation of extracellular matrix (ECM) proteins distorts the hepatic architecture by forming a fibrous scar and impaired organ function [2].

Hepatic stellate cells (HSCs) exist in the space of Disse and account for about 5 to 8 percent of all cells in the liver [3]. The HSCs in response to liver damage plays a crucial role in the liver fibrosis progression and development [4]. Activated HSCs are known to convert to myofibroblast form and cause liver fibrosis by increasing cell proliferation, increasing cell contractivity, deposition of ECM, and upregulation of alpha-smooth muscle actin (α-SMA) [5, 6]. Thus, the suppression of activated HSCs is of great importance in the treatment of anti-fibrotic therapy [7].

Transforming growth factor-β (TGF-β) is the most potent profibrogenic cytokine which can activate HSCs that transmits cytostatic signals to epithelial, neuronal, and immune cells [5, 8]. The molecular mechanism of TGF-β combines with the type II receptor to phosphorylate the type I receptor, which comes in contact with the Smad2 and the Smad3. Smad2 or Smad3 then moves into the nucleus in combination with Smad4 to regulate the transcription [8, 9]. Sequentially, it promotes the generation of collagen through the transactivation of the Smad signaling [8-10].

Mitophagy is an intracellular digestion mechanism that removes damaged or unnecessary mitochondria [11, 12]. It also forms autophagosomes when the mitochondria are damaged and fuses them with the lysosome to selectively remove damaged mitochondria [11, 12]. Mitophagic activity is important for regulating mitochondrial function and maintaining tissue

function in many cells. PINK1/Parkin mitophagy is a key mechanism to contribute mitochondrial quality control by clearance of damaged mitochondria [11-13]. Under normal mitochondrial conditions, PINK1 protein degrades and Parkin is present in the plasma membrane. However, when mitochondrial damage occurs, membrane potential is reduced, PINK1 is stabilized by autophosphorylation, and mitophagy is induced by increasing Parkin recruitment of E3 ubiquitin ligase enzyme [13-15]. Mitopahgy deficiency in liver has been known to affect liver function by causing liver damage due to fatty liver, liver cancer, drugs and ischemic-reperfusion [16-18]. However, there is little studies on the role of mitophagy and its regulatory mechanisms in the activation of HSCs.

In this study, we explored the role of mitophagy in the activation of HSCs and involvement of Parkin. We found that Parkin exists abundantly in HSCs than hepatocytes, and was increased and colocalized with desmin in animal or human fibrotic livers. Moreover, TGF-β induced Parkin expression which promotes mitophagy and mitochondrial sublocalization. In addition, we show that ectopic expression of Parkin enhanced hepatic fibrogenensis which was nullified by Mdivi-1, a mitophagy inhibitor.

II. MATERIALS AND METHODS

1. Materials

Antibodies against Parkin and COX-IV were purchased from Cell signaling technology (Beverly, MA). Plasminogen activator inhibitor-1 (PAI-1) antibody was obtained from BD Bioscience (Mountain View, CA, USA). Anti-LC3B and anti-PINK1 antibodies were provided from NOVUS Biologicals (Littleton, CO). Anti-HSP90 and anti-62 antibodies were supplied from Enzo life sciences (Farmingdale, USA) and Abnova (Teipeh, Taiwan), respectively. Anti-Desmin, and anti-Parkin antibody for immunofluorescence was purchased from Abcam (Cambridge, MA). Horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse antibodies, Mito-SOX and MitoTracker were purchased from Invitrogen (Carlsbad, CA). Rhodamine123 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). α- SMA, and β-actin antibodies, Z-Leu-Leu-Leu-al (MG132), chloroquine (CQ), and actinomycin-D (Act.D), Mdivi-1, Carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), Rotenone were provided from Sigma (St. Louis, MO). TGF-β was obtained from R&D Systems (Minneapolis, MI).

2. Cell culture

LX-2 cells (immortalized human activated HSCs) were generously provided by Dr. S.L. Friedmann (Mount Sinai School of Medicine, NY, USA). Cells were plated in 60 mm plates at 1×10^5 cells per well, and cells were grown to 70-80% confluence. Cells were maintained in DMEM containing 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA), 50 U/ml penicillin/streptomycin at 37℃ in a humidified 5% CO_2 atmosphere. Cells were then washed cold phosphate buffered saline (PBS) before sample preparation.

