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**Basic Study**  
Subcellular distribution of prohibitin 1 in rat liver during liver regeneration and its cellular implication

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**Abstract**  
**BACKGROUND**  
The function of Prohibitin 1 (Phb1) during liver regeneration (LR) remains relatively unexplored. Our previous research identified a down-regulation of Phb1 in rat liver mitochondria 24 h post 70% partial hepatectomy (PHx), as determined by subcellular proteomic analysis.

**AIM**  
This study aims to further investigate Phb1’s potential role during LR.

**METHODS**  
We examined changes in Phb1 mRNA and protein levels, subcellular distribution, and abundance in rat liver during LR following a 70% PHx. We also evaluated mitochondrial changes and apoptosis levels using electron microscopy and flow cytometry. RNA interference-mediated knockdown of Phb1 (PHBi) was performed in BRL-3A cells.

**RESULTS**
Comparing with sham-operation control groups, Phb1 mRNA and protein levels in 70% PHx test groups were down-regulated at 24 h, then up-regulated at 72 h and 168 h. Phb1 mainly located in mitochondria, showed a reduced abundance at 24 h, significantly increased at 72 h and almost recovered to normal at 168 h. Phb1 was also present in nucleus, with continuous increase in abundance observed 72 h and 168 h after 70% PHx. The altered ultrastructure and reduced mass of mitochondria during LR had almost completely recovered to normal at 168 h. PHBi in BRL-3A cells resulted in increased S-phase entry, a higher number of apoptotic cells, and disruption of mitochondrial membrane potential.

CONCLUSION
Phb1 may contribute to maintaining mitochondrial stability and could play a role in regulating cell proliferation and apoptosis of rat liver cells during LR.

INTRODUCTION
It is well known that the liver has the capacity to regenerate and restore its original size and function after 70% partial hepatectomy (PHx), or injury[1,2]. It would be of great clinical implications to develop therapeutic strategies to enhance liver regeneration (LR) or support the liver in its attempt to restore its functional integrity under pathophysiological circumstances[3, 4]. However, the complexity of the regulatory mechanisms of LR, together with our limited understanding of the functional priorities of the hepatocytes have rendered the identification of targets for therapeutic interventions very difficult.

As the hub of energy metabolism, mitochondria have been investigated due to their direct involvement in the process of LR[5]. In an attempt to identify mitochondrial proteins that are correlated with the early phase of LR, using subcellular proteomic analysis in our recent study, our recent study revealed that Prohibitin 1 (Phb1), a
potential tumor suppressor protein, was down-regulated in rat liver mitochondria at 24 h after 70% PHx\textsuperscript{[6]}.

Phb1 is a ubiquitously expressed highly conserved protein among eukaryotes. Previous research has proposed that Phb1 was involved in many cellular processes, such as cell cycle regulation, senescence, transcription regulation, tumor suppression and apoptosis\textsuperscript{[7-11]}. And Phb1 was reported to mainly localize in mitochondria, with its expression up-regulated by mitochondrial stress and down-regulated during cellular senescence\textsuperscript{[12]}. Therefore, Phb1 is thought to have a crucial role in mitochondria function. One study identified a novel function of Phb1 in the maintenance of mtDNA. In Phb1-knockdown cells, the status of mtDNA is altered in several ways\textsuperscript{[13]}. Despite such information, our understanding of the overall functions of Phb1 in mitochondria remains incomplete and its potential role during LR is largely unexplored. LR is a very complicated biological procedure involving various signal transduction pathways and molecular events\textsuperscript{[14, 15]}. Thus, we hypothesized that Phb1 could play a crucial role during LR. This study aims to further investigate Phb1’s function in mitochondria during Rechanges in Phb1 expression, mitochondrial mass and ultrastructure alterations, and the subcellular distribution of Phb1 at 24, 72, and 168 h post 70% PHx in rat liver are examined. Using RNA interference-mediated knockdown of Phb1 (PHBi)), we also analyzed Phb1’s potential functions. Our results revealed differential expression of Phb1 during LR, with its primary localization in mitochondria, where its altered expression may be associated with the recovery of mitochondrial mass and ultrastructure. Phb1 was also present in the nucleus, with a slightly increased abundance during LR. PHBi in BRL-3A cells, a widely employed cell line in hepatic research, led to increased S-phase entry and apoptotic cell count. We also observed a disruption of mitochondrial membrane potential following Phb1 knockdown in BRL-3A cells, mirroring our previous findings. Collectively, these results suggest that Phb1 may contribute to maintaining mitochondrial stability and regulating the cell cycle and apoptosis during LR.
MATERIALS AND METHODS

