Published in final edited form as:

Methods Mol Biol. 2017 ; 1506: 161–178. doi:10.1007/978-1-4939-6506-9_11.

Demonstrating Potential of Cell Therapy for Wilson's Disease with the Long-Evans Cinnamon Rat Model

Fadi Luc Jaber, Yogeshwar Sharma, and Sanjeev Gupta

Abstract

Wilson's disease (WD) is characterized by the inability to excrete copper (Cu) from the body with progressive tissue injury, especially in liver and brain. The molecular defect in WD concerns mutations in *ATP7B* gene leading to loss of Cu transport from the hepatocyte to the bile canaliculus. While drugs, e.g., Cu chelators, have been available for several decades, these must be taken lifelong, which can be difficult due to issues of compliance or side effects. Many individuals may require liver transplantation, which can also be difficult due to donor organ shortages. Therefore, achieving permanent cures via cell or gene therapy are of great interest for WD. Cell therapy is feasible because transplanted hepatocytes can integrate in liver parenchyma and restore deficient functions, including transport of Cu into bile. The availability of authentic animal models that recapitulate hepatic WD, especially the Long-Evans Cinnamon (LEC) rat, has advanced cell transplantation research in WD. We describe requirements for cell therapy in animal models with several standardized methods for studies to test or refine cell therapy strategies in WD.

Keywords

Wilson's disease; Biliary transport; Cell transplantation; Copper; Liver injury; Regeneration

1 Introduction

1.1 Scope of Cell Therapy in WD

In 1912, the renowned neurologist, Samuel Alexander Kinnier Wilson, reported cases of the so-called hepatolenticular degeneration with substantial liver and brain damage. This condition was eventually named after him. Later, Cu toxicosis was found to be central to WD, but it was only in early 1990s that molecular defects in *ATP7B* gene were assigned causal significance for WD [1]. The onset of WD requires homozygous mutations in *ATP7B* alleles, which is relatively rare, and is estimated to occur in only 1–4 cases per 100,000 individuals [2]. No single *ATP7B* mutation is responsible for WD. These mutations tend to be largely sporadic and over 300 *ATP7B* mutations have been annotated to date. The *ATP7B* gene encodes for a P-type ATPase, which is highly conserved during evolution, and is necessary for transporting Cu into biliary excretory pathways exclusive to hepatocytes [3]. The diagnosis of WD is challenging because its onset is typically insidious and patients may come to medical attention only in advanced stages. However, WD offers excellent opportunities for cell/gene therapy in view of specific pathophysiological perturbations that can be resolved by restoring ATP7B protein function.

The hepatic pathology related to Cu toxicosis is complex with persistent oxidative stress, DNA damage, hepatocyte injury, inflammation, fibrosis, decompensation, and eventually death as major clinical consequences. In a small minority of cases (<1–5 %), WD may present with acute liver failure. Other clinical presentations include neuropsychiatric manifestations and motor disorders (tremors, ataxia, dystonia) [2]. WD may manifest with Kayser–Fleischer rings in cornea or with consequences of renal tubular acidosis. Left untreated WD is progressive and fatal. However, medical treatments are available with appropriate use of drugs, such as zinc and chelating agents (penicillamine, trientine, etc.) [2]. Since drugs must be taken daily compliance issues may arise over a life-time. Among alternative therapies, orthotopic liver transplantation (OLT) is most significant, since this can restore Cu excretion. But OLT is hampered by donor organ shortages, technical complexities, cost, and inadequate or no resolution of neurological complications [4].

Therapeutic approaches, such as cell or gene therapy, are of interest in particular for curing WD permanently [5]. These approaches are driven by following major considerations:

1. Successful regeneration of liver with healthy hepatocytes offers opportunities for restoring physiologically regulated ATP7B protein function and Cu excretion;
2. In acute liver failure, by offering metabolic support and paracrine factors to promote liver regeneration, transplanted cells may serve as a bridge to OLT.
3. Brain injury may subside as excessive Cu is cleared by healthy hepatocytes from the body;
4. Liver pathology, e.g., fibrosis, may regress after appropriate replacement of liver with healthy cells and restoration of Cu homeostasis;
5. Quality of life may improve when organ function is restored with cell therapy and drugs are no longer needed for Cu removal.

