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FETAL AND NEONATAL STEM CELLS

Chemokine-Mediated Robust Augmentation of Liver Engraftment: A Novel Approach

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ABSTRACT

Effective repopulation of the liver is essential for successful clinical hepatocyte transplantation. The objective was to improve repopulation of the liver with human hepatocytes using chemokines. We used flow cytometry and immunohistochemistry assays to identify commonly expressed chemokine receptors on human fetal and adult hepatocytes. The migratory capacity of the cells to various chemokines was tested. For in vivo studies, we used a nude mouse model of partial hepatectomy followed by intraparenchymal injections of chemokine ligands at various concentrations. Human fetal liver cells transformed with human telomerase reverse transcriptase were used for intrasplenic cell transplantation. Repopulation and functionality were assessed 4 weeks after transplantation. The receptor CXCR3 was commonly expressed on both fetal and adult hepatocytes. Both cell types migrated efficiently toward corresponding CXC chemokine ligands 9, 10, and 11. In vivo, animals injected with recombinant chemokines showed the highest cell engraftment compared with controls ($p < .05$). The engrafted cells expressed several human hepatic markers such as cytokeratin 8 and 18 and albumin as well as transferrin, UGT1A1, hepatocyte nuclear factor (1α, 1β, and 4α), cytochrome CYP3A1, CCAAT/enhancer binding protein (α and β), and human albumin compared with controls. No inflammatory cells were detected in the livers at 4 weeks after transplantation. The improved repopulation of transplanted cells is likely a function of the chemokines to mediate cell homing and retention in the injured liver and might be an attractive strategy to augment repopulation of transplanted hepatocytes in vivo. STEM CELLS TRANSLATIONAL MEDICINE *2015;4:21–30*

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INTRODUCTION

Extensive preclinical studies and clinical trials have demonstrated the safety and feasibility of hepatocyte transplantation. Nonetheless, its clinical use is restricted due to organ shortage [1] and limited liver repopulation by transplanted hepatocytes [2]. Transplantation of fetal hepatic progenitors or adult hepatocytes is dependent on the successful homing, engraftment, and repopulation of these cells in the liver. Improved understanding of these processes will catalyze the development of therapies to facilitate hepatic progenitor and adult hepatocyte cell transplantation for clinical purposes. Several components of the essential process of hepatocyte homing and engraftment need to be characterized. Cell adhesion molecules and their ligands and the extracellular matrix components participate in the precise regulation of these processes. Consequently, it is important to explore strategies that will increase the efficiency of hepatocyte repopulation and induce the proliferation of the engrafted cells.

Clinical hepatocyte transplantation might be better accomplished using stem and progenitor cells in combination with a strategy that optimizes migration of the cells into the target tissue. Chemokines stimulate stem and progenitor cells through their corresponding chemokine receptors [3]. This may be important for cell homing, retention, and engraftment into the liver [4]. It is likely that the efficiency of engraftment may be dependent on the response of fetal hepatic progenitors and adult hepatocytes, which, in turn, depend on the levels of corresponding receptors expressed on these cells.

In this paper, we used a telomerase-immortalized (hTERT) human fetal liver cell line that gives an unlimited supply of hepatocytes for transplantation [5–9] and that has been reported to retain the morphological and functional characteristics of differentiated hepatocytes, such as the expression of hepatocyte-specific proteins and drug-metabolizing enzymes [10, 11].

Because the expression profile of chemokine receptors on fetal hepatic progenitors and adult hepatocytes is not known, we initially determined the cytokine receptors expressed on the cells. In this study, we hypothesized that exogenous injection of liver cells into a mouse liver that is preconditioned with chemokine receptors may result in

increased homing and retention of transplanted cells. Furthermore, this hypothesis was tested in a nude mouse model with retrorsine-induced liver damage.

Materials and Methods

Cell Isolation and Transfection of Fetal Liver Cells With hTERT

Permission for the present study was granted from the local ethics committee at Sahlgrenska (Gothenburg, Sweden) and Karolinska (Stockholm, Sweden) University Hospitals. Human fetal liver tissues were obtained from aborted fetuses at 6–9.5 weeks of gestation, in accordance with the Swedish guidelines. The study protocol was approved by the local ethics committee. A modified vacuum curettage was performed [12]. Gestational age was estimated according to specific anatomical markers [13]. Gestational age is given as menstrual age. The abortions were performed in pregnancies with no apparent abnormalities, and no fetuses with anomalies were included. Fetal liver was dissected and placed in a sterile tube containing RPMI 1640 medium (Gibco, Grand Island, NY, http://www.invitrogen.com). The liver was then disintegrated into a single-cell suspension by passage through a 70-μm metal mesh. The single-cell suspension was centrifuged at 200*g* for 10 minutes to pellet the cells. All women donating fetal tissue had been serologically screened for syphilis, toxoplasmosis, rubella, HIV-1, cytomegalovirus, hepatitis B and C, parovirus, and herpes simplex types 1 and 2.