1.1 Animals and surgery

Adult male Sprague-Dawley rats (220–250 g) were obtained from the Experimental Animal House at Second Military Medical University (Shanghai, China). The rats were randomly divided into two groups: five served as the sham-operation control group and the other five comprised as the 70% PHx test group. PHx (~70%) was performed according to the method of Higgins et al. The experimental rats were anesthetized by intraperitoneal injection of 2% pentobarbital (40mg/kg). In the test group, the median and left lateral lobes were removed without injuring the remaining liver tissue. The control group underwent a sham operation identical to the test group procedure, but without liver removal. After surgery, the rats were kept on a standard diet until they were euthanized by cervical dislocation under anesthesia.

1.2 Electron microscopy

Liver specimens were fixed with 4% glutaraldehyde in 0.1m sodium cacodylate buffer (pH 7.4) for 4 h at 4 °C. After fixation, they were washed overnight in sodium cacodylate buffer at 4 °C. The specimens were then post-fixed with 1% osmium tetroxide in sodium cacodylate buffer for 1 h at 4 °C, dehydrated in alcohol, embedded in araldite resin, and semithin sections (1 μm) were removed for optical microscopy. Ultra-thin sections were mounted on copper mesh grids and stained with uranyl acetate and lead citrate as described before examination with a Hitachi H-800 electron microscope.

1.3 Separation of rat liver subcellular fractions and Protein preparation

Rat livers were removed and treated as previously reported for nucleus, cytosol and mitochondria isolation. Briefly, the livers were collected and homogenized. Subsequent centrifugation at increasingly higher speeds at 4 °C yielded the following
fractions: nuclear fraction at 1,000 g for 10 min; mitochondrial fraction at 15,000 g for 15 min; and microsomes at 144,000 g for 90 min. The final supernatant was the cytosolic fraction. Purification of mitochondria was performed by Nycodenz® density gradient purification as described [19]. Briefly, the mitochondrial pellets obtained from differential centrifugation were suspended in 12 mL of 25% Nycodenz® and placed onto a discontinuous Nycodenz® gradients consisting of 5 mL of 34% Nycodenz® and 8 mL of 30% Nycodenz® followed by 8 mL of 23% Nycodenz® and finally 3 mL of 20% Nycodenz®. The sealed tubes were centrifuged for 90 min at 52,000 g at 4 ºC. The mitochondria were in the band at the 25%/30% interface which was collected and diluted with the same volume of homogenization buffer and then centrifuged twice at 15,000 g for 20 min. The preparation of each subcellular fraction protein of rat livers was performed as previously described [19]. Protein concentration of each fraction was determined with a Quick Start Bradford Assay Kit (Bio-Rad).

1.4 Western blotting

Protein extracts of each sample were separated on 12% SDS-PAGE and transferred to nitrocellulose membranes (Millipore). The blots were probed by anti-Phb1 antibody (Neomarker) and proteins were normalized with anti-b-actin antibody (Neomarker) or anti-COX IV antibody (Cell Signaling) or anti-Histone H3 antibody (Cell Signaling) and were visualized by Amersham ECL system. The digital image was obtained by scanning the membrane, and then subjected to gray value analysis. For a better understanding of Western blotting results and derived ratio changes, a detailed methodology introduction can be found in the subsequent Figures and Figure Legends.

