Rescue of ATP7B function in hepatocyte-like cells from Wilson’s disease induced pluripotent stem cells using gene therapy or the chaperone drug curcumin

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Directed hepatocyte differentiation from human induced pluripotent stem cells (iPSCs) potentially provides a unique platform for modeling liver genetic diseases and performing drug-toxicity screening in vitro. Wilson’s disease is a genetic disease caused by mutations in the ATP7B gene, whose product is a liver transporter protein responsible for coordinated copper export into bile and blood. Interestingly, the spectrum of ATP7B mutations is vast and can influence clinical presentation (a variable spectrum of hepatic and neural manifestations), though the reason is not well understood. We describe the generation of iPSCs from a Chinese patient with Wilson’s disease that bears the R778L Chinese hotspot mutation in the ATP7B gene. These iPSCs were pluripotent and could be readily differentiated into hepatocyte-like cells that displayed abnormal cytoplasmic localization of mutated ATP7B and defective copper transport. Moreover, gene correction using a self-inactivating lentiviral vector that expresses codon optimized-ATP7B or treatment with the chaperone drug curcumin could reverse the functional defect in vitro. Hence, our work describes an attractive model for studying the pathogenesis of Wilson’s disease that is valuable for screening compounds or gene therapy approaches aimed to correct the abnormality. In the future, once relevant safety concerns (including the stability of the mature liver-like phenotype) and technical issues for the transplantation procedure are solved, hepatocyte-like cells from similarly genetically corrected iPSCs could be an option for autologous transplantation in Wilson’s disease.

INTRODUCTION

Wilson’s disease (WD), also called progressive hepatolenticular degeneration, is an autosomal recessive inborn error of copper metabolism (1). Copper is an essential element to human health, being necessary for many enzymes that act as catalysts in relevant body functions (2). Copper contained in food is absorbed into the portal vein and then loaded into hepatocytes. There, ATP7B (ATPase, Cu²⁺ transporting, beta polypeptide) mediates the secretion on one side into the bile and on the other into the bloodstream after linkage to ceruloplasmin, a protein responsible for organized delivery into...
all tissues (1). Mutations in the ATP7B gene (located in chromosome 13) are responsible for WD, whose prevalence is 1 in 30,000 to 1 in 100,000 (1). When ATP7B function is abolished, copper accumulates in hepatocytes and induces progressive damage by activating a Fenton chemical reaction (3). Later on, it is delivered into the bloodstream without linkage to ceruloplasmin, and damages diverse organs including the basal ganglia of the brain (in particular the lenticular nucleus) (1,3). In consequence, WD normally presents as hepatic and/or neurological disease. Hepatic manifestations include chronic hepatitis with progressive cirrhosis or acute liver failure, while the most frequent neurological manifestations are Parkinsonism, seizures and neurobehavioral changes (1). Notably, multiple ATP7B mutations have been found in WD patients worldwide but some are dominant in specific ethnic groups (4). For example, the H1069Q mutation accounts for 37–63% of WD in Caucasian patients, while in the Chinese population R778L mutation underlies most cases (5,6). Defining the type of mutation is relevant among other things because this influences the clinical manifestations, a circumstance not well understood. In this regard, patients homozygous for the H1069Q mutation have a later onset often dominated by neurobehavioral changes, while R778L mutation predominantly shows as liver disease.

Although current animal models, including the toxic milk mouse, Atp7b−/− mouse and Long–Evans Cinnamon rat, have provided very useful data concerning the pathogenesis of WD, physiological differences between species limit the conclusions (7). These models cannot explain either, the clinical variability and complexity observed among affected WD individuals (6). On the other hand, using primary hepatocytes from liver biopsies of WD patients is not a good option because of difficulty obtaining and culturing these samples, and the fact that these cells may already be damaged. Remarkably, the discovery of induced pluripotent stem cells (iPSCs) by Takahashi and Yamanaka (8,9) offers a revolutionary way to study human diseases (10–13), including liver genetic diseases (14,15). In this report, we aimed to establish an in vitro disease model using iPSCs from WD patients.

