UNIVERSITY OF TURIN

Phd School in life and Health Sciences

Molecular Medicine

XXIX Cycle

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Human liver stem cells transplant extends survival in immunocompromised Crigler Najjar syndrome type I mice by restoring UGT1A1 expression

PhD thesis of: Elvira Famulari

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Human liver stem cells transplant extends survival in immunocompromised Crigler Najjar syndrome type I mice by restoring UGT1A1 expression

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1. Abstract

Background and rationale: Crigler-Najjar Syndrome type I (CNS-I) is a rare recessive disorder caused by rare mutations in the *Ugt1a1* gene, encoding for the UGT1A1 enzyme involved in bilirubin metabolism. Current therapy for CNS-I patients includes extended daily phototherapy and liver transplantation. Treatment with AAV vectors containing a functional version of *Ugt1a1* and primary hepatocyte transplantation have also shown promises in rodent mouse models of the disease. Stem cell-based therapy may, however, offer long-term solutions. We thus aimed at evaluating the therapeutic effect of human adult liver stem cells (HLSC) in immune-compromised Ugt1$^{-/-}$ mice which closely mimic the pathological manifestations observed in CNS-I patients.

Results: Repopulation of decellularized mouse liver scaffolds *ex vivo* with HLSC for 15 days induced differentiation and Ugt1$^{-/-}$ expression and activity in these cells. For the *in vivo* human cell engraftment and recovery experiments, C57Bl/6 Ugt1$^{+/-}$ mice were backcrossed for 8 generations with the immune-compromised NOD SCID Gamma (NSG) mice. Intrahepatic injection of HLSC in five days old NSG Ugt1$^{-/-}$ pups undergoing phototherapy significantly improved their survival compared to PBS-injected controls. Injected HLSC expressed UGT1A1 in the mutant mice liver. Importantly, 6.27 ± 0.83 % of HLA-positive cells were found in mice livers 15 days after cell injection. A significant decrease in bilirubinemia and improvement in brain histology were also observed with HLSC injection in mutant mice compared to controls.

Conclusion: our results show that HLSC can restore UGT1A1 enzyme activity in the NSG Ugt1$^{-/-}$ mice, and are a valid cell-based therapy for the treatment of human CNS-I as well as for other metabolic liver disorders. The mice model employed herein offers a new tool for *in vivo* testing of new sources of human cells.

1. Introduction

The Crigler Najjar syndrome type I (CNS-I; OMIM number 218800) is a rare monogenic recessive disease (0.6 to 1 per 1.000.000 live births) caused by uridine-diphosphate (UDP)-glucuronosyltransferase (UGT) 1A1 enzyme deficiency, which is responsible for bilirubin conjugation in the liver (1). The disease is characterized by severe jaundice (elevated blood levels of bilirubin) since birth and a lifelong risk of bilirubin encephalopathy and death by kernicterus if untreated (2, 3). The clinical management of the disease is very difficult since these patients need 10-12 hours/day of phototherapy (PT). Phototherapy consists of exposure to specific wavelengths of light, energy is absorbed by bilirubin as it circulates in skin capillaries, resulting in conversion of insoluble bilirubin to photoisomers which are water-soluble and readily excreted. However, CNS-I patients respond temporarily to PT, as its effectiveness diminishes with age due to skin thickening and decreased surface/body mass ratio, leading to increased risk of unconjugated bilirubin-induced encephalopathy and permanent brain damage (4). Liver transplantation can improve the prognosis of inherited metabolic diseases, and is currently the only definitive treatment available for CNS-I and other liver diseases (5). However, it is a highly invasive procedure, requires compatible donors and lifelong need of immunosuppression; moreover a 10% of Crigler Najjar syndrome patients, as well as patients of other hepatic diseases with normal liver function, require re-transplantation as determined 5 years after the first intervention (4-7).

As an alternative to liver transplantation, cell-based therapy holds great promise for CNS-I treatment. The metabolic recovery of Gunn rats for short periods has been shown after the transplantation of different cell types. For instance, use of primary hepatocytes or immortalized hepatocytes in irradiated/resected liver and non-treated livers resulted, in some cases, in the long term amelioration of hyperbilirubinemia (up to 12 months) (8). Importantly, human neonatal hepatocyte transplantation also provided long-term (6 months) rescue from unconjugated hyperbilirubinemia in adult Gunn rats

(9). These cells showed better engraftment and repopulation capability after transplantation compared to adult cells. Maerckx *et al.* further demonstrated that differentiated adult progenitor cells caused a decrease in serum bilirubin in the Gunn rat for 27 weeks (10). Other cell types have also been employed in models of CNS-I, and are promising for translation into the clinic. For instance, induced pluripotent stem cells reprogrammed from human skin fibroblasts and induced to differentiate into hepatocyte-like cells *in vitro*, expressed UGT1A1 and constituted 2.5-7.5% of the liver when transplanted into Gunn rats, with a reduction in serum bilirubin levels up to 24 weeks post-transplantation (11). The first infusion of human hepatocytes into the liver of a patient with CNS-I through the portal vein revealed that the cells survived for 11 months and partially ameliorated liver metabolic function (12). Hepatic progenitor cells have also been transplanted into a CNS-I infant through hepatic artery, and a 2 months follow-up showed decrease in total bilirubin and an increase in conjugated bilirubin levels (13). Hepatocytes obtained from other sources of cells still have to be tested in humans and there is also an urgent need to develop animal models capable of receiving human cells to assess the long-term efficacy in the correction of hyperbilirubinemia present in CNS-I.

We have previously reported the isolation and characterization of a population of human liver stem cells (HLSC) (14). HLSC have mesenchymal stem cell characteristics with partial commitment to hepatic cells. HLSC were induced to differentiate in mature hepatocytes, as indicated by the expression of functional cytochrome P450, albumin and urea production, and downregulation of α-fetoprotein expression. *In vivo*, HLSC contributed to regeneration of the liver parenchyma in severe-combined immunodeficient (SCID) mice and also offer protection from death in a lethal model of fulminant liver failure induced by intraperitoneal injection of D-galactosamine and lipopolysaccharide (15). Importantly, these cells require no genetic manipulations for their derivation and

differentiation into hepatocytes, and are currently being employed in an AIFA (Agenzia Italiana del Farmaco)- approved Phase I clinical study in pediatric patients with inborn errors of metabolism at the Liver Transplant Center of the AOU Città della Salute e della Scienza in Turin, Italy (European Clinical Trials Database (EudraCT, https://eudract.ema.europa.eu/). HLSC have been recognized by the European Medical Agency (EMA) as orphan drug for urea cycle disorders and acute liver failure and hence offer great promise for the treatment of other metabolic diseases such as CNSI.

2.1 Crigler Najjar type 1 syndrome

Crigler-Najjar syndrome (CNS) is a rare recessive genetic disease, estimated to affect fewer than 1 in 1 million newborns worldwide, due to mutations affecting the function of the 1A1 isoform of the uridine diphospho glucuronosyltransferase enzyme (UGT1A1). This enzyme is responsible of the glucuronidation of bilirubin molecules that results in an increase in its solubility allowing its excretion in bile. The liver parenchyma and all other metabolic functions are normal, but the patient is at risk of developing severe neurological complications due to the accumulation of bilirubin in the brain. Bilirubin is derived from heme catabolism, when red blood cells are broken down. Bilirubin can be excreted into the bile fluid and, thus, removed from the body only after its glucuronidation in the liver, which converts the toxic, insoluble form of bilirubin, called unconjugated bilirubin, to a nontoxic, soluble form called conjugated bilirubin. This reaction is performed by the UGT1A1 enzyme (16). Consequently, the absence of the enzymatic activity, as in the case of CNS-I, provokes the accumulation of bilirubin in the blood and lipophilic tissues, such as the brain. Bilirubin has an orange-yellow tint, and hyperbilirubinemia causes yellowing of the skin and whites of the eyes (jaundice). Severe unconjugated hyperbilirubinemia can lead to a condition called kernicterus, which is a form of severe brain damage caused by the accumulation of unconjugated bilirubin in the

specific regions of the brain and nerve tissues. Babies with kernicterus are often extremely tired (lethargic) and may have weak muscle tone (hypotonia). These babies may experience episodes of increased muscle tone (hypertonia) and arching of their backs (17). Kernicterus can lead to other neurological problems, including involuntary writhing movements of the body, hearing problems, or intellectual disability. The *Ugt1a1* gene comprises five exons located at the 3' end of the *Ugt1* locus on chromosome 2q37, in which the first one is exclusive to the Ugt1a1 variants present in the Ugt1 cluster. Mutations in the *Ugt1a1* gene may occur in any of the five exons, which either completely (CNS-I) or partially (CNS-II) inactivate this enzyme. CNS Type I is a fatal condition if untreated, indeed patients can die in childhood due to kernicterus. CNS type II is a less severe form of the disease due to a single base pair mutation. CNS-II patients have a partial deficiency of UGT1A1 activity and treatment with phenobarbital is very helpful, indeed it induces UGT1A1 expression acting on its activation pathway, instead it has not effect on CNS-I patients (18).

