Liver-Specific Deletion of Prohibitin 1 Results in Spontaneous Liver Injury, Fibrosis, and Hepatocellular Carcinoma in Mice

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Prohibitin 1 (PHB1) is a highly conserved, ubiquitously expressed protein that participates in diverse processes including mitochondrial chaperone, growth and apoptosis. The role of PHB1 *in vivo* is unclear and whether it is a tumor suppressor is controversial. Mice lacking methionine adenosyltransferase 1A (MAT1A) have reduced PHB1 expression, impaired mitochondrial function, and spontaneously develop hepatocellular carcinoma (HCC). To see if reduced PHB1 expression contributes to the *Mat1a* knockout (KO) phenotype, we generated liver-specific *Phb1* KO mice. Expression was determined at the messenger RNA and protein levels. PHB1 expression in cells was varied by small interfering RNA or overexpression. At 3 weeks, KO mice exhibit biochemical and histologic liver injury. Immunohistochemistry revealed apoptosis, proliferation, oxidative stress, fibrosis, bile duct epithelial metaplasia, hepatocyte dysplasia, and increased staining for stem cell and preneoplastic markers. Mitochondria are swollen and many have no discernible cristae. Differential gene expression revealed that genes associated with proliferation, malignant transformation, and liver fibrosis are highly up-regulated. From 20 weeks on, KO mice have multiple liver nodules and from 35 to 46 weeks, 38% have multifocal HCC. PHB1 protein levels were higher in normal human hepatocytes compared to human HCC cell lines Huh-7 and HepG2. Knockdown of PHB1 in murine nontransformed AML12 cells (normal mouse hepatocyte cell line) raised cyclin D1 expression, increased E2F transcription factor binding to cyclin D1 promoter, and proliferation. The opposite occurred with PHB1 overexpression. Knockdown or overexpression of PHB1 in Huh-7 cells did not affect proliferation significantly or sensitize cells to sorafenib-induced apoptosis. *Conclusion:* Hepatocyte-specific PHB1 deficiency results in marked liver injury, oxidative stress, and fibrosis with development of HCC by 8 months. These results support PHB1 as a tumor suppressor in hepatocytes. (HEPATOLOGY 2010;00:000.)

Prohibitin (PHB) proteins are highly conserved and ubiquitously expressed proteins that have diverse cellular functions.[1,2] Two PHB proteins, PHB1 and PHB2, encoded by genes located on different chromosomes, form a large multimeric complex (PHB complex) that is found largely in the inner mitochondrial membrane where it exerts a chaperone-like function to stabilize newly synthesized mitochondrial

Abbreviations: 4-HNE, 4-hydroxynonenal; AFP, alpha-fetoprotein; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AML12, normal mouse hepatocyte cell line; BrDU, bromodeoxyuridine; ChIP, chromatin immunoprecipitation; EM, electron microscopy; Gapdh, *glyceraldehyde 3-phosphate dehydrogenase; GSTP, glutathione S-transferase Pi; H&E, hematoxylin and eosin; HCC, hepatocellular carcinoma; KO, knockout; MAT 1A, methionine adenosyltransferase 1A; MEF, mouse embryonic fibroblasts; mRNA, messenger RNA; PCNA, proliferating cellular nuclear antigen; PCR, polymerase chain reaction; PDGF, platelet-derived growth factor; PHB1, prohibitin 1; PHB2, prohibitin 2; PI3K, phosphoinositide 3-kinase; Rb, retinoblastoma protein; ROS, reactive oxygen species; SAMe, S-adenosylmethionine; siRNA, small interfering RNA; TIMP1, tissue inhibitor of metalloproteinase 1; VEGF, vascular endothelial growth factor; WT, wild-type.*

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proteins.[3] They are essential for mitochondrial function and biogenesis in yeast.[4] PHB1 is also found in the nucleus, where it has been shown to interact with retinoblastoma protein (Rb) and p53 among other proteins to bring about a change in transcriptional activities of the E2F transcription factor[5] and p53.[6] These nuclear events have been associated with inhibition of cell-cycle progression[5] and induction of apoptosis.[6] In addition, PHB1 is also localized to the plasma membrane of certain cell types and may function as surface receptor, although the ligand(s) remains to be identified, found in circulation, and is found in the gastrointestinal tract (muscularis, muscularis mucosa, and epithelial layers) where it has been implicated to protect against infection and inflammation.[7,8] PHB1 was originally cloned in 1989, identified as having antiproliferative activity, and thought to be a tumor suppressor (hence its name).[9] However, the tumor suppression was later found to be associated with the 3′–untranslated region of PHB1 messenger RNA (mRNA) and not due to the protein itself.[3] The tumor suppressor activity of PHB1 is highly controversial because PHB1 expression is actually higher in many transformed cells and tumors.[1] The most well-characterized function of PHB1 is its role as a mitochondrial chaperone. Although many studies have examined the effects of PHB1 in cell lines and yeast, little is known about the *in vivo* function of these proteins in mammals and nothing has been reported about the function of PHB1 in mammalian liver. The reason for this is that deletion of PHB1 leads to embryonic lethality (Lexicon Knockout Mice Phenotype Data Summary NIH-1165; www.informatics.jax.org/external/ko/lexicon/2210.html).

We found PHB1 to be down-regulated at the protein level from the time of birth in mice lacking methionine adenosyltransferase 1A (MAT1A) and in mice and patients at risk for development of nonalcoholic steatohepatitis.[10] *Mat1a* knockout (KO) mice have increased hepatic oxidative stress, impaired mitochondrial function, and they spontaneously develop steatohepatitis and hepatocellular carcinoma (HCC).[11,12] MAT1A is expressed largely by mature hepatocytes, where it encodes for the enzyme MAT, which is responsible for the biosynthesis of S-adenosylmethionine (SAMe).[13] We found that PHB1 was down-regulated at the protein level when the intracellular SAMe level fell.[10] Because PHB1 was down-regulated in the livers of the *Mat1a* KO mice at birth and persisted up to the development of steatohepatitis,[10] the purpose of the current work was to see if liver-specific *Phb1* KO mice can recapitulate some of the phenotype of the *Mat1a* KO mice, specifically impaired mitochondrial function, oxidative stress, steatohepatitis, and malignant transformation. Our findings show the importance of PHB1 in maintaining normal liver function and support its role as a tumor suppressor in hepatocytes.

Materials and Methods

Materials. Alpha-phosphorus[32] deoxycytidine triphosphate (α-^{32}P-dCTP 3000 Ci/mmol) was purchased from PerkinElmer (Boston, MA). All other reagents were of analytical grade and obtained from commercial sources.

Generation of the Liver-Specific Phb1 KO Mice. Liver-specific *Phb1* KO mouse (C57BL/6J) was developed by serial breeding of $Phb1^{loxP/loxP}$ and *Albumin-Cre*$^{+/+}$(*Alb-Cre*$^{+/+}$) mice as shown in Supporting Fig. 1 and described in detail in Supporting Methods.

