**Regenerative cell therapy for the treatment of
hyperbilirubinemic Gunn rats with fresh and frozen human
iPSCs-derived hepatic stem cells**

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1 Brief communication

Title: Regenerative cell therapy for the treatment of hyperbilirubinemic Gunn rats with fresh and frozen human iPSCs-derived hepatic stem cells

6 **Short running title:** hiPSC-based therapy to treat hyperbilirubinemia

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Competing interests

A.F., F.D. and N.T.H. are now employees of GoLiver Therapeutics, a company developing liver-directed regenerative medicine strategies using pluripotent stem cells.

Abbreviations

AFP, alpha-fetoprotein ; ALB, albumin ; CNS-1, Crigler-Najjar Syndrome type 1 ; GMP, Good Manufacturing Practices ; hESCs, human embryonic stem cells ; hiPSCs, human induced pluripotent stem cells ; HLCs, hepatocyte-like cells ; pHSCs, hepatic stem cells derived from pluripotent stem cells ; LN111, Laminin 111 ; UGT1A1, uridine diphosphate glucuronosyltransferase 1 family polypeptide A1.

Abstract

Pluripotent stem cells have been investigated as a renewable source of therapeutic hepatic cells, in order to overcome the lack of transplantable donor hepatocytes. Whereas different studies were able to correct hepatic defects in animal models, they focused on the most mature phenotype of hepatocyte-like cells (HLCs) derived from pluripotent stem cells, and needed freshly-prepared cells, which limits clinical applications of HLCs.

Here, we report the production of hepatic stem cells (pHSCs) from human induced pluripotent stem cells (hiPSCs) in xeno-free, feeder-free and chemically defined conditions using as extracellular matrix a recombinant laminin instead of Matrigel, an undefined animal-derived matrix. Freshly prepared and frozen pHSCs were transplanted via splenic injection in Gunn rats, the animal model for Crigler-Najjar syndrome. Following cell transplantation and daily immunosuppression treatment, bilirubinemia was significantly decreased (around 30% decrease, $p<0.05$) and remained stable throughout the 6 months study. The transplanted pHSCs underwent maturation *in vivo* to restore the deficient metabolic hepatic function (bilirubin glucuronidation by UGT1A1).

In conclusion, we demonstrate for the first time the differentiation of hiPSCs into pHSCs that 1) are produced using a differentiation protocol compatible with Good Manufacturing Practices, 2) can be frozen and 3) are sufficient to demonstrate *in vivo* therapeutic efficacy to significantly lower hyperbilirubinemia in a model of inherited liver disease, despite their immature phenotype. Thus, our approach provides major advances towards future clinical applications and would facilitate cell therapy manufacturing from human pluripotent stem cells.

Keywords: regenerative medicine, induced pluripotent stem cells, Crigler-Najjar syndrome, hepatocyte-like cells, Gunn rats.

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2 88 **Introduction**

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4 89 Liver transplantation is the only curative option for patients with severe metabolic liver
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6 90 disorders in the terminal stage, but the shortage of donor organs results in an increasing number of
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8 91 patients dying while on the waiting list. Transplantation of hepatocytes isolated from cadaveric
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10 92 livers has emerged as a clinical alternative to liver transplantation [1], including for **Crigler-Najjar**
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13 93 **Syndrome type 1 (CNS-1)** disease, a rare inherited metabolic disease characterized by a natural
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15 94 mutation in the uridine diphosphate glucuronosyltransferase 1 family polypeptide A1 (UGT1A1)
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17 95 enzyme, and causing a complete loss of its activity and a hyperbilirubinemia soon after birth [2].
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19 96 The first successful transplantation of human hepatocytes in a patient with **CNS-1** allowed 50%
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21 97 reduction in bilirubinemia [3], but this approach suffers of some important limitations: the low
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23 98 availability and quality of hepatocytes, their absence of proliferation in vitro due to a rapid
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25 99 dedifferentiation in culture, and their low viability following cryopreservation [4].

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29 100 The discovery of human induced pluripotent stem cells (hiPSCs) and human embryonic
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31 101 stem cells (hESCs) has opened great perspectives as a renewable source of any specialized cells of
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33 102 the body [5]–[7], including for the treatment of liver diseases [8], [9]. Several groups reported the *in*
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35 103 *vitro* differentiation of hESCs and hiPSCs into hepatocyte-like cells (HLCs) using diverse culture
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37 104 conditions mimicking the liver embryonic development stages [10]–[19]. Transplantable HLCs
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39 105 express the main markers of adult hepatocytes (albumin, CYP3A4, UGT1A1) and can engraft in the
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41 106 livers of immunocompromised animals [11], [14], [16], [20]–[22]. However the protocols used to
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43 107 produce transplantable HLCs required a long production process (about 30 days), cells were not
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45 108 frozen prior to cell transplantation, or were not suitable for clinical applications as most of them
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47 109 used Matrigel during the hepatic differentiation, mouse feeder cells and/or viral vectors [23].

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50 110 In the specific context of **CNS-1** disease, Gunn rats recapitulate the genetic and metabolic
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52 111 defects observed in patients [24]. The correction of hyperbilirubinemia in Gunn rats has been
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54 112 observed after transplantation of neonatal hepatocytes [25], hepatic progenitors isolated from liver
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56 113 donors [26], HLCs derived from hiPSCs [27] and recently hiPSC-derived mesenchymal cells [28].

In the present study, we cultured BBHX8 hiPSCs [29] onto recombinant laminin-coated tissue culture dishes to initiate hepatic differentiation and to produce hiPSC-derived hepatic stem cells (pHSCs) in 11 days. Harvested pHSCs could be frozen and, after transplantation into Gunn rats, significantly restored UGT1A1 activity and reduced in the long-term the hyperbilirubinemia.

Material and methods

Maintenance of hiPSCs

The BBHX8 hiPSC [30] line was maintained on Matrigel (BD Biosciences, San Jose, CA, USA)-coated culture wells in mTeSR1 (Stemcell Technologies, Vancouver, BC, Canada) at 37°C in a 5% CO₂ incubator with daily media changes.

