The Establishment of Tumor Necrosis Factor Receptor-associated Protein1 (TRAP1) Transgenic Mice and Severe Fat Accumulation in the Liver of TRAP1 Mice during Liver Regeneration

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SYNOPSIS

Introduction: Tumor necrosis factor receptor-associated protein 1 (TRAP1) is a mitochondrial heat shock protein (HSP), which belongs to HSP90 family. It plays important roles in regulating mitochondrial integrity, protecting against oxidative stress, and inhibiting cell death. Recent studies suggest that TRAP1 is linked to mitochondria and its metabolism. In this study, we established TRAP1 transgenic mice and performed partial hepatectomy (PH) on wild-type (WT) and TRAP1 transgenic mice to investigate the function of TRAP1 during liver regeneration.

Results and Discussion: We found that TRAP1 was highly expressed in liver as well as kidney. In addition, liver regeneration slightly decreased together with increased fatty liver and inflammation at 72 hr after PH in TRAP1 transgenic mice compared with WT control group mice. Concomitantly, we observed decreased levels of p38 protein in TRAP1 transgenic mice compared with WT control group mice. These results suggest that TRAP1 plays a critical role in liver energy balance by regulating lipid accumulation during liver regeneration.

Conclusions and Prospects: To our knowledge, we reported, for the first time, that liver regeneration slightly reduced together with increased fat accumulations after PH in TRAP1 transgenic mice compared with WT control group mice. Concomitantly, we observed decreased levels of p38 protein in TRAP1 transgenic mice compared with WT control group mice. Overexpression of TRAP1 might affect liver regeneration via disturbing mitochondrial function leading to fatty liver *in vivo*.

Key Words: fatty liver; mitochondria; partial hepatectomy; p38; TRAP1

INTRODUCTION

Mitochondria are distinct cellular organelles composed of a double-membrane system. Moreover mitochondria are vitally important organelles, in which the majority of the ATP is synthesized via oxidative phosphorylation. Tumor necrosis factor-associated protein 1 (TRAP1) is a mitochondrial heat shock protein (HSP) belonging to HSP90 family¹ and its expression was also elevated in many cancers. TRAP1 plays important roles in regulating mitochondrial integrity, protecting against oxidative stress, and inhibiting cell death². TRAP1 protects cells from ROS-induced apoptosis and senescence³⁻⁶. In addition, Takamura et al. reported that stable TRAP1 knockdown cells showed abnormal mitochondrial morphology, as well as significant decreases in dynamin-related protein 1 and mitochondrial fission proteins⁷. Zhang et al. reported that TRAP1 protects against cardiac hypertrophy and fibrosis using in vivo mice model⁸. Recent studies demonstrate that TRAP1 is linked to mitochondria and its metabolism and it regulates a metabolic switch between mitochondrial respiration and aerobic glycolysis⁹. These evidences suggest that TRAP1 may play a role in mitochondrial integrity and metabolism.

The liver is one of vital organs in vertebrates and some other animals. Mitochondria are abundant in liver. Moreover, mitochondria are the main energy source in hepatocytes and play a major role in extensive oxidative metabolism and normal function of the liver¹⁰. Liver is the only human internal organ capable of natural regeneration of lost tissue; as little as 30% of a liver

can regenerate into a whole liver. Liver regeneration after partial hepatectomy (PH) is a perfectly orchestrated process of compensatory growth that proceeds in stages¹¹. The main event of liver regeneration is hepatocyte replication followed by mitosis¹².

TRAP1 mRNA and protein are highly expressed in cancer cell lines and tumors^{13,14}, however, little is known about the effect of TRAP1 overexpression on mitochondria after PH conditions. We recently investigated the effect of mitochondrial TRAP1 overexpression in a mouse fibroblast cell line, NIH/3T3 and found that overexpression of TRAP1 resulted in a series of mitochondrial aberrations¹⁵. In order to study the role of TRAP1 in vivo, we established TRAP1 transgenic mice and found that TRAP1 overexpression was predominant in liver tissue. To further elucidate the role of TRAP1 on liver, we investigated the effect of mitochondrial TRAP1 overexpression on liver regeneration under PH conditions.