Human fetal hepatocytes were isolated by magnetic cell sorting, as described by us previously [14–18]. The method is based on a negative selection of this population using a depletion cocktail including antibodies to 12 lineage-specific cell-surface antigens: anti-CD2, anti-CD3, anti-CD14, anti-CD16, anti-CD19, anti-CD24, anti-CD36, anti-CD38, anti-CD45RA, anti-CD56, anti-CD66b, anti-glycophorin A. The negative fraction was used as fetal hepatocytes.

All transfection studies were carried out at the Karolinska Institute using unsorted cells. The cells (8.5 weeks of gestation) were transfected using the pCDM7/EF1α/hTERT/PAC plasmid and the Amaxa Nucleofector system (Lonza, Walkersville, MD, http://www.lonza.com) (supplemental online data). We cultured and characterized, in detail, cells from one of the bulk clones obtained (hFL161/hTERT) (supplemental online data).

Adult Hepatocytes

Adult hepatocytes were freshly isolated from three different organ donors after permission from the relatives. The procedure for isolation was carried out according to the standard protocol [19]. Isolated hepatocytes were cultured in Williams' Medium E without phenol red (Sigma-Aldrich, Stockholm, Sweden, http://www.sigmaaldrich.com), further supplemented with 10% AB serum, 5% (vol/vol) L-glutamine (Invitrogen, Carlsbad, CA, http://www.invitrogen.com), 5% (vol/vol) PEST, 5% (vol/vol) of nonessential amino acids, 5% (vol/vol) sodium pyruvate, vascular endothelial growth factor (5 ng/ml; Invitrogen), interleukin-6 (2 ng/ml; Invitrogen), hepatocyte growth factor (30 ng/ml; Invitrogen), epidermal growth factor (20 ng/ml; Millipore, Solna, Sweden, http://www.millipore.com), and basic fibroblast growth factor (10 ng/ml; Invitrogen). Freshly isolated cells or cells in passage 2 were used for all analyses.

Immunohistochemistry and Immunocytochemistry for In Vitro Study

Fresh frozen sections (5-μm thickness) of adult liver (n = 3) and fetal liver (n = 3) were used. Immunohistochemistry was performed using the biotin-peroxidase complex method. Briefly, slides were incubated with primary antibodies CCR1, CCR2, CCR3, CCR4, CCR5, and CCR6 and CXCR1, CXCR2, CXCR3, CXCR4, and CXCR6 (1:200 in phosphate-buffered saline; R&D Systems, Oxon, U.K., http://www.rndsystems.com) overnight at 4°C. The slides were then processed as per standard protocol [18].

Immunofluorescent staining of the cultured human fetal liver cells transformed with human telomerase reverse transcriptase (hFL4TERT) was performed, as described in our earlier study [18], using antibodies specific for human cytokeratins (CK8, CK18, and CK19), human hepatocyte antigen (1:100; all antibodies from Santa Cruz Biotechnology, Santa Cruz, CA, http://www.scbt.com), human c-Met (1:100; Fitzgerald Industries International, Acton, MA, http://www.fitzgerald-fii.com), α-fetoprotein (1:500, Abcam, Cambridge, U.K., http://www.abcam.com), and human albumin (ALB; 1:100; Bethyl Inc., Montgomery, TX, http://www.bethyl.com).

Flow Cytometric Analysis

Fresh primary human adult hepatocytes and hFL4TERT (passages 6, 24, and 50) were analyzed for chemokine receptor expression by flow cytometry. Unconjugated primary antibodies CCR1, CCR2, CCR3, CCR4, CCR5, and CCR6 and CXCR1, CXCR2, CXCR3, CXCR4, and CXCR6 (all from R&D Systems) were used. Respective fluorescein isothiocyanate isotype controls were used as negative controls. Further procedures were performed as described in our earlier study [18]. Details are provided in the supplemental online data (supplemental online Table 2).

Cell Migration Assay

Recombinant human chemokine ligands (R&D Systems) CXCL9, CXCL10, and CXCL11 were used to study chemotaxis of hFL4TERT. A migration assay was assessed with a standard Transwell migration system (Corning Enterprises, Corning, NY, http://www.corning.com) using the Chemicon ECM508 assay kit (Millipore) (supplemental online data; supplemental online Table 3). Migration of cells to recombinant human chemokine ligands CXCL9 (0.1, 0.5, and 1.0 μg/ml), CXCL10 (0.02, 0.05, and 0.1 μg/ml), and CXCL11 (5, 10, and 10 ng/ml) and cocktails (lower and high concentration of each ligand; supplemental online data; supplemental online Table 3) were studied.

In Vitro Functionality Testing of hFL4TERT

Cell lysates were prepared from 1×10^6 hFL4TERT using cell extraction buffer (FNN0011; Invitrogen). Protein and urea concentrations were measured using the Bradford method and a quantitative colorimetric urea assay [20], respectively.