The various ways by which transplanted cells with or without additional modifications to express gene constructs, might induce liver regeneration or ameliorate hepatic fibrosis or inflammation, are summarized (Fig. 1). For WD, curative approaches will require successful engraftment and proliferation of transplanted cells, besides the availability of cellular functions required to excrete Cu from the body. This necessitates that transplanted hepatocytes are integrated in liver parenchyma with restoration of bile canalicular network, which is normally disrupted during enzymatic release of donor hepatocytes. On the other hand, temporary liver support could be achieved by transplanting cells into nonhepatic locations, e.g., ectopic sites, such as peritoneal cavity, where vascularization may proceed when suitable scaffolds are included [6]. However, in this case Cu cannot be excreted outside the body since bile must travel through blood to the native liver for excretion, but ATP7B protein will be absent in the WD liver. If cell transplantation could ameliorate liver injury or inflammation, another benefit could be realized. Also, the potential of additional cell populations, e.g., liver progenitor cells, stem cell-derived cells, etc., is of considerable interest. Taken together, the concepts for cell therapy in WD are particularly exciting in view of the availability of suitable animal models for cell transplantation studies.

1.2 Translational Requirements for Cell Therapy in WD

Although hepatocyte transplantation has been undertaken in people for multiple acquired or genetic conditions, clinical trials of cell therapy are yet to be implemented in WD [7, 8]. The clinical course of WD is such that patients typically come to medical attention only when liver or brain injury is already established [2]. Prospective diagnosis of WD through genetic or other testing is rare. Therefore, a major requirement for cell transplantation research is that animals should be treated under similar conditions of preexisting or established disease. The parameters for evaluating liver-directed cell therapy should be as follows. Here, the issue of neurological damage is not considered since available animal models do not reproduce WD-associated brain damage.

1. Establishing cell transplantation regimen (i.e., delivery route, frequency, cell dosing). For integration of transplanted cells in the liver parenchyma, cells are best deposited in liver sinusoids, such as by transplantation into either portal vein or its upstream vessels. In small animals, this is most simply achieved by transplanting cells into splenic pulp followed by blood flow-related migration of cells into splenic vein and then into the portal vein, which take place instantaneously.
2. Tracking the fate of transplanted cells with suitable reporters to define mechanisms in cell engraftment, proliferation and liver repopulation. Normally, transplanted hepatocytes are mostly cleared (>70 % cells) by cell transplantation-induced inflammation but strategies to curb these cell losses are being developed [9].
3. To achieve suitable extent of liver repopulation in WD (e.g., 30–50 % of the liver) clinically applicable strategies for inducing proliferation in transplanted cells must be identified in the context of Cu toxicosis. Studies of transplanted cell proliferation in animal models of WD provide significant information regarding the efficacy of preconditioning regimens although those currently useful in animals, e.g., radiation, chemicals, and surgery [10 – 12], are not readily translated for clinical trials in people.
4. Establishing correction of Cu transport in bile through *ATP7B* expression by noninvasive methods that identify bile Cu excretion will be a great advantage for clinical trials, as was recently demonstrated in rat and mouse models of WD [13 – 15].
5. Improvements in parameters of liver injury, inflammation or fibrosis are important for establishing therapeutic benefits. This may require interval liver biopsies or suitable blood tests and/or imaging parameters.
6. The ability to transplant allogenic or xenogenic cells will require effective immunosuppressive regimens both in animals and people. Animals in appropriate immunodeficient backgrounds will be helpful for immunological research, although this area is evolving and is beyond the scope of the discussion here.

1.3 Animal Models for Cell Therapy in WD

The most significant models of interest for WD concern the Long-Evans Cinnamon (LEC) rat, which arose in a colony of Long-Evans Agouti (LEA) rats through natural deletion of the distal 3' region of *Atp7b* gene [16], and the *Atp7b*^{-/-} knockout mouse, which was created by inactivating the *Atp7b* gene [17]. Unlike various other animal models of WD, including large animals, these animals reproduce *Atp7b*-dependent Cu toxicosis with significant liver damage over time. However, neurological damage associated with WD is observed in neither LEC rat nor *Atp7b*^{-/-} mouse for unknown reasons. On the other hand, as these animals may be crossed with additional animal strains to localize transplanted cells, their utility for cell transplantation research becomes amplified. For instance, transplantation of healthy hepatocytes into mouse or rat strains lacking expression of the cell surface protein, dipeptidyl peptidase 4 (DPPIV), has been highly effective for localizing transplanted cells and studying mechanisms in transplanted cell engraftment, proliferation or function [9]. These DPPIV - animals serving as recipients for syngeneic mouse or rat donor cells for cell transplantation studies are available in our laboratory. Transplanted cells may also be localized in LEC rats or *Atp7b*^{-/-} mice by other approaches, e.g., by staining for healthy versions of native ATP7B protein or by using stains for fluorescence of exogenous transgene reporters, such as green fluorescent protein (GFP), or another such marker, as has been done in many instances. Of course, it should be noteworthy that in some instances immune responses against transgene reporters, e.g., against GFP, may introduce confounding variables since transplanted cells may simultaneously be cleared. Transplantation of allogeneic donor cells in LEC rats or *Atp7b*^{-/-} mice allows studies of rejection mechanisms whereas after crossing of these animals into immunodeficient strains, additional models may be obtained for studies of further immunological mechanisms and also for pursuing xenografts.