1.5 Cell culture

The normal rat liver cell line BRL-3A was obtained from the Shanghai Institute of Biochemistry and Cell Biology (China). The BRL-3A cells were maintained as a
monolayer in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/mL of penicillin, and 100 mg/mL of streptomycin. The cells were maintained at 37°C in an atmosphere with 5% CO₂.

1.6 RNA interference

Duplex small interfering RNA (siRNA) was obtained from GeneChem (Shanghai, China). The siRNA sequence targeting rat prohibitin 1 was 5’-GCCAGAUUUGUGGGAAAtt-3’ (sense) and 5’-UUUCCACCACAAUCUGGCtt-3’ (antisense). A nonsense duplex was used as the control (mock). BRL-3A cells were plated on 6-well plates with antibiotic-free DMEM overnight and transfected with siRNA by Lipofectamine2000 (Invitrogen) according to the manufacturer’s protocol. The final concentration of siRNA duplex was 100 nm. Six hours after transfection, the medium was switched to DMEM supplemented with antibiotics.

1.7 RT-PCR and quantitative RT-PCR

Total RNA of each sample was isolated by Trizol reagent (Invitrogen) according to the manufacturer’s protocol. After treatment with DNase I, each RNA sample was reverse-transcribed with random primers (dN6) following the manufacturer’s protocol (MBI Fermantas, Vilnius, Lithuania). The single-stranded cDNA was then used in quantitative real-time PCR to evaluate the relative expression levels of Phb1 (5’-GCCGTGGAAGCCAAACAG-3’ and 5’-TTCTTCTGCTGCTAGCGCTTT-3’), compared to β-actin (5’-ATGGTGGGTATGGGTCAAG-3’ and 5’-TGGCTGGGTTGGTGAAGTC-3’) used as an internal control for determining cell number and metabolic status. Quantitative real-time PCR (ABI7300, Applied Biosystems) was done with the SYBR Green I reagents (TOYOBO) and the primers were designed according to the ABI manufacturer’s protocol. Forty cycles of PCR were
performed with cycling conditions of 15 s at 95 °C, 60 s at 60 °C. The real-time PCR signals were analyzed with LightCycler 3.5 software (Roche Diagnostics).

1.8 Flow cytometry

Cells were stained with propidium iodide (PI; BD Clotech) as previously described[20]. A suspension of 1 × 10⁴ cells was analyzed for each DNA histogram, and from the analysis of DNA histograms, the percentages of cells in different phases of cell cycle were evaluated. Flow cytometry was performed on a FACSCalibur and analyzed using CellQuest software (BD Bioscience).

The Annexin V / PI method was used to quantify numbers of apoptotic cells. Cells were washed twice with PBS and stained with Annexin V and PI for 20 minutes at room temperature. The level of apoptosis was determined by measuring the fluorescence of the cells by flow cytometry analysis.

1.9 Statistical Analysis

The data presented are the means ± SD of three independent experiments. Statistical significance was estimated with Student’s t-test for unpaired observations. A P-value of less than 0.05 was considered significant.

RESULTS
2.1 Altered expression of Phb1 mRNA and protein abundance during LR

Phb1 mRNA expression was examined by real-time PCR. Compared with control group, the expression of Phb1 mRNA in 70% PHx test group was 0.46-fold decrease at 24 h, and a 1.54-fold and 1.89-fold increase at 72 h and 168 h respectively (Figure 1A). Western blotting showed that Phb1 protein expression during LR was 0.54-fold
decrease at 24 h, 1.48-fold and 1.73-fold increase at 72 h and 168 h after 70% PHx respectively (Figure 1B), which was consistent with the expression of Phb1 mRNA.

2.2 Subcellular distribution and protein abundance alteration in each subcellular fraction of Phb1 during LR

Previous observations suggest that examining the subcellular distribution of Phb1 might yield important information about physiological or pathological processes that are taking place in cells. To verify the cellular distribution pattern of Phb1 during LR, we fractionated cytosolic, mitochondrial and nuclear fractions of rat liver cells and performed Western blotting analysis. The purity of subcellular fractionation was controlled by several marker proteins (Figure 2A). Results showed that Phb1 was mainly located in mitochondria and its abundance was 0.47-fold reduction at 24 h, 1.47-fold induction at 72 h and almost recovered to normal at 168 h after 70% PHx (Figure 2B). Phb1 was also located in nucleus and its abundance was increased during LR after 70% PHx (Figure 2C). No Phb1 was found in cytosol in our results.