The ammonia content and superoxide dismutase (SOD) activity were measured using a quantitative colorimetric assay kit (AA0100; Sigma-Aldrich) and a spectrophotometer-based assay kit (OxisResearch, Foster City, CA, http://www.oxisresearch.com), respectively.

Transplantation of hFL4TERT in Nude Mice

Animal experiments were approved by the local ethics committee and performed in accordance with national and institutional

regulations. C57BL/6 nude mice (n = 28) of approximately 22- to 28-g body weight were used and divided in different groups, as shown in supplemental online Table 1. Briefly, acute liver injury was induced by a single intraperitoneal injection of 0.7 g/kg of D-galactosamine (Sigma-Aldrich). After 36 hours, under general anesthesia, all mice underwent partial hepatectomy (PH; resection of the left lobe, ~30% of the liver volume). Immediately after PH, 250 μl of the cocktail of CXCL9, CXCL10, and CXCL11 (supplemental online Table 1) were injected into the liver parenchyma at multiple sites, followed immediately by intrasplenic injection of hFL4TERT (2×10^6 cells per mouse). Approximately, 50 μl of the cocktail was injected at 5 sites. The animals were divided into various groups: group 1 was control group without any treatment; group 2 was sham control (D-galactosamine plus PH); group 3 was sham control for chemokine ligands; in group 4, cells were transplanted without chemokine ligands; in groups 5 and 6, cells were transplanted with low and high chemokine concentrations, respectively; and in group 7, without PH, cells were transplanted with low chemokine concentration. The concentration of chemokine ligands injected was determined in initial in vitro experiments using the Transwell migration assay. After securing hemostasis, the abdominal incision was closed, and animals were monitored closely until recovery from anesthesia.

Mouse liver-tissue retrieval, sample processing, and immunohistological methods used were as described in the earlier study [18].

Liver sections from transplanted mice were single stained for human albumin and double stained for human nuclei and albumin positivity (human nuclei$^+$/ALB$^+$) or Ki-67/CK8 positivity (Ki-67$^+$/CK8$^+$) using immunofluorescence. Goat anti-human albumin (1:200), mouse anti-human nuclei (1:100), Ki-67 (1:200, Abcam), and anti-human CK8 (1:200) were added and incubated overnight at 4°C. Further protocols were performed as described in our earlier study [18].

Human Albumin Quantification

Serum taken from transplanted animals at 4 weeks was analyzed for human albumin with a sandwich enzyme-linked immunosorbent assay (Bethyl Inc.), according to the manufacturer's protocol [18].

Western Blot Analyses

Tissue lysates from all transplanted animals were used to detect the presence of UGT1A1, CCAAT/enhancer binding protein-α (cEBP-α) and cEBP-β, β-tubulin, transferrin (Abcam), CYP3A1, CYP3A4, hepatocyte nuclear factor-1β (HNF-1β), and HNF-4α (Santa Cruz Biotechnology) by standard Western blotting (supplemental online data). Western blot bands were quantified by densitometry using the Quantity One (4.6.9) image analysis software (Bio-Rad, Hercules, CA, http://www.bio-rad.com).

Real Time-Polymerase Chain Reaction TaqMan

Total RNA was isolated from liver tissue from transplanted mice and human liver according to the manufacturer's protocol (Norgen Biotek Corp, Thorold, ON, Canada, https://norgenbiotek.com), and 1 μg of total RNA was reverse transcribed into cDNA at 50°C using an Advantage first-strand synthesis kit (Clontech, Mountain View, CA, http://www.clontech.com). The cDNA samples were subjected to polymerase chain reaction (PCR) amplification using primers specific for human albumin, AFP, CK19, CYP3A4, CYP3A7, HNF-1α, HNF-1β, HNF-4α, and G6PD (supplemental online Table 4). Samples obtained from three mice (albumin, CK19, G6PD) and four mice (HNF-1α, HNF-1β, and HNF-4α) per group were run in triplicate for PCR. Relative expressions of genes (albumin, CK19, HNF-1α, HNF-1β, and HNF-4α) were calculated by diving with G6PD densitometry values. Further details are given in the supplemental online data.

Quantification of Engrafted hFL4TERT

Engrafted and proliferating hFL4TERT in the mouse livers were counted in 500 serial sections of 5-μm thickness. Fresh frozen sections were single stained for human albumin and double stained for human ALB/nuclei or Ki67/CK8 antibody, respectively. We counted cells from three to five portal or central regions on each section from the liver of each mouse in the different groups. At a magnification of ×40, the visual field of the CCD camera corresponds to a volume of 0.0003 mm^3 (i.e., area of 0.06 mm^2 and height of 0.005 mm). The number of engrafted cells on a 1-mm^3 section was extrapolated from sections viewed and calculated as the average number of cells per section divided by volume (0.0003 mm^3).