For simplicity in reporting methods for the uninitiated, we focus on transplantation of freshly isolated healthy hepatocytes via the spleen, which has been widely adopted for cell transplantation studies in mice and rats.

2 Materials

All chemicals and reagents should be obtained from validated commercial suppliers. We use sterile deionized water in laminar flow cabinets to avoid microbial contamination of solutions. In preparing buffers and performing liver perfusion we pay careful attention to pH, temperature, and conditions for gassing as described elsewhere in protocols from our institution [18]. Typically, we isolate primary hepatocytes from 8- to 12-week-old healthy donor rats or mice by two-step perfusion of the liver with either mixture of bacterial collagenases and other enzymes or blend of recombinant collagenase (Liberase TL, Roche Life Sciences, Indianapolis, IN, USA). Waste disposal, including that of sharps, should be done in conformity with institutional guidelines and regulations.

2.1 Surgical Supplies

All instruments and surgical supplies are autoclaved before use as appropriate.

1. Straight scissors (3–5 in.).

2. One pair of iris scissors.
3. Non-traumatic forceps (large and small; full curve).
4. Angiocaths of 18-19G (rat) or 23-25G (mouse).
5. PE 60 tubing (Dow Corning, Auburn, MI, USA) for use as cannulae for bile collection.
6. Hemostats.
7. Sterile cotton-tipped applicators.
8. Cotton gauze.
9. 70 % ethanol.
10. Povidone–iodine solution.
11. Absorbable vicryl sutures.
12. Wound closing kit (9 mm clips and applicator; Stoelting Co., Wood Dale, IL, USA).
13. Fur clippers.
14. Perfusion components (*see below in Subheading 2.5*).
15. Peristaltic pump capable of delivering 4–60 mL/min flow rates through silastic tubing for gas exchange.
16. Anesthetic and analgesic drugs: isoflurane, ketamine, xylazine, and acepromazine.

2.2 Cell Processing Supplies

1. 15–50 mL sterile serological tubes.
2. Roswell Park Memorial Institute (RPMI)-1640 medium or Dulbecco's Modified Eagle's Medium (DMEM).
3. Phosphate buffered saline, pH 7.2 (PBS).
4. 100–200 mL Sterile glass beakers.
5. 100 mm diameter sterile petri dishes for liver dissociation.
6. To separate dissociated hepatocytes from tissue, 80- μ m dacron mesh (Tetko Inc., Briarcliff Manor, NY, USA). This mesh is autoclaved beforehand and then secured to the top of autoclaved glass funnels with either rubber bands or paper tape.
7. To count cells and determine viability: 0.4 % trypan blue.
8. Neubauer Chamber.
9. Cell culture supplies with cell culture-grade plastic dishes.

10. Fetal bovine serum and cell culture-grade antibiotics (penicillin and streptomycin).
11. Refrigerated centrifuge.

2.3 Tissue Sectioning and Staining Supplies

1. Glass or plastic containers for formalin fixation (*see Note 1*).
2. Cryopreservation vials for storing frozen tissue.
3. Methylbutane for tissue freezing (*see Note 2*).
4. Cryostat.
5. Fixation of tissue sections with acetone/chloroform or absolute ethanol.
6. Charged glass microscopy slides and glass coverslips.
7. Harris hematoxylin and eosin stains.
8. Enzyme histochemistry for DPPIV activity: Gly-Pro-4-methoxy- β -naphthylamide (dissolved in *N,N*-dimethylformamide), Fast blue B salt.
9. Antibodies for identifying cell proliferation: Ki-67 (catalog number 550609) (BD Biosciences, San Jose, CA, USA) (*see Note 3*).
10. Microscopes with objectives for 40 $\times$, 100 $\times$, 400 $\times$, and 1000 $\times$ magnification and image acquisition capacity.
11. Image analysis software (e.g., ImageJ, NCI, Bethesda, MD, USA).

2.4 Animals

1. Cell donors (8–12 week old healthy inbred LEA rats or healthy inbred C57BL/6 mice) (*see Note 4*).
2. Wilson's disease animals as cell recipients (6–10 week old LEC rats or *Atp7b*^{-/-} mice in C57BL/6 background).
3. Certified facilities for animal housing and care in compliance with institutional, state and national regulations. Animals are typically housed under 14-h light and 10-h dark cycles with free access to food (standard rodent chow containing 11.8 mg copper/kg) and tap water.

2.5 Perfusion Buffers [18]

1. 10 $\times$ Leffert's buffer: 100 mM HEPES, 30 mM KCl, 1.3 M NaCl, 10 mM NaH₂PO₄, 100 mM D-glucose. Use 0.22 μ m filter to sterilize, store at 4 °C.
2. 1 $\times$ Leffert's buffer: 100 mL 10 $\times$ Leffert's buffer, 900 mL deionized or distilled H₂O. Adjust pH to 7.4. Use 0.22 μ m filter to sterilize and store in water bath at 37 °C until perfusion.