RESULTS

We obtained skin samples from a Chinese Han patient diagnosed with WD in a local hospital. Fibroblasts were expanded in vitro and the genomic DNA extracted and sequenced to confirm a homozygous R778L mutation in exon 8 of ATP7B gene, the Chinese hotspot mutation (Fig. 1A and B) (6). WD fibroblasts were reprogrammed to iPSCs using retroviruses that express the four Yamanaka factors individually (9) (Fig. 1C). Two different approaches were used [KSR + valproic acid (VPA) or defined fetal bovine serum (dFBS) + vitamin C (Vc) + VPA], which yielded ~0.5 and ~2% efficiency of human embryonic stem cell (ESC)-like colonies, respectively (16,17) (Fig. 1C). After picking and expanding several colonies from both protocols, three iPSC clones (WD iPSC-1 and WD iPSC-3 from KSR + VPA and WD iPSC-2 from dFBS + Vc + VPA) were selected for further characterization. These WD iPSCs were positive for alkaline phosphatase (AP) activity (Fig. 1D) and expressed pluripotent markers including the surface antigens SSEA-3, SSEA-4, TRA-1-81 and TRA-1-60, and the endogenous ESC-transcription factors OCT4, SOX2, NANOG, REX1, as assessed by immunofluorescence microscopy (Fig. 1D) and quantitative real-time RT–PCR (qPCR) analysis, respectively (Fig. 1E). Integration of the exogenous transgenes in the genomic DNA of WD iPSCs was verified by semi-quantitative PCR and qPCR showed that their expression was silenced (Figs 1F and 2A). The karyotype was normal (Fig. 2B) and OCT4 and NANOG proximal promoters were demethylated (Fig. 2C). Moreover, short tandem repeat (STR) analysis confirmed that WD iPSCs are originated from WD donor fibroblasts and not contamination with other cell lines grown in our laboratory (Fig. 2D). Global gene expression profiling with DNA microarrays also showed that WD iPSCs clustered together with human ESCs (H9) when compared with donor fibroblasts (Fig. 2E). In addition, their pluripotent properties were assessed by embryoid body (EB) (Fig. 3A and B) and teratoma formation (Fig. 3C), both of which produced derivatives of the three germ layers including rather complex structures in the case of teratomas. The morphology and growth characteristics of WD iPSCs remained unchanged up to passage 40 in this study. Therefore, we have successfully reprogrammed WD fibroblasts into cells that are pluripotent and stably display human ESC-like characteristics.

Next, we differentiated WD iPSCs into hepatocyte-like cells using a previously established protocol (Fig. 4A) (18) simulating normal liver development through three main phases: definitive endoderm, hepatic progenitors and hepatocyte-like cells. Appearance of markers corresponding to the three phases was verified by immunofluorescence microscopy (Fig. 4A). The number of albumin (ALB) positive cells after the final maturation phase was high (60–80%) as measured by immunofluorescence and cell counting or flow cytometry (Supplementary Material, Fig. S1A and B). Cells positive for alpha 1 anti-trypsin (A1AT) and the asialoglycoprotein receptor (ASGPR) were less abundant, reaching ~20% and 18% respectively (Supplementary Material, Fig. S1A and C). We then performed qPCR for phase I and phase II genes, which mostly encode for liver-specific enzymes (e.g. cytochrome oxidases) with a key role in detoxifying compounds (19), and measured other relevant liver-related genes as well (Fig. 4B, C and D and Supplementary Material, Fig. S1D). Primary human hepatocytes (PHH) cDNA was used as positive control. Expression levels varied among the different genes but were in general significantly lower in hepatocyte-like cells from WD iPSCs than in PHH, and this was particularly obvious for phase I and II genes (Fig. 4B and C). ATP7B mRNA was instead rather similarly expressed in hepatocyte-like cells from WD iPSCs than in PHH, and levels were higher than in undifferentiated iPSCs (Fig. 4D). Similar qPCR results for the whole panel of genes were observed using iPSCs from control fibroblasts IMR90 (90 iPSC-2) and H9 ESCs (Supplementary Material, Fig. S1D). On the other hand, ATP7B protein was undetectable in lysates from undifferentiated WD iPSCs and displayed high expression in hepatocyte-like cells from WD iPSCs and PHH, as assessed by western blotting (Fig. 4E). ALB also showed significant expression in WD hepatocyte-like cells (with variation between iPSC clones) by western blotting but levels were
lower than in PHH (Fig. 4E). The fact that except for ATP7B, hepatocyte-like cells from ESCs and iPSCs (including WD) do not recapitulate the gene expression profile of PHH is in agreement with multiple studies on ESCs/iPSCs (20–22). Nevertheless, relevant liver cell functions are still present, although in general they are reduced compared with PHH. For example, we observed accumulation of glycogen by periodic acid Schiff (PAS) staining (Fig. 5A and Supplementary Material, Fig. S2A), significant ALB and urea secretion (Fig. 5B and C and Supplementary Material, Fig. S2B and C), and also detected substantial inducible cytochrome P450 activity upon phenobarbital addition (variable between the two tested clones) (Fig. 5D and Supplementary Material, Fig. S2D). Notably, immunofluorescence microscopy showed co-expression of ALB and ATP7B (Fig. 6A) and negligible staining in undifferentiated iPSCs (Fig. 6B), reinforcing the potential utility of our iPSCs to model WD disease in vitro. ATP7B had a perinuclear distribution in hepatocyte-like cells derived from normal iPSCs and co-localized with the trans-Golgi network marker p230 (Fig. 6B and C), but in hepatocyte-like cells from WD iPSCs such distribution was altered (Fig. 6B and C). This is in agreement with a previous report over-expressing the R778L mutation of ATP7B in HEK293T cells (23), though to the best of our knowledge the phenomenon had not yet been documented with endogenous ATP7B. Altered localization of mutated R778L ATP7B is thought to be a consequence of abnormal protein folding and retention in the endoplasmic reticulum (23). Interestingly, addition of the pharmacological chaperone curcumin partially rescued ATP7B distribution in hepatocyte-like cells from WD iPSCs (Fig. 6B and C), also supporting previous work using HEK293T cells (23). Curcumin did not noticeably change