CNS-I affected patients have a quality of life deeply impaired due to prolonged photherapy treatment, that impedes normal life conditions. The only cure for the disorder is orthotopic liver transplantation (OLT), a very invasive procedure with many risks. Consequently, these limitations make necessary alternative treatments for Crigler Najjar type I syndrome.

2.2 Gunn rat and other animal models for inherited deficiency of bilirubin glucuronidation

The Gunn rat model has been used for many years in the study of CNS and potential therapies. It is characterized by a single mutation on the *Ugt1A1* gene that results in a truncated protein without enzymatic activity due to the introduction of an in-frame premature stop codon (20).

Charles Kenneth Gunn first discovered mutant rats in 1934 in a Wistar rat colony maintained at the Connaught Laboratory in Toronto, Canada. Gunn rats pups and adult animals have a yellowish discoloration of the skin (jaundice) (21). In this model, many experimental protocols have been tested, aiming at developing efficient alternative treatments to liver transplantation for CNS-I. Gunn rats have been largely used for gene therapy but also for cell therapy, giving important information about the management of the pathology (22).

Two murine model were developed. Bortolussi et al in 2012 generated a new model of CNS in C57B1/6 background. Ugt1$^{-/-}$-C57B1/6 mice developed jaundice 36 hours after birth and presented a yellowish color of the skin. After 5 days from birth the lack of bilirubin-glucuronidation activity leaded to high levels of unconjugated bilirubin in plasma and tissues inducing death in the 50% of mice. Mutant mice displayed kernicterus and severe motor impairment. Survival of mutant mice was extend to >20 days treating them with phototherapy after two days from birth (λ=450nm, 12h/d). (23).

2.3 Alternative treatments to OLT

We can individuate two types of treatment for CNS-I that are alternative to liver transplantation: **gene therapy** and **cell therapy**.

- **Gene therapy**

Gene therapy is the therapeutic delivery of nucleic acids, in this case a correct copy of the *Ugt1a1* gene, into patient's cells as a drug to treat disease, in order to obtain a permanent and therapeutic expression levels of the enzyme. Gene therapy has long been considered as a potential alternative treatment for CNS-I, as it would alleviate the demand for organ donors and could bring a definitive cure without the side effects of a life-long immunosuppressive regimen (24).

CNS-I is an ideal candidate disease for liver gene therapy thanks to the fact that the liver is histologically normal and accessible to systemically administered vectors and also because partial gene correction should be sufficient for providing major clinical benefit. More-over, the facts that the liver is highly vascularized and the largest organ of the body make this organ a good candidate for gene therapy (24).

Adeno-associated virus (AAV) vectors are a promising tool for liver gene therapy. Although wild-type AAV integrates into the human genome, recombinant AAV vectors are usually considered as nonintegrative vectors, even though integration may occur in some instances. Bortolussi et al, applied AAV liver-specific gene therapy to a lethal mouse model of CNS-I, and demonstrated that a single neonatal *hUgt1a1* gene transfer was successful for the therapy (24). Indeed they demonstrated that after neonatal AAV transduction, all AAT-*hUgt1a1*-treated mutant animals survived showing an important and clinically relevant reduction in plasma bilirubin during the first months of treatment (70–80% reduction). This reduction was maintained up to the end of the experimental protocol (50% reduction 17 months post injection) and also the histological and functional features of the AAV-treated mice were normal (24). Montenegro et al, demonstrated that also Adenoviral gene transfer efficiency is comparable to AAV for treatment of CNS I in Gunn rat and that, compared with AAV, less AdV genomes were needed for complete correction of hyperbilirubinemia (25).

Gene therapy has the great ability to replace defective cells, and different research groups demonstrated its effectiveness for CNS-I in Gunn rat and also in other animal models (23, 24). Nevertheless gene therapy presents some limitation, such as the risk to damage the human gene pool, giving rise to other disorders.

- **Cell therapy and stem cell therapy**

Liver cell transplantation is an attractive technique to treat liver-based metabolic diseases, as in the case of CNS, where the goal is to reduce serum bilirubin levels within safe limits

and to alleviate phototherapy requirements to improve quality of life. Hepatocytes transplantation can acts as a bridge therapy for patients waiting for a whole organ, avoiding risks of surgery. (26). More-over the cell requirement for transplantation may be lower in inherited metabolic liver diseases, where the aim is to replace a single deficient enzyme, in particular in CNS where the repopulation rate necessary to observe a metabolic efficacy is just 5% (16). Fox et al, demonstrated for the first time the potentiality of liver cell therapy as a new alternative treatment. They demonstrated that transplantation of hepatocytes, derived from a healthy liver donor, could be safely infused through the portal vein for engraftment into the patient's liver. Transplanted hepatocytes survived for more than 11 months, partially correcting a liver-based metabolic disorder of a 10 years old CNS-I patient (12). The infusion of 7.5 $x10^9$ viable liver cells in the patient resulted in a significant decrease of bilirubin levels. Moreover UGT1A1 enzyme activity was detected in the host liver and glucuronjugates were found in bile confirming the integration of functional hepatocytes (12). Nevertheless at present hepatocytes transplantations presents different limitations, as impaired donor cell quality and poor repopulation rates. Enhancement of liver cell engraftment capacity is another challenge. Engraftment depends on liver cell quality and host liver environment. Indeed, although cryopreserved/thawed hepatocytes have been shown to possess in vivo replicative potential restricted in time, their in vitro functionality remains lower than that of freshly isolated hepatocytes (12). Furthermore with the current protocols, cryopreservation/thawing of hepatocytes induces cell alterations and mitochondrial defects (26).

Tolosa et al demonstrated that, because of their attachment capability and expression of adhesion molecules as well as the higher proportion of hepatic progenitor cells, neonatal hepatocytes may have an advantage over adult cells. After injection of adult or neonatal hepatocytes in Gunn rat, they demonstrated that neonatal hepatocytestransplantation

results in long-term correction and also in a better engraftment and repopulation capability 3 days and 6 months after transplantation, in comparison with adult cells (9). Indeed, neonatal hepatocytes presented good resistance to cryopreservation with post thawing viability and attachment capability similar to those of freshly isolated cells as well as very good functional performance. Additionally, they showed that neonatal hepatocytes proliferate more than adult hepatocytes, and this could be responsible for long-term effects in the Gunn rat when it is transplanted (9).

As stem cells were recently described to have a hepatocyte differentiation potential, these are currently considered with growing interest for liver cell therapy as alternative to hepatocyte transplantation (15). The most potent candidates are mesenchymal stem cells isolated from various tissues (15). Stem cells display partial hepatocyte-like functionality and further advance is necessary to consider such cell types for therapy.

Maerckx et al tested the potentiality of human liver stem/progenitor cells for Crigler Najjar syndrome injecting them into Gunn rats. They demonstrated that these cells were able to conjugate bilirubin *in vitro* and to restore a deficient metabolic function in hyperbilirubinemic rats (10).

Subsequently, Yong Chen et al, transplanted hepatocyte-like cells (iHeps) differentiated from human induced pluripotent stem cells (iPSCs) in Gunn rat. Preconditioning liver lobe by irradiation and treating it with tracolimus, to provide a proliferative advantage of exogenous cells and minimize the of rejection respectively, they demonstrated that injected iHeps are able to engraft, proliferate and express UGT1A1 in the rat liver, reducing bilirubin serum level (11).