3. Primary Hepatic Stellate cells and Hepatocytes isolation

Hepatocytes and HSCs were isolated from the liver of 8-week-old mice (Oriental Bio, Sungnam, South Korea). After intubation in the portal vein, the livers were perfused *in situ* with Ca^{2+}-free Hank's balanced saline solution (HBSS) at 37℃ for 15 min and then perfused with the solution containing 0.05% collagenase and Ca^{2+} for 15 min at a flow mice of 10 mL/min. The perfused livers were minced, filtered through 70 μM cell strainer (BD Bioscience), and centrifuged at 50 g for 3 min to separate the supernatant and pellet. Hepatocytes were resuspended in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/mL streptomycin, 5 mM HEPEs, and 10 nM dexamethasone. HSCs were isolated according to the previously published method. Briefly, the supernatant was further centrifuged at 500 g for 10 min, resuspended in Ficoll plus Percoll (1:10, GE Healthcare, IL, USA), and centrifuged at 1400 g for 17 min. HSCs were collected from the interface. Quiescent HSCs were cultured for 0 day, and activated HSCs were cultivated for 7 days.

4. Patient sample

Human fibrotic or non-fibrotic liver samples obtained from 10 cancer patients were separated by a histologic examination and ultrosonography at Chosun University Hospital in South Korea, as used in the previous study. The study protocol was approved by the institutional review board of Chosun Medical Center (#2013-04-005).

5. Immunoblot analysis

Protein extraction, SDS polyacrylamide gel electrophoresis, and immunoblot analysis were performed as previously reported. Briefly, the cell lysates were separated by electrophoresis in 7.5% and 12% gels and electrophoretically transferred to a nitrocellulose membrane (GE Healthcare, IL, USA). The nitrocellulose membrane was incubated with the indicated primary antibody and then incubated with a horseradish peroxidase-conjugated secondary antibody (Invitrogen). Immunoreactive protein was visualized by enhanced chemiluminescence

detection kit (Amersham Biosciences, Buckinghamshire, UK). Equal protein loading was verified using β-actin.

6. RNA isolation and RT-PCR analysis

Total RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's instructions. The RNA (2 μg each) was reverse-transcribed using an oligo (dT) $_{16}$ primers to obtain cDNA. The cDNA was obtained amplified using a high-capacity cDNA synthesis kit (Bioneer, Daejeon, Korea) with a thermal cycler (Bio-Rad, Hercules, CA, USA). Amplified products were separated by using 2% agarose gel, stained with ethidium bromide (Sigma, St. Louis, MO, USA), and visualized in gel documentation system (Fujifilm, Tokyo, Japan). Primer sequences were as follows: human Parkin sense 5′-TCCTTCCTGCTGTCAGTGTG-3′, and antisense 5′-GCAGAGACCGTGGAGAAAAG-3′; human GAPDH sense 5′-GAAGGTGAAGGTCGGAGTC-3′, and antisense 5′-GAAGATGGTGATGGGATTTC-3′ mRNA expression data was normalized to GAPDH.

7. Immunofluorescence

LX-2 cells were treated with 2 ng/ml TGF-ß and 5 μM CCCP (positive control) for 6h. To identify depolarizing mitochondria, the cells were simultaneously stained with 200 nM Mito-tracker for 30min at 37℃ in a humidified 5% CO_2 atmosphere. The treated cells were fixed with 4% paraformaldehyde solution, followed by permeabilization with 0.1% Triton X-100. The cell samples were immunostained with antibodies directed against Parkin for 1 h , followed by incubation with Alexa Fluor 555 goat anti-rabbit IgG (Invitrogen). After incubation, the samples were coverslips with mounting media. The samples were examined using a laser-scanning confocal microscope (A1, Nikon instruments Inc., NY, USA).

8. Mitochondrial fractionation

Cell fractionation was performed using a mitochondria isolation kit (Invitrogen, #89874). Briefly, cells were scraped in PBS and centrifuged at 850 × *g* for 2 min. Following the addition of reagent A provided by the kit, cells were mixed by vortex and incubated on ice for 2 min. After addition of reagent B, incubate tubes on ice for 5 minutes, and vortex. Reagent C was added to the homogenized lysates and centrifugation was performed at 700 × *g* for 10 min. The supernatant from first centrifugation was further centrifuged at 3000 × *g* for 15 min. The pellet and the supernatant from the second centrifugation containing intact mitochondrial and cytosolic proteins, respectively, were used for western blot analysis.