2.3 Alterations of mitochondria during LR

Mitochondrial mass and ultrastructure alterations during LR were observed in order to determine whether Phb1 changes were associated with mitochondrial stabilization or biogenesis. The mitochondrial mass was quantified by examining the protein contents of mitochondrial fractions[21] extracted from equivalent weights (1g) of liver tissues from each experimental group. The results indicated an increase in mitochondrial protein contents during LR with 5.37±0.08 mg, 6.38±0.10 mg and 7.16±0.16 mg at 24 h, 72 h and 168 h respectively, after 70% PHx. Notably, the mitochondrial protein contents at 168 h within the 70% PHx test group closely mirrored that of the control group [Fig. 3A].

Mitochondrial ultrastructure alterations during LR were observed by electron microscopy. The mitochondrial morphologies of control livers, depicted in Figure 3B
upper panel (SH 24 h, SH 72 h, SH 168 h), were characterized by a consistent basic architecture featuring a folded internal membrane and a dense matrix. The alterations in mitochondrial ultrastructure following 70% PHx were showed in Figure 3B bottom panel. At 24 h after 70% PHx (PH 24 h), the mitochondria displayed a significant swelling, a reduction in the number of cristae, a dilated and paled matrix, an absence of dense granules, and clear matrix compartment vacuolization. At 72 h after 70% PHx (PH 72 h), only slight changes with moderate distension were seen in mitochondrial morphology. By 168 h post 70% PHx (PH 168 h), the mitochondria had large returned to their normal in morphology, rich in cristae, and with an electron-dense matrix. These alterations in mitochondrial ultrastructure have been associated with changed in mitochondrial function during LR[17].

2.4 PHBi leads to an increase in the number of apoptotic cells

To down-regulate Phb1 cellular expression, RNA interference-mediated knockdown of Phb1 (PHBi) was performed in BRL-3A cells. PHBi resulted in a dramatic reduction in both Phb1 mRNA and protein level compared with that of the control group (mock). Detailed results are available in our previous publication[4].

Previous reports have suggested that Phb1 could serve an antiapoptotic role in undifferentiated granulosa cells[22]. In this study, to evaluate whether Phb1 is involved in modulating apoptosis in rat liver cells, flow cytometry was used to evaluate percentage of apoptotic cells by Annexin V / PI staining. As shown in Figure 4A, Phb1 knockdown cells displayed a 1.56-fold increase in the percentage of apoptotic cells compared with controls.

2.5 PHBi leads to an increased S-phase entry

We investigated whether the decrease of Phb1 by PHBi had any effect on cell growth and proliferation. The cell cycle distribution in Phb1 knockdown cells showed a 1.26-
fold increase in the S-phase compared to control cells (Figure 4B). Although the increase in the S-phase was not dramatic, the difference was statistically significant. Nuell et al also previously reported a cell cycle modulatory role of Phb1, indicating that Phb1 could function as a negative cell cycle regulator.[23]

DISCUSSION

Phb1, a potential tumor suppressor protein, was initially cloned due to its ability to induce a G1/S arrest. Phb1 has been proposed to be involved in numerous cellular processes. However, most studies to date have focused on the role of Phb1 in various types of tumors, with its role during LR remaining largely unexplored. In recent years, some studies have explored the role of Phb1 in liver injury and liver cancer.[16, 24-27]. However, the role of Phb1 in LR remained unstudied.