Figure 1. Generation of patient specific-iPSCs with mutated ATP7B. (A) Scheme showing homozygous G–T mutation in position 2333 of the ATP7B gene in WD fibroblasts. NCBI accession number for the gene is included. (B) This mutation results in an R778L amino acid change that affects the normal folding of ATP7B protein. (C) Scheme depicting the process of iPSC generation, phase contrast photographs of donor WD fibroblasts and the resulting iPSCs (a colony emerged on the original plate is shown) are displayed. (D) AP staining and immunofluorescence microscopy for the indicated ESC-like markers in a representative WD iPSC clone (iPSC-3); nuclei are stained in blue with DAPI (also hereafter). Scale bars indicate 100 μM. (E) qPCRs showing high expression of endogenous (endo) ESC transcription factors in selected WD iPSC clones. Values (mean of three replicates) are referred to donor WD fibroblasts; H9 ESCs were the positive control. (F) qPCR showing silencing of the exogenous transgenes (Tg) in selected WD iPSC clones. Values (mean of three replicates) are referred to WD fibroblasts transduced with the exogenous factors and extracted at Day 6; donor fibroblasts were the negative control.
ATP7B expression by western blotting (Fig. 6D). The abnormal distribution of ATP7B and the correction upon curcumin treatment were likewise verified using cells derived from another WD iPSC clone (data not shown). Hence, our WD iPSCs can be differentiated into hepatocyte-like cells that display some characteristics of native hepatocytes but not others. Moreover, these cells have abnormal localization of ATP7B as expected according to the underlying mutation, and this phenomenon is susceptible to correction using a chaperone drug.

We then aimed to develop a method that measures copper-export capacity in control and WD hepatocyte-like cells, so that this could be used as a platform to explore more accurately the ways (genetic or pharmacological) that correct the abnormality (Fig. 7A). Differentiated cells were cultured in medium supplemented with 100 μM copper for 24 h, washed and the medium replaced by copper-free medium. The copper-export pattern was investigated in the following 9 h by collection of cell-culture supernatants at different time points and detection using an atomic absorption spectrophotometer, and represented as copper accumulated in the supernatant. For these experiments, we used hepatocyte-like cells produced from H9 ESCs, two WD iPSC clones and normal iPSCs (90 iPSC-2). In all cases, data of copper secretion were normalized at the end of the time course by detaching the differentiated hepatocyte-like cells and counting ATP7B positive cells by flow cytometry (Fig. 7B). WD hepatocyte-like cells from the two different iPSC clones showed a significantly reduced copper-export activity compared with the controls (Fig. 7C and D). These controls showed as well some variability as expected for cells of different genetic background. We also over-expressed a codon-optimized
version (to improve expression as the cDNA is large) of \( \text{ATP7B} \) into WD hepatocyte-like cells using a self-inactivating lentiviral vector (24,25). This vector is very similar in design to the one used in a recent successful gene therapy protocol by Cartier et al. (26). As a control for our \( \text{ATP7B} \) vector, we infected cells with a lentiviral vector expressing only GFP. Western blot verified over-expression of ATP7B protein (data not shown). In parallel, we tested the effect of curcumin, encouraged by its potential to improve ATP7B localization (Fig. 6B and C). \( \text{ATP7B} \) lentiviral vectors and treatment with curcumin both improved copper export in WD hepatocyte-like cells (Fig. 7C and D). We also measured copper export activity in undifferentiated ESCs/iPSCs and found it to be negligible (Supplementary Material, Fig. S2E). Altogether these data support the validity of our experimental setup and the quality of our hepatocyte-like cells from WD iPSCs to model the disease phenotype.