These promising results and the enormous potential of stem cells to differentiate in adult cells, make them excellent candidates in cell therapy for metabolic liver disease such as Crigler Najjar type I syndrome.

2.4 Human liver stem cells

Human liver stem cells are a population of mesenchymal stem cells residents in the human liver. HLSC showed the presence of several stem cell markers, as vimentin ,Oct 4, but also some liver specific markers. Herrera et al, isolated and characterized normal adult human liver stem cells, with multiple differentiating capabilities, distinct from those of oval stem cells. HLSC expressed several mesenchymal but not hematopoietic stem cell markers and albumin, α-fetoprotein, and CK18, indicating a partial commitment to the hepatic lineage (14). Similarly to MSCs, HLSC underwent endothelial and osteogenic differentiation. This is maybe due to the fact that HLSC may represent a mesenchymal population modified by the influence of the environment in establishing the phenotype of MSC (14). Furthermore HLSC are able to differentiate into both mature hepatocytes and insulin-producing islet-like cells.

The same group also tested the potentiality of HLSC in regenerative medicine, injecting them into a mouse model with fulminant liver failure (FLF), demonstrating that they are able to improve survival of fulminant liver failure mice, inhibiting liver necrosis, apoptosis and enhancing liver regeneration (15).

They also observed a long-term engraftement of HLSC in liver mice, and their ability to differentiate in vivo (15). HLSC have been also used to re-cellularize a de-cellularized liver scaffold, generating a functional humanized liver. The tridimensional structure of the native matrix and the preservation of bioactive molecules promoted differentiation and maturation of HLSC to functional hepatocytes. In this condition HLSC lost the embryonic markers and increased the expression of hepatic markers as albumin; more over after 4 days of differentiation HLSC were metabolically active, as demonstrated by the presence of urea nitrogen in the culture medium (27).

The other important potentiality of HLSC, is that manipulating them is really manageable. HLSC are easily obtainable from small surgical samples or from biopsies of

human adult liver. Herrera et al isolated HLSC form healthy liver tissue digesting it with collagenase. They cultured the cell population obtained for two weeks. At this time point hepatocytes started to die and colonies of HLSC were evident; colonies were first cultured as single colony in an individual well and than transferred and expanded in flask (14).

HLSC can be expanded in culture at variance of normal adult hepatocytes, could be used to re-cellularize liver scaffold and exhibit a great proliferation potential, remaining stable for over 30 culture passages (27).

These characteristics make the HLSC potential candidates for the generation of functional hepatocytes to be used in regenerative medicine, but also in cell therapy for liver metabolic diseases as Crigler Najjar type I syndrome.

2. Materials and Methods

3.1 Generation of NSG Ugt $^{-/-}$ mouse model and phototherapy

To reach the goal of this work, we decided to create a new model for CNS, using an NSG background. Both Gunn rat and C57B1/6 Ugt1$^{-/-}$ CNS animal model, described in the introduction, have a higher risk of rejection if transplanted with human cells being immune-competent. For this reason we have generated a new mouse model by introducing the Ugt1 mutation into immune-compromised mice (NOD SCID IL2Rgamma, NSG), capable of receiving human cells without the necessity of immune suppression to prevent graft rejection. NSG mice do not express the *Prkdc* gene nor the X-linked *Il2rg* gene, in this way they lack of B and T cells and NK cells. This severe immunodeficiency allows the mice to better accept human cells as human CD34+ hematopoietic stem cells, patient derived xenograft or adult stem cells and tissues (28).

NSG Ugt$^{+/-}$ mice were obtained by backcrossing previously described C57Bl/6 Ugt1$^{+/-}$ mice with wild type NOD^{scid} gamma, NOD^{scid} IL2Rg^{null}, NOD^{scid} IL2Rgamma^{null}, NSG (JAX 005557)(wt) mice for 7 generations (23,24). For each generation, Ugt1$^{+/-}$ mice were selected by genotyping for the NSG background (https://www.jax.org/strain/005557) and Ugt1a1 mutation as previously described (Table S1) (24). NSG Ugt $^{-/-}$ mice were then derived by crossing NSG Ugt $^{+/-}$ mice. Mice were bred under specific-pathogen-free conditions and allowed free access to regular chow (standard diet 4RF21, Mucedola srl) and water. All animals received humane care according to the criteria outlined in "Guide for the Care and Use of Laboratory Animals, 8th edition" (29). To perform PT under sterile conditions, we devised special cages with blue LED lights (450 nm) applied on all sides in order to deliver uniform irradiation (HLMP-CB3A-UV0DD, 20Ma, 3.2V, 450nm, Avago Technologies). A timer connected to the cage ensured 14 hours' daily exposure to blue light (λ=450nm) during the light cycle.

This study was approved by the local ethical committee and the Italian Ministry of Health (number 1110/2015-PR).

3.2 Cell culture and recellularization of mice liver bioscaffold

We used HLSC given to us from our collaborators (Prof. Camussi group), isolated as previously described (14). HLSC were cultured in α-MEM/EBM-1 (3:1) (Invitrogen, Carlsbad, CA, USA) media supplemented with L-glutamine (5 mM), Hepes (12 mM, pH 7.4), penicillin (50 IU/ml), streptomycin (50 μg/ml) (all from Sigma-Aldrich) and fetal calf serum (FCS) (10%) (Invitrogen) and characterized as previously described (30). HLSC (2.5×10^6) were differentiated in a rotary cell culture system as previously described in order to analyze hepatic gene expression (14). Functional hepatocytes were further derived from HLSC for assessment of UGT1A1 expression by recellularizing acellular mouse liver bioscaffolds as previously described (27). Briefly, 12×10^7 HLSC, cultured for at most 6 passages, were seeded in the bioscaffolds and cultured at 37°C and 5% CO_2 for 15 days. The medium was changed every other day during the entire experiment. Human UGT1A1 expression was analyzed by immunofluorescence using rabbit antibody against the protein (Ugt1A, sc-25847, Santa Cruz Biotechnology) and was revealed using FITC-conjugated anti-rabbit secondary antibody (Invitrogen).

3.3 HLSC transplantation in NSG Ugt1$^{-/-}$ mice

NSG Ugt1$^{-/-}$ pups underwent PT as from 2 days after birth, when phenotype is visible, and 1×10^5 HLSC resuspendend in 10 microliters of PBS, stained with DiL (1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate, Thermo Fisher Scientific) according to manufacturer's instructions were injected directly in the liver parenchyma of 5 days old pups. PT-treated PBS-injected mice were used as negative control. The experiment was stopped at 21 days.

3.4 Serum bilirubin measurement

Serum bilirubin levels were measured in NSG Ugt1$^{-/-}$ mice injected with HLSC at Day 21 using Bilirubin, Total, kit (BQ kits, Inc.) as previously described (31).

NSG Ugt1$^{-/-}$ mice undergoing PT till postnatal day 8 and sibling wt littermate mice were used as negative controls.

3.5 RNA extraction, PCR and Real-time PCR

RNA was extracted using the PureLink RNA kit (Ambion) and cDNA prepared using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Target gene expression was analyzed by Real-time PCR (qRT-PCR) and normalized to endogenous 18s expression as previously described (32). Primers used for qRT-PCR are listed in Table S1.

3.6 Western blotting

Total protein was extracted from cells using lysis buffer containing 1% Triton X-100 supplemented with protease inhibitors (Complete Mini, Roche) and 50 microgram samples were separated by 4-15% SDS-PAGE (Biorad). Antibodies used were rabbit anti-Ugt1A antibody (Ugt1A, sc-25847, Santa Cruz Biotechnology) and mouse anti-vimentin (in-house). Densitometric analysis was performed using the volume analysis tool of ImageLab software (Biorad Laboratories Inc).

3.7 UGT1A1 enzyme activity

Differentiated HLSC were assessed for UGT1A1 enzyme activity (33). Briefly, mouse liver bio-scaffolds were replenished with HLSC (80 x10^6 cells) and maintained in culture for 15 days as previously described (Navarro-Tableros et al., 2015). The bioscaffolds were then pulverized in liquid nitrogen, and homogenized in ice-cold PBS.