Hepatic Differentiation in vitro

Cells were plated on laminin 111 (5µg/ml; Biolamina, Sundbyberg, Sweden) in RPMI 1640 supplemented with B27 serum-free supplement (Life technologies, Carlsbad, CA, USA)(RPMI/B27) and 100ng/ml Activin A (Miltenyi Biotec, Paris, France) for 5 days, as well as 50ng/ml WnT3A (R&D systems, Minneapolis, MN, USA) the 2 first days, and 10µM rock inhibitor (Stemcell Technologies, Vancouver, BC, Canada) the first day. Media was changed every day. Then cells were cultured for 2 days in RPMI/B27 supplemented with 10ng/ml fibroblast growth factor 10 (FGF10) (Miltenyi Biotec, Paris, France) and 10ng/ml bone morphogenetic protein 4 (BMP-4)(R&D systems, Minneapolis, MN, USA). Cells were then split and plated on 5µg/ml laminin 111 in RPMI/B27 supplemented with 10ng/ml FGF10, 10ng/ml BMP-4 for 3 days, with 10µM rock inhibitor for the first day. Finally, cells were cultured in hepatocyte culture medium (HCM)(Lonza, Bâle, Switzerland) supplemented with 20ng/ml hepatocyte growth factor (HGF) and 20ng/ml oncostatin M (OSM) (Miltenyi Biotec, Paris, France) for 4 days. Thereafter, no HGF was added anymore and the media was changed every 2 days. For cryopreservation, pHSCs were detached from the plates using TrypLE (Life technologies, Carlsbad, CA, USA) for 10 min at 37°C, and centrifuged for 10 min at 400xg at 4°C (Megafuge 40R, Thermo Fisher Scientific, St Herblain,

France) and at 4°C before resuspension of the cell pellet in CryoStor™ CS10 (StemCell Technologies, Vancouver, BC, Canada) at 6 millions/ml. Cells are then frozen per batch of 6 millions cells in 2mL cryotubes (Corning, Wiesbaden, Germany) 16 hours at -80°C in a CoolCell freezing container (Corning, Wiesbaden, Germany), before transfer to liquid nitrogen. For cell thawing, 1 ml of frozen cells were thawed in a 37°C water bath (Waterbath WNB 14, Memmert, Büchenbach, Germany) and mixed with 9 ml of mTeSR1 (Stemcell Technologies, Vancouver, BC, Canada), centrifuged for 10 min at 400xg and at 4°C before resuspension of cell pellet in saline solution (0,5 mL). Cells are then kept on ice and transplanted maximum 2h after thawing. –for cell transplantation.

RT-qPCR

Total mRNA was isolated from cultured cells using the RNeasy minikit (Qiagen, Courtaboeuf, France) specifications or TRIzol® (Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions. Analyses of transcripts were performed with a ViiA7 sequence detection system using Power EXPRESS One-Step SYBR®GreenER™ Kit (Life Technologies, Carlsbad, CA, USA) starting from 10ng mRNA. Primers sequences were as follows: AFP-F:GCTTGGTGGTGGATGAAACA; AFP-R:TCCTCTGTTATTTGTGGCTTTTG; ALB-F:GCACAGAATCCTTGGTGAACAG; ALB-R:ATGGAAGGTGAATGTTTTCAGCA; CK19-F:CTCCCGCGACTACAGCCACT; CK19-R:TCAGCTCATCCAGCACCTG; FOXA2-F:GCACTCGGCTTCCAGTATG; FOXA2-R:CACGTACGACGACATGTTCA; GAPDH-F:AATCCCATCACCATCTTCCA; GAPDH-R:TGGACTCCACGACGTACTCA; HNF4-F:TGGACAAAGACAAGAGGAACC; HNF4-R:ATAGCTTGACCTTCGAGTGC; SOX17-F:TTTCATGGTGTGGGCTAAGG; SOX17-R:CGGCCGGTACTTGTAGTTG; OCT4-F:GTGGAGGAAGCTGACAACAA; OCT4-R:CAGGTTTTCTTTCCCTAGCT; NANOG-F:TCCAACATCCTGAACCTCAG; NANOG-R:TCCTGGATGTTCTGGGTCTG.

Flow Cytometry

Cells were fixed with 0.25% paraformaldehyde for 30 min at 4°C, permeabilized with 0.2% Tween-

20 for 15 min at 37°C, and blocked with 3% BSA/PBS for 30 min at 4°C in the presence or absence of primary antibodies against OCT4 (AlexaFluor 647 Mouse anti-OCT3/4, BD Biosciences, San Jose, CA, USA), NANOG (PE Mouse anti-human-NANOG, BD Biosciences, San Jose, CA, USA), FOXA2 (PE Mouse anti-human-FOXA2, BD Biosciences, San Jose, CA, USA), AFP (AlexaFluor 488 Mouse Anti-human-AFP, BD Biosciences, San Jose, CA, USA), SOX17 (Allophycocyanin Goat anti-hSOX17, R&D, Minneapolis, MN, USA) and ALB (FITC Rabbit Anti-human-ALB, Dako, Carpinteria, CA, USA). The analysis was performed with a FACSCanto II (BD Biosciences, San Jose, CA, USA) and Flow Jo software (Tree star, Ashland, OR, USA).

Immunofluorescence Assay

Cultured cells were fixed with 4% paraformaldehyde for 30 min at room temperature, permeabilized with 0.5% Triton X-100 in PBS for 15 min and blocked with 3% BSA-0.1% Triton in PBS for 15 min. The cells were incubated with rabbit antibody against human HNF4 α (Santa Cruz Biotechnology, Le Perray en Yveline, France) overnight at 4°C, 30 min at room temperature with Alexa 555 anti-rabbit secondary antibody (Abcam, Paris, France), counterstained with DAPI, and observed under a Zeiss fluorescent microscope (Axiocam ICM1 model).

Transplantation of hepatocytes in Gunn rats

Homozygous (j/j) Gunn rats (age: 8-9 week-old) were **bred** in the animal facilities of Nantes University Medical School (Nantes, France), and were subjected 24h before transplantation to a two-third partial hepatectomy to create an environment optimal for cell transplantation [31]. Briefly, rats were anesthetized with isoflurane general anesthesia (3% [v/v] in air) and positioned in dorsal decubitus. Laparotomy was performed, then median and left lobes were ligatured before the ablation of the right lobe and closure of the abdomen wall. 1×10^7 fresh (N=10 rats, 2 males and 8 females) or frozen (N=6 rats, 3 males and 3 females) pHSCs after 11 days of differentiation were resuspended in 0.5 mL saline solution and injected with a 29G insulin syringe into the inferior splenic pole of rats immunosuppressed daily with intraperitoneal injection of tacrolimus at 0.2 mg/kg from the day before transplantation for 3 days and at 0.1 mg/kg thereafter.