RESULTS

Establishment of TRAP1 transgenic mice

We established TRAP1 transgenic mice as described in Materials and Methods. To confirm that TRAP1 vector was inserted in transgenic mice, we performed genomic DNA PCR analysis. As shown in Figure 1A, specific products for TRAP1 appeared only in TRAP1 transgenic mice both two lines. It has been known that TRAP1 is a mitochondrial protein. Therefore, we determined whether overexpression of TRAP1 was targeted to mitochondria via subcellular fractionation in livers from wild type and TRAP1

Figure 1. Establishment of TRAP1 transgenic mice. (A) Genomic DNA PCR against TRAP1 in wild type and TRAP1 transgenic mice. NT: no template, NC: normal mouse genomic DNA, PC: positive control and TRAP1 transgenic mice (#1 and #2: transgenic mouse line number). (B) TRAP1 expression in mitochondrial fraction. Tissues were fractionated into cytosolic (cyto) and mitochondrial (mito) fractions, and subjected to Western blotting against TRAP1. (C) TRAP1 expression in wild type (WT) and TRAP1 transgenic mice tissues. Protein levels were analyzed via Western blotting using specific antibody for human TRAP1. (B; brain, H; heart, Lv; liver, K; kidney, PC; positive control). (D) Measurement of Body weight in WT and TRAP1 transgenic mice (n = 5). (E) Morphology of tissues of WT and TRAP1 transgenic mouse. Representative organs were dissected from mice and fixed. Sections were obtained from paraffin-embedded tissue samples, stained with hematoxylin and eosin (H&E), and examined under a microscope and photographed. (B: brain; × 100, H: heart, Lv: liver, and K: kidney, × 200)

transgenic mice respectively via Western blot analysis using human specific TRAP1 antibody. In a previous report⁴, we observed that β -actin could be utilized as a cytosolic marker compared with a mitochondrial marker. Therefore, β -actin was used as a cytoplasmic marker protein in Figure 1B. Although small amount of TRAP1 was detected in cytosolic fraction, most TRAP1 was found in mitochondria. We assessed the tissue distribution of TRAP1 in these mice. Brain, heart, liver and kidney tissues from WT and TRAP1 transgenic mice were lysed and Western blot analysis against TRAP1 was performed in those tissues as described in Materials and Methods. As shown in Figure 1C, TRAP1 was significantly overexpressed in liver and kidney of TRAP1 transgenic mice. Next, we measured body weight under physiological condition in WT and TRAP1 transgenic mice. Figure 1D shows that there was no significant difference between two types of mice group. We also dissected representative tissues including liver from WT or transgenic mice and performed hematoxylin and eosin (H&E) staining. As shown in Figure 1E, there was no significant difference between WT and TRAP1 transgenic mice. These data suggested that TRAP1 transgenic mice were successfully generated and their physiological conditions were not impact by overexpression of TRAP1 although these analyses were limited in some tissues.

Liver regeneration in WT and TRAP1 transgenic mice after PH

Since TRAP1 was highly expressed in liver, which is rich in mitochondria, we utilized partial hepatectomy (PH) model system to evaluate whether overexpression of TRAP1 affects liver re-

generation. After operation with or without two-thirds PH as mentioned in Materials and Methods, we measured body weight and liver weight in WT and TRAP1 transgenic mice. There was no significant difference in body weight between WT and TRAP1 transgenic mice 72 hr after sham- or PH-operated groups (Figure 2A; upper panel). Difference in liver weight also was not significant either between WT and TRAP1 transgenic mice 72 hr after sham operation (Figure 2A; lower left panel). However, we found that liver weight was significantly decreased in PH-operated TRAP1 transgenic mice compared with PH-operated wild type mice 72 hr after operation (Figure 2A; lower right panel, $P = 0.044$). Figure 2B shows the ratio of liver weight to body weight in WT and TRAP1 mice at the time point of 24, 48, and 72 hr after PH ($P = 0.062$). Interestingly, as shown in Figure 3, TRAP1 transgenic mice developed severe fatty livers 48 hr (60%; three of five mice) and 72 hr (40%; two of five mice) after PH but not in WT control mice (0%; zero of five mice). These results suggest that overexpression of TRAP1 decreased in vivo liver regeneration in response to PH.