In this study, we found Phb1 mRNA and protein expression underwent concordant changes during LR after 70% PHx. Compared to sham-operation control groups, 70% PHx test groups showed down-regulation of Phb1 mRNA and protein expression at 24 h, and up-regulation at 72 h and 168 h (fig. 1). A previous study found that the gene encoding Phb1 might have additional anti-proliferative effects that do not require translation.[21]. Manjeshwar et al reported that the 3’UTR of the Phb1 gene encoded a functional RNA that arrested cell-cycle proliferation between the G1 and S phases.[28]. In light of previous reports, we propose that Phb1 might regulate cell proliferation during LR in a complex manner, potentially involving mechanisms mediated by both Phb1 mRNA and protein.

The well-characterized function of Phb1 is as a chaperone involved in the stabilization of mitochondrial proteins. Mitochondrial-localized Phb1 is confirmed as a high-molecular-weight hetero-complex (ring-shaped structure) by single particle structures.[7]. The interaction of no assembled respiratory chain subunits with the Phb1 complex has led to the proposal of a chaperone activity of Phb1 during the biogenesis of the respiratory chain.[29]. Recently, Phb1 was reported to be essential for normal mitochondrial development, and Phb1 deficiency was showed to be associated with
deficient mitochondrial biogenesis\textsuperscript{[30]}, PHBi showed enhanced sensitivity to anthralin-induced cell death due to enhanced loss of mitochondrial membrane potential in psoriatic lesions\textsuperscript{[31]}. Mitochondria are the center of energy metabolism and play a crucial role in regulating cell life. Various stimuli can induce dysfunction and structural injury in mitochondria, which triggers a series of cellular events ultimately leading to apoptosis or necrosis. We found Phb1 was mainly located in mitochondria in rat liver, and its abundance underwent a 0.47-fold reduction at 24 h, a 1.47-fold induction at 72 h, and nearly recovered to normal level at 168 h after 70\% PHx (Figure 2B). Interestingly, mitochondria showed significant changes in the ultrastructure at 24 h, and nearly recovered to normal at 168 h after 70\% PHx (Figure 3B). The reduced mitochondrial mass also nearly recovered to normal at 168 h after 70\% PHx. Mitochondrial membrane potential is an important parameter of mitochondrial function. In our previous study, we found that knockdown of Phb1 in BRL-3A cells resulted in disruption of mitochondrial membrane potential, implying a potential role of Phb1 in maintaining mitochondrial integrity\textsuperscript{[6]}. Ross \textit{et al} also reported that siRNA -mediated knockdown of Phb1 in Kit225 cells resulted in disruption of mitochondrial membrane potential and Phb1 proteins were novel phosphoproteins up-regulated during T cell activation that function to maintain mitochondrial integrity\textsuperscript{[32]}. In this study, using PHBi, we also observed that Phb1 knockdown cells exhibited a 1.56-fold increase in the number of apoptotic cells (Figure 4A). Although these results provide evidence for a functional role of Phb1 in suppressing apoptosis in rat liver cells, the involved molecular mechanism(s) remain unknown. It is likely that the mechanism by which knockdown of Phb1 results in apoptosis targets the mitochondria in agreement with previous findings\textsuperscript{[22]}. Altogether, these results suggest that Phb1 has a role in regulating stabilization of mitochondria during LR, which might affect mitochondrial function.