Control rats (N=9, 4 males and 5 females) received the same surgical procedures, including daily injection of tacrolimus, but were not transplanted (neither injected with saline solution). Buprenorphine (0,01 mg/kg) was administrated as an analgesic before and after surgery. Animal experiments were performed in a dedicated room from the animal facility. All the animal care and procedures performed were approved by the Animal Experimentation Ethics Committee of the Pays de la Loire region, France, in accordance with the guidelines from the French National Research Council for the Care and Use of Laboratory Animals (Décret n° 2013-118, Directive 2010/63/UE).

Liver function tests

Blood was drawn from retro-orbital sinus, and serum total bilirubin and alanine aminotransferases (ALAT) were measured in the biochemistry department of Nantes University hospital (Nantes, France), as previously described [32]. Enzymatic activity of UGT1A1 was assayed on splenic homogenates by the diazotation method of Black et al. [33], using liver homogenates from wild type rats as a positive control. Data were expressed as a percentage of the activity measured just before transplantation.

Histology and Immunohistochemistry

Histology and immunohistochemistry were performed by APEX platform (Oniris, Nantes). Paraffin-embedded liver sections (5µm) were incubated with human UGT1A1 (Abgent, San Diego, CA, USA) antibody. Liver sections were counterstained with hematoxylin. The percentage of UGT1A1-expressing cells was calculated as the percentage of positively stained cells in 10 fields at x40 magnification (i.e., at least 3000 cells).

Teratoma assay

2×10⁶ pHSCs in 30uL Matrigel were subcutaneously injected into the testis capsule of immunodeficient IL2rg Knockout rats (5 pHSCs rats and 2 positive control rats injected with iPS cells), sourced from the TRIP platform [34], and bred in the animal facility of the INSERM UMR 1064 of the University of Nantes (Nantes, France). Observations for teratoma formation were followed for 6 months after the cell injection. Animals were sacrificed at 6 months, or before when

a weight increase of more than 10% occurred, or when any behavioral signs of pain or suffering became evident.

Statistical analysis

Serum bilirubin levels in individual rats were determined in triplicate and expressed as percent of pre-transplant values. For each time point results were compared with those in the control group at the same time point using Student's unpaired two-tailed t test. Data are expressed as mean values $\pm$ SEM. Statistical analysis was made using the GraphPad Prism® 5.04 software (GraphPad Software, San Diego, CA). A p-value < 0.05 was considered to be statistically significant.

Results

Recombinant laminin 111 is efficient at inducing the differentiation of hiPSCs into hepatic stem cells

We modified and combined previously reported protocols [12], [16], [35] to develop a GMP-compatible hepatic differentiation protocol to produce HLCs from BBHX8 hiPSCs [29], [36] (Fig.1), using laminin 111 (LN111), expressed in fetal liver, as a matrix [35], [37].

qPCR (Fig.2A, N=3) and FACS (Fig.2B, N=3) experiments confirmed that Oct4 and Nanog, (markers of pluripotency) were maximally expressed at day 0, whereas the differentiation into definitive endoderm (SOX17, FOXA2) was optimal at day 5, with 80-90% of the cells strongly expressing these markers (Fig.2).

By day 11, almost all cells were positive for markers expressed in hepatic stem cells (HNF4a, CK19), but negative for alpha-fetoprotein (AFP), a marker of hepatoblasts, and albumin (ALB) marker of mature hepatocytes (Fig.2A-C)[38]. In parallel, they lost their expression of SOX17 and FOXA2, evidencing a phenotype of hepatic stem cells (Fig.2A-B). Finally, the hepatic markers AFP and ALB appeared progressively along the differentiation process towards HLCs, with a maximal expression after 21 days (Fig.2A). To use these cells for cell therapy, we then decided to focus on day 11-pHSCs, because of the short culture duration (> 20 days for HLCs), and

because they can easily and efficiently be frozen in a GMP-compatible cryopreservation solution. The injection of these cells in the testis of immunodeficient rats (IL2rg KO rats; [34]) did not induce the formation of teratoma (N=5) contrary to iPS cells (N=2), demonstrating their loss of pluripotency potential and therefore their suitability for cell transplantation (Supplementary Fig.1).

Transplantation of hiPSCs-derived pHSCs into Gunn rats

Freshly prepared pHSCs were transplanted in Gunn rats (N=10). Serum levels alanine aminotransferase (ALAT) values in treated rats were not different from pre-operative values (that is (before hepatectomy and transplantation), indicating that animal recovered well from surgical procedures (Supplementary Fig.2). Serum bilirubin levels showed a progressive reduction of baseline bilirubin levels in the treated group, that thereafter persisted throughout the study at a level of $28 \pm 5\%$ decrease compared to the initial pre-operative level, and was statistically significant (Fig.3A, discontinuous line) ($P < 0.05$). Serum bilirubin in the treated group was $57 \pm 5 \mu\text{M}$ at time of sacrifice versus $84 \pm 11 \mu\text{M}$ for pre-transplantation values ($p = 0.005$), and noteworthy, similar results were also obtained after transplantation of frozen pHSCs (N=6) (Fig.3A, dotted line). In contrast, serum bilirubin in the control group remained elevated with values up to $150 \mu\text{M}$ (60% increase in bilirubinemia) (N=9) (Fig.3A, continuous line).

Immunohistochemistry revealed the presence in the liver of engrafted cells expressing the human specific antigen UGT1A1 showing that transplanted pHSCs matured into adult hepatocytes (Fig. 3B upper panel). As no selective growth advantage was given to transplanted pHSCs, only few UGT1A1 expressing-human hepatocytes were detected in the liver as expected (less than 0.1% of the liver), explaining the absence of detection of human serum proteins like albumin (data not shown). Interestingly, we also detected matured pHSCs expressing the human UGT1A1 enzyme in the spleen of rats transplanted with freshly prepared and frozen pHSCs (1-2 % positive cells) (Fig.3B lower panel). Coherently with this result, the measurement of UGT1A1 activity in the spleen extracts of these animals showed a significant elevated activity compared to extracts of control rats (Fig.3C), whereas no activity was measured in liver extracts.