**DISCUSSION**

We have described the generation of WD iPSCs that can be used for *in vitro* disease modeling. Hepatocyte-like cells from WD iPSCs do not fully recapitulate all aspects of mature human hepatocytes, but this is a general problem of hepatocyte-like cells derived from ESCs/iPSCs (20–22). Besides, they faithfully reproduce the main disease phenotype, namely copper-export defect, *in vitro*. The failure to mimic other specific liver functions does not represent in principle a caveat and in the future this may be overcome by improved tissue-culture methods (19). Recently, two groups reported iPSCs produced from liver genetic diseases including Crigler–Najjar syndrome, A1AT deficiency, familial hypercholesterolemia, glycogen storage disease and others (14,15). Rashid et al. (14) also described the existence of an *in vitro* phenotype for A1AT deficiency, familial hypercholesterolemia and glycogen storage disease type 1a based on the comparison with wild-type hepatocyte-like cells from control iPSCs. This work is relevant but cannot exclude that epigenetic abnormalities might affect the behavior of iPSCs and make them unable to overcome the phenotype upon genetic manipulation or drug treatment. For example, Kim et al. (27) described a tendency to accumulate epigenetic alterations in iPSCs. Reduced differentiation ability into specific lineages of iPSCs compared with ESCs has also been reported (28). However, our WD iPSCs displayed rather comparable differentiation into hepatocyte-like cells as human ESCs. The differentiated cells shared similar functional characteristics as well, except for a striking functional phenotype in copper transport that is not simply consequence of abnormal reprogramming as it could be reversed with gene therapy or drug treatment. To the best of our knowledge, our study is the first to report functional correction of hepatocyte-like cells derived from iPSCs of a patient with liver genetic disease.

Whole-liver transplantation is currently an option for WD but appropriate donors are scarce and the procedure risky. Cartier et al. (26) used a lentiviral self-inactivating vector to correct the disease in *in vitro* disease modeling. This vector is very similar in design to the one used in a recent successful gene therapy protocol by Cartier et al. (26). As a control for our \( \text{ATP7B} \) vector, we infected cells with a lentiviral vector expressing only GFP. Western blot verified over-expression of \( \text{ATP7B} \) protein (data not shown). In parallel, we tested the effect of curcumin, encouraged by its potential to improve \( \text{ATP7B} \) localization (Fig. 6B and C). \( \text{ATP7B} \) lentiviral vectors and treatment with curcumin both improved copper export in WD hepatocyte-like cells (Fig. 7C and D). We also measured copper export activity in undifferentiated ESCs/iPSCs and found it to be negligible (Supplementary Material, Fig. S2E). Altogether these data support the validity of our experimental setup and the quality of our hepatocyte-like cells from WD iPSCs to model the disease phenotype.
Figure 4. Generation and characterization of hepatocyte-like cells from WD iPSCs. (A) Scheme showing the stepwise protocol used for producing hepatocyte-like cells from WD iPSCs and the time needed. Below, phase contrast and immunofluorescence photographs for representative markers corresponding to each of the three major steps. Images correspond to WD iPSC-3. Scale bars of FOXA2, HNF4a, AFP (alpha-fetoprotein), A1AT and ASGPR indicate 50 μM, others 100 μM. (B–D) qPCR analysis for the indicated genes in WD iPSCs and derived hepatocyte-like cells, PHH cDNA was used as control. Values (mean of three replicates) are referred to human liver. A representative experiment is shown. (E) Representative western blot for ATP7B and ALB in the same cell types. Actin was used as loading control. The quantification (normalized by the actin levels) is shown below.
scaffold-supporting structure. Indeed, achieving appropriate vascularization of the transplanted islands may be sufficient. From a different perspective, an early general step for treating WD patients is the administration of copper chelators such as penicillamine. However, this has side effects and some patients do not respond well (1). Hence, it is desirable to find drugs that have an impact on ATP7B’s defective function rather than its consequence (copper accumulation). Our results using curcumin are encouraging, but this drug has limited oral bioavailability (30). High throughput screening using modifications of our copper-export assay may thus be relevant to find an alternative compound. Remarkably, there are over 300 identified ATP7B mutations that determine differences in clinical manifestations and possibly different susceptibility to drug administration as well (4,6). In the future, generation of iPSCs from a varied panel of patients with WD and differentiation into hepatocytes or neurons would be important to help establish phenotype/genotype correlations and predict drug susceptibility. Hepatocyte-like cells from WD iPSCs may also be helpful for studying signaling pathways activated by accumulated copper that trigger liver damage and an inflammatory response, and this may be another way to discover drugs that can treat the disease. Taken together, our study provides a valuable advance for studying and correcting WD that may be as well a good model for doing the same with other genetic liver diseases.