9. Mitochondrial membrane potential assay

LX-2 cells were treated with or without TGF-β (2 ng/ml) or Rotenone (10 μM) as a positive control at 37℃ for 18 h, then incubated with Rho-123 (0.05 ng/ml) at 37℃ for 30 minutes. The cells were harvested by trypsinization and washed with PBS. The intensity of the fluorescence in the cells was measured using a fluorescence microplate reader (Gemini XPS, Molecular Device, Sunnyvale, CA). Rho-123 fluorescence was used channel FL1-A.

10. Mitochondrial ROS level

LX-2 cells were treated with or without TGF-β (2 ng/ml) at 37℃ for 15 minutes or a positive control, Rotenone (10 μM) at 37℃ for 1 h, then incubated with Mito-SOX (10 μM) at 37℃ for 30 minutes. The harvested by trypsinization and washed PBS. The intensity of the fluorescence in the cells was measured using a fluorescence microplate reader (Gemini XPS, Molecular Device, Sunnyvale, CA) Mito SOX fluorescence was used channel FL2-A.

11. Statistical analysis

One way analysis of variance (ANOVA) was used to assess statistical significance of differences among treatment groups. For each statistically significant effect of treatment, the Newman–Keuls test was used for comparisons between multiple group means. The data were expressed as means ± S.E.

III. RESULTS

1. Parkin was up-regulated in HSCs and in fibrotic liver samples

First, we compared Parkin expression in different types of hepatic cells and found that Parkin expressed higher in HSCs than in hepatocytes (Fig 1A). To evalutate the link between Parkin and hepatic fibrosis, we examined Parkin levels in animal fibrosis models. To induce liver fibrosis, CCl_4 dissolved in olive oil (10% v/v) was intraperitoneally injected (0.5 mg/kg) into male ICR mice three times a week for 2 weeks. BDL model is the common bile duct near its attachment to the duodenum was exposed and ligated with 6.0 silk for 2 weeks. In sham-operated mice, the bile duct was dissected and similarly manipulated, but no ligation was made. Parkin expressions were increased in the livers from CCl_4-treated mice as compared to vehicle-treated mice (Fig 1B). PINK1 levels were also upregulated in fibrotic liver samples. The similar tendency was observed in liver samples of BDL-induced mice (Fig 1C). In confocal microscopy for Parkin staining were increased in fibrotic liver samples and co-localized with α-SMA or Desmin (Fig 1D and 1E), confirmative of Parkin overexpression in HSCs of fibrotic livers. These data indicated that the expression of Parkin was increased in HSCs under fibrotic stimulation.