Statistical Analysis

Data are presented as mean values with standard deviations. Statistical analysis of in vitro functionality and human albumin in serum was performed using the two-tailed Student's t test. PCR densitometry and quantification of transplanted cells were analyzed using one-way analysis of variance followed by Tukey's test for post correction. A p value $<.05$ was considered statistically significant. Statistical analysis was performed using SPSS 17.0 software (IBM Corp., Armonk, NY, http://www-01.ibm.com/software/analytics/spss/) and Graph Pad Prism version 4.0 (GraphPad Software, Inc., San Diego, CA, http://www.graphpad.com), respectively.

RESULTS

Characterization of hFL4TERT

Immunocytochemical analysis of hFL4TERT showed intense expression of albumin, α-fetoprotein, c-Met, and CK18 markers. Cells were also positive for CK19 and hepatocyte antigen. Expression of CK8 in hFL4TERT was weak (supplemental online Fig. 1).

Functionality of hFL4TERT

Metabolic and free radical scavenging capacity of hFL4TERT was assessed by measuring ammonia content, urea content, and SOD activity (Table 1). Adult hepatocytes were used as control. In general, hFL4TERT ammonia and urea levels were significantly lower than those in adult hepatocytes. SOD activity in hFL4TERT was 0.67 U/mg protein, which was slightly more than one-tenth of the value of adult hepatocytes.

Chemokine Receptor Expression in Human Fetal and Adult Livers

The expression of CCR1 through CCR6, CXCR1 through CXCR4, and CXCR6 was examined using immunohistochemistry (Fig. 1) and flow cytometry (supplemental online Fig. 2; supplemental online Table 2). Fetal livers were positive for the expression of

Table 1. Biochemical analysis of cell lysates from hFL4TERT and adult hepatocytes

Cells	Ammonia (mg/ml)	Urea (mg/l)	Superoxide dismutase (U/mg protein)
hFL4TERT	0.16 ± 0.002[a]	0.80 ± 0.02[a]	0.67 ± 0.05[a]
Adult hepatocytes	0.60 ± 0.06	6.40 ± 0.25	6.09 ± 2.04

Statistical analysis was done using Student's *t* test. Results are shown as mean ± SD.
[a]Compared with adult hepatocytes ($p < .05$).
Abbreviation: hFL4TERT, human fetal liver cells transformed with human telomerase reverse transcriptase.

CCR5, CXCR3, and CXCR4 but negative for all other chemokine receptors tested (Fig. 1A). In contrast, adult livers were weakly positive for CCR1 and CXCR6; strongly positive for CCR5, CCR6, CXCR1, CXCR2, CXCR3, and CXCR4; and negative for CCR2, CCR3, and CCR4 (Fig. 1B; supplemental online Table 2). The receptors that were commonly expressed in both fetal and adult livers were CXCR3, CXCR4, and CCR5. Fetal liver CXCR3 expression was uniformly observed in the boundaries of hepatic cords, whereas in the adult liver, it was observed only in the portal lobule (Fig. 1B). We found that the expression of CXCR3 and CXCR4 was consistent and reproducibly present in hepatocytes using either method. Consequently, we decided to study in vitro and in vivo effects of the corresponding ligands to CXCR3, namely, CXCL9, CXCL10, and CXCL11, on human hepatocytes.

In Vitro Chemotactic Effects of CXC Chemokine Ligands 9, 10, and 11 on hFL4TERT and Adult Hepatocytes

We studied the in vitro migratory capacity of hFL4TERT (supplemental online Fig. 3) and adult hepatocytes (supplemental online Fig. 3) expressing CXCR3 to its corresponding ligands CXCL9, CXCL10, and CXCL11. In general, lower numbers CXCL9 (8,312.5 ± 203 [16% ± 0.4%]), CXCL10 (7,375 ± 293 [13.0% ± 0.5%]), and CXCL11 (9,937.5 ± 453 [16.85% ± 1.03%]) of adult hepatocytes migrated toward different concentrations of these ligands compared with fetal hepatocytes. The hFL4TERT demonstrated a migratory capacity of 85.44 ± 1.3% and 86.48% ± 5.7% toward CXCL10 (0.05 μg/ml) and CXCL11 (20 ng/ml), respectively (supplemental online Table 3), and 69.50% ± 3.6% and 63.75% ± 3.9% toward chemokine cocktails C-1 (i.e., low concentration) and C-2 (i.e., high concentration), respectively (Fig. 2A, 2B).

In Vivo Effects of CXCL9, CXCL10, and CXCL11

Repopulation of Injured Livers Transplanted With hFL4TERT

Repopulation of nude mouse livers with hFL4TERT was assessed using immunohistochemistry for human-specific antibodies such as AFP, c-Met, CK8, CK18, CK19, and hepatocyte-specific antigen (Fig. 3; supplemental online Fig. 4). Interestingly, liver sections from mice receiving cocktails C-1 (low concentration) and C-2 (high concentration) (i.e., groups 6 and 7, respectively) showed human cells positive for CK18 and CK19 near bile ducts. Cells expressing hepatocyte-specific antigen were found more in groups 5–7 compared with group 4 (Fig. 3).