3. 10× EGTA solution: 1.9 g EGTA, 100 mL 10× Leffert's buffer, 800 mL H₂O dest. Use 10 N NaOH for titration of pH to 7.4. Adjust the final volume to 1 L. Use 0.22 µm filter to sterilize and store at 4 °C.
4. 1× EGTA solution: 30 mL 10× EGTA solution, 270 mL 1× Leffert's buffer. pH 7.4. Use 0.22 µm filter to sterilize, keep at 37 °C.
5. 100× CaCl₂ solution: 27.9 g CaCl₂ to 1 L deionized or distilled H₂O. Use 0.22 µm filter to sterilize, store at 4 °C.
6. Perfusion and washing buffer with CaCl₂ solution: 6 mL 100× CaCl₂ solution for adding to 600 mL 1× Leffert's buffer, pH 7.4. Use 0.22 µm filter to sterilize 300 mL, store on ice.
7. Just before use prepare liberase in 300 mL buffer containing CaCl₂ solution (*see* Subheading 2.5, **item 6**), using 7 unit liberase/100 mL for mouse and 14 units liberase/100 mL for rat. pH 7.4. Use 0.22 µm filter to sterilize, keep at 37 °C.

3 Methods

3.1 Handling of Donor Hepatocytes

1. Hepatocytes are isolated by two step perfusion of the liver starting with 1× Leffert's buffer, followed by 1× EGTA solution and finally perfused with liberase containing CaCl₂ solution for digestion.
2. Hepatocytes are separated by differential centrifugation in washing buffer under $50 \times g$ for 1 min at 4 °C thrice. Finally, cells per suspended in medium without serum (DMEM or RPMI-1640).
3. An aliquot of cells is analyzed with 10× dilution using PBS and stained with 0.4 % trypan blue. Cells are counted manually in Neubauer chamber and the fraction excluding trypan blue is recorded.
4. Cells are used for transplantation studies only when viability exceeds 75 %. The concentration of cells is adjusted to $20\text{--}40 \times 10^6$ per mL DMEM or RPMI-1640 without serum.
5. Periodically, the ability of hepatocytes to attach in cell culture dishes is tested for quality control. This requires use of cell culture medium with 10 % fetal bovine serum and antibiotics.
6. Tubes containing cells are kept immersed in wet ice until transplantation.
7. Cells are transplanted into animals within 2 h after cell isolation.

3.2 Surgery for Cell Transplantation

1. Animals are allowed normal access to food and water.
2. Surgery is performed under aseptic techniques with autoclaved instruments.
3. The animal is anesthetized with either inhaled isoflurane to effect or intraperitoneal injection of ketamine–xylazine mixture (*see* **Note 5**) with

monitoring of eye blinking, corneal reflexes and toe-pinch withdrawal responses, etc., to ensure surgical plane of anesthesia has been reached prior to incisions.

4. The animal is placed sideways with left side of the abdomen facing upward and is gently secured to the platform with surgical adhesive tape.
5. The subcostal region of upper left abdomen is prepared by clipping fur and povidone–iodine is applied with a piece of gauze held in a hemostat or forceps, and excess is removed with 70% ethanol.
6. To access spleen, a 1 cm laparotomy incision is made in the subcostal region overlying the splenic area. The spleen is isolated and brought out of the abdomen by gently holding its mesentery with non-traumatic forceps.
7. To secure hemostasis and avoid leakage of injected cells, a ligature of 4-0 absorbable vicryl is applied approximately 0.5 cm from the lower tip of spleen and gently tightened without damaging tissue. A portion of splenic mesentery is typically included in this ligature.
8. Cells are drawn into 1 mL syringe with 25G needle (*see Note 6*).
9. Cells are injected directly into the splenic pulp (Fig. 2). The needle is inserted into the lower pole of the spleen inside the ligature for hemostasis. Cells are injected as a bolus over 12–15 s with observation for subcapsular entry, excessive splenic distension, or passage of needle through the splenic pulp with hemorrhage or spillage of cells into the peritoneal cavity. Blanching of splenic pulp during injection of cells is to be expected.
10. Previously placed ligature around lower pole of the spleen is tightened further, if needed, when the needle is withdrawn. Hemostasis is ensured by observation of the injection site for a few seconds.
11. The spleen is gently returned to the abdomen with a sterile surgical cotton-tipped applicator stick.
12. The inner abdominal wall is closed with absorbable sutures.
13. The skin incision is closed with surgical clips using designated applicator.
14. An analgesic is administered, animals are kept warm with heating lamp, and observed until recovery from anesthesia (*see Note 7*).
15. Animals are returned to their cages with resumption of normal food and water intake and usual activities. Postoperative care is provided. Surgical clips are typically removed after 7 days.