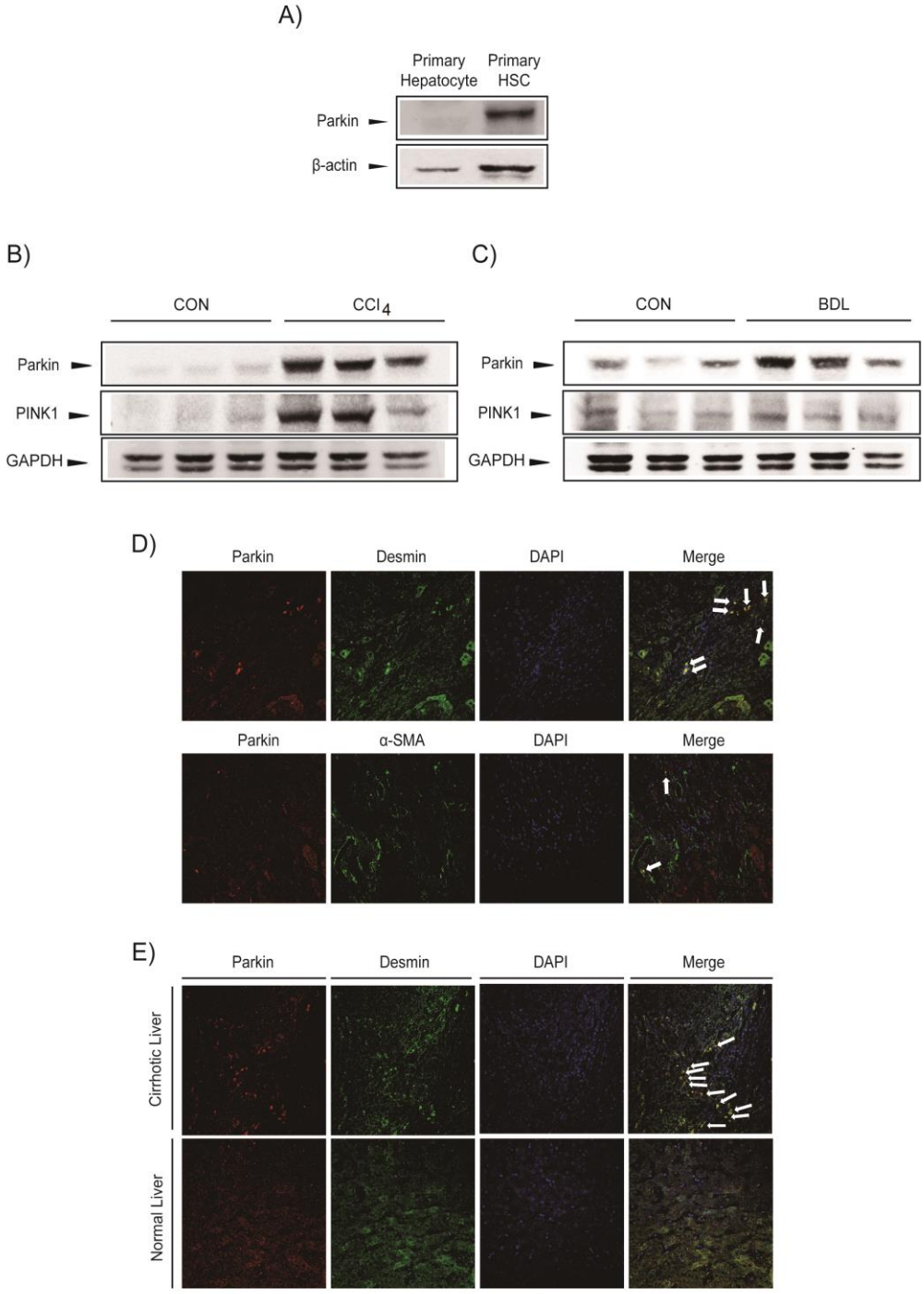


Figure 1. Induction of Parkin in activated HSCs or fibrotic liver samples

(A) Parkin expression in primary murine hepatocyte and quiescent hepatic stellate cells (HSCs). (B and C) Immunoblotting for Parkin and PINK1 in CCl_4-treated (B) or BDL-induced mice liver. Protein levels were assessed by immunoblot analysis. GAPDH was used to assess equal protein loading. (D) Immunostaining for Parkin and α-SMA in CCl_4-injeted mice liver section (magnification: 400X). White arrows indicate co-localization of Parkin and α-SMA. (E) Immunostaining for Parkin and Desmin in human cirrhotic and adjacent normal liver samples (magnification: 400X). White arrows indicate co-localization of Parkin and Desmin

2. Parkin is overexpressed *in vitro* or *ex vivo* under fibrotic stimulation

Next, we investigated Parkin expression after TGF-β treatment in LX-2 cells (immortalized human HSC cells) for different time periods and concentrations. Parkin was increased after 0.5-12 h of TGF-β treatment and was highest expression at 12 h (Fig. 2A). In addition, Parkin was gradually induced by TGF-β stimulation up to at 2 ng/ml of TGF-β (Fig. 2B). Furthermore, we extracted HSCs from the mice injected with vehicle or CCl_4. Parkin level was mildly up-regulated in primary HSCs from CCl_4–injected mice (Fig 2C). These results support evidence that Parkin is overexpressed in HSCs during hepatic fibrogenesis.