Immunofluorescence was used to detect single staining of human albumin, double-positive cells expressing human-specific CK8/Ki-67 or human nuclear antigen/ALB. Double fluorescence staining for human-specific CK 8/Ki67 indicated presence of proliferating hFL4TERT cells. The number of double-positive cells expressing CK8/Ki-67 was not significantly different (Fig. 3) among all transplanted groups (groups 4–7). Cell colonies expressing human nuclei and ALB were significantly higher in group 6. Interestingly, in group 5, we observed integration of hFL4TERT in the mouse bile ducts.

Human albumin concentration in serum was highest (74.8 ± 0.9 ng/ml) in group 6, followed by group 5 (70.9 ± 0.70 ng/ml). Groups 4 and 7 showed similar concentrations of human albumin in serum. Normal and sham animals did not show detectable levels of human albumin in serum (Table 2).

Presence of Human-Specific Protein Expression in Injured Mouse Livers

Human hepatocyte-specific expression of transferrin, UGT1A1, CYP3A1, HNF-1β, HNF-4α, cEBP-α, and cEBP-β markers were detected in mouse liver using Western blotting. Expression of β-tubulin was used as loading control. Relative expressions of proteins (transferrin, UGT1A1, CYP3A1, HNF-1β, HNF-4α, cEBP-α, cEBP-β) were calculated by dividing with β-tubulin densitometry values. Livers from normal mice and sham-transplanted mice did not show any detectable human proteins (Fig. 4A; supplemental online Figs. 5, 6). Data presented in the supplemental online data and supplemental online Table 5A show that animals receiving cocktail C-2 (group 6) and C-1 (group 5) had 3.05- and 1.65-fold increased expression of CYP3A1 (important human drug metabolizing enzyme), respectively, in comparison with chemokine control (group 7). Expression of human hepatocyte-specific functional markers UGT1A1, HNF-1β, HNF-4α, cEBP-α, and cEBP-β in group 5 showed 0.32-, 0.25-, 0.40-, 0.12-, and 0.43-fold increases, respectively, compared with group 4. Transplanted group 6 showed 0.05-, 0.09-, 0.24-, and 0.17-fold increases in HNF-1β, HNF-4α, cEBP-α, and cEBP-β, respectively, compared with group 4 (Fig. 4B). Furthermore, animals in group 5 demonstrated higher protein expressions of transferrin, UGT1A1, HNF-1β, HNF-4α, and cEBP-β (3.35-, 2.56-, 2.06-, 1.8-, and 1.29-fold increases, respectively) compared with group 6.

Presence of Human-Specific Gene Markers Using Real-Time PCR Analysis in Injured Mouse Livers

We demonstrated human hepatocyte-specific gene expression in transplanted animals with the help of real time-PCR (RT-PCR). Expression of human ALB, CK19, HNF-1α, HNF-1β, HNF-4α, CYP3A4, and CYP3A7 mRNA in mouse livers of transplanted animals was determined by real-time PCR using primers specific for the human sequences (Fig. 4C; supplemental online Table 4). Normal human liver and hFL4TERT mRNAs were used as positive controls, whereas normal nude mice and sham-group mRNAs were used as negative controls. We used G6PD as a housekeeping gene to demonstrate human-specific gene expression in different transplanted groups. The data obtained (supplemental online data; supplemental online Table 4) from hFL4TERT transplanted mouse livers and human liver show similar expression pattern; however, the expression of CK19 was not detected and may be due to low numbers of cells expressing this marker. Cytochrome P450 (CYP3A4 and CYP3A7) expression was not detected in transplanted animal groups (supplemental online Table 5B).

Number of Transplanted hFL4TERT Present in Injured Mouse Livers

After 4 weeks of transplantation, we investigated the distribution of these transplanted hepatocytes, as demonstrated by