All experiments were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Southern California. Mice aged between 3 and 46 weeks were used for the experiments. Please see Supporting Methods for details of specimen handling. Isolated hepatocytes were obtained by the Cell Culture Core of the USC Research Center for Liver Diseases as described.[14]

Cell Cultures, PHB1 Knockdown or Overexpression. A normal mouse hepatocyte cell line, AML12, was purchased from American Type Culture Collection (ATCC, Manassas, VA), whereas HepG2 and Huh-7

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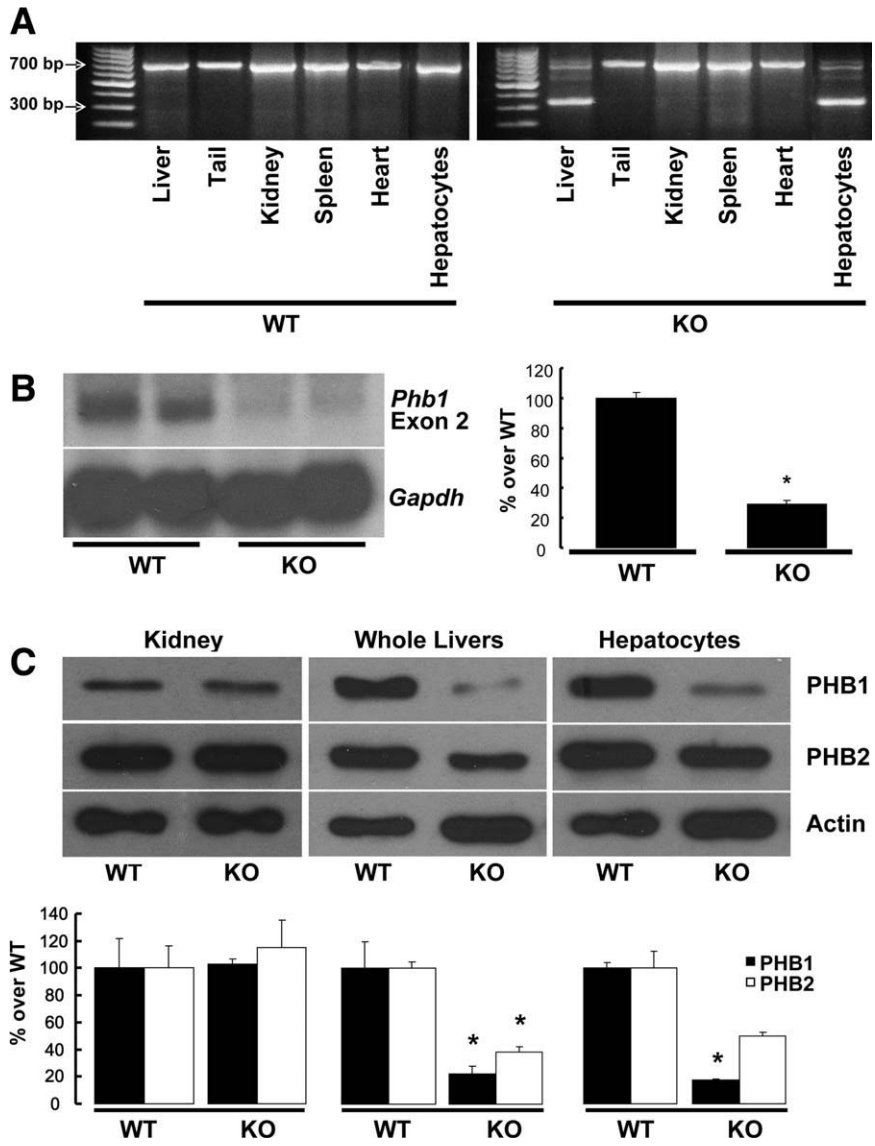

Fig. 1. Confirmation of deletion of *Phb1* in DNA, mRNA, and protein levels. (A, left) Genotyping of *Phb1* exon 2 region including loxP flanking *Phb1* exon 2 sites shows the same size of bands (predicted 731 bp) in all of the organs tested in WT control mice. (A, right) However, deletion of loxP flanking *Phb1* exon 2 sequence (predicted 334 bp) is detected only in liver and hepatocytes but not in other organs from liver-specific *Phb1* KO mice, which indicated that *Phb1* exon 2 is deleted only in the liver of liver-specific *Phb1* KO mice. (B, left) Northern blots of total RNA from the livers of WT control and liver-specific *Phb1* KO mice show that *Phb1* exon 2 is significantly removed in the livers of liver-specific *Phb1* KO mice compared to those of WT control littermates. (B, right) The graph on the right shows densitometric changes in percent of WT control, which is expressed as mean ± SEM from 2 in each group, $*P < 0.01$. (C, top) Protein expression of PHB1 and PHB2 by western blots shows only PHB1 protein expression is dramatically decreased in whole livers and hepatocytes, but not in kidney. (C, top) PHB2 expression is also decreased along with PHB1 protein in the whole livers and hepatocytes in liver-specific *Phb1* KO mice. (C, bottom) The graph shows densitometric changes in percent of WT control, which is expressed as mean ± SEM from two mice each for kidneys and hepatocytes and eight mice each for livers, $*P < 0.01$. SEM, standard error.

cells were provided by the Cell Culture Core and cultured in recommended media in a humidified incubator at 37°C and CO_2 at 5%. Cells with passage number <18 were used for the experiments. Primary human hepatocytes were obtained from CellzDirect (Pittsboro, NC). Cells were washed with phosphate-buffered saline three times and protein was extracted for western blot analysis as described below.

The predesigned small interfering RNA (siRNA) targeting mouse *Phb1* (sense sequence: AGAGCGAGCGGCAACAUUUtt or AGAAACCAAUUAUCUUUGAtt) and negative control siRNA were purchased from Ambion (Austin, TX). AML12 and Huh-7 cells in six-well plates (0.2×10^6 cells/well); the cells were transfected using RNAiMax (5 μL/well) from Invitrogen (Carlsbad, CA) with PHB1 siRNA (12 nM) or negative control siRNA for 18 hours (AML12) and 48 hours (Huh-7) for mRNA or protein expression or 24 hours (AML12) and 48 hours (Huh-7) for proliferation or apoptosis assays, following the manufacturer's manual.

Phb1 overexpression vector (PHB1-pcDNA3.1) and negative control empty vector were kindly provided by Dr. Mehta (Illinois Institute of Technology Research Institute, Chicago, IL). Transient transfection was done using 3 μL of Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and 1.4 μg of target plasmid per well of six-well plates. After 4 hours, the transfection medium was changed to normal medium and cells were cultured for an additional 44 hours for mRNA, protein expression, proliferation, or apoptosis assays.

Nucleic Acid Isolations. Genomic DNA for genotyping was isolated from hepatocytes and various organs by the method of Strauss.[15] Total RNA was

isolated from livers, AML12, and Huh-7 cells using Trizol reagent (Invitrogen) and then purified by total RNA isolation kit (Bioland Scientific LLC, Cerritos, CA) following the manufacturer's manuals.

Genotyping. Genotyping was determined by polymerase chain reaction (PCR) and is described in detail in Supporting Methods.

Gene and Protein Expression. Northern blot analysis, autoradiography and densitometry were done as described.[12] The specific probes for mouse *Phb1* exon 2 and glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) were designed to correspond to published mRNA sequences from +52 to +154 (*Phb1*, NM_008831.3) and +221 to +810 (*Gapdh*, NM_008084), respectively. Both were labeled with α-^{32}P-dCTP using a random primed DNA labeling kit (DECAprime II, Ambion Inc., Austin, TX) as described.[12] Results of northern blot analysis were normalized to *Gapdh*.

See Supporting Methods for details of the microarray analysis, which examined differential mRNA expression profiles and quantitative real-time PCR for confirmation.

Protein expression was examined using western blot analysis performed as described[16] using anti-PHB1, PHB2, and β-actin antibodies (Abcam, Cambridge, MA).

Chromatin Immunoprecipitation (ChIP) Assay. Please see Supporting Methods for details.

Histology, Immunohistochemistry, Immunocytofluorescence, and Electron Microscopy (EM). Histologic examination was done in blinded fashion as to the genotype or age of the animal. Please see Supporting Methods for details of these procedures.

Plasma Alkaline Phosphatase (ALP), Bilirubin, and Alanine Aminotransferase (ALT) Levels. Plasma bilirubin, ALT, and ALP were determined by total bilirubin kit (Thermo Electron Corp., Waltham, MA), ALT reagent (Raichem; Cliniqa Corp., San Marcos, CA), and ALP assay kit (Biovision Inc., Mountain View, CA), respectively, following the manufacturer's protocols.

Plasma and Hepatic Triglyceride and Cholesterol Levels. The lipid portion of the liver was extracted by the method of Folch et al.[17] using chloroform/methanol (2/1, vol/vol) as solvent matrix. Evaporated and reconstituted lipid from liver and frozen and thawed plasma were subjected to the assays for cholesterol and triglyceride using commercial kits (Thermo DMA, Louisville, CO) following the manufacturer's manuals.