Although it has been reported that Phb1 is primarily located in mitochondrial\textsuperscript{[12,30,33,34]}, other studies have reported that Phb1 is also located in the nucleus\textsuperscript{[35,36]}. In this study, we found Phb1 was located in nucleus as well as mitochondria in rat liver and its abundance increased during LR (Figure 2B). Previous studies reported that Phb1 was
present in the nucleus and interacted with transcription factors important in cell cycle progression\textsuperscript{[35, 36]}. In this study, using PHBi, we observed that Phb1 knockdown cells showed an increased S-phase entry (Fig. 4B). The involvement of Phb1 in the cell cycle was also observed in a prostate cancer cell line, where down-regulation of Phb1 led to a slight increase in cell cycle entry from G1 to S\textsuperscript{[30]}. Although the majority of data suggest that Phb1 has an antiproliferative effect by interacting with both the p53 and pRb pathways in the nucleus\textsuperscript{[5, 37]}, it appears that Phb1 can also have anti-apoptotic effects. In osteosarcoma cells, Phb1 was identified as a gene with down-regulated expression in response to cytotoxic drugs, and the transient overexpression of the Phb1 coding sequence significantly reduced cytotoxic drug-induced apoptosis in these cells\textsuperscript{[38]}. In this study, we also observed that Phb1 knockdown cells showed an increase in the number of apoptotic cells (Figure 4A). It has been reported that the subcellular localization of Phb1 may depend on the cell type examined and its physiological status, and Phb1 might have distinct but overlapping functions in each of these cellular compartments\textsuperscript{[39]}. Although there is controversy concerning the function of nuclear-localized Phb1, in combination with previous reports, we suggest that the up-regulated Phb1 in nucleus in rat liver cells might have a function, at least in part, in regulating cell cycle progression of rat liver cells. It might regulate the balance between proliferation and apoptosis during LR after 70% PHx, but this needs further investigation.

CONCLUSION

In summary, our results demonstrate that Phb1 plays two roles in the LR process: one is to regulate cell cycle and apoptosis, and the other is to regulate and maintain mitochondrial stability. Whether the two effects are directly linked or show two different effects remains unclear. Further in-depth studies will aid in us better understanding the complexities and roles of Phb1 in the LR process.

ARTICLE HIGHLIGHTS

Research background
It would be of great clinical implications to develop therapeutic strategies to enhance liver regeneration (LR) or support the liver in its attempt to restore its functional integrity under pathophysiological circumstances. However, the complexity of the regulatory mechanisms of LR, together with our limited understanding of the functional priorities of the hepatocytes have rendered the identification of targets for therapeutic interventions very difficult.

Research motivation
Phb1 is a ubiquitously expressed highly conserved protein among eukaryotes. Previous research has proposed that Phb1 was involved in many cellular processes. And Phb1 was reported to mainly localize in mitochondria, with its expression up-regulated by mitochondrial stress and down-regulated during cellular senescence. Therefore, Phb1 is thought to have a crucial role in mitochondria function. One study identified a novel function of Phb1 in the maintenance of mtDNA. In Phb1-knockdown cells, the status of mtDNA is altered in several ways. Despite such information, our understanding of the overall functions of Phb1 in mitochondria remains incomplete and its potential role during LR is largely unexplored. LR is a very complicated biological procedure involving various signal transduction pathways and molecular events. Thus, we hypothesized that Phb1 could play a crucial role during LR.

Research objectives
This study aims to further investigate Phb1’s function in mitochondria during Rechanges in Phb1 expression, mitochondrial mass and ultrastructure alterations, and the subcellular distribution of Phb1 at 24, 72, and 168 h post 70% PHx in rat liver are examined. Using RNA interference-mediated knockdown of Phb1 (PHBi), we also analyzed Phb1’s potential functions.

Research methods
1 Materials and methods
1.1 Animals and surgery
1.2 Electron microscopy
1.3 Separation of rat liver subcellular fractions and Protein preparation
1.4 Western blotting
1.5 Cell culture
1.6 RNA interference
1.7 RT-PCR and quantitative RT-PCR
1.8 Flow cytometry
1.9 Statistical Analysis

Research results
2.1 Altered expression of Phb1 mRNA and protein abundance during LR
2.2 Subcellular distribution and protein abundance alteration in each subcellular fraction of Phb1 during LR
2.3 Alterations of mitochondria during LR
2.4 PHBi leads to an increase in the number of apoptotic cells
2.5 PHBi leads to an increased S-phase entry

Research conclusions
In summary, our results demonstrate that Phb1 plays two roles in the LR process: one is to regulate cell cycle and apoptosis, and the other is to regulate and maintain mitochondrial stability.

Research perspectives
Whether the two effects are directly linked or show two different effects remains unclear. Further in-depth studies will aid in us better understanding the complexities and roles of Phb1 in the LR process.

ACKNOWLEDGEMENTS
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