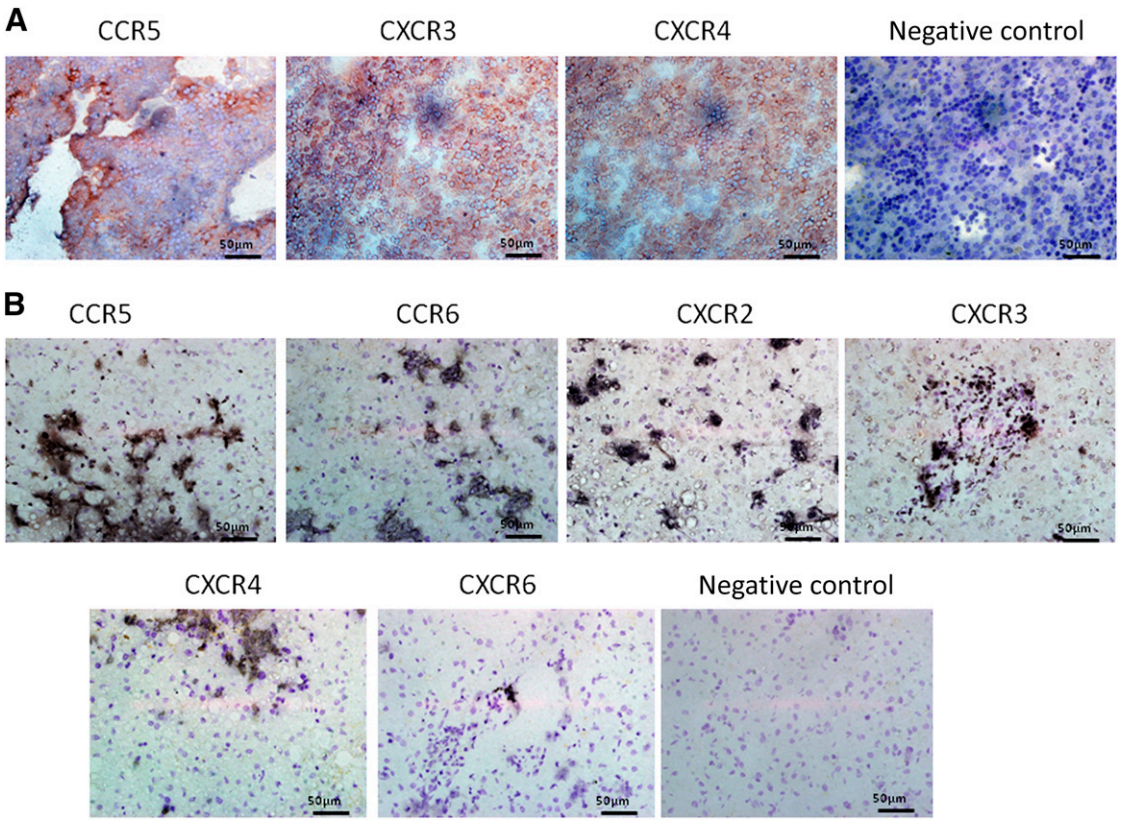

Figure 1. Immunohistochemical staining for chemokine receptors in human fetal and adult livers. **(A):** Fresh frozen sections of human fetal livers were examined for expression of chemokine receptors and were found to be positive for CCR5, CXCR3, and CXCR4 (brown staining). **(B):** Fresh frozen sections of human adult livers showed positive expression for CCR5, CCR6, CXCR2, CXCR3, and CXCR4 (black staining). Only anti-mouse secondary antibodies were used as negative control. We found that the expression of CXCR3 was the most reliable and reproducible in both cell types. Magnification, ×40. Scale bar = 50 μm.

immunofluorescence staining for human nuclei$^+$/ALB$^+$, human nuclei$^+$, and Ki67$^+$/CK8$^+$ in the transplanted livers. At 4 weeks after cell transplantation, numerous large clusters of double-positive hepatocytes (human nuclei$^+$/ALB$^+$) were present in transplanted groups (Figs. 3, 5A). In addition, we found single positive cells (human nuclei$^+$) without albumin (Fig. 3). The location of the engrafted cells between groups 5 and 6 were same, with scattered engrafted cells in the centrilobular and periportal regions.

Group 6 had the highest magnitude of liver repopulation. The total numbers of albumin-positive cells were higher in group 6 (83,971.54 $\pm$ 3,710.06), followed by groups 5, 4, and 7. The number of transplanted cells not producing albumin were higher in group 5 (61,052.47 $\pm$ 4,907.22), followed by groups 6, 4, and 7 (Fig. 5C). However, transplanted cells behaving as hepatocytes by producing albumin were found to be significantly higher in group 6 compared with group 7 (p = .0003), group 5, and group 4 ($p < .05$), respectively. No significant difference was observed for albumin-negative cells or mitotic human cells among transplanted groups (Fig. 5B, 5C).

Discussion

In this paper, for the first time, we report a novel approach to augment repopulation of injured livers using CXC chemokines. Taking advantage of the important role of chemokines in cell homing, retention, and engraftment of cells, we locally injected chemokines into the liver parenchyma to successfully achieve robust repopulation of damaged livers with transplanted hepatocytes. Based on the results obtained, we believe these chemokines may have favored retention and survival of transplanted hepatocytes expressing the corresponding chemokine receptors. In fact, CXC chemokines have been shown to induce proliferation of hepatocytes both in vitro and in vivo [21, 22]. Furthermore, treatment of mice with CXC chemokines increased hepatocyte proliferation and liver regeneration after partial hepatectomy [23]. Successful clinical outcome of hepatocyte cell transplantation depends on sufficient repopulation of the liver. Wider use of clinical hepatocyte transplantation can be a reality, provided strategies to improve repopulation are established by reducing cell loss during and after cell transplantation. Supplying chemokine in combination with the hepatocytes may be one strategy for increasing repopulation.