3.3 Cell Transplantation Procedure for Engraftment Analysis

1. Animals are not prepared beforehand with liver conditioning regimens.
2. Cells are transplanted as in Subheading 3.2.
3. Groups of animals ($n = 3–6$) are observed for interval analysis, typically 1 day, 3 days, and 7 days after cell transplantation.

4. For cell engraftment analyses, animals are sacrificed, liver is harvested, and samples from each lobe of the liver are either frozen in methylbutane cooled to -80°C or fixed in formalin for embedding in paraffin and subsequent sectioning. Tissue sections of 4–6 μm thickness are then processed in conventional ways for hematoxylin and eosin staining for evaluation of tissue integrity or processed for histochemical or immunostaining procedures for detecting transplanted cells (e.g., DPPIV staining) (*see Note 8*).
5. Morphometric analysis of cell numbers and distributions in vascular spaces or parenchyma of liver lobule are performed (*see Subheading 3.5*).

3.4 Cell Transplantation Procedure for Proliferation Analysis

1. Animals are either without prior conditioning or are subjected to liver preconditioning regimens to induce transplanted cell proliferation. A frequently employed preconditioning regimen is to give rats 30 mg/kg i.p. of the pyrrolizidine alkaloid, retrorsine, twice at 6 and 8 weeks of age, followed by partial hepatectomy after another 4 weeks with cell transplantation either immediately after partial hepatectomy or up to 7 days after partial hepatectomy, which requires another surgical procedure, of course (*see Note 9*).
2. Cells are transplanted as in Subheading 3.2.
3. Groups of animals ($n = 3\text{--}6$ each) are established for interval analysis, e.g., 1 month, 3 months, 6 months or 12 months after cell transplantation (*see Note 10*).

3.5 Localization of Transplanted Cells and Morphometric Analysis

1. Tissue sections are stained for reporters (e.g., DPPIV) to enable localization of transplanted cells in recipients (*see Note 11*). Alternatives include staining for ATP7B protein in donor hepatocytes [13] (Fig. 3) (*see Note 12*).
2. For morphometric analysis of cell engraftment, the number of transplanted hepatocytes in the liver is analyzed from multiple animals (*see Note 13*). A minimum of 50 consecutive portal areas per liver lobe and a minimum of 250 consecutive portal areas per animal are scored for transplanted cells.
3. For transplanted cell proliferation, multiple images of sections per liver lobe per animal are captured at 40 $\times$ magnification. Imaging software is used to analyze liver area occupied by transplanted cells. The extent of liver repopulation is determined by measuring the liver area occupied by transplanted cells as a fraction of the entire liver area examined.
4. Statistical methods for data analysis are utilized with P values (*see Note 14*).

3.6 Assays for Tissue Injury Grading and Related Assays

1. H&E staining of tissue sections is used to identify differences in healthy and Wilson's disease conditions with histological parameters [19] (Table 1) (*see Note 15*). This takes into account unique aspects of Cu toxicosis, which include polyploidy (enlarged cell nuclei containing greater than diploid DNA), steatosis

(cell injury), apoptosis (cell loss), or mitosis (regenerative activity) (Fig. 4) (*see Note 16*). The final histological grade yields substantial differences in healthy livers (typical grade 0-1) and WD livers (maximal injury grade of 13) to provide a range for changes over time.

2. Serological assays for liver tests (albumin, aminotransferases, bilirubin, alkaline phosphatase, ceruloplasmin) (*see Note 17*).

3.7 Assays for *Atp7b* mRNA Expression After Cell Transplantation

1. Liver samples are collected by snap freezing in liquid nitrogen and stored at -80°C .
2. *Atp7b* mRNA expression analysis is performed by reverse transcription—polymerase chain reaction (RT-PCR) [10], ideally by quantitative assays (*see Note 18*).
3. Detection of *Atp7b* expression in liver by RT-PCR typically requires significant liver repopulation and is usually insufficient for evaluating cell engraftment alone.