Discussion

We demonstrated the differentiation of hiPSCs into hepatic stem cells (HNF4a⁺, CK19⁺, AFP⁻, ALB⁻) using chemically defined conditions, with xeno-free, feeder-free media, recombinant factors and recombinant matrix support, such that clinical application can be considered. We show for the first time the efficacy of hiPSC-based regenerative medicine to treat the CNS-1 hepatic metabolic disease, with a GMP-compatible hepatic differentiation protocol and using frozen pHSCs.

Some previous publications already showed the participation of LN111 to the hepatocyte differentiation of hiPSCs, but include a step on matrigel-coated dish during the hepatic differentiation protocol [35], or as a mixture with LN521 and without *in vivo* validation [39]. On the contrary, we developed a protocol of differentiation that uses only recombinant LN111 for the production of transplantable hepatocytes-like cells.

These cells were able to induce a significant decrease in bilirubinemia in icteric adult Gunn rats, sustained for a long period (6 months). In addition, no teratoma was observed 6 months after direct injection of cells in the testis of immunodeficient rats (Supplementary Fig.1), suggesting the absence of residual undifferentiated hiPSCs in the cell preparation. While the teratoma assay was performed in different conditions (different rat model and site of injection) and would need complementary experiment such as cell injection in the liver, the absence of tumor formation in transplanted rats maintained on long term (more than 4 months) however suggests the absence of tumorigenicity.

Our study indicates that hiPSCs specified into hepatic stem cells (negative for UGT1A1 expression, data not shown) were capable of repopulating the liver and matured *in situ* at a level sufficient to ensure metabolic hepatic functions. The maximum therapeutic efficacy was observed around 2 months after cell injection, which is comparable to other studies following transplantation of HLCs or human neonate hepatocytes [25][27]. pHSCs were also able to survive and differentiate

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2 296 in the spleen, and were there still able to fulfill their metabolic role as evidenced by the UGT1A1
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4 297 activity (Fig.3C). Indeed the spleen can provide an adequate environment for hepatocyte
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6 298 engraftment, proliferation, maturation and functionality [40].
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9 299 Recent studies described the production of transplantable HLCs using GMP-compatible hepatic
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11 300 differentiation protocol [41][42], but culture times were about 30 days versus 11 days for pHSCs.
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13 301 Takayama et al. demonstrated a very efficient cryopreservation of HLCs using a GMP-compatible
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16 302 cryopreservation solution, but cells functionality was not confirmed *in vivo* [41]. While our study
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18 303 did not allow to compare pHSCs efficiency to the one of HLCs, our data show that pHSCs can
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20 304 efficiently be transplanted in Gunn rats to correct hyperbilirubinemia, using a protocol asking two
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23 305 times less time, and in absence of liver preconditioning that would give a selective growth
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25 306 advantage to transplanted hepatocytes. Interestingly, even if that would need further validation, the
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27 307 transplantation efficacy seems to be comparable when using freshly prepared or frozen pHSCs.
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30 308 Moreover the therapeutic efficacy was similar to the cell therapy approach using primary
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32 309 hepatocytes [25].
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34 310 Thus, in order to transpose this finding into clinical settings, a clinical scenario could be
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36 311 easily imagined by replacing partial hepatectomy by partial liver embolization to stimulate liver
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39 312 regeneration and cell engraftment, as we showed in non human primates [43]. Alternatively partial
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41 313 liver irradiation prior to cell transplantation could also be considered [44]. In parallel to consider the
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43 314 use of those cells as universal cell donors some optimization will be needed to avoid their
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46 315 recognition by the immune system, like the invalidation of HLA expression [45].
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52 53 318 Conclusion

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55 319 There is a clinical demand for frozen human hepatocytes to treat inherited liver diseases,
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58 320 including **CNS-1**. Thanks to a relatively simple, fast and GMP-compatible hepatic differentiation
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60 321 protocol to produce therapeutic cells, we propose a new and immediately available unlimited source

of frozen hepatic stem cells starting from human induced pluripotent stem cells, that could be a safe clinical alternative to liver and hepatocytes transplants, and that beyond CNS-1 disease could also be used for the transplantation in other hepatic defects.

For Peer Review

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Figure legends

Figure 1. Protocol for the hepatic differentiation of human induced pluripotent stem cells (hiPSCs). Protocol to differentiate hiPSCs into hepatic stem cells (pHSCs), used for the transplantation, or more mature hepatocyte-like cells (HLCs), including the different media (brackets), cytokines (upper frame) and matrix (lower frame) used.

Figure 2. Characterization of differentiation markers during the conversion of hiPSCs into hepatic stem cells (pHSCs). (A) Hepatic differentiation was monitored over time from Day 0 (D0), Day 5 (D5), Day 11 (D11), Day 21 (D21) to Day 30 (D30) by RT-qPCR analyses for mRNA expression of pluripotency (OCT4, NANOG), endoderm (FOXA2, SOX17) and hepatic markers (HNF4A, CK19, AFP, ALB). The relative gene expression was calculated using the $2^{-\Delta\Delta C_t}$ quantification method after normalization to GAPDH values, and expressed as the fold change in mRNA levels in a given sample relative to undifferentiated cells (N=3, mean $\pm$ SEM). The expression of the same markers was confirmed at the protein level through FACS analysis (N=3) (B) or immunofluorescence staining (C). The scale bar represents 100 μ m.

Figure 3. Transplantation of iPSC-derived hepatic progenitors into the liver of Gunn rats. (A) Serum bilirubin levels in Gunn rats. Bilirubinemia data are represented as change in serum bilirubin from baseline (compared to pretransplant levels) over time (mean $\pm$ SEM), in control rats (N=9, continuous line) and rats transplanted with fresh (N=10, discontinuous line) or frozen (N=6, dotted line) pHSCs. (B) Histochemical analysis of human UGT1A1 expression in the liver (upper panel) or spleen (lower panel) of Gunn rats transplanted with fresh or frozen pHSCs. The scale bar represents 20 μ m. (C) UGT1A1 activity was measured by the diazotation method in spleen extracts of transplanted rats (N=9) and expressed as percentage to normal liver activity.

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2 471 **Supplementary Figure 1. Absence of Teratoma after injection of iPSC-derived hepatic stem**
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4 472 **cells in rat testis of IL2rg KO rats.** Six months after their injection in rat testis, iPSCs generated
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6 473 teratoma (N=2) whereas iPSC-derived hepatic stem cells did not (N=5).
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11 475 **Supplementary Figure 2. Postoperative measurement of alanine amino transferase (ALAT)**
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13 476 **activity in the serum of transplanted rats.** ALAT activity was measured in the serum of control
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16 477 mice and mice transplanted with fresh and frozen iPSC-derived hepatic stem cells, at different time
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Brief communication

Title: Regenerative cell therapy for the treatment of hyperbilirubinemic Gunn rats with fresh and frozen human iPSCs-derived hepatic stem cells

Short running title: hiPSC-based therapy to treat hyperbilirubinemia

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Competing interests

A.F., F.D. and N.T.H. are now employees of GoLiver Therapeutics, a company developing liver-directed regenerative medicine strategies using pluripotent stem cells.