Because the chemokine receptor expression of hepatocytes is not fully reported, we initiated this study by determining the chemokine receptor profile of adult and fetal hepatocytes. We found that, in general, immature fetal hepatocytes express a limited set of these receptors, whereas adult hepatocytes express most of the receptors tested. We also detected consistent and reproducible expression of the receptor CXCR3 on fetal and adult hepatocytes using two different methods. For convenience and simplicity, we decided to proceed further by using the corresponding ligands of CXCR3. Because CXCR3 has been reported

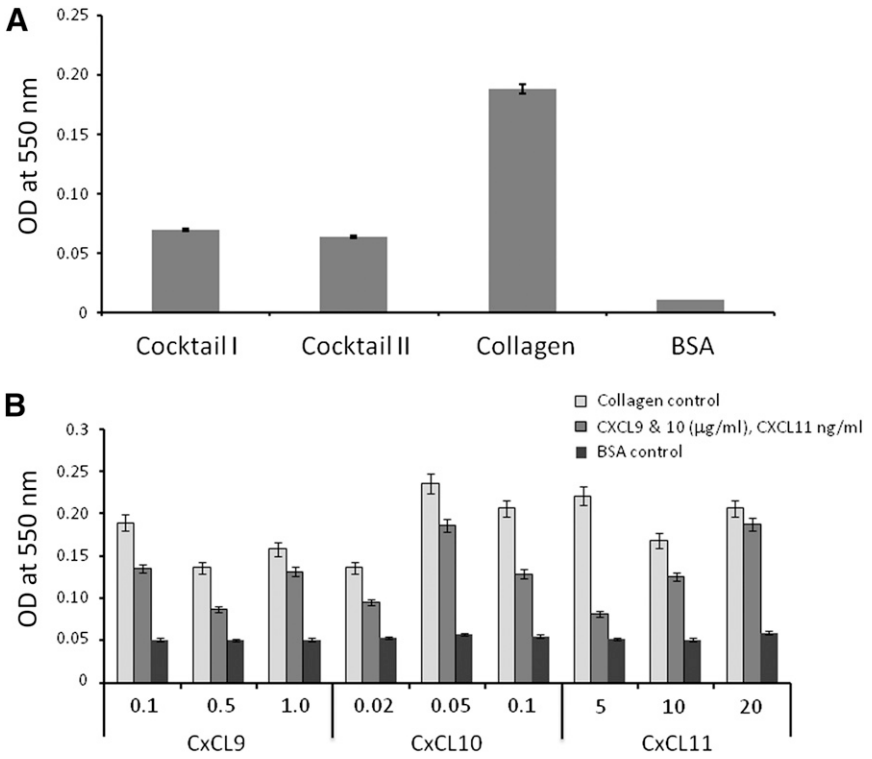

Figure 2. QCM colorimetric cell migration assay. **(A):** Migration assays were performed by stimulation of human fetal liver cells transformed with human telomerase reverse transcriptase (hFL4TERT) with a cocktail of various concentrations of chemokine ligands to the receptor CXCR3. Cocktail 1: CXCL9 (0.5 μg/ml) plus CXCL10 (0.05 μg/ml) plus CXCL11 (10 ng/ml). Cocktail 2: CCL9 (1.0 μg/ml) plus CXCL10 (0.1 μg/ml) plus CXCL11 (20 ng/ml). Collagen and bovine serum albumin-coated surfaces were used as positive and negative controls. No significant difference in migration of cells to any chemokine cocktails (i.e., C-1 and C-2) was observed. **(B):** Migration assay of hFL4TERT was also performed using the individual chemokines at various concentrations: CXCL9 at 0.1, 0.5, and 1.0 μg/ml; CXCL10 at 0.02, 0.05, and 0.1 μg/ml; CXCL11 at 5, 10, and 20 ng/ml. The concentrations determined to be optimal were those giving the highest optical density values of migrated cells. Abbreviations: BSA, bovine serum albumin; OD, optical density.

to have three main CXC ligands, namely, CXCL9, CXCL10, and CXCL11, we decided to use a cocktail of these ligands in different predetermined concentrations (based on initial experiments). We aimed to test whether the ligands of CXCR3 expressed on hepatocytes would be able to augment hepatocyte cell transplantation. All further studies were carried out using immortalized fetal cells instead of primary adult hepatocytes for reasons of logistics and easy access. We characterized the immortalized cell line (hFL4TERT) and found that the cells expressed many of the major hepatic markers such as ALB, α-fetoprotein, CK19, hepatocyte-specific antigen, c-Met, CK8, CK18, and CK19. However, these cells showed negligible metabolic functionality in hFL4TERT compared with adult hepatocytes, indicating the immature nature of these fetal cells and confirming our earlier results [18], in which we also observed similar low metabolic activity in normal fetal hepatocytes.