Following centrifugation to eliminate debris and nuclei, the resulting supernatant was centrifuged at 100 000 g for 60 min at 4 °C, as previously described (33). The microsomal pellet was resuspended in buffer (50 mM Tris-HCl, pH 7.4, 10 mM MgCl2, 1mM phenylmethylsulfonyl fluoride), and the protein concentration was determined by the Bradford method. UGT1A1 activity was then measured using 0.2 mg/ml protein and UGT Glo assay following the manufacturer's protocol (Promega) and as previously described (34). Microsomes derived from wt mouse liver were used as positive control.

3.8 Immunohistochemistry and histology

Liver, brain and blood were taken at indicated time points. Liver lobes of wt and PT-treated NSG Ugt1$^{-/-}$ mice at Day 8 and Day 21 PT and HLSC-treated NSG Ugt1$^{-/-}$ mice were included in OCT and processed for immunofluorescence studies. Formalin-fixed, paraffin-embedded liver sections were also stained with hematoxylin/eosin. Brain sections were also stained with hematoxylin/eosin. DiL-labeled cells were tracked using a Zeiss microscope and Apotome software. UGT1A1 and DiL colocalization was performed using Leica TCS SP5-AOBS 5-channel confocal system (Leica Microsystems) equipped with a 405nm diode, an argon ion, a 561nm DPSS and a HeNe 633nm lasers. Liver sections were imaged using a HCX PL APO 63x/1.4 NA or 40x/1.25 NA oil immersion objective.

3.9 Flow cytometry

Depletion of B and T lymphocytes and natural killer (NK) cells in NSG Ugt1$^{-/-}$ mice generated by the backcrosses were verified using FITC anti-mouse CD3ε (T lymphocytes, clone 145-2C11, BioLegend), PerCP/Cy5,5 anti-mouse/human CD45/B220 (B-lymphocytes, clone RA3-6B2, BioLegend) and APC anti-mouse CD335 (NKp46) (NK cells, clone.29A1.4, BioLegend) by flow cytometry (Beckton Dickinson). HLSC engraftment was assessed by injecting 1x10^5 HLSC in the liver of 5 days old NSG

wt mice and analyzing the percentage of HLSC present after 16 days of cell injection using PE Mouse anti-Human HLA-A2 (clone BB7.2, BD Pharmigen™) by flow cytometry. Briefly, mouse livers were perfused with Liver Perfusion solution followed by Liver Digest medium as per manufacturer's protocol (ThermoFisher Scientific). Cells were stained with anti-HLA antibodies and data were analysed using the CellQuest software (BD FACSCalibur).

3.2.1 Statistical analyses

Data are expressed as mean ± standard deviation (s.d) and are representative of at least 3 independent experiments. Statistical differences (where n>3) were determined by a 2-tailed Student's *t* -test (* P <0.05, ** P <0.01, *** P <0.001).

3. Results

4.1 HLSC express UGT1A1 protein *ex vivo*

We first assessed whether HLSC could express UGT1A1. HLSC induced to differentiate in a rotary cell culture system expressed detectable mRNA levels of UGT1A1 as well as albumin, with respect to undifferentiated cells (Fig. 1). However, the UGT1A1 protein was barely detectable using this system. We thus used an *ex vivo* differentiation system which further enhances HLSC differentiation. Mouse livers were decellularized and the scaffold was repopulated with HLSC as we previously reported (27). The scaffolds were maintained for 15 days to evaluate hepatocyte differentiation and UGT1A1 expression (27). Importantly, immunofluorescence analysis on mouse liver bioscaffolds revealed that HLSC started expressing UGT1A1 as from 7 days of differentiation and that there was a substantial increase after 15 days of differentiation (Figure 2A). Empty scaffolds and undifferentiated HLSC were used as negative control. Gene expression analysis of HLSC confirmed differentiation and UGT1A1 expression after 15 days in the bioscaffold versus undifferentiated cells (Figure 2B). Importantly, bilirubin conjugation activity was detected in HLSC differentiated *ex vivo* in liver scaffolds for 15 days which was compared to wt mouse liver (Figure 2C). The data revealed that *ex vivo* differentiated HLSC have 14.7% of UGT1A1 activity with respect to wt mice whole liver (100%).

4.2 HLSC engraft in NSG wt mice livers *in vivo*

To analyze HLSC engraftment *in vivo* in a NSG background, 2 months old wt mice were injected intraparenchymally with $1x10^6$ DiL-labeled HLSC. Five days after cell injection, immunfluorescence analysis showed DiL-positivity in the livers of HLSC-injected mice (Figure 3A). To know the percentage of cells that colonize NSG mouse liver, NSG wt mouse were injected with $1x10^5$ cells at 5 days after birth. Whole liver

was further analysed in 21 days-old NSG wt mice (16 days after injection) using anti-HLA-A2 antibodies that revealed the presence of human cells. Interestingly, an average of 6.27 ± 0.83 % of cells was positive for HLA-A2 in 21 days-old HLSC-injected NSG wt mouse livers (Figure 3B) compared to untreated mouse livers used as controls.

4.3 Characterization of NSG Ugt$^{-/-}$ mice phenotype

The UGT1A1 mutation was shifted into the NSG genetic background by crossing C57Bl/6 Ugt1$^{+/-}$ with NSG wt mice. The resulting Ugt$^{+/-}$ mice from each generation were crossed with NSG wt mice for 7 generations. Thereafter, NSG Ugt$^{-/-}$ mice were obtained by breeding NSG Ugt$^{+/-}$ mice. NSG Ugt$^{-/-}$ mice had the visible appearance of jaundice, as evidenced by orange skin colour, at postnatal Day 2 (Figure 4A) and at Day 5 under PT (Figure 4B) as confirmed by PCR analysis of tail DNA (Figure 4C) and Western blot of liver protein extracts (Figure 4D). NSG Ugt$^{-/-}$ pups without phototherapy treatment can survive between two and three days (Figure 5). At day 2 they present a smaller body than the others normal pups and have difficulties to feed on. To improve their health conditions and survival we devised special cages to deliver PT to NSG Ugt$^{-/-}$ (Figure 4E). We applied light led (λ=450nm) around a specific-pathogen-free conditions cage (on the lid and on all the sides), performing little holes in the plastic of cage in correspondence of each single led, in order to remove the plastic screen and to have an effective irradiation at 450nm. With PT, NSG Ugt$^{-/-}$ mice survival is prolonged till 14-16 days (Fig. 5). In the last days NSG Ugt$^{-/-}$ were emaciated and dehydrated, they had severe motility impairment and a significant reduction in body mass (as shown from videos and in Figure 6 A, Day 14 and Day16). This improvement till 16 days with PT has been crucial for our work, indeed it allowed us to manipulate mice and to inject them.

4.3 HLSC injection improves NSG Ugt$^{-/-}$ mice survival

NSG Ugt$^{-/-}$ mice were treated with phototherapy starting from Day 2 after birth (Figure 6B). HLSC were directly delivered (as described in Materials and Methods) in the liver parenchyma of 5 days old mice, when the liver is still visible through the skin. It is really crucial that the injection occurs in the liver, where HLCS can receive liver matrix stimuli to enhance the differentiation into hepatocyte-like cells. After the injection, mice were maintained with cells and under PT till Day 16, that is the maximum survival time of mutant mice treated only with PT. At Day 16 PT was stopped and HLSC injected mice were sacrificed at Day 21, 5 days after removal stop of PT treatment and 16 days after injection. All mice analysed after cell injection were comparable to the wt littermates, with normal weight and no apparent motor deficits (Figure 6 A Day 19, Day 20). All untreated NSG Ugt$^{-/-}$ mice and those treated with vehicle alone did not survive beyond 16 days of phototherapy. As shown in Figure 5, there was a significant improvement in survival with phototherapy (mean survival: 15 days) *versus* non-treated NSG Ugt$^{-/-}$ mice (mean survival: 2 days). Importantly, total bilirubin levels decreased by two-fold in NSG Ugt$^{-/-}$ mice treated with HLSC compared to untreated ones (Figure 6). In order to assess the degree of UGT1A1 expression and cell grafting in HLSC-injected NSG Ugt$^{-/-}$ mice, liver sections from treated mice were analyzed by immunohistochemistry with anti-Ugt1a1 antibodies. As shown in Figure 7A, there was an intense and localized HLSC-induced UGT1A1 expression in the liver of 21 days old PT-treated NSG Ugt$^{-/-}$ mice compared to PT-treated PBS-injected controls. (Figure 7A i, ii, and iii *versus* iv, respectively). Human liver sections used as positive control showed diffuse UGT1A1 staining compared to the localized staining in HLSC-injected NSG Ugt1$^{-/-}$ mice (Figure 7A v *versus* i, ii, iii, respectively). DiL-positivity and UGT1A1 immunoreactivity colocalized in the liver of DiL-stained HLSC-

injected NSG Ugt$^{-/-}$ mice (Figure 7B). These data show that injected cells engrafted in NSG Ugt1$^{-/-}$ mice livers *in vivo* and restored UGT1A1 expression.