Cell Proliferation and Apoptosis Assays. Cell proliferation in PHB1 silenced cells for 24 hours or 48 hours was measured by the incorporation rate of bromodeoxyuridine (BrDU) into DNA using a BrDU assay kit (CalBiochem, San Diego, CA) as described[18] with 3000 cells per well in 96-well plates and 4 hours or 1 hour of BrDU incorporation time for AML12 or Huh-7 cells, respectively. Apoptosis was measured by Hoechst staining as described[18] in Huh-7 cells treated with sorafenib (10 μM, last 24 hours of knockdown or overexpression).

Statistical Analysis. Data are given as mean ± standard error. Statistical analysis for the microarray data is described separately, in that section. For the rest of the results, statistical analysis was performed using unpaired Student *t* test. For changes in mRNA and protein levels, the ratios of various genes and proteins to the housekeeping gene or protein densitometric values were compared. Significance was defined by $P < 0.05$.

Results

Generation of the Liver-Specific Phb1 KO Mice. Following the scheme shown in Supporting Fig. 1, liver-specific *Phb1* KO was generated. Of the 120 mice genotyped from 18 litters of heterozygous mating ($Phb1^{loxP/+}$; $Alb\text{-}Cre^{+/-}$), 10% were liver-specific KOs ($Phb1^{loxP/loxP}$; $Alb\text{-}Cre^{+/+}$ or $Alb\text{-}Cre^{+/-}$), 73% were heterozygotes ($Phb1^{loxP/+}$), and 17% were wild-type (WT) ($Phb1^{+/+}$).

The deletion of *Phb1* occurred only in the liver of 3-week-old KO mice (Fig. 1A). However, deletion was not complete at this age, as a faint band remains that corresponds to the WT gene in both liver and isolated hepatocytes. Consistent with this, northern blot analysis using *Phb1* exon 2 as the complementary DNA (cDNA) probe shows an 80% reduction in *Phb1* mRNA level (Fig. 1B) and western blot analyses show a comparable reduction in the PHB1 protein level in the liver and hepatocytes but not in the kidney (Fig. 1C). The PHB2 protein level was also reduced, but to a lesser degree, to 30% to 40% of controls in the liver and hepatocytes (Fig. 1C).

Physical and Biological Changes in the Liver-Specific Phb1 KO Mice. From the time of birth, there is variability in the weight and health of the KO mice. 15% of the pups (115/768) died before weaning (3 weeks old). Although most were not genotyped, of the ones that died before weaning and were examined, all were liver-specific KOs. KOs that survived past 3 weeks weighed less than WT control littermates and this difference persisted up to 14 weeks of age (Supporting Figs. 2 and 3). The relative liver to body

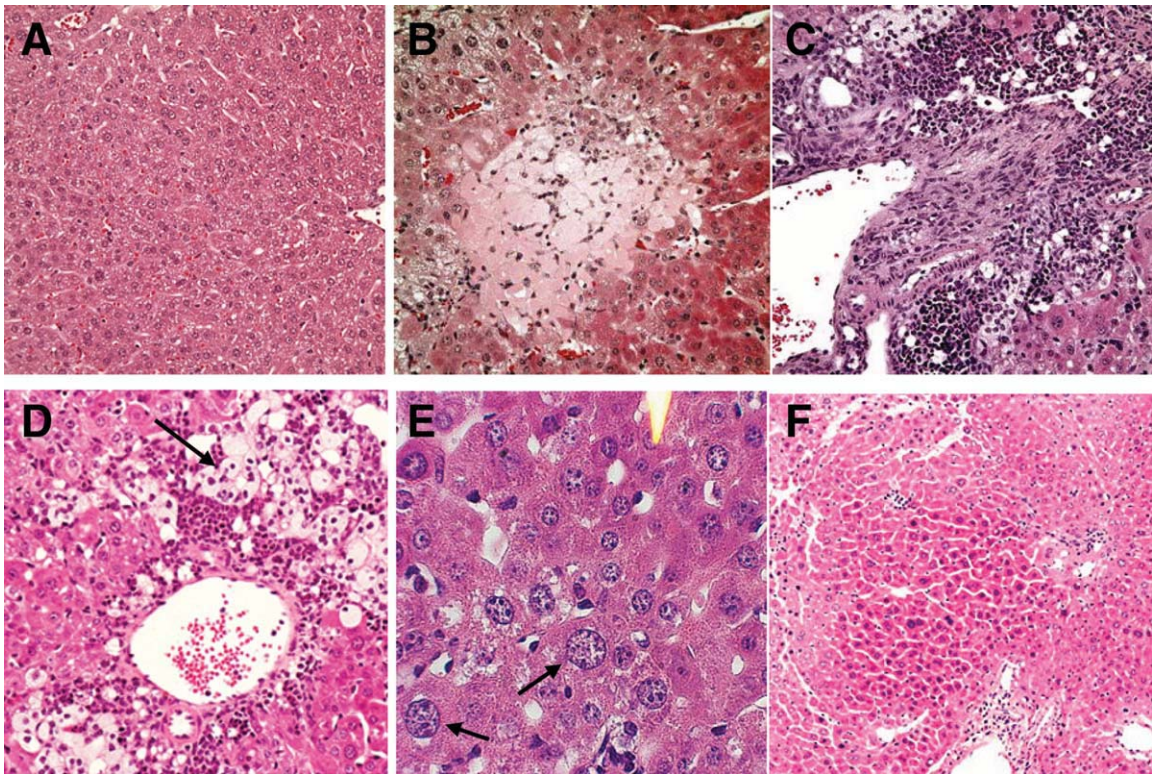

Fig. 2. Histologic changes in liver-specific *Phb1* KO livers. (A-D) (×400), (E) (×800), and (F) (×200) show H&E staining of livers from 3-week old WT (A) control and (B-E) liver-specific *Phb1* KO mice (except for F, 14-week-old KO). Characteristic findings in liver-specific *Phb1* KO livers are (B) foci of necrosis, (C) increased inflammatory cell infiltrate, (D) bile duct metaplasia as indicated by arrow, and (E) anisocytosis (varying sizes) of hepatic nuclei as indicated by arrows, consistent with hepatocyte dysplasia. (F) Consistent with this, a 14-week-old KO mouse exhibits dysplastic nodule.

weight was higher in the KO mice (Table 1). At 3 weeks of age, many KO mice appeared ill, and liver injury is biochemically evident (Table 1). Liver injury is confirmed histologically by marked necrosis and inflammation seen throughout the liver (Fig. 2B,C). There is also bile duct metaplasia (Fig. 2D), anisocytosis of hepatic nuclei (Fig. 2E), and positive staining for OV-6, an oval cell marker (Fig. 3B), and glutathione S-transferase Pi (GSTP) (Fig. 3D), a preneoplastic marker in the 3-week-old KO liver.

Mat1a KO mice have higher hepatic triglyceride levels[11] and develop steatohepatitis.[12] This prompted us to measure lipid levels in the liver-specific *Phb1* KO mice. Liver-specific *Phb1* KO mice have elevated plasma cholesterol levels, but their hepatic cholesterol levels and both plasma and hepatic triglyceride levels were unchanged from WT controls (Table 1).

As the mice grew older, by 14 weeks hepatic nodules can be seen in some liver sections but not on gross examination (Fig. 2F). By 38 weeks, many KO livers stain positive for alpha-fetoprotein (AFP) (Fig. 3F). Because PHB1 is a mitochondrial chaperone protein, we examined mitochondrial morphology by EM. Supporting Fig. 4A,B shows that mitochondria in the 3-week-old KO liver appear swollen and many have no discernible cristae. Positive 4-hydroxynonenal (4-HNE) staining from increased lipid peroxidation in the KO liver, as compared to WT control liver (Supporting Fig. 4C,D), is consistent with impaired mitochondrial function. As the KO mice grew older, there was progressive apoptosis, as shown by activated caspase-3 staining (Fig. 4, top row), persistent proliferation as indicated by proliferating cellular nuclear antigen (PCNA) staining (Fig. 4, middle row), and progressive fibrosis on reticulin staining (Fig. 4, bottom row). Based on histologic examination, no frank cancer was noted in eight KO mice on a normal diet by 14 weeks. However, by 20 weeks, all mice have multiple liver nodules on gross examination of the liver (Fig. 5B); between the ages of 35 and 46 weeks 38% (5/13 mice; 1/5 male, and 4/8 female) have multifocal HCC (Fig. 5C,D).