3.8 Assays for Liver and Bile Copper Content

1. To measure liver Cu levels, liver tissue samples are stored frozen.
2. To measure stimulated bile Cu excretion, bile is collected by cannulating bile duct before and after intrasplenic injection of Cu-histidine as a bolus [20] (Fig. 5).
3. Bile duct cannulation requires anesthesia of the animal for up to 1 h or longer with isoflurane or ketamine–xylazine.
4. After skin preparation, a midline laparotomy incision is made in upper abdomen extending from below the xiphoid process to below the line of the liver.
5. The duodenal loop is gently pulled out of the incision, common bile duct is identified and isolated with gentle dissection.
6. The lower end of the common bile duct is ligated with 3-0 thread.
7. A second ligature is placed around the middle part of the common bile duct for securing the cannula later.
8. A nick is made approximately 1 cm above the entry of common bile duct into the duodenum with an iris scissors to allow insertion of cannula fashioned from PE-60 tubing.
9. The cannula is gently advanced by 2–3 cm in bile duct toward the liver and secured by tying previously placed ligature. After successful cannulation, bile begins to flow immediately and should be clear without blood. If blood is seen this would indicate trauma to the duct, especially if bleeding persists.

10. Bile is typically collected for 1 h with sampling before and after tracer dose of Cu.
11. Tissue and bile copper are measured by graphite furnace atomic absorption spectroscopy. In preparation for spectroscopy, tissues are desiccated at 65 °C under vacuum overnight. The assays are performed with solubilization of Cu in bile and tissues with nitric acid.

3.9 Noninvasive Positron Emission Tomography (PET) with Radio-Cu for Demonstrating Mobilization of Cu from Liver

1. This method offers rapid and dynamic assessment of Cu excretion over short- or long-term by noninvasive imaging of biliary copper excretion.
2. Animals are studied by dynamic PET recordings after a tracer dose of (64)Cu complexed to L-histidine is administered to animals either via tail vein or spleen [13–15] (Fig. 6).
3. Loss of hepatic (64)Cu activity is examined by clinically used image analysis software [13–15].
4. This is a specialized procedure that requires access to a cyclotron for generating (64)Cu, along with equipment for imaging small animals and expertise in nuclear medicine procedures.

4 Notes

1. Liver tissue fragments of ~0.5 cm size are fixed in formalin, embedded in paraffin, and sectioned to 4–5 µm thickness on rotary microtome.
2. Liver tissue fragments of ~0.5 cm size are placed in prechilled methylbutane at –80 °C overnight. Each fragment is mounted on pieces of cork for cryosectioning.
3. For Ki-67 immunostaining, liver sections are fixed in 4 % paraformaldehyde in PBS, pH 7.4, blocked in 3 % goat serum for 1 h, incubated for 1 h with mouse anti-Ki-67 (1:150 dilution), and then for 1 h with peroxidase-conjugated goat anti-mouse IgG (1:300) (Sigma-Aldrich). Color is developed with diaminobenzidine (DAB) (catalog number K3467; Dako North America, Carpinteria, CA, USA).
4. Various strains of donor animals may be used for allogenic cell transplantation. Animal models based on reporter constructs in donor cells (e.g., GFP or another marker) can be effective for localization of transplanted cells. Human cells may also be examined for xenografting with use of human-specific probes for localization of transplanted cells although this will require suitable immunodeficient backgrounds of recipient animals.
5. Combine 1.5 mL of 100 mg/mL xylazine and 10 mL of 100 mg/mL ketamine for rats.

6. Syringes may be drawn in advance prior to surgery and kept on ice.
7. The animal can be placed on a thermal pad for heating (Braintree Scientific).
8. Additional analyses for ischemic injury, inflammation, Kupffer cell activation, and gene expression may be conducted at this stage.
9. Retrosine is a plant-derived pyrrolizidine alkaloid which impairs liver regeneration by forming DNA adducts and confers proliferative advantage to undamaged transplanted hepatocytes. Alternative methods for preconditioning liver include radiation combined with hepatotoxic chemicals, partial hepatectomy, ischemia–reperfusion injury and portal vein ligation, and also combinations of monocrotaline (another plant-derived pyrrolizidine alkaloid) and drugs (rifampicin, phenytoin) or monocrotaline and partial hepatectomy.
10. At least three animals should be analyzed per condition.
11. For analysis, 4–5 μm -thick cryosections are mounted on glass slides, air-dried overnight and fixed for 10 min in acetone/chloroform (vol/vol). Sections are incubated for 20–30 min in DPPIV substrate followed by counterstaining with hematoxylin or another nuclear stain. Sections are dried and mounted in glycerol for microscopy.
12. If transplanted cells are tagged with transgenic reporters (e.g., GFP), immunostaining for these proteins may be performed at this stage.
13. Analysis is preferably conducted by blinded observers; all liver lobes should be included for comparisons. If one lobe is chosen, the same lobe should be analyzed in all animals.
14. Significances are analyzed by t test, chi-square test, or analysis of variance (ANOVA) with Tukey's test or another ad hoc test for pair-wise comparisons of means. *P* values <0.05 are considered significant.
15. Liver samples are fixed in 10 % buffered formalin. Paraffin-embedded sections are prepared and stained with hematoxylin–eosin using standard procedures.
16. These alterations are typically graded by an experienced histopathologist. The grades are summed with maximal possible score of 13 (*see* Table 1).
17. Assays are conducted with standard automated clinical systems or commercial kits.
18. *Atp7b* and beta-actin mRNAs are coamplified by reverse transcription polymerase chain reaction (RT-PCR) with commercial kit (Access RT-PCR, Promega Corp., Madison, WI, USA). *Atp7b* primers spanning a region absent in LEC rats are 5'CCATCTCCAGTGACATCAG (forward) and 5'AGTCCCAATAGCAATGCC (reverse). Rat β -actin primers spanning an intervening intron are used simultaneously: 'AGGCATACAGGGACAACAC (forward) and 5'GGAGAAGATTTGGCACCCAC (reverse).