MATERIALS AND METHODS

Cell culture

WD patient fibroblasts were obtained from a dermal biopsy of a middle age Chinese Han male; consent form is available upon request. The Ethics Committee of the Guangzhou Institutes of Biomedicine and Health approved the procedure. R778L mutation was confirmed by sequencing of genomic DNA; primers are listed in Supplementary Material, Table S1. Fibroblasts were maintained in DMEM (Invitrogen, Rockville, MD, USA) + 10% (vol/vol) FBS (Hyclone, Logan, UT, USA). Then $4 \times 10^4$ fibroblasts at passage...
Defective subcellular localization of ATP7B in hepatocyte-like cells from WD iPSCs. (A) Representative double immunofluorescence staining shows that most if not all hepatocyte-like cells from WD iPSCs (iPSC-3) co-express ALB and ATP7B. Scale bars indicate 50 µm.

(B) Representative double immunofluorescence staining for ATP7B and p230 in IMR90 iPSCs (90 iPSC-2) and WD iPSCs (WD iPSC-3) and their corresponding hepatocyte-like cells. The two proteins co-localize in the trans-Golgi network in hepatocyte-like cells derived from normal iPSCs but not WD iPSCs (similar results were obtained with iPSC-1, data not shown). Curcumin partially restored ATP7B localization in hepatocyte-like cells from WD iPSCs (similar results were obtained with iPSC-1, data not shown). Zoomed images are shown in the corner for all cell types and conditions. Scale bars indicate: 90 iPSC-2, WD iPSC-3 and WD iPSC-3-Hep panels = 50 µm, 90 iPSC-2-Hep and WD iPSC-3-Hep panels = 25 µm. (C) Quantification of cells showing co-localization of ATP7B and p230. Five random fields were counted and the mean calculated, a representative experiment is shown. (D) Representative western blotting shows that expression of ATP7B in hepatocyte-like cells from a WD iPSC clone does not change significantly upon treatment with curcumin.
3 were transduced in fibroblast medium with a cocktail of retroviruses as reported before (11,12). At Day 2, post-infection medium was changed to DMEM/F12 (Invitrogen) + 20% dFBS (Hyclone) + Vc (sodium L-ascorbate, 50 μg/ml; Sigma, St Louis, MO, USA). At Day 6, infected cells were seeded on feeders (mitotically inactivated-murine embryonic fibroblasts) and cultured in standard human ESC medium consisting of DMEM/F12 plus 20% Knockout serum replacement.