Because *Phb1* KO mice develop HCC, we next compared PHB1 protein expression in normal primary human hepatocytes to that of human HCC cell lines Huh-7 and HepG2. The protein level of PHB1 is

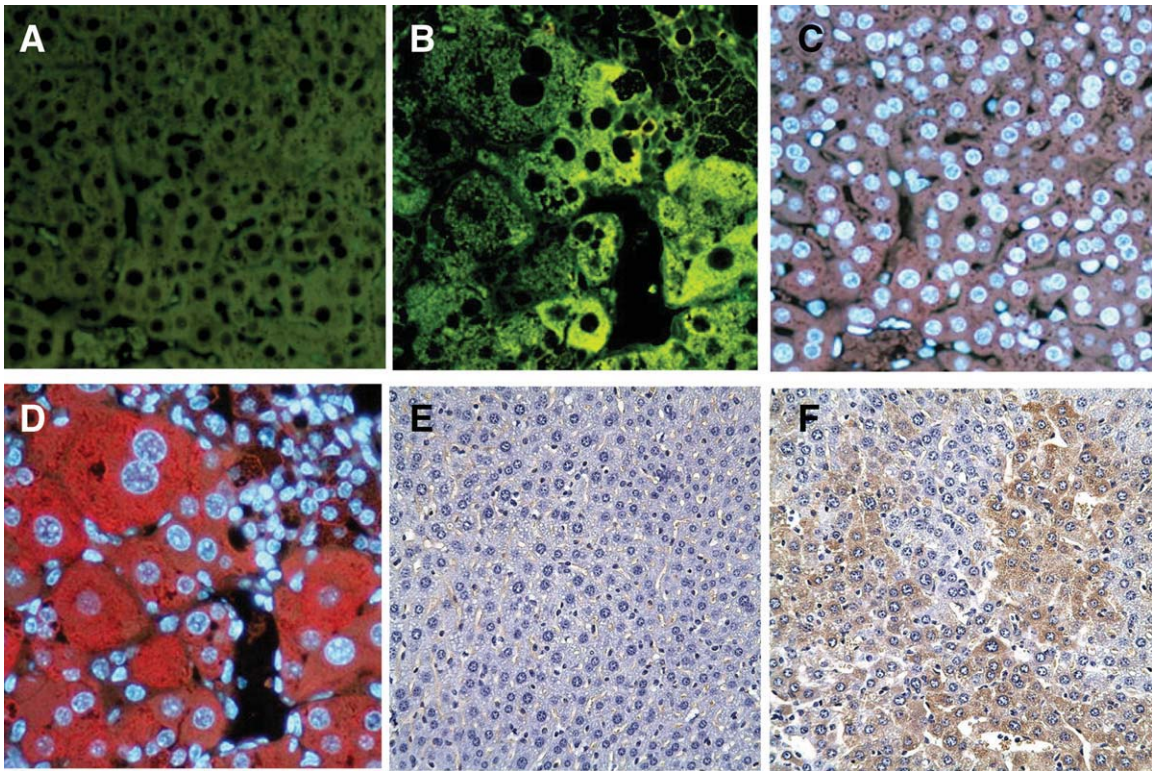

Fig. 3. Liver-specific *Phb1* KO livers exhibit increased staining for oval cell and preneoplastic markers. OV6 staining is (A) negative in 3-week old WT control (×800) but (B) positive in 3-week-old KO mice (bright green staining, ×800). GSTP staining with DAPI co-staining for nuclei shows (C) negative GSTP in 3-week-old WT control (×800) but (D) positive cells in 3-week old KO (red staining, ×800). (B) Note these GSTP-positive cells are also OV6 positive. In A and C, the same microscopic field is shown as in B and D with double OV6/GSTP immunofluorescent staining. AFP staining was negative in 38-week-old (E) WT control (×400) but strongly positive in (F) KO (×400). DAPI, 4′S,6-diamidino-2-phenylindole.

markedly decreased in the two liver cancer cell lines (Fig. 5E).

Differential Gene Expression in the Three-Week-Old Liver-Specific Phb1 KO Mice Livers. Differential gene expression was examined using microarray. Supporting Fig. S5 shows the heat map generated from the microarray data demonstrating the striking difference in gene expression between KO and controls. Note the controls were very similar regardless of age. Microarray analysis (25,000 genes) revealed that 402 genes were up-regulated and 182 genes were down-regulated (fold-change > 2.0; $P < 0.05$) (see Supporting Tables 2 and 3 for complete list). Quantitative real-time PCR confirmed these changes in more than 15 genes (Table 2). Many of the genes differentially expressed in *Phb1* KO mice liver are involved in growth such as H19, CDC20, PRC1, IGF2B, cyclin D1 (CCND1), EGFR1, RASAL1, and SRC (Table 2). Several genes involved in fibrogenesis are also markedly up-regulated, including many collagen genes and tissue inhibitor of metalloproteinase 1 (TIMP1). Interestingly, many enzymes are markedly down-regulated, including several cytochrome P450 (CYP450) family members and uridine diphosphoglucuronate (UDP) glycosyltransferase (Table 2). Genes differentially expressed fall into many different pathways including angiogenesis, cytoskeletal regulation, signaling pathways involved in epidermal growth factor receptor, heterotrimeric G-protein, inflammation, integrin, interleukin, p53, phosphoinositide 3-kinase (PI3K), platelet-derived growth factor (PDGF), Ras, and

Table 1. Physical and Biological Changes in Three-Week-Old Liver-Specific *Phb1* KO Mice

	WT	KO	P
Relative liver weight (%)	4.4 ± 0.2	5.4 ± 0.2	0.004
Plasma bilirubin (μmol/L)	8.7 ± 1.8	46.3 ± 9.1	0.01
Plasma ALT (U/L)	29.8 ± 5.1	250 ± 33.2	0.00003
Plasma ALP (U/L)	134 ± 12.4	532 ± 112	0.04
Plasma cholesterol (mg/dL)	136 ± 6.5	233 ± 23	0.002
Hepatic cholesterol (μg/μg DNA/g liver)	1.8 ± 0.2	1.2 ± 0.6	0.4
Plasma triglyceride (mg/dL)	141 ± 13.6	140 ± 14.9	0.95
Hepatic triglyceride (μg/μg DNA/g liver)	3.0 ± 0.4	1.7 ± 0.8	0.15

Results are expressed as mean ± SEM from 3 to 10 WT controls and 6 to 15 liver-specific *Phb1* KO mice.

SEM, standard error.