Abbreviations

AFP, alpha-fetoprotein ; ALB, albumin ; CNS-1, Crigler-Najjar Syndrome type 1 ; GMP, Good Manufacturing Practices ; hESCs, human embryonic stem cells ; hiPSCs, human induced pluripotent stem cells ; HLCs, hepatocyte-like cells ; pHSCs, hepatic stem cells derived from pluripotent stem cells ; LN111, Laminin 111 ; UGT1A1, uridine diphosphate glucuronosyltransferase 1 family polypeptide A1.

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Abstract

Pluripotent stem cells have been investigated as a renewable source of therapeutic hepatic cells, in order to overcome the lack of transplantable donor hepatocytes. Whereas different studies were able to correct hepatic defects in animal models, they focused on the most mature phenotype of hepatocyte-like cells (HLCs) derived from pluripotent stem cells, and needed freshly-prepared cells, which limits clinical applications of HLCs.

Here, we report the production of hepatic stem cells (pHSCs) from human induced pluripotent stem cells (hiPSCs) in xeno-free, feeder-free and chemically defined conditions using as extracellular matrix a recombinant laminin instead of Matrigel, an undefined animal-derived matrix. Freshly prepared and frozen pHSCs were transplanted via splenic injection in Gunn rats, the animal model for Crigler-Najjar syndrome. Following cell transplantation and daily immunosuppression treatment, bilirubinemia was significantly decreased (around 30% decrease, $p<0.05$) and remained stable throughout the 6 months study. The transplanted pHSCs underwent maturation *in vivo* to restore the deficient metabolic hepatic function (bilirubin glucuronidation by UGT1A1).

In conclusion, we demonstrate for the first time the differentiation of hiPSCs into pHSCs that 1) are produced using a differentiation protocol compatible with Good Manufacturing Practices, 2) can be frozen and 3) are sufficient to demonstrate *in vivo* therapeutic efficacy to significantly lower hyperbilirubinemia in a model of inherited liver disease, despite their immature phenotype. Thus, our approach provides major advances towards future clinical applications and would facilitate cell therapy manufacturing from human pluripotent stem cells.

Keywords: regenerative medicine, induced pluripotent stem cells, Crigler-Najjar syndrome, hepatocyte-like cells, Gunn rats.

Introduction

Liver transplantation is the only curative option for patients with severe metabolic liver disorders in the terminal stage, but the shortage of donor organs results in an increasing number of patients dying while on the waiting list. Transplantation of hepatocytes isolated from cadaveric livers has emerged as a clinical alternative to liver transplantation [1], including for Crigler-Najjar Syndrome type 1 (CNS-1) disease, a rare inherited metabolic disease characterized by a natural mutation in the uridine diphosphate glucuronosyltransferase 1 family polypeptide A1 (UGT1A1) enzyme, and causing a complete loss of its activity and a hyperbilirubinemia soon after birth [2]. The first successful transplantation of human hepatocytes in a patient with CNS-1 allowed 50% reduction in bilirubinemia [3], but this approach suffers of some important limitations: the low availability and quality of hepatocytes, their absence of proliferation *in vitro* due to a rapid dedifferentiation in culture, and their low viability following cryopreservation [4].

The discovery of human induced pluripotent stem cells (hiPSCs) and human embryonic stem cells (hESCs) has opened great perspectives as a renewable source of any specialized cells of the body [5]–[7], including for the treatment of liver diseases [8], [9]. Several groups reported the *in vitro* differentiation of hESCs and hiPSCs into hepatocyte-like cells (HLCs) using diverse culture conditions mimicking the liver embryonic development stages [10]–[19]. Transplantable HLCs express the main markers of adult hepatocytes (albumin, CYP3A4, UGT1A1) and can engraft in the livers of immunocompromised animals [11], [14], [16], [20]–[22]. However the protocols used to produce transplantable HLCs required a long production process (about 30 days), cells were not frozen prior to cell transplantation, or were not suitable for clinical applications as most of them used Matrigel during the hepatic differentiation, mouse feeder cells and/or viral vectors [23].

In the specific context of CNS-1 disease, Gunn rats recapitulate the genetic and metabolic defects observed in patients [24]. The correction of hyperbilirubinemia in Gunn rats has been observed after transplantation of neonatal hepatocytes [25], hepatic progenitors isolated from liver donors [26], HLCs derived from hiPSCs [27] and recently hiPSC-derived mesenchymal cells [28].

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2 114 In the present study, we cultured BBHX8 hiPSCs [29] onto recombinant laminin-coated
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4 115 tissue culture dishes to initiate hepatic differentiation and to produce hiPSC-derived hepatic stem
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6 116 cells (pHSCs) in 11 days. Harvested pHSCs could be frozen and, after transplantation into Gunn
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9 117 rats, significantly restored UGT1A1 activity and reduced in the long-term the hyperbilirubinemia.
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14 119 **Material and methods**

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16 120 **Maintenance of hiPSCs**

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18 121 The BBHX8 hiPSC [30] line was maintained on Matrigel (BD Biosciences, San Jose, CA, USA)-
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21 122 coated culture wells in mTeSR1 (Stemcell Technologies, Vancouver, BC, Canada) at 37°C in a 5%
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23 123 CO² incubator with daily media changes.
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25 124 **Hepatic Differentiation in vitro**