4.4 HLSC injection reduces brain injury in NSG Ugt1$^{-/-}$ mice

Improvement in brain histology was observed in HLSC-treated NSG Ugt1$^{-/-}$ mice compared to PT-treated ones. The number of eosinophilic neurons in the hippocampus, indicative of neuronal injury and subsequent cell death, was absent in 8 days old wt mice, and significantly reduced in 21 days old HLSC-treated NSG Ugt$^{-/-}$ mice compared to 8 days old PT-treated controls (Figure 8A and Figure 8B). No eosinophilic neurons were found in HLSC-treated NSG Ugt$^{-/-}$ mouse cerebellum compared to PT-treated mice.

For serum bilirubin and brain and liver histology assessment, 21 days old HLSC-injected NSG Ugt$^{-/-}$ mice could not be compared with 21 days old control NSG Ugt$^{-/-}$ mice as the latter did not survive beyond 16 days despite PT.

4. Discussion

Adult liver stem cells can have the potential to differentiate into hepatocyte-like cells both *in vitro* and *in vivo*, and can thus be infused into recipient livers to restore metabolic function (35). The use of autologous stem cells to correct liver function in monogenic liver diseases is considered to be a very promising approach (36). However, this strategy, which depends on the efficient delivery of the therapeutic gene into the transplanted cells, still requires further studies for long term or curative purposes. Importantly, no HLA matching is required for cell transplant in the liver, allowing the use of ample donor-derived stem cells for cell therapy (37). We thus explored the efficacy of HLSC in rescuing CNS-I phenotype in immune-compromised $Ugt1^{-/-}$ mice through the restoration of liver UGT1A1 expression and activity.

SCID is the most widely used mouse strain for human cell injection *in vivo*. However, it has been shown that IL-2R common gamma-chain deficient NSG mice support CD34+ human stem cell engraftment at much higher levels than SCID controls. In fact, NSG mice engrafted with human mobilized blood stem cells provided a new *in vivo* long-lived model of robust multilineage human hematopoietic stem cells engraftment (38). We thus generated an immune-compromised mouse model of the Crigler Najjar syndrome by crossing $Ugt1^{+/-}$ with NSG mice to derive NSG $Ugt1^{-/-}$ mice. It is expected that these mice show better long term engraftment of HLSC with respect to existing CNS I animal models, hence providing a valid preclinical model for studying the efficacy of transplanted human cells in restoring metabolic function.

The highly proliferative state of the liver in very young animals can provide the right cues for better understanding of the cell engraftment mechanisms, increasing effectiveness of cell therapy in the neonatal/pediatric period and preventing tissue damage that may not be possible to correct later in life. Importantly, around 5% of replacement of the total liver mass may improve metabolic disorders, and 10% may normalize liver function (35). For instance, in humans, infusion of 5% of the liver mass efficiently

lowered bilirubin levels in CNS I patients (12,39, 40). In our model, by directly delivering the HLSC to newborn NSG wt mice livers, we found 6.27 ± 0.83 % of engrafted human cells 15 days post-injection. The potency of HLSC was proven in *ex vivo* culture as well as by *in vivo* differentiation after transplantation in mice by their ability to express UGT1A1 enzyme and to lower circulating bilirubin levels. Importantly, bilirubin conjugation activity was observed in HLSC differentiated in bioscaffolds *versus* wt liver (14.7% versus 100%, respectively), in agreement with previous studies showing that cell lines bear lower UGT enzyme activity compared to the whole liver or primary hepatocytes (41).

Moreover, maturation of HLSC into hepatocytes *in vivo* is observed as early as 15 days after cell injection as shown by our data on UGT1A1 expression in the liver of knockout mice. Cells were injected in our model at postnatal Day 5 when the liver is growing, with significant hepatocyte proliferation, which renders the microenvironment ideal for receiving human HLSC, despite the fact that CNS I livers are healthy and HLSC-derived hepatocytes do not have any adaptative advantage over the resident hepatocytes. The expression of the UGT1A1 enzyme in the neonatal period is enhanced upon differentiation of HLSC under these conditions (42). Brain damage, mainly in the hippocampus of the NSG Ugt1$^{-/-}$ mice, was reduced upon HLSC injection with respect to PT-treated controls, showing that these cells efficiently prevented the pathological effects of unconjugated bilirubin when injected early in the newborns (43, 44).

Therapies based on gene and cell transfer techniques are mostly in the preclinical phase. It is crucial to evaluate the safety, potency, absence of tumorigenicity and toxicity, as well as *in vivo* biodistribution of the cells in animal models before their clinical development (35). Cell-free, AAV-mediated gene therapy for CNS I have proved to be very effective in rescuing bilirubin-induced neonatal lethality in Ugt1$^{-/-}$ mice (24, 45,46).

Scalable production of HLSC in GMP (Good Manufacturing Process) is now possible and has allowed performing the first-in-human Phase 1 clinical study in neonates with urea-cycle disorders. In the near future, HLSC-derived products (extracellular vesicles), employed as drug and prepared under GMP conditions, may solve the problem related to ABO blood group incompatibility and long term engraftment (30, 47).

Our novel immune-compromised Ugt1$^{-/-}$ mice offers a platform for *in vivo* testing of candidate human cells for the long-term cure of CNS-I. Compared to the Gunn rat, these mice offer the advantages of not requiring immunosuppression regimens for transplanting human cells, and of being lethal, hence closely mimicking the human disease, if therapy is not efficiently delivered early after birth. This model can be further employed to *in vivo* test the long term safety and efficacy of other human cells before their clinical study (48).

Our data demonstrated that HLSC injection can seriously improve NSG Ugt1$^{-/-}$ healthy conditions. These mice have dramatic fate, indeed if untreated they die after two days from birth. With PT they can survive till 14-16 days, but without any other treatment they result really emaciated and suffering. After HLSC transplant they have a strong recovery of health conditions an can survive almost till 21 days, when they present a normal phenotype, normal body mass and motility, comparable to the wt. For these first experiments on NSG Ugt1$^{-/-}$ mice injected with HLSC, we decided to stop therapy at 21 days because we thought it was already an excellent result to draw first informations about the behavior of HLSC in our new CNS-I mouse model, and about the efficiency of UGT1A1 enzyme. In the next future it will be really important to evaluate the long-term engraftment and effect of HLSC, to know how a single HLSC injection can improve NSG Ugt1$^{-/-}$ survival.

Based on data obtained from the immune-compromised Ugt1$^{-/-}$ mice used in our study, we believe that HLSC could develop into a therapeutic opportunity for pediatric patients with metabolic disorders, such as CNS-I.