Figure 7. Detection of copper-export defect in hepatocyte-like cells from WD iPSCs. (A) Schematic representation of the protocol used for detecting copper export in hepatocyte-like cells produced from iPSCs or ESCs. (B) FACS analysis showing the number of ATP7B positive cells in a representative copper-export experiment using hepatocyte-like cells from WD iPSCs (including WD iPSC-3-Hep, WD iPSC-3-Hep-ATP7B, WD iPSC-3-Hep-GFP, WD iPSC-3-Hep-curcumin), normal iPSCs and ESCs. Only one item for each cell type is shown; an isotype control antibody was used for selecting the gate. (C and D) Detection of exported copper in the cell supernatant after copper supplementation for 24 h to the indicated hepatocyte-like cells. Export was measured at different time points. A representative experiment (same as in B for C) is shown with samples measured in triplicate.
(KSR, Invitrogen), 50 IU/ml penicillin/streptomycin (Hyclone), 1 mM glutamine (Invitrogen), 0.1 mM non-essential amino acids (Invitrogen), 0.1 mM beta-mercaptoethanol (Invitrogen) and 8 ng/ml bFGF (Shenzhen Symmix Industry, Shenzhen, China). VPA (1 mM; Merck, Darmstadt, Germany) was added from Day 7–20. Human ESC-like colonies appeared around Day 20 post-infection and were picked manually at around Day 30. Picked iPSCs and human ESCs were routinely maintained on feeders in human ESC medium or on Matrigel (BD, Franklin Lakes, NJ, USA) in mTeSR1 (Stemcell Technologies, Madison, WI, USA). PHH were purchased from Lonza (Walkersville, USA) and cultured on rat-tail type 1 collagen-coated plates with HCM™ Bullet-Kit (Lonza) according to the manufacturer’s instructions. RNA extraction [with Trizol (Invitrogen)], protein lysate extraction Kit (Lonza) according to the manufacturer’s instructions. Microarrays were scanned and data analyzed using the Partek Genomic Suite 6.5 software (Partek, St Louis, MO, USA). Data have been deposited in the GEO database (accession number GSE24997). For EB differentiation, iPSCs on feeders were treated with dispase (Invitrogen) and collected by scraping. After centrifugation, cell pellets were re-suspended in human ESC medium or on Matrigel (BD, Franklin Lakes, NJ, USA) in mTeSR1 (Stemcell Technologies, Madison, WI, USA). PHH were purchased from Lonza (Walkersville, USA) and cultured on rat-tail type 1 collagen-coated plates with HCM™ Bullet-Kit (Lonza) according to the manufacturer’s instructions. RNA extraction [with Trizol (Invitrogen)], protein lysate extraction and functional assays using PHH were performed or started at Day 2 of culture.

iPSC characterization

AP staining, transgene integration, karyotyping, STR analysis and bisulfate sequencing were performed as described (16). Genomic DNA was extracted using the DNeasy Tissue kit (Qiagen, Hilden, Germany). qPCR was performed using a Thermal Cycler DiceTM Real Time System (ABIT300, ABI, Foster, CA, USA) and SYBR Green Premix EX TaqTM (Takara, Shiga, Japan); β-actin was used for normalization and all items were measured in triplicate. qPCR and semi-quantitative PCR primers used in this study are summarized in Supplementary Material, Table S1 (31). DNA microarrays were performed with Affymetrix GeneChip HuGene 1.0 ST array (Affymetrix, Santa Clara, USA) according to the manufacturer’s instructions. Microarrays were scanned and data analyzed using the Partek Genomic Suite 6.5 software (Partek, St Louis, MO, USA). Data have been deposited in the GEO database (accession number GSE42997). For EB differentiation, iPSCs on feeders were treated with dispase (Invitrogen) and collected by scraping. After centrifugation, cell pellets were re-suspended in human ESC medium without bFGF and grown for 8 days in non-adherent dishes. EBs were transferred to gelatin-coated dishes to allow differentiation for another 8 days before processing for immunofluorescence analysis. For teratomas, 2 × 10⁶ WD iPSCs were injected subcutaneously or intramuscularly into immune-compromised NOD-SCID mice. Tumors were excised 8 weeks later and fixed, embedded in paraffin, sectioned and stained with hematoxylin/eosin.

Immunofluorescence microscopy

Cells were fixed in 4% paraformaldehyde for 30 min, washed, blocked and permeabilized in blocking solution (PBS containing 3% normal goat serum and 0.2% Triton X-100) for 30 min. Then they were incubated with primary antibodies in blocking solution at 4°C overnight, washed twice and incubated with the corresponding secondary antibodies for 1 h at room temperature. Cells were washed twice and stained with DAPI (Sigma) for 5 min, and then photographed using a LEICA DMI6000B microscope (Leica Microsystems GmbH, Wetzlar, Germany). All primary antibodies used in this article are listed in Supplementary Material, Table S1.