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27 125 Cells were plated on laminin 111 (5µg/ml; Biolamina, Sundbyberg, Sweden) in RPMI 1640
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30 126 supplemented with B27 serum-free supplement (Life technologies, Carlsbad, CA,
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32 127 USA)(RPMI/B27) and 100ng/ml Activin A (Miltenyi Biotec, Paris, France) for 5 days, as well as
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34 128 50ng/ml WnT3A (R&D systems, Minneapolis, MN, USA) the 2 first days, and 10µM rock inhibitor
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36 129 (Stemcell Technologies, Vancouver, BC, Canada) the first day. Media was changed every day.
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39 130 Then cells were cultured for 2 days in RPMI/B27 supplemented with 10ng/ml fibroblast growth
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41 131 factor 10 (FGF10) (Miltenyi Biotec, Paris, France) and 10ng/ml bone morphogenetic protein 4
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43 132 (BMP-4)(R&D systems, Minneapolis, MN, USA). Cells were then split and plated on 5µg/ml
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45 133 laminin 111 in RPMI/B27 supplemented with 10ng/ml FGF10, 10ng/ml BMP-4 for 3 days, with
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47 134 10µM rock inhibitor for the first day. Finally, cells were cultured in hepatocyte culture medium
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50 135 (HCM)(Lonza, Bâle, Switzerland) supplemented with 20ng/ml hepatocyte growth factor (HGF) and
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52 136 20ng/ml oncostatin M (OSM) (Miltenyi Biotec, Paris, France) for 4 days. Thereafter, no HGF was
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55 137 added anymore and the media was changed every 2 days. For cryopreservation, pHSCs were
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57 138 detached from the plates using TrypLE (Life technologies, Carlsbad, CA, USA) for 10 min at 37°C,
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60 139 and centrifuged for 10 min at 400xg at 4°C (Megafuge 40R, Thermo Fisher Scientific, St Herblain,

France) before resuspension of the cell pellet in CryoStor™ CS10 (StemCell Technologies, Vancouver, BC, Canada) at 6 millions/ml. Cells are then frozen per batch of 6 millions cells in 2mL cryotubes (Corning, Wiesbaden, Germany) 16 hours at -80°C in a CoolCell freezing container (Corning, Wiesbaden, Germany), before transfer to liquid nitrogen. For cell thawing, 1 ml of frozen cells were thawed in a 37°C water bath (Waterbath WNB 14, Memmert, Büchenbach, Germany) and mixed with 9 ml of mTeSR1 (Stemcell Technologies, Vancouver, BC, Canada), centrifuged for 10 min at 400xg and at 4°C before resuspension of cell pellet in saline solution (0,5 mL). Cells are then kept on ice and transplanted maximum 2h after thawing.

RT-qPCR

Total mRNA was isolated from cultured cells using the RNeasy minikit (Qiagen, Courtaboeuf, France) specifications or TRIzol® (Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions. Analyses of transcripts were performed with a ViiA7 sequence detection system using Power EXPRESS One-Step SYBR®GreenER™ Kit (Life Technologies, Carlsbad, CA, USA) starting from 10ng mRNA. Primers sequences were as follows: AFP-F:GCTTGGTGGTGGATGAAACA; AFP-R:TCCTCTGTTATTTGTGGCTTTTG; ALB-F:GCACAGAATCCTTGGTGAACAG; ALB-R:ATGGAAGGTGAATGTTTTTCAGCA; CK19-F:CTCCCGCGACTACAGCCACT; CK19-R:TCAGCTCATCCAGCACCCCTG; FOXA2-F:GCACTCGGCTTCCAGTATG; FOXA2-R:CACGTACGACGACATGTTCA; GAPDH-F:AATCCCATCACCATCTTCCA; GAPDH-R:TGGACTCCACGACGTACTCA; HNF4-F:TGGACAAAGACAAGAGGAACC; HNF4-R:ATAGCTTGACCTTCGAGTGTC; SOX17-F:TTTCATGGTGTGGGCTAAGG; SOX17-R:CGGCCGGTACTTGTAGTTG; OCT4-F:GTGGAGGAAGCTGACAACAA; OCT4-R:CAGGTTTTCTTTTCCCTAGCT; NANOG-F:TCCAACATCCTGAACCTCAG; NANOG-R:TCCTGGATGTTCTGGGTCTG.

Flow Cytometry

Cells were fixed with 0.25% paraformaldehyde for 30 min at 4°C, permeabilized with 0.2% Tween-20 for 15 min at 37°C, and blocked with 3% BSA/PBS for 30 min at 4°C in the presence or absence

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2 166 of primary antibodies against OCT4 (AlexaFluor 647 Mouse anti-OCT3/4, BD Biosciences, San
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4 167 Jose, CA, USA), NANOG (PE Mouse anti-human-NANOG, BD Biosciences, San Jose, CA, USA),
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6 168 FOXA2 (PE Mouse anti-human-FOXA2, BD Biosciences, San Jose, CA, USA), AFP (AlexaFluor
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8 488 Mouse Anti-human-AFP, BD Biosciences, San Jose, CA, USA), SOX17 (Allophycocyanin
9 169 Goat anti-hSOX17, R&D, Minneapolis, MN, USA) and ALB (FITC Rabbit Anti-human-ALB,
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11 170 Dako, Carpinteria, CA, USA). The analysis was performed with a FACSCanto II (BD Biosciences,
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13 171 San Jose, CA, USA) and Flow Jo software (Tree star, Ashland, OR, USA).
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18 173 **Immunofluorescence Assay**
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22 permeabilized with 0.5% Triton X-100 in PBS for 15 min and blocked with 3% BSA-0.1% Triton
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25 176 Cruz Biotechnology, Le Perray en Yveline, France) overnight at 4°C, 30 min at room temperature
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27 177 with Alexa 555 anti-rabbit secondary antibody (Abcam, Paris, France), counterstained with DAPI,
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29 178 and observed under a Zeiss fluorescent microscope (AxioCam ICM1 model).
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34 180 **Transplantation of hepatocytes in Gunn rats**
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36 181 Homozygous (j/j) Gunn rats (age: 8-9 week-old) were bred in the animal facilities of Nantes
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38 University Medical School (Nantes, France), and were subjected 24h before transplantation to a
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41 183 Briefly, rats were anesthetized with isoflurane general anesthesia (3% [v/v] in air) and positioned in
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43 184 dorsal decubitus. Laparotomy was performed, then median and left lobes were ligatured before the
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45 185 ablation of the right lobe and closure of the abdomen wall. 1x10⁷ fresh (N=10 rats, 2 males and 8
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47 186 females) or frozen (N=6 rats, 3 males and 3 females) pHSCs after 11 days of differentiation were
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49 187 resuspended in 0.5 mL saline solution and injected with a 29G insulin syringe
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51 188 into the inferior splenic pole of rats immunosuppressed daily with intraperitoneal injection of
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53 189 tacrolimus at 0.2 mg/kg from the day before transplantation for 3 days and at 0.1 mg/kg thereafter.
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55 190 Control rats (N=9, 4 males and 5 females) received the same surgical procedures, including daily
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injection of tacrolimus, but were not transplanted (neither injected with saline solution). Buprenorphine (0,01 mg/kg) was administrated as an analgesic before and after surgery. Animal experiments were performed in a dedicated room from the animal facility. All the animal care and procedures performed were approved by the Animal Experimentation Ethics Committee of the Pays de la Loire region, France, in accordance with the guidelines from the French National Research Council for the Care and Use of Laboratory Animals (Décret n° 2013-118, Directive 2010/63/UE).