Pathological and morphological analysis of WT and TRAP1-overexpressed liver tissues after 72 hr of PH

Next, we analyzed liver tissues in WT and TRAP1 transgenic mice after PH. Livers were dissected from WT mice and TRAP1 transgenic mice 72 hr after sham operation or PH, respectively and fixed in 10% formalin. Sections were obtained from paraffin-embedded tissue samples, stained with H&E, and examined under a microscope. Figure 3A shows that there was an increase of fat accumulation in TRAP1 transgenic mice compared

Figure 2. Liver regeneration in WT and TRAP1 transgenic mice after PH. (A) Body weight and liver weight at the time point of 72 hr after sham or PH in WT and TRAP1 transgenic mice. (B) Liver weight per body weight was calculated after 24 hr, 48 hr, and 72 hr after PH in wild type and TRAP transgenic mice, respectively ($n = 3-5$). Sham-operated mice were treated without liver resection. (C) Fat accumulations in liver after 72 hr of PH. Livers were dissected from mice after 72 hr of PH in wild type (WT) and TRAP1 transgenic mice. Representative livers were photographed.

Figure 3. Pathological analysis of liver tissues after 72 hr of PH in wild type (WT) and TRAP1 transgenic mice. (A) Hematoxylin and eosin (H&E) staining. Livers were dissected from mice and fixed. Sections were obtained from paraffin-embedded tissue samples, stained with H&E, and examined under a microscope and photographed ($\times 400$). (B) Electron microscopy. Liver tissues were fixed as described in Materials and Methods. Samples were visualized under electron microscope ($\times 10,000$). Arrows indicate lipid accumulation.

Figure 4. Western blot analysis of livers after 72 hr of PH in wild type (WT) and TRAP1 transgenic mice. (A) Proteins from liver tissues were extracted, and subjected to Western blotting against specific antibodies. (B) and (C) Intensities of images from (A) was measured using Image J program, normalized by ERK and represented as relative expression to sham group of wild type mice. * $P < 0.05$, ** $P < 0.01$ vs. wild type mice

with WT control mice 72 hr after PH. Since TRAP1 is a mitochondrial protein and mitochondria are abundant in liver, we examined TRAP1-induced morphological change of mitochondria via electron microscope. As described in Materials and Methods, liver tissues were fixed and visualized under electron microscope. We could not detect significant morphological alterations in mitochondria between WT and TRAP1 mice during liver regeneration. Instead, we found increased lipid droplets in TRAP1 transgenic mice compared with WT control mice 72 hr after PH similar to H&E staining result (Figure 3B).

Expression levels of p27 and p38 in WT and TRAP1-overexpressed liver tissues 72 hr after PH

Since MAPK such as p38¹⁶ and ERK and p27¹⁷ participates in cell cycle regulation, and function as a suppressor of cell proliferation during PH, we determined phosphorylation of p38 and ERK in WT control mice and TRAP1 transgenic mice during liver regeneration after sham-operation or PH. Figure 4A shows that phosphorylation of p38 and ERK was not detected both in

WT control mice and TRAP1 transgenic mice 2 hr after PH. However, total p38 protein levels were decreased in TRAP1 transgenic mice 72 hr after PH. There was no significant difference in total ERK protein levels between WT and TRAP transgenic mice. We also examined expression levels of p27 under the same condition. Moreover, expression of p27 protein decreased both in WT and TRAP1 transgenic mice 72 hr after PH compared with sham-operation; however, there was no difference between WT and TRAP1 transgenic mice.