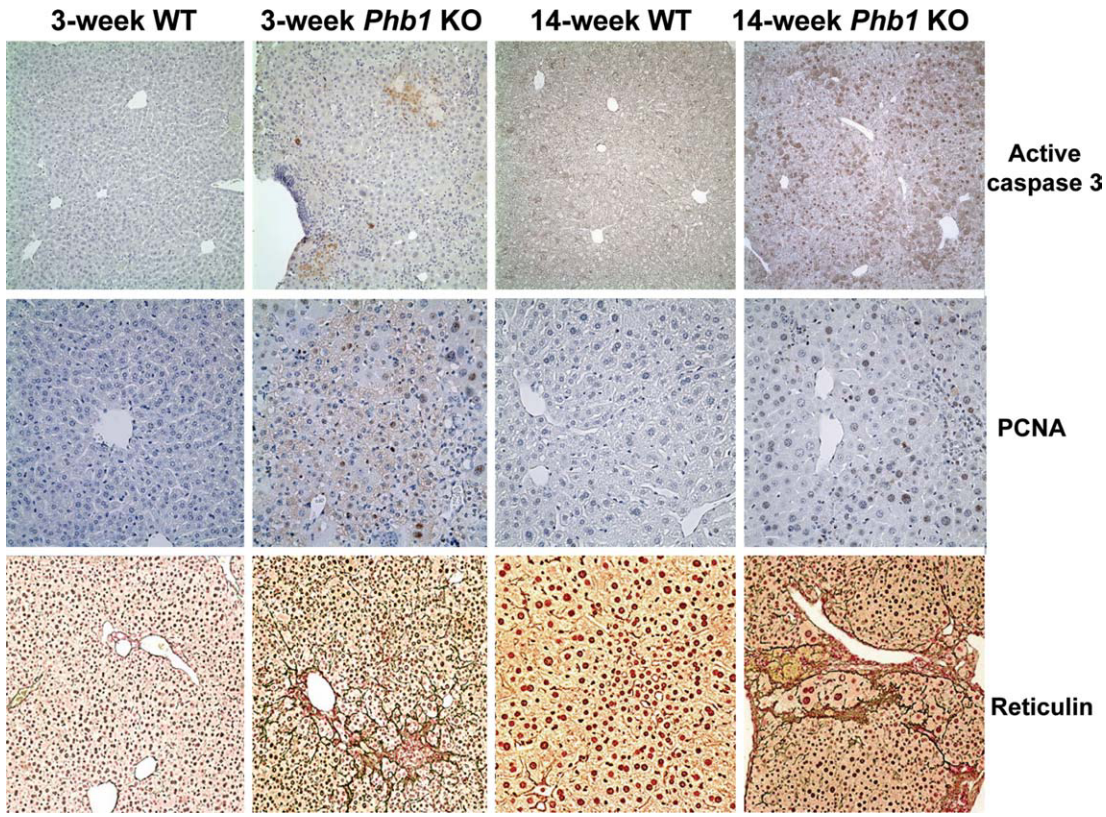

Fig. 4. Changes in liver-specific *Phb1* KO livers from 3 to 14 weeks of age. Apoptosis is detected by immunohistochemical staining for active caspase-3 (top row, ×200). Slightly increased number of active caspase-3–positive cells (brown staining) was found in the liver of 3-week-old liver-specific *Phb1* KO mouse, which progressed as the mice aged. Proliferation is detected by immunohistochemical staining for PCNA (middle row, ×400). Liver-specific *Phb1* KO mice have more PCNA-positive cells (brown staining) regardless of their age than those of their control littermates. Fibrosis is detected by reticulin staining (bottom row, ×200 except for 14-week-old WT, ×400). Significant amount of reticulin is accumulated in the liver of 3-week-old liver-specific *Phb1* KO mouse, which progressed in the 14-week-old KO livers.

vascular endothelial growth factor (VEGF). Supporting Tables S6 to S19 describe changes in mRNA level based on different signaling pathways and biological functions.

Localization of PHB1 in Hepatocytes. PHB1 subcellular localization in hepatocytes has not been examined. Using confocal microscopy, Supporting Fig. 6A shows that the bulk of PHB1 is localized in the mitochondria but there is also staining in the nuclei of normal mouse hepatocytes. PHB1 staining is diminished in both compartments in the hepatocytes isolated from the KO mouse (Supporting Fig. 6B). Both mitochondrial and nuclear staining can also be seen in AML12 cells (Supporting Fig. 6C).

Effects of Acute PHB1 Knockdown in Nontransformed AML12 Cells. To better assess whether the changes observed in the KO mice are due to direct or indirect effects (compensatory proliferation in response to injury) of PHB1 deficiency, we employed acute knockdown with siRNA against *Phb1* in nontransformed AML12 cells. After 18 hours of siRNA treatment, the efficiency of PHB1 knockdown is about 90% (Fig. 6A) at the mRNA level whereas PHB1 protein level fell by only 30% (Fig. 6B). After 18 hours of siRNA treatment, a number of the genes picked up on *in vivo* microarray analysis also exhibited a similar change, but with much smaller magnitude, for instance, cyclin D1 (CCND1) was increased 64% instead of four-fold. Some of the genes exhibited similar magnitude of change as in the *in vivo* microarray, such as KRT18, which increased by 69% and p53, which increased by 48%. Some of the genes actually showed the opposite change as in the *in vivo* microarray, such as CYP4A12A, which more than doubled instead of being 20-fold reduced, and CYP2F2, which increased by 31% instead of a 10-fold reduction (Fig. 6C; Supporting Table 3). Acute reduction of PHB1 for 24 hours resulted in a 50% increase in cell proliferation (Fig. 6D).

To see if the effect of PHB1 knockdown on cyclin D1 expression may be exerted at the level of E2F binding to its consensus sites on the cyclin D1 promoter, we performed ChIP analysis comparing E2F binding to different regions of the promoter that