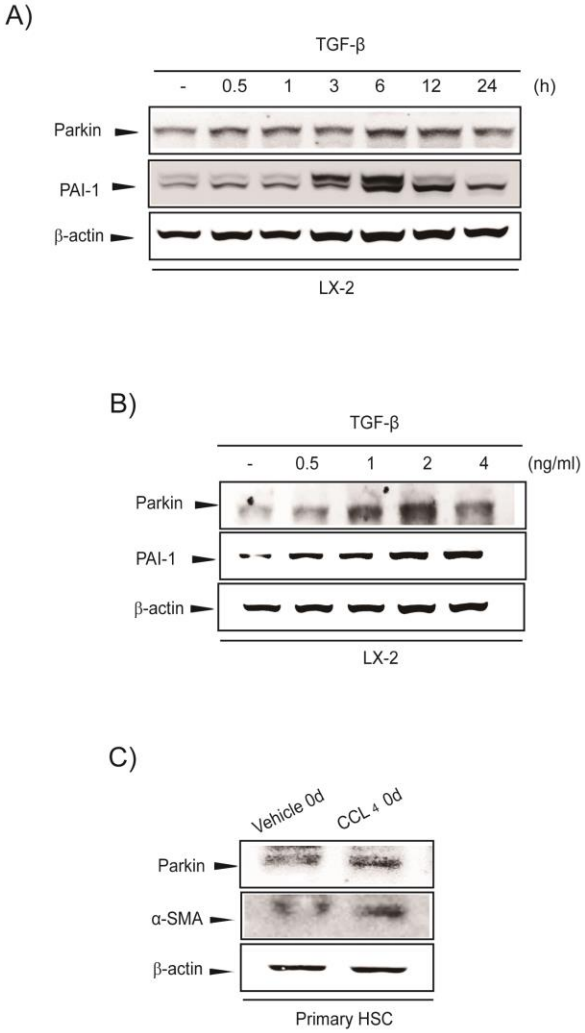


Figure 2. Parkin induction upon stimulation of hepatic fibrogenesis in HSCs

(A) The time courses of Parkin expression in LX-2 cells treated with TGF-β (2 ng/ml). (B) The effect of various concentrations of TGF-β on Parkin induction in LX-2 cells. Parkin protein was immunoblotted in the lysates of cells incubated with 0.5-4 ng/ml TGF-β for 12 h. (C) Parkin level in primary HSCs from CCl_4-treated mice. Mice were injected with 1 mg/kg CCl_4 for 24 h. Primary HSCs were isolated, and Parkin and α-SMA (a HSC activation marker) expressions were assessed by immunoblotting.

3. Regulatory mechanisms of TGF-β-induced Parkin overexpression

To verify whether Parkin was regulated in a transcriptional level, we checked the Parkin mRNA level in TGF-β-incubated LX-2 cells. The mRNA level of Parkin was increased 1-3 h of TGF-β treatment and peaked at 1 h. The Parkin mRNA level then gradually decreased up to 12 h treatment (Fig 3A). Furthermore, we treated to LX-2 cells with a transcription inhibitor, actinomycin D (ActD) or ActD alone or co-treatment with TGF-β. TGF-β-mediated Parkin induction was attenuated by presence of ActD (Fig 3B). Therefore, these results showed that Parkin was transcriptionally regulated by TGF-β. To reveal whether posttranscriptional regulation is involved Parkin induction, we incubated LX-2 cells with a proteasomal inhibitor (MG132, MG) or a lysosomal inhibitor (chloroquine, CQ) under TGF-β treatment. Interstingly, TGF-β-related Parkin overexpressions were also affected by MG or CQ (Fig. 3C and 3D). Hence, we supposed Parkin expression was controlled by transcriptional or posttranscriptional manner.

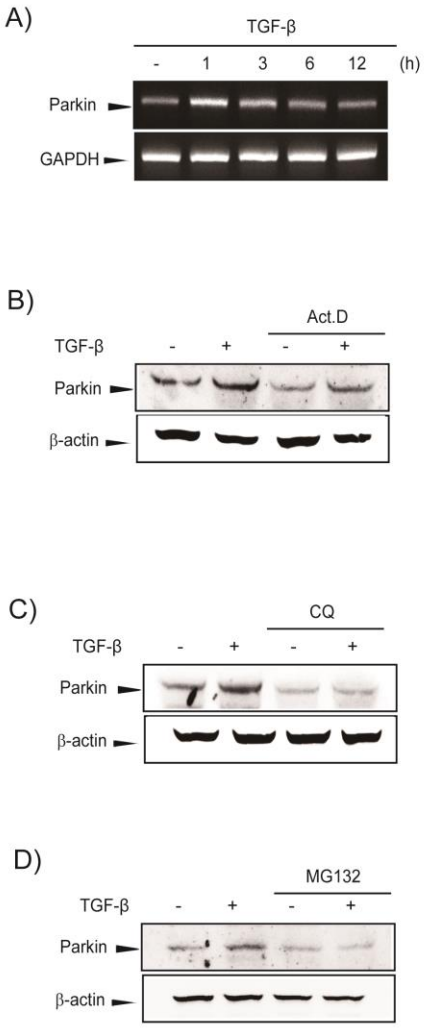


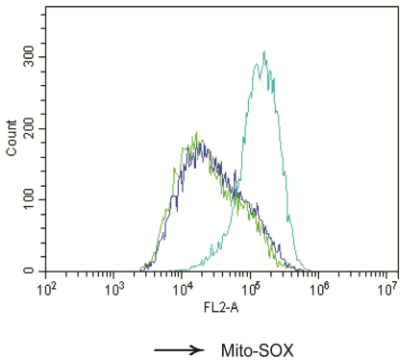
Figure 3. The Parkin induction by either transcriptional or post-transcriptional mechanism