Acknowledgments

Supported in part by NIH grants, R01-DK088561, R01-DK071111, and P30-DK41296.

References

1. Bull PC, Thomas GR, Rommens JM, Forbes JR, Cox DW. The Wilson disease gene is a putative copper transporting P-type ATPase similar to the Menkes gene. *Nat Genet.* 2014; 5:327–337.
2. Ala A, Walker AP, Ashkan K, Dooley JS, Schilsky ML. Wilson's disease. *Lancet.* 2007; 369:397–408. [PubMed: 17276780]
3. Kim BE, Nevitt T, Thiele DJ. Mechanisms for copper acquisition, distribution and regulation. *Nat Chem Biol.* 2008; 4:176–185. [PubMed: 18277979]
4. Weiss KH, Schäfer M, Gotthardt DN, Angerer A, Mogler C, Schirmacher P, Schemmer P, Stremmel W, Sauer P. Outcome and development of symptoms after orthotopic liver transplantation for Wilson disease. *Clin Transplant.* 2013; 27:914–922. [PubMed: 24118554]
5. Gupta S. Cell therapy to remove excess copper in Wilson's disease. *Ann NY Acad Sci.* 2014; 1315:70–80. [PubMed: 24820353]
6. Bandi S, Joseph B, Berishvili E, Singhanian R, Wu YM, Cheng K, Gupta S. Perturbations in ataxia telangiectasia mutant signaling pathways after drug-induced acute liver failure and their reversal during rescue of animals by cell therapy. *Am J Pathol.* 2011; 178:161–174. [PubMed: 21224054]
7. Hansel MC, Gramignoli R, Skvorak KJ, Dorko K, Marongiu F, Blake W, Davila J, Strom SC. The history and use of human hepatocytes for the treatment of liver diseases: the first 100 patients. *Curr Protoc Toxicol.* 2014; 12:1–23.
8. Gramignoli R, Vosough M, Kannisto K, Srinivasan RC, Strom SC. Clinical hepatocyte transplantation: practical limits and possible solutions. *Eur Surg Res.* 2015; 54:162–177. [PubMed: 25633583]
9. Viswanathan P, Kapoor S, Kumaran V, Joseph B, Gupta S. Etanercept blocks inflammatory responses orchestrated by TNF- α to promote transplanted cell engraftment and proliferation in rat liver. *Hepatology.* 2014; 60:1378–1388. [PubMed: 24844924]
10. Malhi H, Irani AN, Volenberg I, Schilsky ML, Gupta S. Early cell transplantation in LEC rats modeling Wilson's disease eliminates hepatic copper with reversal of liver disease. *Gastroenterology.* 2002; 122:438–447. [PubMed: 11832458]
11. Malhi H, Joseph B, Schilsky ML, Gupta S. Development of cell therapy strategies to overcome copper toxicity in the LEC rat model of Wilson disease. *Regen Med.* 2008; 3:165–173. [PubMed: 18307400]
12. Joseph B, Kapoor S, Schilsky ML, Gupta S. Bile salt-induced pro-oxidant liver damage promotes transplanted cell proliferation for correcting Wilson disease in the LEC rat model. *Hepatology.* 2009; 49:1616–1624. [PubMed: 19185006]
13. Bahde R, Kapoor S, Bhargava KK, Schilsky ML, Palestro CJ, Gupta S. Positron emission tomography with copper-64-histidine for noninvasive diagnosis of biliary copper excretion in LEC rat model of Wilson's disease. *J Nucl Med.* 2012; 53:961–968. [PubMed: 22577234]
14. Peng F, Lutsenko S, Sun X, Muzik O. Positron emission tomography of copper metabolism in the Atp7b $-/-$ knock-out mouse model of Wilson's disease. *Mol Imaging Biol.* 2012; 14:70–78. [PubMed: 21327972]
15. Bahde R, Kapoor S, Bhargava KK, Palestro CJ, Gupta S. Diagnosis of abnormal biliary copper excretion by positron emission tomography with targeting of 64copper-asialofetuin complex in LEC rat model of Wilson's disease. *Am J Nucl Med Mol Imaging.* 2014; 4:537–547. [PubMed: 25250203]
16. Wu J, Forbes JR, Chen HS, Cox DW. The LEC rat has a deletion in the copper transporting ATPase gene homologous to the Wilson disease gene. *Nat Genet.* 1994; 7:541–545. [PubMed: 7951327]
17. Lutsenko S. Atp7b $-/-$ mice as a model for studies of Wilson's disease. *Biochem Soc Trans.* 2008; 36(Pt 6):1233–1238. [PubMed: 19021531]
18. Neufeld DS. Isolation of rat liver hepatocytes. *Methods Mol Biol.* 1997; 75:145–151. [PubMed: 9276266]