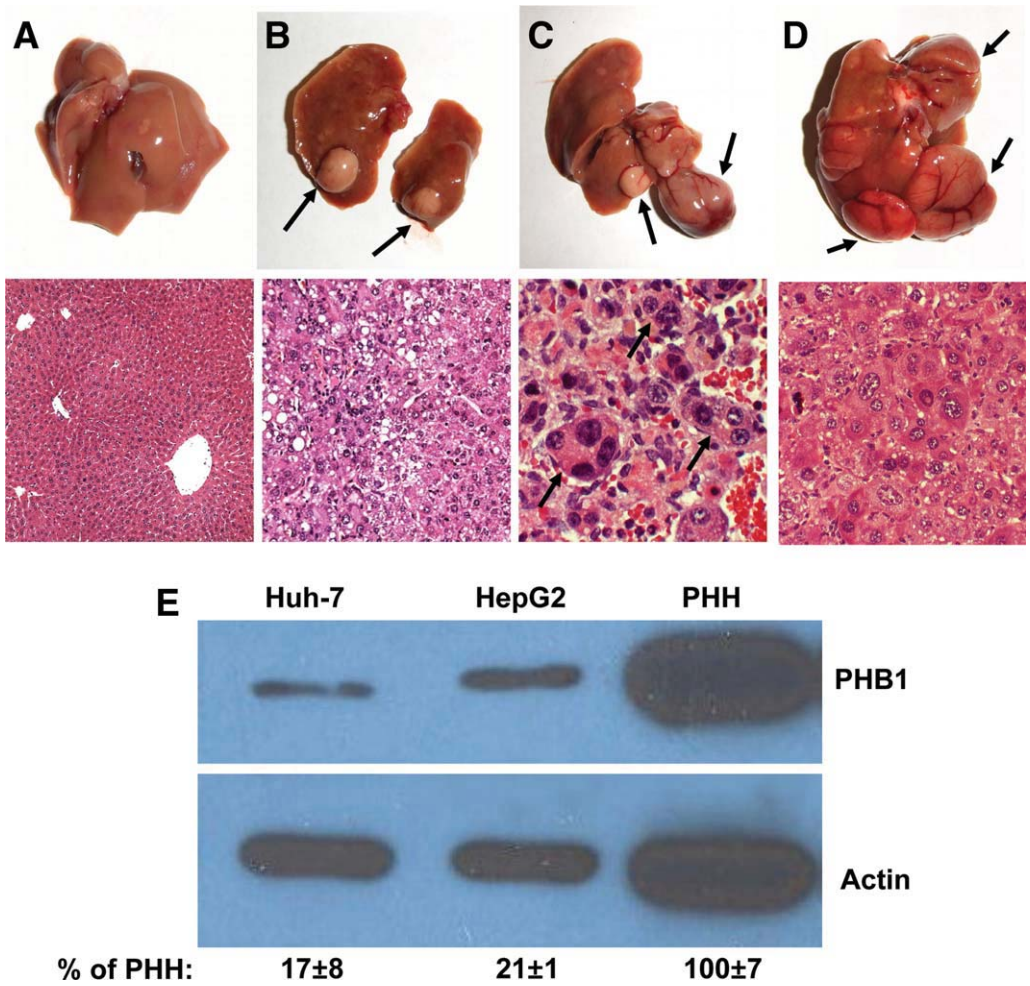

Fig. 5. Development of HCC in the liver-specific *Phb1* KO mice and PHB1 expression in human liver cancer cell lines. Histologic changes on H&E are shown below the gross picture of each liver. (A) No significant change in the liver of WT mouse on gross or histologic examination (×200). (B) Multiple nodules (arrows) on the surface of the liver of a 35-week-old KO mouse, one of which shows well-differentiated HCC with fat deposit on H&E staining (×400). (C) Different stages of HCC development in the liver of a 46-week-old KO mouse (arrows), where neoplastic cells (arrows) are clearly observed on H&E staining (×800). (D) Multifocal HCC in a 38-week-old KO mouse by gross examination (arrows) and on H&E (×600). (E) PHB1 protein level as determined by western blot analysis is much lower in Huh-7 and HepG2 cells as compared to normal PHH. Densitometric analyses from three independent experiments are shown below the blot and are expressed as mean ± SEM percent of PHH; $P < 0.05$ between Huh-7 or HepG2 and PHH. PHH, primary human hepatocytes; SEM, standard error.

contain E2F binding sites. E2F binding increased (Fig. 6E), particularly in region −513 to −697 (500 ± 12% of scrambled control from three experiments, $P < 0.05$) of the cyclin D1 promoter in cells where PHB1 expression was reduced. E2F binding to the other regions also increased significantly but to a much lesser degree (150% to 200%).

Effects of PHB1 Overexpression. Overexpression of PHB1 in AML12 cells reduced proliferation (Fig. 7B,D). However, whereas overexpression in Huh-7 cells tended to lower proliferation, it was not statistically significant (Fig. 7A,C).

PHB1 Expression and Sensitivity to Sorafenib. To see if PHB1 expression in liver cancer cells can affect sensitivity to sorafenib, Huh-7 cells were treated with siRNA against *PHB1* or overexpression vector to raise PHB1. This was then followed by sorafenib treatment. Apoptosis and proliferation were measured thereafter. PHB1 knockdown did not sensitize Huh-7 cells to sorafenib-induced apoptosis or inhibition in proliferation (Fig. 8). Overexpression of PHB1 also had no influence on sorafenib-induced apoptosis or inhibition of proliferation (data not shown).

Discussion

MAT is an essential enzyme for survival as it is responsible for the biosynthesis of SAMe, the principal biological methyl donor and, in mammalian liver, a precursor of GSH.[13] *MAT1A* is one of two MAT genes

Table 2. Confirmation of Gene Expression Changes by QRT-PCR

Genes	Accession Number	Microarray Fold Changes	*P*	QRT-PCR Fold Changes	SEM	*P*
H19	NR_001592.1	146.8	<0.0000001	252.8	48.3	0.002
CDC20	NM_023223.1	14.8	0.000001	25.3	12.7	0.076
LPL	NM_008509.2	14.5	0.0009	3.5	0.9	0.04
PRC1	NM_145150.1	11.4	0.000006	36.4	6.3	0.03
ANXA2	NM_007585.3	11.1	0.000005	10.1	1.5	0.001
TUBB2B	NM_023716.2	8.7	0.000001	3.1	0.2	0.004
TIMP1	NM_011593.2	5.1	0.00004	11.2	2.0	0.002
IGF2BP3	NM_023670.2	5.1	0.0005	2.5	0.3	0.02
GADD45A	NM_007836.1	4.7	0.001	5.8	1.2	0.007
CCND1	NM_007631.2	4.0	0.002	4.0	0.5	0.006
EGR1	NM_007913.5	3.4	NA	4.6	0.9	0.06
RASAL1	NM_013832.3	3.2	0.000003	58.1	8.3	0.0005
SRC	NM_001025395.2	2.8	0.0003	2.8	0.4	0.02
KRT18	NM_010664.1	2.2	0.00003	1.5	0.1	0.03
CD83	NM_009856.1	1.8	0.0001	2.4	0.5	0.034
COL4A1	NM_009931.1	6.0	0.0007	NA	NA	NA
COL6A1	NM_009933.2	5.4	0.0006	NA	NA	NA
COL5A1	NM_015734.1	4.2	0.0003	NA	NA	NA
COL4A2	NM_009932.2	3.8	0.0002	NA	NA	NA
COL1A1	NM_007742.2	3.4	0.0003	NA	NA	NA
COL6A2	NM_146007.1	1.9	0.0003	NA	NA	NA
COL1A2	NM_007743.2	NA	NA	6.1	0.6	0.007
p53	NM_011640.1	NA	NA	1.5	0.2	0.02
CYP4A12A	NM_177406.3	−110.4	<0.0000001	−20.4	8.0	0.04
CYP2F2	NM_007817.2	−9.7	0.0001	−42.0	9.2	0.0001
UGT3A2	NM_144845.3	−8.7	0.000002	−35.5	10.1	<0.0000001

QRT-PCR was done in three WT controls and seven *Phb1* KOs. See Supporting Table S1 for gene names.
QRT-PCR, quantitative real-time PCR; SEM, standard error.

that encode for the catalytic subunit of MAT that is largely expressed in normal differentiated mammalian liver.[13] The expression and activity of hepatic MAT falls in patients with liver disease due to lower MAT1A mRNA level and inactivation of the MAT1A-encoded isoenzymes.[13] This work was originally prompted by our observation that *Mat1a* KO mice have reduced PHB1 protein level from birth that persisted up to 8 months of age.[10] Because PHB1 is known to stabilize mitochondrial proteins, we speculated that reduced PHB1 might have led to impaired mitochondrial function, oxidative stress, and susceptibility to many liver injuries in *Mat1a* KO mice.[10–12] *Mat1a* KO mice also develop HCC spontaneously.[11] Whether reduced PHB1 could have contributed to this was unclear because there is tremendous controversy with regard to PHB1's role as a tumor suppressor.[1] Although the functional role of the PHB complex as a mitochondrial chaperone is well characterized, particularly in yeast,[1,3] whether it plays a similar role in mammals *in vivo* has been unclear because *Phb1* and *Phb2* knockout mice are lethal embryonically (www.informatics.jax.org/external/ko/lexicon/2210.html).[19] Development of the liver-specific *Phb1* KO mice has allowed us to address whether reduced PHB1 contributes to the *Mat1a* KO phenotype and, more importantly, to study the role of PHB1 in normal liver physiology.

Using the albumin promoter to drive Cre expression has resulted in hepatocyte-specific deletion of *Phb1*. However, at 3 weeks of age the deletion was not complete. This is consistent with known efficiency of the albumin-Cre transgene as reported by Postic and Magnuson,[20] which was 40% immediately after birth, 60% at 1 week, and 75% at 3 weeks. Deletion of liver-specific *Phb1* resulted in striking liver injury very early on. Because the proportion of homozygotes (KO) is much lower than the expected 18.8% (25% chance from $Phb1^{lox/lox}$ and 75% from $Alb\text{-}Cre^{+}$, or $0.25 \times 0.75 = 0.188$) based on Mendelian genetics, we suspect there is fetal wastage. This is plausible as albumin can be expressed very early during mouse development, stage 7 to 8 somites.[20] In yeast, it is known that PHB1 and PHB2 are interdependent.[3] Thus, loss of one results in the loss of the other. Whether this is also true in higher organisms was unclear. Our results show that this is also true in mammalian liver as PHB2 was also reduced (although to a lesser degree) when PHB1 was markedly reduced.