(A) RT-PCR analysis. LX-2 cells were treated with 2 ng/mL TGF-β for 1-12 h. mRNA levels of Parkin were determined by RT-PCR using GAPDH as an internal control. (B) Effect of actinomycin D (ActD) on Parkin mediated by TGF-β in LX-2 cells. The cells were treated with 5 μg/mL of ActD with or without TGF-β treatment. The level of Parkin was determined after 2 ng/mL TGF-β treatment for 12 h. (C) The effect of lysosomal inhibition on Parkin induced by TGF-β. LX-2 cells were pretreated with 10 μM of chloroquine (CQ) for 12 h in the presence or absence of 1 ng/ml TGF-β, and then Parkin level in the cell lysates was evaluated by immunoblotting. (D) The effect of proteasome inhibition on TGF-β-related Parkin induction. LX-2 cells were pretreated with the 10 μM of MG132 for 12 h in the presence or absence of 1 ng/ml TGF- β, and then Parkin expression was examined by immunoblotting.

4. Unaffected mitochondria function by TGF-β stimulation

We investigated whether TGF-β affects mitochondrial function in LX-2 cells. First, we measured mitochondria ROS (mtROS) accumulation by using Mito-Sox. When we treated with TGF-β (2 ng/ml, for 15 min) in LX-2 cells, mtROS level was not changed (Fig 4A). But Rotenone (10 μM, for 1 h), a mitochondria complex I inhibitor used as a positive control, increased mtROS. Next, we analyzed mitochondrial membrane potential (MMP) change of the LX-2 cells by staining with mitochondria sensitive Rhodamine 123 (Rho-123). While Rotenone (10 μM, for 18h) increased disruption of MMP, but TGF-β (2 ng/ml, for 18 h) treatment was not affected MMP. Hence, we may conclude that TGF-β stimulation did not affect the mitochondria function of LX-2 cells.

A)



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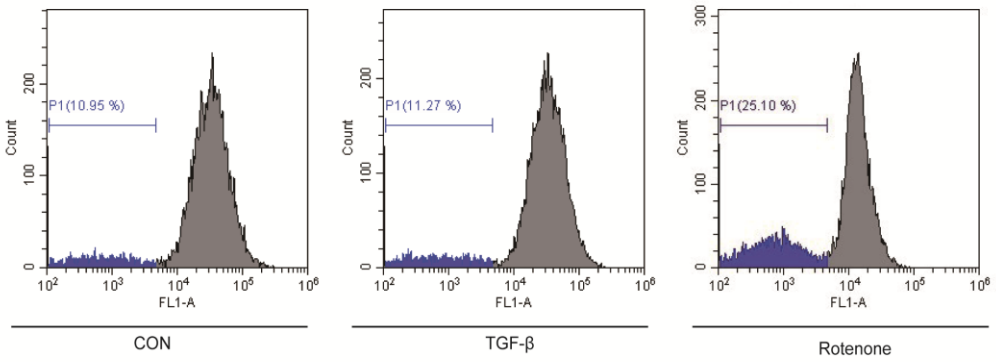

Figure 4. The effect on mitochondria function by TGF-β in LX-2 cells

(A) Representative histogram of flow cytometry analysis of mitochondrial ROS with Mito-Sox. LX-2 cells were treated with or without 2 ng/ml TGF-β at 37℃ for 15 minutes, or 10 μM Rotenone at 37℃ for 1 h, then incubated with Mito-SOX (10 μM) at 37℃ for 30 minutes. The cells were analyzed by FACS (green: con, blue: TGF-β, sky-blue: Rotenone). (B) The loss of MMP was measured by Rhodamine-123 (Rho-123) staining and analyzed by flow cytometry. Cells were treated with TGF-β 2 ng/ml or positive control Rotenone 10 μM for 18 h, and then loaded with Rho-123 (0.05 ng/ml) for 30 min. The inserted histogram demonstrated a left shift of histogram peak representing the decrease of Rho-123 fluorescence intensity due to the loss of MMP. Percentage of cells with reduced fluorescence intensity was calculated.