19. Malhi H, Bhargava KK, Afriyie M, Volenberg I, Schilsky ML, Palestro CJ, Gupta S. 99mTc-mebrofenin scintiscanning for evaluating liver disease in a rat model of Wilson's disease. *J Nucl Med.* 2002; 43:246–252. [PubMed: 11850492]
20. Schilsky ML, Irani AN, Gorla GR, Volenberg I, Gupta S. Biliary copper excretion capacity in intact animals: correlation between ATP7B function, hepatic mass and copper excretion. *J Biochem Mol Toxicol.* 2000; 14:210–214. [PubMed: 10789499]

Fig. 1.

Depiction of cell therapy mechanisms for WD. The ability to cure disease with replacement of ATP7B protein is a primary mechanism of interest. Mechanisms of secondary interest are those that may offer supportive and ameliorative benefits

Fig. 2.

Schematic representation of steps in cell transplantation for WD. The initial step in this process concerns isolation of healthy donor cells with suitable viability (70 % or greater). In small animals, injecting cells into splenic pulp is a convenient method for depositing cells in liver sinusoids. The final step is to analyze outcomes related to the fate of transplanted cells

Fig. 3. Localization of transplanted cells in liver with immuno staining for native ATP7B protein. (a) Representative image indicates ATP7B protein expression in hepatocytes throughout the healthy liver of a donor LEA rat. (b) Liver of a LEC rat 3 months after transplantation of donor LEA rat hepatocytes indicating clusters of proliferating cells. The LEC rat had been preconditioned with retrorsine and partial hepatectomy to accelerate proliferation in transplanted hepatocytes. (c) LEC rat without cell transplantation showing absence of ATP7B staining. Modified from ref. [13]. ATP7B was localized by immunostaining

Fig. 4. Characteristic histological findings in LEC rat liver. Representative images of normal liver (**a**) and liver of a LEC rat with Cu toxicosis (**b**). In LEC rat, note marked enlargements in nuclear size indicating polyploidy (*long arrow*), as well as apoptosis (*arrowheads*), steatosis (*asterisk*), and occasional mitoses (*small arrows*). Grading of these parameters is helpful for evaluation of cell therapy outcomes. H&E staining, original magnification 400×

Fig. 5. Cannulating bile duct in small animals for biliary Cu excretion. The procedure requires insertion of cannula fashioned from polyethylene tubing into common bile duct. **(a)** Indicates the final assembly of cannula and bile collection. **(b–d)** Individual steps in bile duct cannulation are presented schematically, including ligation of the distal end of common bile duct and placement of an encircling ligature toward its proximal end after the duct has been isolated **(b)**, nicking of the bile duct in preparation for inserting the cannula **(b)**, and securing of the cannula with tying of the proximal ligature **(c)**. After collecting baseline samples of bile, tracer doses of Cu are injected, followed by further timed collections of bile. Liver samples are also collected at the end. Cu content is measured by atomic absorption spectrometry

Fig. 6. PET imaging for Cu mobilization from the liver. Shows retention of activity 1 h after administration of (^{64}Cu)-histidine complex. **(a)** In LEA rat liver activity was excreted in bile and decreased over this period. **(b)** In LEC rat liver activity increased due to the absence of Cu excretion in bile. This procedure offers noninvasive ways to evaluate Cu excretion from liver and thus to establish whether cell/gene therapy would be successful. Modified from ref. [13]

Table 1
Histological grading for liver injury in WD models

Histologic parameter	Severity of lesion	Nuclei affected (% total)	Score
Polyploid cells	Absent	0	0
	Mild	0–10	1
	Moderate	11–25	2
	Marked	26–50	3
	Severe	>50	4
Microvesicular or macrovesicular steatosis	Absent	0	0
	Mild	0–10	1
	Moderate	11–25	2
	Marked	26–50	3
	Severe	>50	4
Apoptosis ^a	Absent	0	0
	Mild	1–5	2
	Marked	>5	4
Mitosis	Absent	Not applicable	0
	Present	Not applicable	1

^aNumber of apoptotic bodies per high power field under 400× magnification
 Table modified from ref. [19]