Many of the liver-specific *Phb1* KO mice died before weaning and at only 3 weeks of age there is

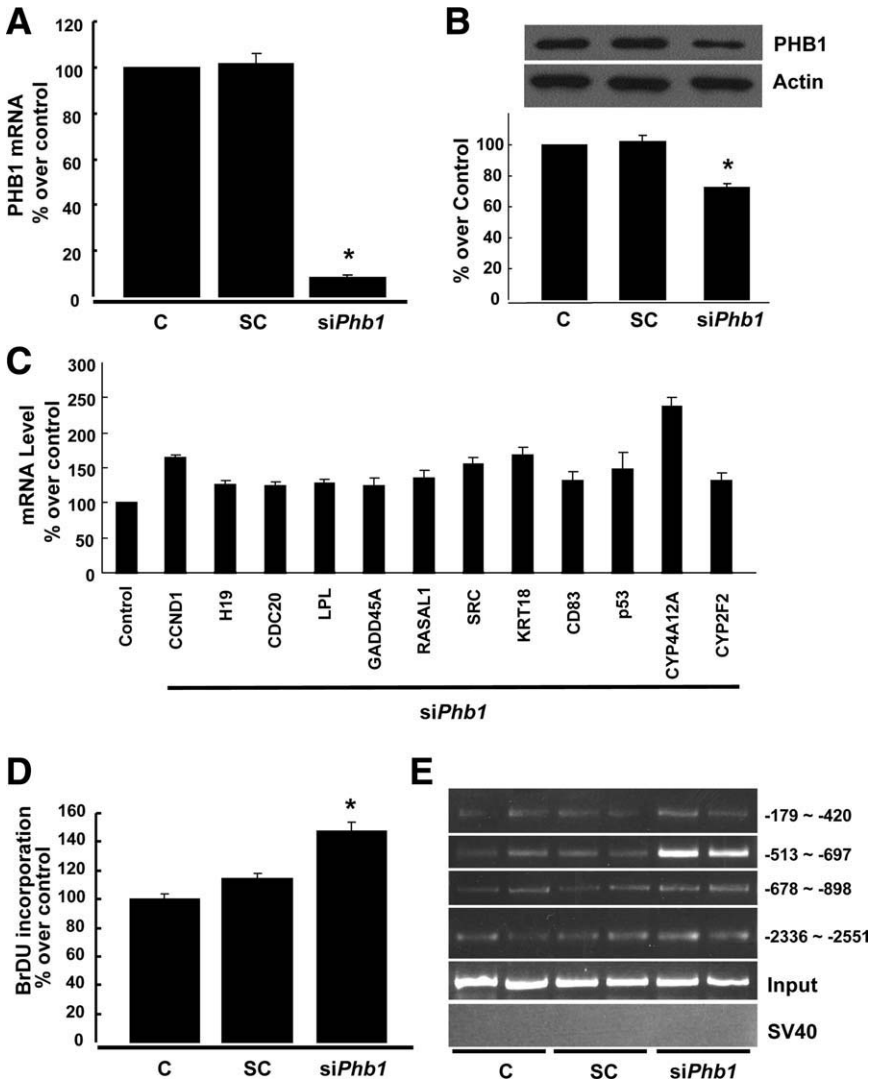

Fig. 6. Effects of *Phb1* siRNA treatment in the AML12 cell line. AML12 cells were treated with siRNA of *Phb1* for 18 hours (expression) or 24 hours (growth and ChIP). PHB1 mRNA level was reduced by 90% as compared to (A) control and SC (n = 6) and (B) PHB1 protein expression decreased by 30% (n = 3). (C) mRNA levels in *Phb1* siRNA–treated AML12 cells for the genes that are up-regulated or down-regulated most in the livers of liver-specific *Phb1* KO mice (n = 4-6); all mRNA expression changes are statistically significant against control and/or SC ($P < 0.05$; Supporting Table 3). (D) *Phb1* siRNA–treated AML12 cells have increased proliferation rate measured by BrDU incorporation into DNA for 4 hours (n = 4). *$P < 0.005$ versus control and SC. (E) The effect of *Phb1* siRNA on E2F binding to different regions of the cyclin D1 promoter (numbers are in reference to the transcription start site). E2F binding is increased in all four regions but particularly in the region −513 to −697. These changes were measured densitometrically and were significantly different from SC control with $P < 0.05$. SC, scrambled siRNA.

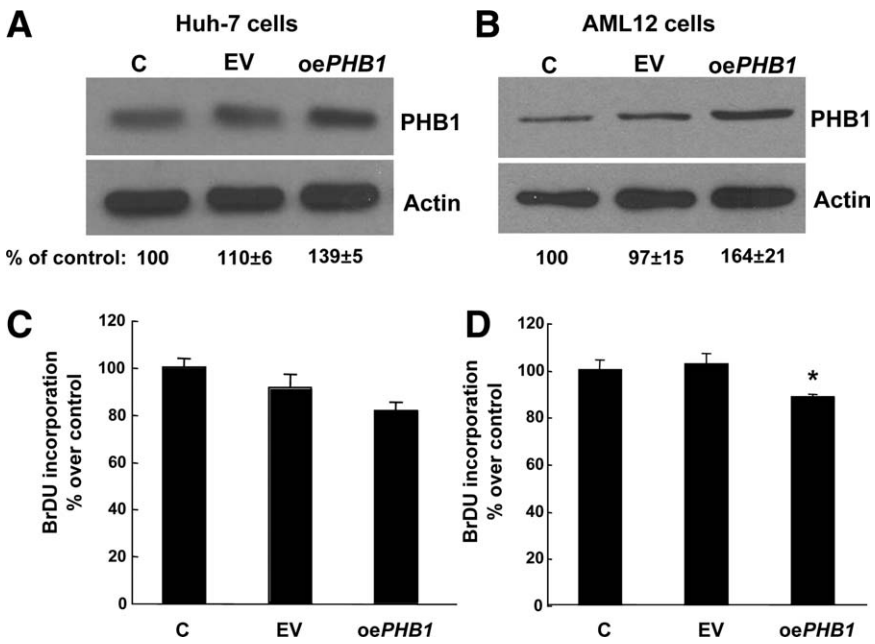

Fig. 7. Effects of PHB1 overexpression in the AML12 and Huh-7 cell lines. AML12 and Huh-7 cells were treated with the human PHB1-pcDNA3.1 PHB1 overexpression plasmid for 48 hours. (A,B) The efficiency of PHB1 overexpression in protein level by western blot. PHB1 protein level increased by 35% to 60% Huh-7 or AML12 cells treated with the overexpression vector (oe*PHB1*) as compared to EV control, respectively. Densitometric analyses from three independent experiments are shown below the blots and are expressed as mean ± SEM percent of control, $P < 0.05$ between oe*PHB1* and EV. Proliferation was measured by BrDU incorporation and are shown in (C) and (D). AML12 cells overexpressing PHB1 had a significant (D) decrease in proliferation as compared to control and EV, whereas (C) no significant change was found in Huh-7 cells. *$P < 0.05$ versus control and EV. EV, empty vector; SEM, standard error.