5. Figures

Figure 1

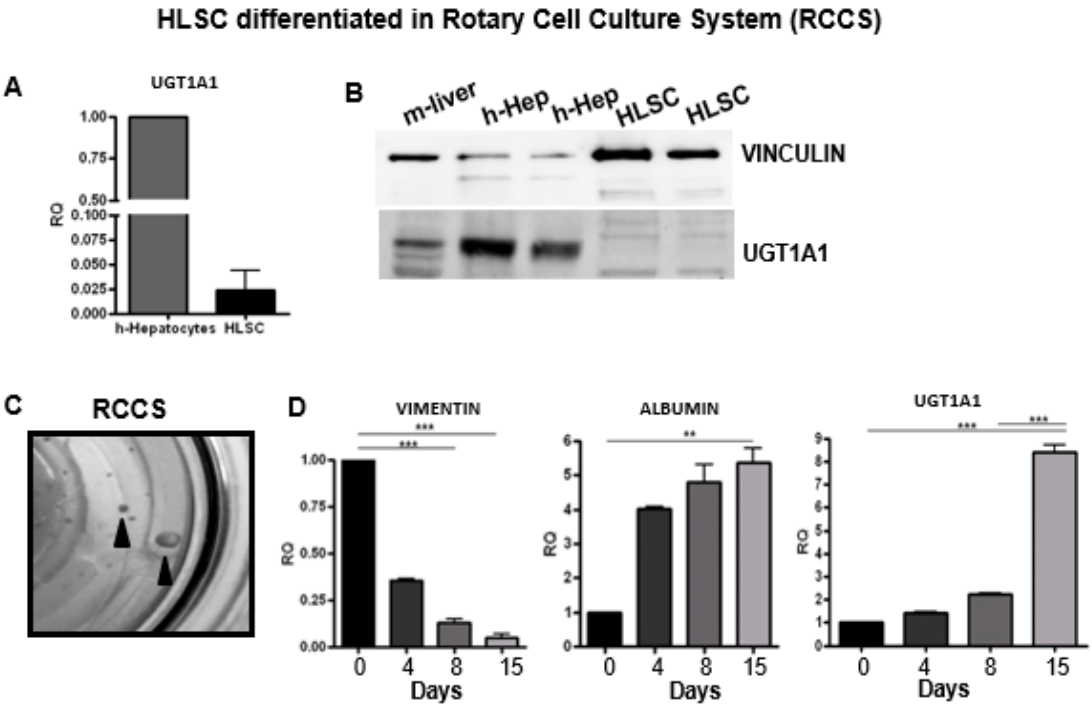

Fig. 1: Differentiation of HLSC in rotary cell culture system (RCCS) *in vitro*. A. Expression of UGT1A1 mRNA in undifferentiated HLSC was assessed by qRT-PCR *versus* human hepatocytes. B. Western blot analysis of UGT1A1 protein in mouse liver (m-liver) and human hepatocytes (h-Hep) *versus* undifferentiated HLSC. C. The rotary cell culture system (RCCS) showing aggregates of differentiating HLSC (arrowheads). D. Analysis of vimentin, albumin and UGT1A1by qRT-PCR upon hepatocyte differentiation of HLSC at different time points.

Figure 2

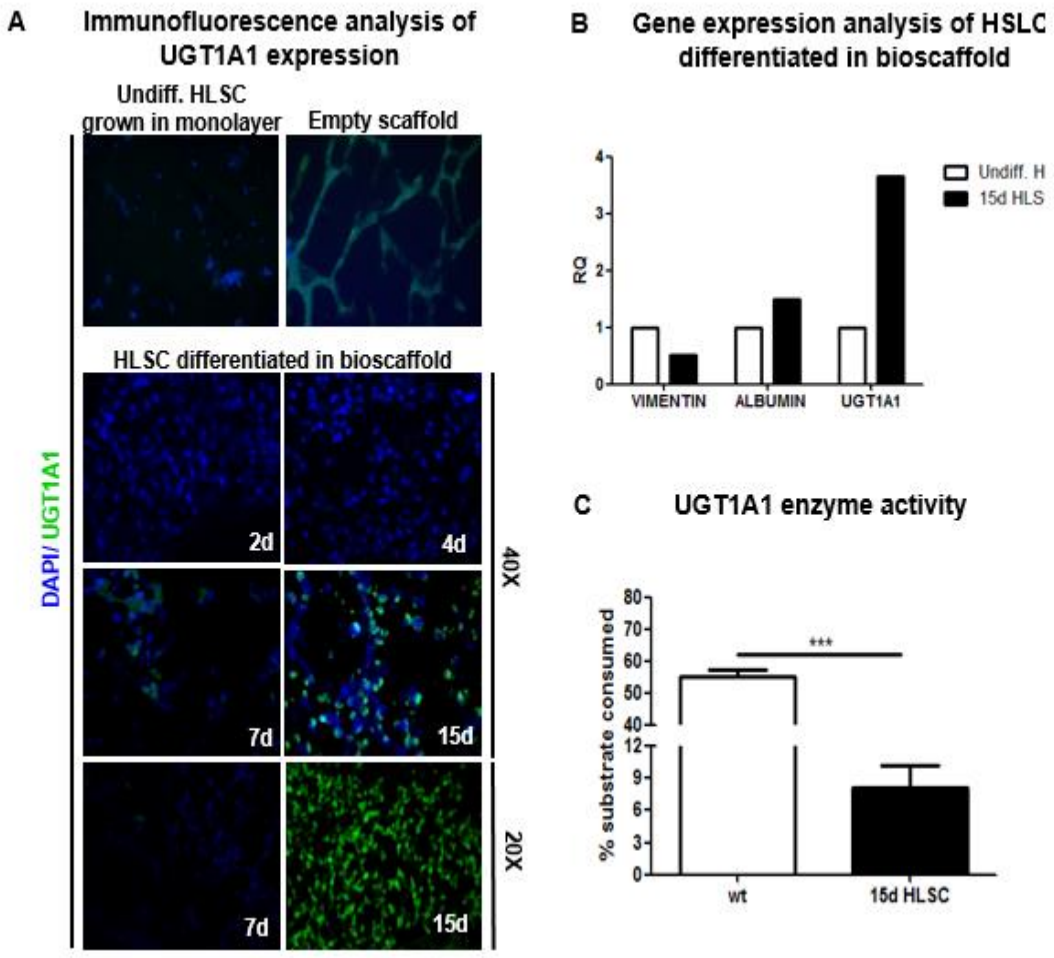

Figure2: Analysis of UGT1A1 expression *ex vivo* in mice liver bioscaffolds. A. Immunofluorescence analysis of HLSC differentiation in mice liver scaffolds at the indicated time points (days, d) showing human UGT1A1 expression (Magnification: 40X for all time points; 40X and 20X for 7d and 15d). Undifferentiated (Undiff.) HLSC grown in monolayer and empty scaffolds were used as controls. B. Representative qRT-PCR analysis of mesenchymal marker (vimentin) and hepatic marker (albumin, Ugt1a1) expression upon hepatocyte differentiation of HLSC at different time points. C. UGT1A1 enzyme activity measured in HLSC cultured for 15 days in bioscaffolds *versus* wt mouse liver.