Liver function tests

Blood was drawn from retro-orbital sinus, and serum total bilirubin and alanine aminotransferases (ALAT) were measured in the biochemistry department of Nantes University hospital (Nantes, France), as previously described [32]. Enzymatic activity of UGT1A1 was assayed on splenic homogenates by the diazotation method of Black et al. [33], using liver homogenates from wild type rats as a positive control. Data were expressed as a percentage of the activity measured just before transplantation.

Histology and Immunohistochemistry

Histology and immunohistochemistry were performed by APEX platform (Oniris, Nantes). Paraffin-embedded liver sections (5µm) were incubated with human UGT1A1 (Abgent, San Diego, CA, USA) antibody. Liver sections were counterstained with hematoxylin. The percentage of UGT1A1-expressing cells was calculated as the percentage of positively stained cells in 10 fields at x40 magnification (i.e., at least 3000 cells).

Teratoma assay

2×10⁶ pHSCs in 30uL Matrigel were subcutaneously injected into the testis capsule of immunodeficient IL2rg Knockout rats (5 pHSCs rats and 2 positive control rats injected with iPS cells), sourced from the TRIP platform [34], and bred in the animal facility of the INSERM UMR 1064 of the University of Nantes (Nantes, France). Observations for teratoma formation were followed for 6 months after the cell injection. Animals were sacrificed at 6 months, or before when a weight increase of more than 10% occurred, or when any behavioral signs of pain or suffering

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4 219 **Statistical analysis**

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6 220 Serum bilirubin levels in individual rats were determined in triplicate and expressed as percent of
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11 222 the same time point using Student's unpaired two-tailed t test. Data are expressed as mean values $\pm$
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13 223 SEM. Statistical analysis was made using the GraphPad Prism® 5.04 software (GraphPad Software,
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16 224 San Diego, CA). A p-value < 0.05 was considered to be statistically significant.

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20 226 **Results**

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23 227 **Recombinant laminin 111 is efficient at inducing the differentiation of hiPSCs into**
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25 228 **hepatic stem cells**

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28 229 We modified and combined previously reported protocols [12], [16], [35] to develop a
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30 230 GMP-compatible hepatic differentiation protocol to produce HLCs from BBHX8 hiPSCs [29], [36]
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32 231 (Fig.1), using laminin 111 (LN111), expressed in fetal liver, as a matrix [35], [37].

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34 232 qPCR (Fig.2A, N=3) and FACS (Fig.2B, N=3) experiments confirmed that Oct4 and Nanog,
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37 233 (markers of pluripotency) were maximally expressed at day 0, whereas the differentiation into
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39 234 definitive endoderm (SOX17, FOXA2) was optimal at day 5, with 80-90% of the cells strongly
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41 235 expressing these markers (Fig.2).

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44 236 By day 11, almost all cells were positive for markers expressed in hepatic stem cells
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46 237 (HNF4a, CK19), but negative for alpha-fetoprotein (AFP), a marker of hepatoblasts, and albumin
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48 238 (ALB) marker of mature hepatocytes (Fig.2A-C)[38]. In parallel, they lost their expression of
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51 239 SOX17 and FOXA2, evidencing a phenotype of hepatic stem cells (Fig.2A-B). Finally, the hepatic
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53 240 markers AFP and ALB appeared progressively along the differentiation process towards HLCs,
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55 241 with a maximal expression after 21 days (Fig.2A). To use these cells for cell therapy, we then
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58 242 decided to focus on day 11-pHSCs, because of the short culture duration (> 20 days for HLCs), and
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60 243 because they can easily and efficiently be frozen in a GMP-compatible cryopreservation solution.

The injection of these cells in the testis of immunodeficient rats (IL2rg KO rats; [34]) did not induce the formation of teratoma (N=5) contrary to iPS cells (N=2), demonstrating their loss of pluripotency potential and therefore their suitability for cell transplantation (Supplementary Fig.1).

Transplantation of hiPSCs-derived pHSCs into Gunn rats

Freshly prepared pHSCs were transplanted in Gunn rats (N=10). Serum levels alanine aminotransferase (ALAT) values in treated rats were not different from pre-operative values (that is before hepatectomy and transplantation), indicating that animal recovered well from surgical procedures (Supplementary Fig.2). Serum bilirubin levels showed a progressive reduction of baseline bilirubin levels in the treated group, that thereafter persisted throughout the study at a level of $28 \pm 5\%$ decrease compared to the initial pre-operative level, and was statistically significant (Fig.3A, discontinuous line) ($P < 0.05$). Serum bilirubin in the treated group was $57 \pm 5 \mu\text{M}$ at time of sacrifice versus $84 \pm 11 \mu\text{M}$ for pre-transplantation values ($p = 0.005$), and noteworthy, similar results were also obtained after transplantation of frozen pHSCs (N=6) (Fig.3A, dotted line). In contrast, serum bilirubin in the control group remained elevated with values up to $150 \mu\text{M}$ (60% increase in bilirubinemia) (N=9) (Fig.3A, continuous line).

Immunohistochemistry revealed the presence in the liver of engrafted cells expressing the human specific antigen UGT1A1 showing that transplanted pHSCs matured into adult hepatocytes (Fig. 3B upper panel). As no selective growth advantage was given to transplanted pHSCs, only few UGT1A1 expressing-human hepatocytes were detected in the liver as expected (less than 0.1% of the liver), explaining the absence of detection of human serum proteins like albumin (data not shown). Interestingly, we also detected matured pHSCs expressing the human UGT1A1 enzyme in the spleen of rats transplanted with freshly prepared and frozen pHSCs (1-2 % positive cells) (Fig.3B lower panel). Coherently with this result, the measurement of UGT1A1 activity in the spleen extracts of these animals showed a significant elevated activity compared to extracts of control rats (Fig.3C), whereas no activity was measured in liver extracts.