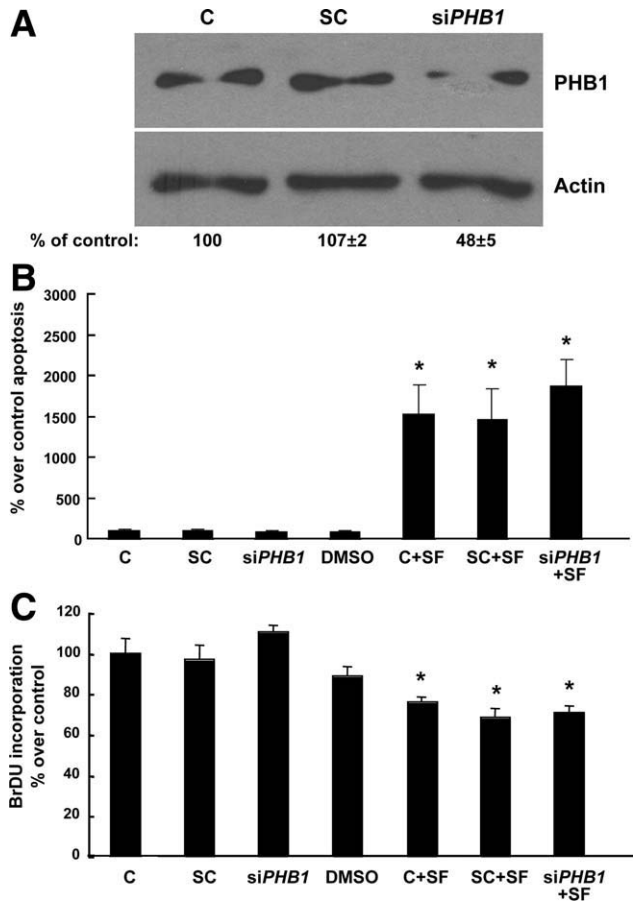

Fig. 8. Effects of *Phb1* siRNA and SF treatments in Huh-7 cells. Huh-7 cells were treated with *Phb1* siRNA or SC for 48 hours and sorafenib was added during the last 24 hours. (A) PHB1 siRNA reduced PHB1 protein level by 50% as shown on western blot (n = 3), results are mean ± SEM densitometric values expressed as percent of control). (B) Percentage over control apoptosis in Huh-7 cells treated with si*PHB1* or SC control, with or without SF. Randomly selected ×10 optic magnification fields were taken and the ratio of apoptotic cells to normal cells was calculated in four fields in each group. si*PHB1* treatment did not affect apoptosis or sensitize cells to apoptosis induced by SF. (C) Proliferation changes in similarly treated Huh-7 cells. SF inhibited proliferation and thus was not affected by si*PHB1*. *$P < 0.05$ versus DMSO control. DMSO, dimethylsulfoxide; SF, sorafenib.

biochemical and histological evidence of marked liver injury. Histologically, the liver is characterized by necrosis and inflammation at 3 weeks. There is also increased apoptosis, which progressed as the mice grew to 14 weeks. Consistent with its known role as a mitochondrial chaperone, marked reduction in PHB1 resulted in abnormal mitochondrial morphology and oxidative stress. There is also increased proliferation, as indicated by PCNA staining. Interestingly, as early as 3 weeks of age, there is already increased staining for OV-6 and GSTP, oval cell and preneoplastic markers, respectively. By 14 weeks, dysplastic hepatic nodules were evident microscopically and by 20 weeks, all mice have multiple hepatic nodules on gross examination. By 35 to 46 weeks, more than one-third of the mice developed multifocal HCC. Because increased proliferation and stem cell expansion observed in the livers of the KO mice may be due to a compensatory response to injury, we examined the effect of acute reduction in PHB1 on cell proliferation in nontransformed AML12 cells. Acute loss of PHB1 in a nontransformed hepatocyte resulted in increased proliferation whereas overexpression of PHB1 resulted in the opposite. Although PHB1 expression also tended to have a similar effect in Huh-7 cells, changes were not statistically significant. Taken together, these observations would support a role for PHB1 as a tumor suppressor, at least in normal hepatocytes. It is possible that the effect of PHB1 on growth is different in normal versus malignant hepatocytes as many signaling pathways are altered in cancer. This is an area that will require further investigation.

There is compelling data from several laboratories using a variety of techniques that support presence of PHB1 in the nucleus in steroid-responsive cells (breast and prostate cancer cell lines) and physical interaction with Rb, E2F, and p53, as well as other proteins to repress the transcriptional activity of E2F[5,21,22] and activate the transcriptional activity of p53.[6] Our results show that in hepatocytes, a small amount of PHB1 can be found in the nucleus. Furthermore, reduced PHB1 expression in AML12 cells resulted in increased E2F binding to the cyclin D1 promoter and cyclin D1 expression. However, if PHB1 inhibits cell-cycle progression and induces apoptosis, as these studies suggest, why would it be highly expressed in many human cancers? Although it is highly expressed in many types of cancer,[23] it has not been well studied in HCC and whether it is functionally normal has not been examined. Indeed, we found much higher PHB1 protein level in normal human hepatocytes when compared to two human liver cancer cell lines. PHB1 expression level also does not equate function. A recent study found liver cells and transgenic mice that express hepatitis C virus core protein have increased mitochondrial PHB1 protein level but despite higher level, it is functionally impaired.[24] Thus, it would be of interest to see if there is a difference in subcellular localization of PHB1 in various cancers and whether it is functionally intact. Consistent with this notion, a recent study found lung cancer cells that have increased membrane-associated PHB1 (cell surface and microsomal membrane fractions) were more resistant to paclitaxel[23] and interestingly, total whole-cell levels of PHB1 were unchanged. We did not observe any difference in

sensitivity to sorafenib in Huh-7 cells when PHB1 expression was varied. Thus, the effect of PHB1 may be cancer-specific and cell line–specific.

The microarray data revealed that many pathways are affected by reduced PHB1 expression. Many are growth-related and oncogenes. Interestingly, cyclin D1 and PCNA, which are up-regulated in *Mat1a* KO[25] and *Phb1* KO livers, are known E2F targets.[26] These findings, along with our current observations, would support PHB1's repressive role on E2F transcriptional activity in mouse liver. We also examined whether these genes are similarly affected after acute knockdown of PHB1 in AML12 cells. Although many of the growth-related genes are similarly affected, the magnitude of change is much smaller than *in vivo*. This may be partly due to the fact that PHB1 protein level fell only by 30% after 18 hours of siRNA treatment, even though the mRNA level fell by 90%. PHB1 protein appears to be quite stable, with a half-life that exceeds 10 hours.[27] Interestingly, CYP4A12A and CYP2F2 are both increased in the AML12 cells following PHB1 knockdown, which is the opposite of what happened in the KO livers. This likely reflects some adaptive changes that occurred *in vivo* and the influence of the *in vivo* microenvironment.

Recently, conditional deletion of *Phb2* using mouse embryonic fibroblasts (MEFs) from homozygous $Phb2^{fl/fl}$ embryos transduced with Cre-recombinase was reported.[19] Loss of PHB2 in MEFs was accompanied by loss of PHB1, confirming their interdependence in the mammalian system. Loss of PHB2 resulted in aberrant mitochondrial cristae morphogenesis and increased apoptosis, which is similar to *Phb1* KO. However, loss of PHB2 in MEFs led to impaired cellular proliferation.[19] Given that these two proteins function as a complex at least in the mitochondria, it is intriguing that they should have such different effects on growth. Our findings are consistent with an earlier report; during liver regeneration in rats, where the expression of PHB1 is abundant in quiescent hepatocytes and nearly absent during the 3-hour to 12-hour period following two-thirds partial hepatectomy, and returning to normal levels at 24 hours.[28] These changes correlated with entry of hepatocytes into the cell cycle and support the notion that a fall in PHB1 facilitates cell-cycle entry and proliferation.

Based on the findings thus far, reduced PHB1 expression that occurs in the *Mat1a* KO livers can contribute to liver injury, increased oxidative stress, impaired mitochondrial function, expansion of liver progenitor cells, and development of HCC in the *Mat1a* KO mouse model.[10–12,29] However, whether it also contributes to the susceptibility to develop fatty liver in the *Mat1a* KO mice[12] is not clear. Although there is no evidence for increased fat accumulation in *Phb1* KO livers up to 14 weeks of age, there is increased plasma cholesterol level, which may signal impairment in cholesterol uptake by the liver. This possibility will require further investigation.

In summary, liver-specific deletion of *Phb1* results in marked liver injury at an early age that is characterized by necrosis, apoptosis, swollen mitochondria, oxidative stress, fibrosis, and increased expression of progenitor cell and preneoplastic markers. Multifocal HCC occurs by 8 months. Marked reduction of PHB1 alters the expression of genes involved in multiple cellular pathways, from growth, inflammation, and xenobiotic metabolism. Our study demonstrates for the first time a vital role for PHB1 in normal liver physiology and supports PHB1 as a tumor suppressor in liver.

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