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5. TGF-β induces mitophagy, translocates Parkin into mitochondria

We evaluated whether TGF-β induce mitophagy and Parkin localization to the mitochondria during mitophagy. First, we observed that LC3B and p62 expressions as mitophagic markers. Levels of the LC3B-II and p62 were increased after TGF-β incubation as compared with control (Fig 5A). When cells were exposed to Mdivi-1 (10 μM), a division/mitophagy inhibitor, increased LC3BII level by TGF-β was diminished (Fig 5B). The same tendency of Parkin1 expression was observed. Thus, it is suggested that a Parkin-mediated mitophagy occurs when LX-2 cells were incubated with TGF-β. Furthermore, we demonstrated that Parkin translocation to the mitochondria was significantly enhanced in the mitochondrial fraction of TGF-β-treated LX-2 cells (Fig 5C). Consistently, Parkin translocation to the mitochondria was enhanced in TGF-β-stimulated LX-2 cells as in CCCP (a well-known mitophagy inducer)-incubated cells with MitoTracker Green (Fig 5D). Hence, these data imply that TGF-β-induced Parkin mediates mitophagy, localized to the mitochondria, and subsequently induces hepatic fibrogenesis.

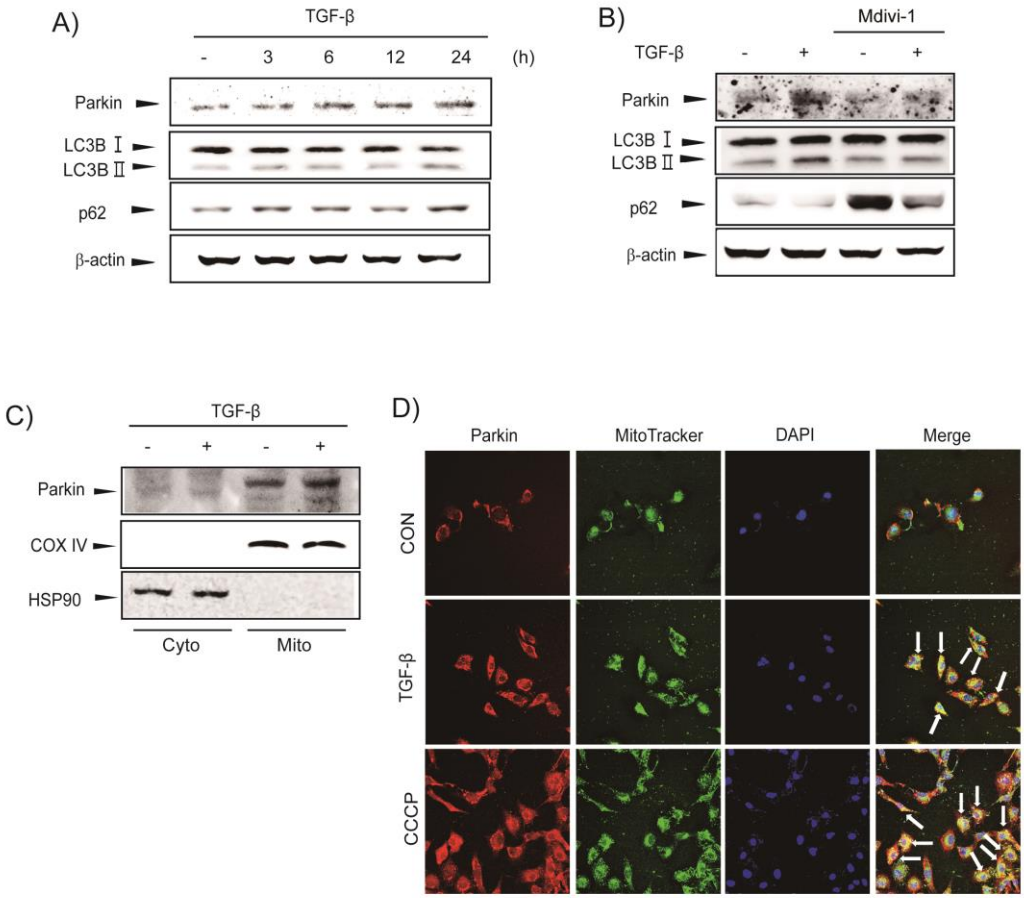

Figure 5. Effect of TGF-β on mitophagy in LX-2 cell

(A) The effect of TGF-β on mitophagic activity. Western blot of Parkin, LC3B, and p62 in LX-2 cells treated with 2 ng/mL TGF-β for various times (0, 3, 6, 12, 24 h). (B) The effect of Mdivi-1 on TGF-β-mediated mitophagy. Levels of LC3B and p62 was detected after 2 ng/mL TGF-β sequential 10 μM Mdivi-1 treatment. (C) Parkin expression in cytosolic and mitochondria fractions of TGF-β-incubated LX-2 cells. COX IV was used as a mitochondria marker, and HSP 90 was used as a cytosolic marker. (D) Immunostaining for Parkin in 2 ng/mL TGF-β-stimulated LX-2 cells. The cells were stained with MitoTracker Green (200 nM), fixed with 4% paraformaldehyde and washed with PBS. The cell samples were incubated with anti-Parkin at 37℃ for 1 h and finally incubated with secondary antibody and DAPI at RT for 1 h (red: mitochondria, green: Parkin, blue: DAPI).

IV. DISCUSSION

In this study, we explored the role of Parkin-mediated mitophagy in HSCs activation. We found that Parkin exists abundantly in HSCs than in hepatocytes. Parkin was upregulated in hepatic fibrosis animal model (CCl_4 injected or bild duct ligated mice liver) and colocalized with desmin in fibrotic animal or human livers. TGF-β-induced Parkin expression was transcriptionally and post-transcriptionally regulated in HSCs. Moreover TGF-β-induced Parkin expression promotes mitophagy and mitochondrial localization.