DISCUSSION

It has been shown that TRAP1, the mitochondrial Hsp90 homolog, is highly expressed in many cancers. It plays many important roles in regulating mitochondrial integrity, protecting against oxidative stress, and inhibiting cell death². In addition, stable TRAP1 knockdown cells showed abnormal mitochondrial morphology, as well as significant decreases in mitochondrial fission proteins. These results suggest that TRAP1 regulates the

expression of fission proteins and affects mitochondrial function⁷. A recent report showed that TRAP1 protects against cardiac hypertrophy and fibrosis in vivo mice model¹⁸. However, it remains unknown about the effects of TRAP1 overexpression *in vivo*. In the present study, we generated TRAP1 transgenic mice and investigated the function of TRAP1 *in vivo* during liver regeneration. We observed the differential tissue distribution of TRAP1 expression in TRAP1 transgenic mice. To drive ectopic gene expression in various *in vivo* contexts, we utilized a constitutive promoter of elongation factor-1 alpha which consists of two isoforms eEF1A1 and eEF1A2. In mammalian, eEF1A1 is ubiquitously expressed, whereas eEF1A2 expression is switched-on in adult life in specialized tissues such as skeletal muscle, cardiomyocytes and nervous system¹⁸. Hence, we speculate that protein expression level varies in mice unlike in the cell lines and TRAP1 expression levels varied in tissues of transgenic mice.

We demonstrated that liver regeneration slightly reduced together with increased fat accumulation and inflammation at 72 hr after PH in TRAP1 transgenic mice compared with WT control group mice. Concomitantly, we observed decreased p38 protein levels in TRAP1 transgenic mice compared with WT control group mice.

It has been reported that p38 participates in cell cycle regulation, functioning as a suppressor of cell proliferation and tumorigenesis. Campbell et al. reported that p38 is activated in normal liver, however, it is rapidly inactivated starting 30 minutes after PH, and is re-activated by 12 hr¹⁶. In the present study, we observed that p38 protein levels were significantly decrease of in TRAP1 mice at 72 hr after PH compared with WT mice group. However, we could not observe activation of p38 at 24, 48, and 72 hr after PH in both WT and TRAP1 mice groups. We speculate a possibility that we examined activation of p38 at late time after PH. Yu et al., recently suggested the complicated modulation of fatty acid synthase (FASN) degradation by concerted action of p38, E3 ligase, and Shp2¹⁹. They reported that p38 is activated by fasting and it stimulates FASN protein degradation in mice. Considering that p38 protein level decreased after PH in TRAP1 transgenic mice together with fatty liver, it suggested that there is a link between p38 and fatty liver during PH in TRAP1 transgenic mice, which requires further study. Besides p38, p27 has also been involved in regulating re-entry of residual hepatocytes into the cell cycle upon loss of liver tissue by PH. Rohlfing et al. reported that PH in rats results in immediate down-regulation of p27 in residual liver tissue by transcriptional and post-translational processes¹⁷. Similarly, we also observed that p27 protein levels were reduced in both WT and TRAP1 mouse at 72 hr after PH.

Interestingly and unexpectedly, we found that liver regeneration was slightly decreased together with increased fat accumulation and inflammation at 72 hr after PH in TRAP1 transgenic

Table 1. Summary of fatty liver incidence after PH.