Western blotting

Cells were washed twice with PBS, scraped off and lysed in RIPA [50 mM Tris-Cl, 70 mM 2-mercaptoethanol, 2% sodium dodecylsulfate (SDS)] supplemented with protease inhibitor cocktails (RocheApplied Science, Indianapolis, USA). Gel was lysates were subjected to 10% polyacrylamide–SDS gel electrophoresis and transferred to a nitrocellulose membrane. After blocking with 5% non-fat dried milk in TBST (50 mM Tris–HCl at pH 8.0, 150 mM NaCl, 0.1% Tween 20), the membrane was incubated with the TBST-diluted primary and secondary antibodies. Signals were detected with Amer sham ECL Advance Western Blotting Detection Kit (Amer sham Pharmacia Biotech, Piscataway, NJ, USA). Quantification was performed using Quantity One Software (BioRad). Antibodies used for western blotting are listed in Supplementary Material, Table S1.

Flow cytometry

The differentiated cells were trypsinized (0.05% trypsin; Invitrogen) for 10 min and resuspended in fibroblast medium. The single cells were fixed in 4% paraformaldehyde for 15 min and permeabilized in 0.2% Triton X-100 for 20 min for ALB and ATP7B detection except for ASGPR. Both primary and secondary antibody incubation were carried out according to the manufacturer’s instruction. Control samples were stained with isotype-matched control antibodies. After washing, the cells were resuspended and preceded for flow cytometry (BD FACSAria, San Jose, CA, USA). The antibodies used for flow cytometry are listed in Supplementary Material, Table S1.

Plasmids

Retroviral plasmids producing human OCT4, SOX2, KLF4 and c-MYC transcription factors were purchased from Addgene (Cambridge, MA, USA). The 4.3 kbp ATP7B cDNA was codon optimized (adapted to preferred human codon usages to improve expression) and cryptic polyadenylation and splice sites as well as RNA secondary motifs were deleted. The cDNA was synthesized (GeneArt, Regensburg, Germany) in two parts and later united to yield an ATP7B cDNA flanked by AgeI and SalI sites. ATP7B was inserted into a third-generation self-inactivating lentiviral vector (pRRL.PPT.SF.GFPpre) (24,25), driven by a spleen focus-forming virus U3 promoter and equipped with a woodchuck post-transcriptional regulatory element for improved viral titer and expression. For better detection, an IRES co-expression cassette with an EGFP fluorescent marker was introduced as an SalI fragment. The BsrGI site of EGFP also incorporates a BsrGI fragment of the blastidicin resistance gene (from LeGO-G/BSD, kindly provided by Kristoffer Weber and Boris Fehse, Hamburg, Germany). To produce infectious ATP7B lentiviral particles, HEK293T cells cultured on 10 cm dishes were transfected with pRRL.PPT.SF.ATP7B.i2GFPBsd.pre together with the packaging plasmids.
psPAX2 and pMD2.G. Viral supernatants were harvested on two consecutive days starting 48 h after transfection; these supernatants were administered in four rounds from Day 8 to 11 during hepatocyte-like differentiation.

Hepatocyte-like cell differentiation
The protocol used for in vitro differentiation towards hepatocyte-like cells has been described previously (18). Activin A and oncostatin M (OsM) were purchased from R&D System (Minneapolis, MN, USA), BMP2, FGF4, HGF and KGF from PeproTech and dexamethasone (Dex) from Enzo Life Sciences (Farmingdale, USA). RPMI 1640, N2 and B27 were purchased from Invitrogen. ALB secretion was performed using a kit from Koma Biotech (Seoul, South Korea). Urea was detected using a urea nitrogen determination system kit (Kehua Dongling, Shanghai, China) by ultraviolet-glutamic dehydrogenase method after incubating cells with medium containing 5 mM of NH₄Cl (Sigma) for 24 h. Cytochrome P450 activity was detected using P450-Glo™ Assay from Promega (Madison, USA). PAS staining was performed using a kit purchased from Polysciences (Warrington, USA). Curcumin was purchased from Sigma and added at 5 μM for 48 h. Phenobarbital was purchased from Guangdong BangMin Pharmaceutical (Guangzhou, China).

Copper measurement
At Day 23 after induction, differentiated cells were treated with 100 μM of CuCl₂ (Sigma) for 24 h and then washed three times with PBS before copper-free medium was added. In the following 9 h, the cell-culture supernatant was sampled every 1.5 h for copper measurement with atomic absorption spectrophotometer (Hitachi, Tokyo, Japan). Where indicated, cells were pre-treated with curcumin. Accumulated copper concentrations at each time point were normalized to the number of ATP7B positive cells analyzed by flow cytometry (BD FACSARia) using a red fluorochrome.

Statistical analysis
Analysis was performed with one-way ANOVA and the Bonferroni post hoc test using SPSS 13.0 software.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

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