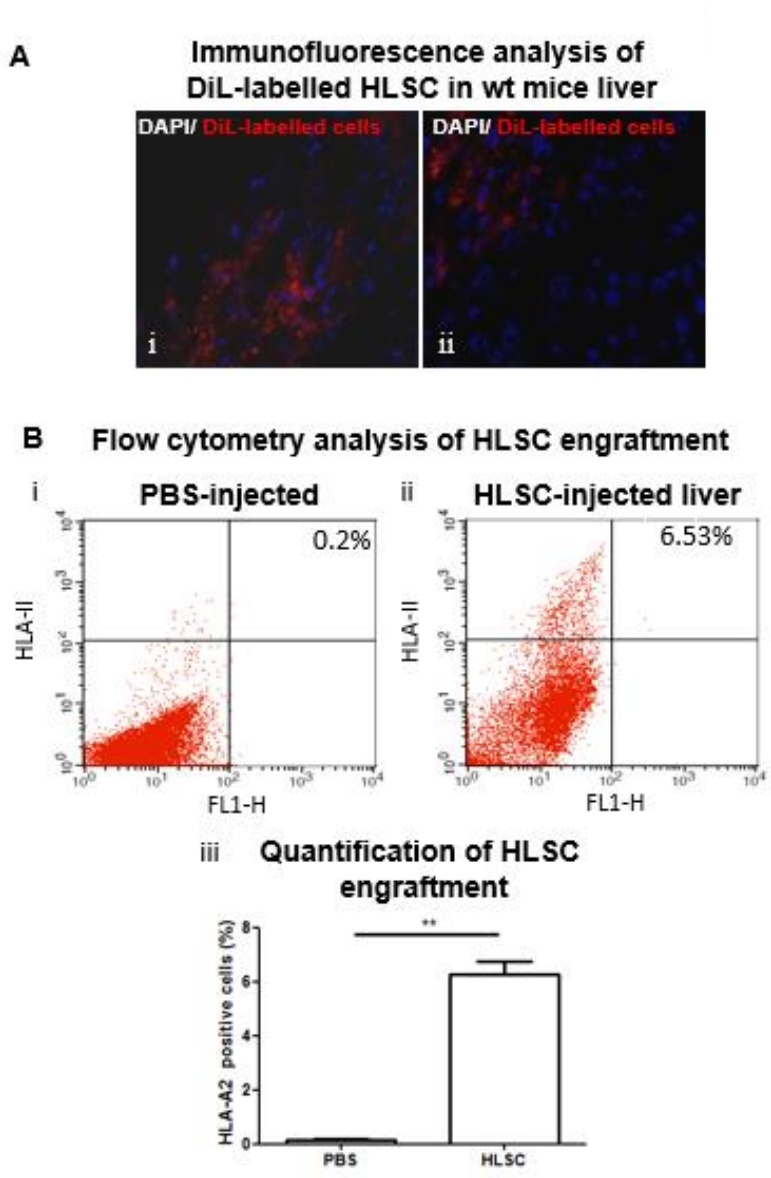

Figure 3: HLSC injection in NSG wt mice. A. Immunofluorescence analysis of DiL-labeled HLSC in a 2 month old wt mouse liver (i, ii). B. Percentage engraftment of HLSC in 21 days old NSG mice livers analyzed by flow cytometry (i-ii) with respect to 21 days old non-injected wt controls (i). Each panel corresponds to a single animal. Graph shows percentage of HLSC engraftment (n=3).

Figure 4

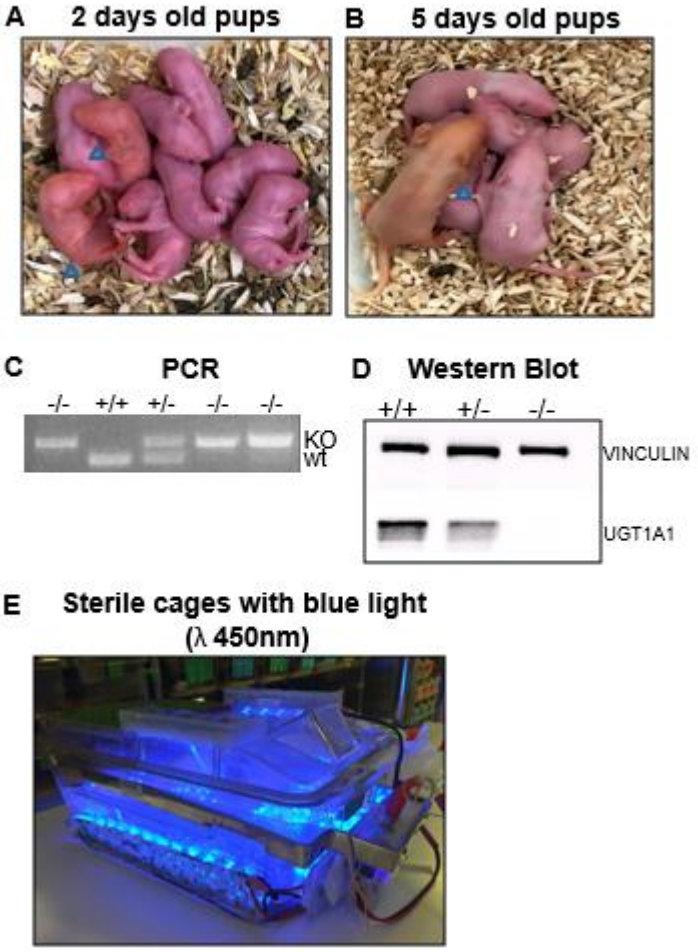

Figure 4: Generation and characterization of NSG Ugt1$^{-/-}$ mice. A. Two days old litters showing NSG Ugt1$^{-/-}$ mice (yellowish, arrowheads). B. Five days old litters with NSG Ugt1$^{-/-}$ mice under phototherapy (PT) (yellowish, arrowheads) C. Genotyping of mice by PCR (KO = knockout; wt = wild type). D. Analysis of UGT1A1 expression by western blot. E. Sterile cages were devised with blue lights for PT of NSG mice.

Figure 5

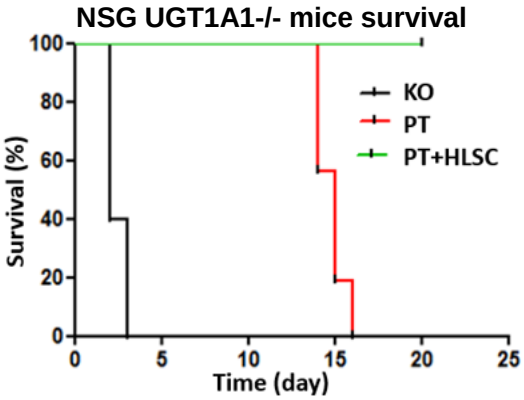

Figure 5: Survival of NSG Ugt1$^{-/-}$ mice. Untreated knockout (KO, n=20) *versus* PT-treated (n=13) and PT + HLSC-treated (n=3) NSG Ugt1$^{-/-}$ mice).

Figure 6

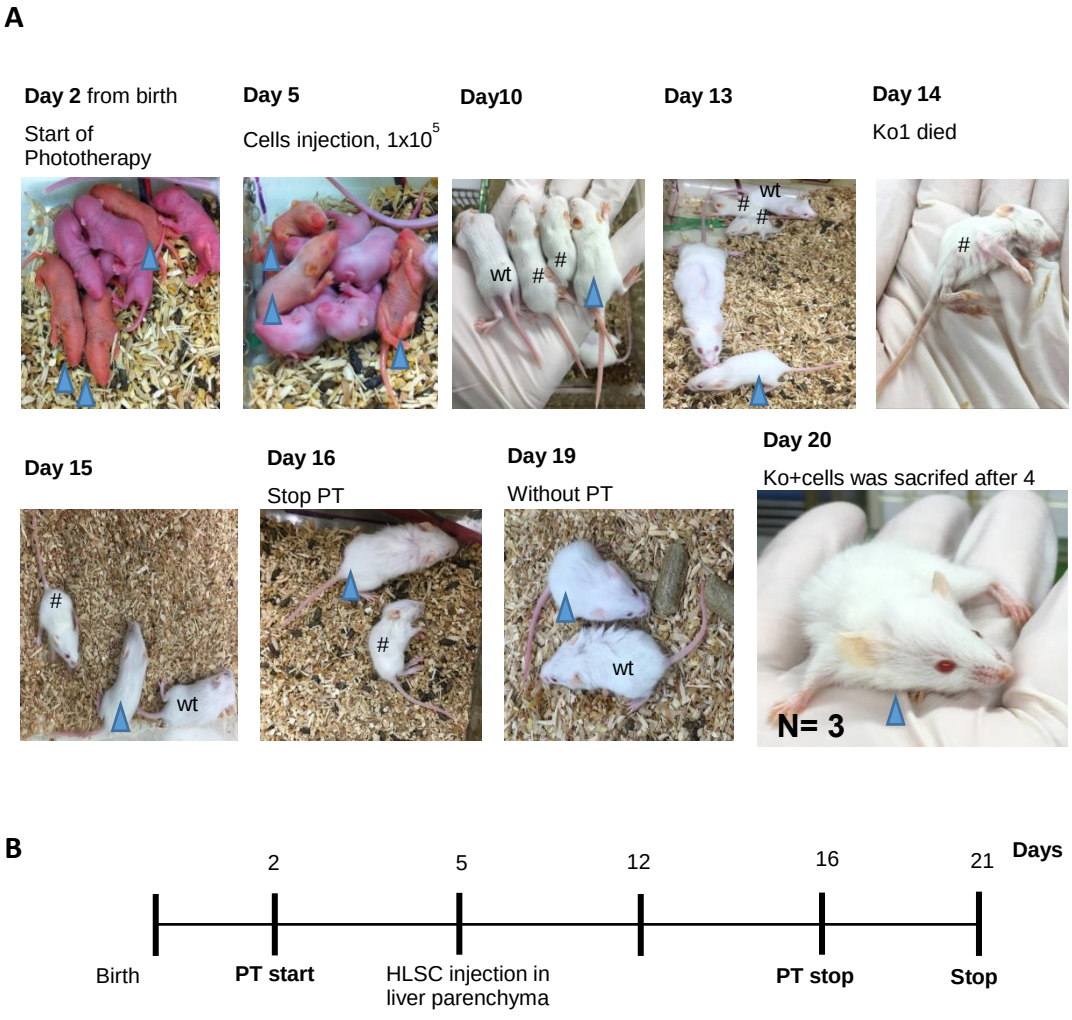

Figure 6: HLSC injection in NSG Ugt1$^{-/-}$ mice. A. Photographs of NSG Ugt1$^{-/-}$ mice treated with HLSC, representative of three independent experiments, are shown. KO mice (arrowheads) are shown at neonatal Day 2 (before PT initiation) and Day 5 (undergoing PT and before HLSC injection). At Day 10, PT-treated KO mice (#) and PT-treated, HLSC-injected KO mice (arrowheads) are shown compared to a wt mouse (wt). HLSC-injected KO mice (arrowhead) at Day 19 and Day 21 post-injection are shown. B. Schedule of PT and HLSC injection in NSG Ugt1$^{-/-}$ mice.