	Sham	24 h	48 h	72 h
WT	0/5	0/5	0/5	0/5
TRAP1	0/5	0/5	3/5	2/5

Number of mice with fatty liver were counted at the time point of 24, 48, and 72 hr after sham or PH in WT and TRAP1 transgenic mice (n = 5).

mice compared with WT control group mice. The incidence of fatty liver in TRAP1 transgenic mice was significantly higher (about 50%) than that of WT control mice (0%) at 48 and 72 hr after PH (Table 1). We also observed increased fat accumulation via electron microscopy in TRAP1 mice at 72 h after PH. Fatty liver is a condition that large vacuoles of triglyceride fat accumulate in liver cells via the process of steatosis. It has been shown that the most common liver disease, affecting one-third of the overall population, is nonalcoholic fatty liver disease (NAFLD), which is characterized by an intrahepatic accumulation of lipids. NAFLD can evolve into nonalcoholic steatohepatitis (NASH) in the presence of oxidative stress and inflammation. Moreover, NASH is a serious risk factor for disabling and deadly liver diseases such as cirrhosis and hepatocellular carcinoma²⁰. Colak et al. reported distinct molecular mechanisms for early hepatocellular carcinoma (HCC) and they developed a rat model of liver regeneration post-hepatectomy, as well as liver cells undergoing malignant transformation and compared them to normal liver using a microarray approach and observed that over 70% of signature genes including p53 and p38 were cancer-related²¹. It has been reported altered mitochondrial functions in a variety of chronic liver diseases including alcohol-induced liver disease, nonalcoholic fatty liver disease, viral hepatitis, and cholestasis¹⁰.

A recent study suggests that TRAP1 regulates a metabolic switch between mitochondrial respiration and aerobic glycolysis as well as mitochondrial fusion/fission. TRAP1-deficiency promotes an increase in mitochondrial respiration and fatty acid oxidation and reactive oxygen species together with suppression of glucose metabolism⁹. Moreover, recent our report suggests that overexpression of TRAP1 leads to aberrations of mitochondria¹⁵. Considered that TRAP1 is an important regulator of not only mitochondrial structure but also metabolism, it is possible to speculate from our findings that overexpression of TRAP1 has effects on mitochondria and a series of metabolic process such as fatty acid oxidation leading to fatty liver during liver regeneration after PH *in vivo*. It requires further studies to elucidate the mechanism of fatty liver in TRAP1 transgenic mice after PH and the expression of TRAP1 in human fatty liver samples.

CONCLUSION AND PROSPECTS

In summary, we investigated the function of TRAP1 during liver

regeneration after PH on WT and TRAP1 transgenic mice. To our knowledge, we reported, for the first time, that liver regeneration slightly reduced together with increased fat accumulations after PH in TRAP1 transgenic mice compared with WT control group mice. Concomitantly, we observed decreased levels of p38 protein in TRAP1 transgenic mice compared with WT control group mice. These results suggest that TRAP1 plays a critical role in liver energy balance by regulating lipid accumulation during liver regeneration. Overexpression of TRAP1 might affect liver regeneration via disturbing mitochondrial function leading to fatty liver *in vivo*.

MATERIALS AND METHODS

Vector preparation

pcDNA3.1-TRAP1 plasmid containing human full length TRAP1 was prepared via subcloning into the pcDNA3.1 vector, under the control of the EF1 α -promoter (Macrogen, Inc., Korea) with the original pOTB7-TRAP1 vector (the Korea Research Institute of Bioscience and Biotechnology, Korea).

Production of human TRAP1 transgenic mice

Standard microinjection procedures were used for transgenic mice production (Macrogen, Inc., Korea). Briefly, fertilized mouse eggs were flushed from the oviducts of superovulated C57BL/6NCrjBgi mice, and male pronuclei were injected with pcDNA3.1-TRAP1 (2 ng/ μ L) that had been linearized with the PI SceI enzyme at its cognate restriction site located in the pcDNA3.1 vector. The injected eggs were reimplanted in the oviducts of pseudo-pregnant ICR recipient females. At 3 weeks of age, the animals were tested for the presence of the transgene by PCR analysis. 100 μ g of genomic DNA was amplified via PCR, as follows: denaturation for 5 minutes at 94°C, 35 cycles of denaturation for 30 seconds at 94°C, 30 seconds of annealing at 60°C, and 30 seconds of extension at 72°C, and a final extension for 10 minutes at 72°C, using specific primers spanning human TRAP1 and EF-1 α promoter region (sense: 5'-gggagctcaaatgaggac-3' and antisense: 5'-gatggtgatggtgccttct-3'). The specific PCR products were resolved on 1% agarose gels, and visualized via ethidium bromide staining.