Discussion

We demonstrated the differentiation of hiPSCs into hepatic stem cells (HNF4a⁺, CK19⁺, AFP⁻, ALB⁻) using chemically defined conditions, with xeno-free, feeder-free media, recombinant factors and recombinant matrix support, such that clinical application can be considered. We show for the first time the efficacy of hiPSC-based regenerative medicine to treat the CNS-1 hepatic metabolic disease, with a GMP-compatible hepatic differentiation protocol and using frozen pHSCs.

Some previous publications already showed the participation of LN111 to the hepatocyte differentiation of hiPSCs, but include a step on matrigel-coated dish during the hepatic differentiation protocol [35], or as a mixture with LN521 and without *in vivo* validation [39]. On the contrary, we developed a protocol of differentiation that uses only recombinant LN111 for the production of transplantable hepatocytes-like cells.

These cells were able to induce a significant decrease in bilirubinemia in icteric adult Gunn rats, sustained for a long period (6 months). In addition, no teratoma was observed 6 months after direct injection of cells in the testis of immunodeficient rats (Supplementary Fig.1), suggesting the absence of residual undifferentiated hiPSCs in the cell preparation. While the teratoma assay was performed in different conditions (different rat model and site of injection) and would need complementary experiment such as cell injection in the liver, the absence of tumor formation in transplanted rats maintained on long term (more than 4 months) however suggests the absence of tumorigenicity.

Our study indicates that hiPSCs specified into hepatic stem cells (negative for UGT1A1 expression, data not shown) were capable of repopulating the liver and matured *in situ* at a level sufficient to ensure metabolic hepatic functions. The maximum therapeutic efficacy was observed around 2 months after cell injection, which is comparable to other studies following transplantation of HLCs or human neonate hepatocytes [25][27]. pHSCs were also able to survive and differentiate in the spleen, and were there still able to fulfill their metabolic role as evidenced by the UGT1A1

activity (Fig.3C). Indeed the spleen can provide an adequate environment for hepatocyte engraftment, proliferation, maturation and functionality [40].

Recent studies described the production of transplantable HLCs using GMP-compatible hepatic differentiation protocol [41][42], but culture times were about 30 days versus 11 days for pHSCs. Takayama et al. demonstrated a very efficient cryopreservation of HLCs using a GMP-compatible cryopreservation solution, but cells functionality was not confirmed *in vivo* [41]. While our study did not allow to compare pHSCs efficiency to the one of HLCs, our data show that pHSCs can efficiently be transplanted in Gunn rats to correct hyperbilirubinemia, using a protocol asking two times less time, and in absence of liver preconditioning that would give a selective growth advantage to transplanted hepatocytes. Interestingly, even if that would need further validation, the transplantation efficacy seems to be comparable when using freshly prepared or frozen pHSCs. Moreover the therapeutic efficacy was similar to the cell therapy approach using primary hepatocytes [25].

Thus, in order to transpose this finding into clinical settings, a clinical scenario could be easily imagined by replacing partial hepatectomy by partial liver embolization to stimulate liver regeneration and cell engraftment, as we showed in non human primates [43]. Alternatively partial liver irradiation prior to cell transplantation could also be considered [44]. In parallel to consider the use of those cells as universal cell donors some optimization will be needed to avoid their recognition by the immune system, like the invalidation of HLA expression [45].

Conclusion

There is a clinical demand for frozen human hepatocytes to treat inherited liver diseases, including CNS-1. Thanks to a relatively simple, fast and GMP-compatible hepatic differentiation protocol to produce therapeutic cells, we propose a new and immediately available unlimited source of frozen hepatic stem cells starting from human induced pluripotent stem cells, that could be a safe

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2 322 clinical alternative to liver and hepatocytes transplants, and that beyond CNS-1 disease could also
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4 323 be used for the transplantation in other hepatic defects.
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For Peer Review

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Figure legends

Figure 1. Protocol for the hepatic differentiation of human induced pluripotent stem cells (hiPSCs). Protocol to differentiate hiPSCs into hepatic stem cells (pHSCs), used for the transplantation, or more mature hepatocyte-like cells (HLCs), including the different media (brackets), cytokines (upper frame) and matrix (lower frame) used.

Figure 2. Characterization of differentiation markers during the conversion of hiPSCs into hepatic stem cells (pHSCs). (A) Hepatic differentiation was monitored over time from Day 0 (D0), Day 5 (D5), Day 11 (D11), Day 21 (D21) to Day 30 (D30) by RT-qPCR analyses for mRNA expression of pluripotency (OCT4, NANOG), endoderm (FOXA2, SOX17) and hepatic markers (HNF4A, CK19, AFP, ALB). The relative gene expression was calculated using the $2^{-\Delta\Delta C_t}$ quantification method after normalization to GAPDH values, and expressed as the fold change in mRNA levels in a given sample relative to undifferentiated cells (N=3, mean $\pm$ SEM). The expression of the same markers was confirmed at the protein level through FACS analysis (N=3) (B) or immunofluorescence staining (C). The scale bar represents 100 μ m.

Figure 3. Transplantation of iPSC-derived hepatic progenitors into the liver of Gunn rats. (A) Serum bilirubin levels in Gunn rats. Bilirubinemia data are represented as change in serum bilirubin from baseline (compared to pretransplant levels) over time (mean $\pm$ SEM), in control rats (N=9, continuous line) and rats transplanted with fresh (N=10, discontinuous line) or frozen (N=6, dotted line) pHSCs. (B) Histochemical analysis of human UGT1A1 expression in the liver (upper panel) or spleen (lower panel) of Gunn rats transplanted with fresh or frozen pHSCs. The scale bar represents 20 μ m. (C) UGT1A1 activity was measured by the diazotation method in spleen extracts of transplanted rats (N=9) and expressed as percentage to normal liver activity.

Supplementary Figure 1. Absence of Teratoma after injection of iPSC-derived hepatic stem cells in rat testis of IL2rg KO rats. Six months after their injection in rat testis, iPSCs generated teratoma (N=2) whereas iPSC-derived hepatic stem cells did not (N=5).

Supplementary Figure 2. Postoperative measurement of alanine amino transferase (ALAT) activity in the serum of transplanted rats. ALAT activity was measured in the serum of control mice and mice transplanted with fresh and frozen iPSC-derived hepatic stem cells, at different time points after surgery.

Figure 1

Figure 2

Figure 3

**Supplementary
Figure 1**
Xenotransplantation

Supplementary Figure 2