Figure 7

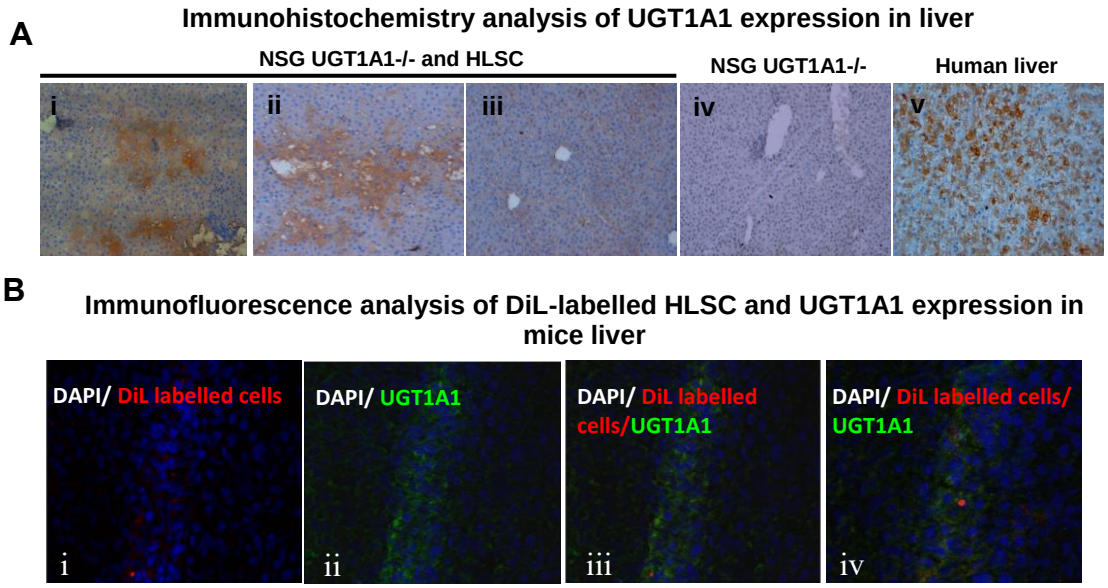

Figure 7: HLSC tracking in NSG Ugt1$^{-/-}$ mice. A. UGT1A1 expression *in vivo* in 21 days old mouse livers was analyzed by immunohistochemistry. UGT1A1-positive areas of livers from NSG Ugt1$^{-/-}$ treated with HLSC are shown in i-ii. UGT1A1-negative areas shown in another section of NSG Ugt1$^{-/-}$ mouse liver treated with HLSC (iii). Non-treated NSG Ugt1$^{-/-}$ (iv) mice and human liver sections (v) were used as negative and positive controls, respectively. B. Immunofluorescence analysis of 21 days old NSG Ugt1$^{-/-}$ mice livers after injection of 1X10^5 HLSCs for the presence of DiL-labeled cells (i) and UGT1A1 protein expression (ii) and merged images (iii). HLSC and UGT1A1 colocalization at higher magnification (60x) is shown in iv.

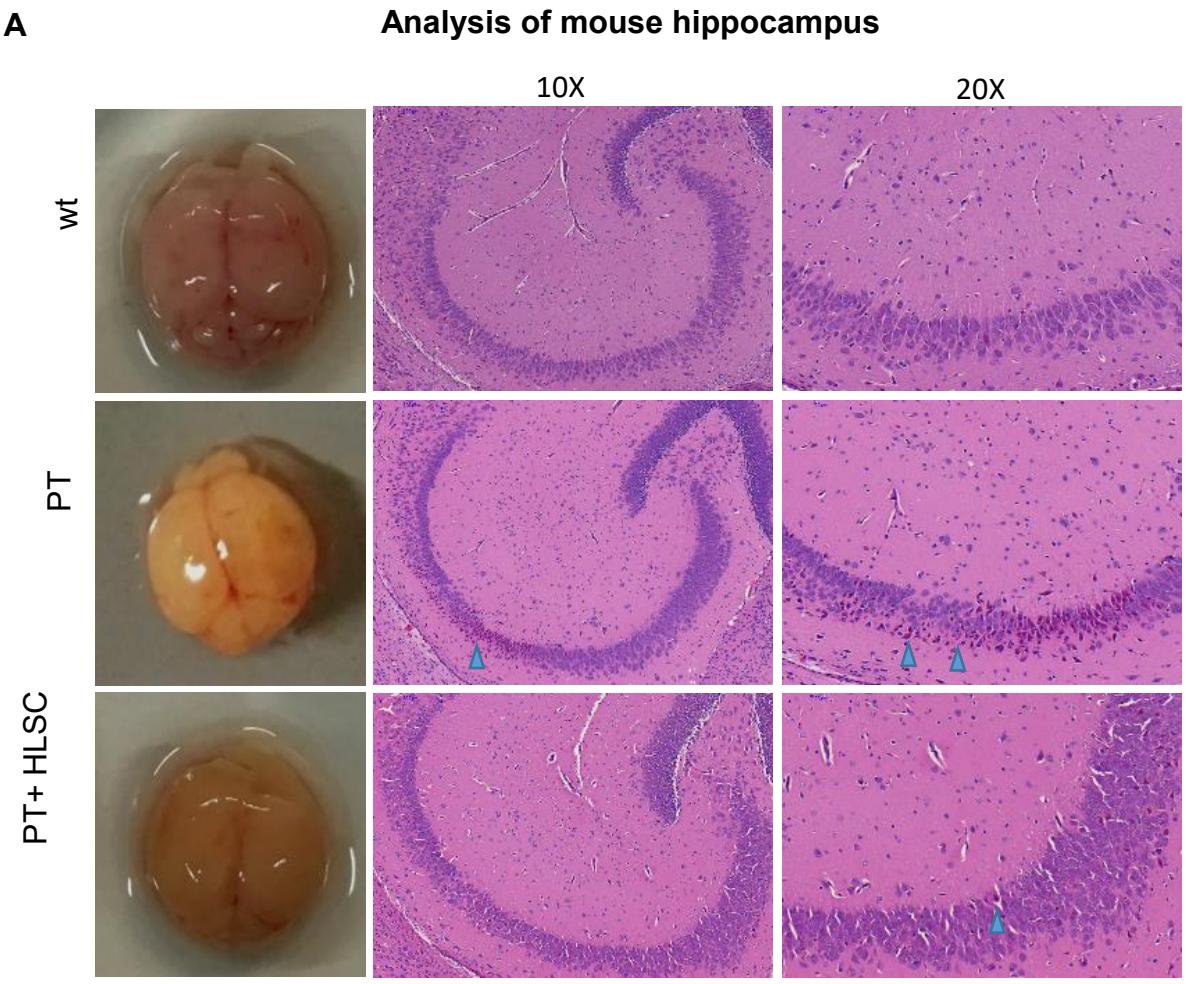

Figure 8: Analysis of mice brain. A. Representative images of brains and hematoxylin/eosin (H/E)-stained hippocampal sections of PT-treated HLSC-injected NSG Ugt1$^{-/-}$ mice (PT + HLSC) compared to wt and PT-treated NSG Ugt1$^{-/-}$ (PT) controls. Arrowheads show eosinophilic neurons. B. Quantification of eosinophilic neuron on 3 fields/ section.

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