Animals

We obtained the approval for all animal experiments from Seoul National University Council (SNU-070328-1). Mice (C5BL/6N) were housed in cages (in groups of 5 mice) with food and water freely available and under a standard specific pathogen free (SPF) zone. Humidity (50 $\pm$ 10%) and temperature (22 $\pm$ 2°C) were kept constant, and a 12 hr light/dark cycle was used (lights on, 7:00 a.m. to 7:00 p.m.). All tests were conducted during the light cycle, and the procedures were per-

formed in accordance with guidelines.

Partial hepatectomy

Partial hepatectomy (PH) was performed on 6- to 8-week-old male TRAP1^{+/-} and their wild type TRAP1^{-/-} littermates under anesthesia using the mixture of zoletil 50 and rompun²². Mice (n = 5) were sacrificed at different time points and the liver remnants were removed, weighed, and frozen or processed for molecular and histological experiments, respectively. Sham-operated mice were treated as above without liver resection.

Western blotting

Tissues were lysed in a buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% sodium dodecyl sulphate (SDS), complete protease inhibitor cocktail, 1 mM phenylmethylsulfonyl fluoride, and 1 mM NaF, for 30 minutes on ice. After homogenizing, lysates were centrifuged, quantitated with protein assay reagent (Bio Rad), and subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred to nitrocellulose membranes (S&S). The membranes were blocked with 5% (w/v) nonfat dry milk in 1 $\times$ Tris buffered saline containing 0.1% Tween-20 (TBS-T), for 1 hr at room temperature. Each of the proteins was detected with its specific antibody (anti-mouse TRAP1 antibody from NeoMarkers, anti-mouse β -actin and ERK antibodies from Santa Cruz Biotechnology and anti-rabbit phospho-ERK (Thr202/Tyr204) antibody, anti-rabbit phospho-p38 and p38 antibodies, anti-rabbit p27 antibody from Signaling). The antibody-antigen complex was detected with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology), and visualized via the standard chemiluminescence technique, which was conducted in accordance with the manufacturer's provided instructions (Pierce). Images were obtained on LAS 3000 (Fujifilm).

Subcellular fractionation

The cytosolic and the mitochondrial fractions were isolated with digitonin (Sigma). In brief, tissues were lysed in lysis buffer (80 mM KCl, 250 mM sucrose, 200 μ g/mL digitonin in PBS) for 5 minutes on ice. After 5 minutes of centrifugation at 10,000 g, the supernatant was used as a cytosolic fraction. The pellets were resuspended in buffer containing 50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.2% TritonX-100, and 0.3% NP-40, and incubated for 10 minutes on ice. The supernatants were used as a mitochondrial fraction after 5 minutes of centrifugation at 10,000 g. The samples of each fraction were then quantitated using protein assay reagent (Bio-Rad).

Hematoxylin and eosin staining

Various organs were dissected from mice and fixed in 10% formalin. Sections were obtained from paraffin-embedded tissue

samples, stained with hematoxylin and eosin (H&E), and examined under a microscope and photographed. Pathological analysis was carried out in the Department of Pathology Seoul National University.

Electron microscopy

Liver tissues were fixed with 2.5% glutaraldehyde, and post-fixed in 1% buffered osmium tetroxide. After dehydration with a graded ethanol series and embedding in Epoxy resin (Poly bed 812 kit), ultra-thin sections were prepared using an ultra-microtome (RMC products-MTXL) equipped with a diamond knife. Heavy metal staining was conducted with 4% uranyl acetate and lead citrate solutions, and samples were visualized under electron microscope (Tokyo, Japan) at a magnification ($\times 10,000$).

Statistical Analysis

Student's t-tests were employed in order to compare the differences between two different groups. *P* value of < 0.05 was considered statistically significant.

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