Parkin is the RING-type E3 ubquitin ligase, which is controlled by the interaction between the N-terminal Ubl domain and the C-terminus of the protein [19]. Parkin works with PTEN-induced kinase 1 (PINK1) to facilitate the removal of damaged mitochondria through the mitophagy [12, 13, 20]. When the mitochondrial membrane potential is impaired, PINK1 accumulates on the surface of the mitochondria and phosphorylates ubiquitin in the Parkin Ub-like domain (Ubl) at Ser65 to promote Parkin activation and translocation into the mitochondria [21, 22]. Then, damaged mitochondria is removed through mitophagy as the various mitochondria membrane protein is ubiquitination [23, 24]. Thus, Parkin E3 ubquitin ligase activity is a key molecule that regulates mitophagy.

Our current study showed that TGF-β-induced Parkin expression was transcriptionally regulated. TGF-β, a key cytokine that causes liver fibrosis, signaling is divided into the canonical pathway and the non-canonical pathway. Canonical TGF-β signals are transduced from the cell surface to the cytoplasm, and then translocated into the nucleus, a process that involves ligands (TGF-β), receptors (TGFBR2/1), receptor-activated Smads (Smad2/3), common Smad (Smad4), and the inhibitory Smad (Smad7) [25, 27]. Non-canonical pathways are signaling pathways mediated through a series of phosphorylation of MEKK, MEK, and MAPK [26, 27]. This signaling activates HSCs and promotes transcription of ECM including collagen, to increase production and accumulate to cause fibrosis. Therefore, it is necessary to

investigate which transcription factor activation is required for TGF-β-induced Parkin expression. Molecular mechanism study of TGF-β-induced Parkin expression is performing in our lab. Parkin expression was also regulated in post-transcriptional ways that identified by treatment with proteasome inhibitor, MG132 and lysosome inhibitor, chloroquine (CQ). However, study regarding role of the ubiquitin-proteasome system in regulating TGF-β-induced Parkin expression is needed and thus further studies are still required to understand and identify the exact mechanism and the physiological role about Parkin about ubiquitination.

Recently it was found that TGF-β induces prolonged mitochondrial ROS generation through decreased complex IV activity with senescent arrest in Mv1Lu cells [28]. Moreover, it has been reported that TGF-β led to epithelial-to-mesenchymal transition via inhibiting mitochondrial functions in A549 cells [29]. Therefore, we investigated whether TGF-β affects mitochondria function and leads to mitophagy in HSCs. However, TGF-β did not affect the mitochondria function in HSCs as evidenced by analysis of mitochondrial ROS production and mitochondrial membrane potential. Thus, Parkin induced by TGF-β did not directly affect mitochondrial function, but contributed to maintaining mitochondrial homeostasis and mitophagy through direct Parkin/PINK1 pathway.

In conclusion, Parkin was exclusively distributed in non-parenchymal cells HSCs. TGF-β-induced Parkin expression promoted HSCs activation and mitophagy in Parkin/PINK1 pathway. Moreover, mitophagy through Parkin induction by TGF-β leads to HSCs activation. These finding present that Parkin may act as a prevention and therapeutic target of liver fibrosis.

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