We next tested the in vitro migratory capacity of CXCR3 expressing fetal hepatocytes in a Transwell assay toward CXCL9, CXCL10, and CXCL11.We could demonstrate in vitro that these cells efficiently migrated toward different concentrations of these chemokines. Cocktails of these chemokines induced increased cell migration, which may be due to a synergistic effect of the three chemokines. Furthermore, using a nude mouse model with D-galactosamine-induced liver damage, we injected these into the parenchyma of the injured mouse livers to study their in vivo effects on the transplanted hepatocytes. A suitable dose of the relevant chemokines was injected into the livers of these animals, followed by cell transplantation. Analysis at 4 weeks after transplant showed an abundant number of albumin-positive transplanted cells in the mouse livers of chemokine cocktail groups compared with sham and control groups. It is likely that increased adhesion of the transplanted cells to the chemokine ligands may result in better survival and retention of these cells in the liver and may in turn lead to robust repopulation. Results from Western blot and RT-PCR analysis demonstrated that the transplanted cells expressed several important hepatic transport proteins and hepatic transcription factors [24, 25]. High engraftment of human hepatocytes in knockout mouse models can be achieved by serial transplantation, but it involves major surgical issues [26–29]. The advantage of our procedure is that a single injection of 2×10^6 hFL4TERT is sufficient to achieve robust repopulation.

Interestingly, studies demonstrate that liver injury and partial hepatectomy [30] or liver injury and exogenous chemokine injections alone may not be sufficient to induce augmented engraftment of transplanted cells. It is reported that levels of CXC cytokines are increased 3- to 5-fold after 70% hepatectomy, indicating that PH results in only low to moderate levels [21]. In the present study, animals showing the highest engraftment cell levels were those with liver injury plus PH plus additional doses of exogenous hepatic CXCL9, CXCL10, and CXCL11. Our results also demonstrate that chemokines are essential components not only in the process of engraftment but also in proliferation of hepatocytes,

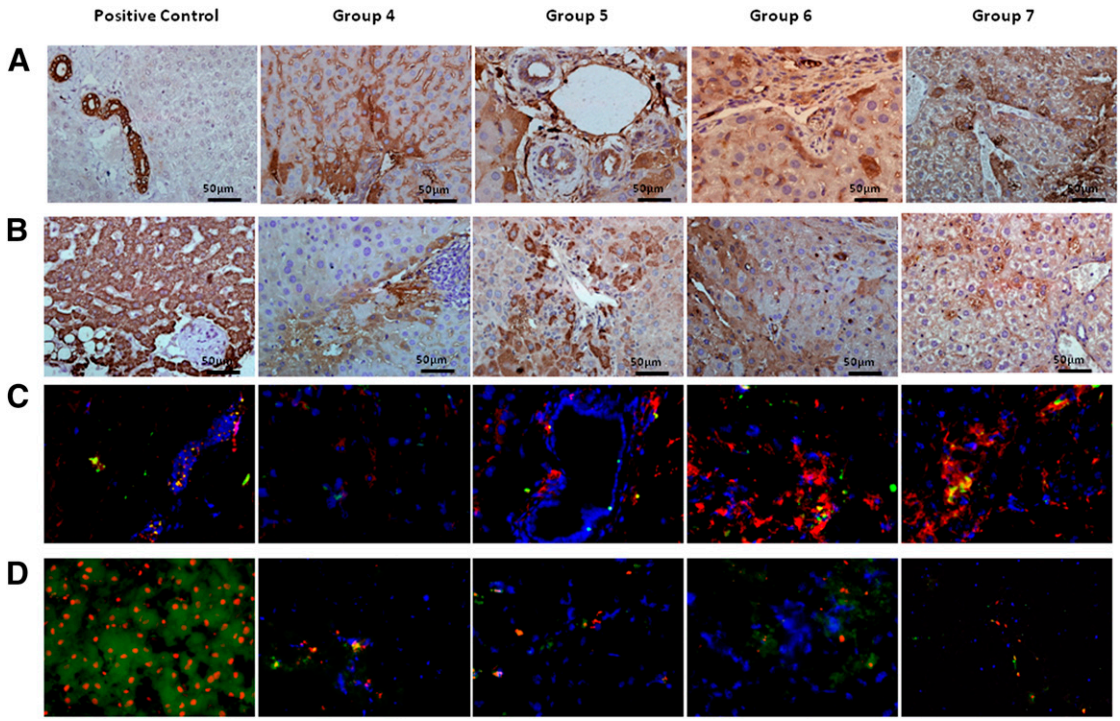

Figure 3. Transplantation of human fetal liver cells transformed with human telomerase reverse transcriptase (hFL4TERT) in nude mice. D-Galactosamine-treated C57BL/6 nude mice underwent partial hepatectomy (40%) followed by chemokines injected in remaining lobes. Transplantation of hFL4TERT (2×10^6 cells per mouse) was done intrasplenically. Biopsy from human liver cancer tissue served as positive control and from a sham group was a negative control. **(A, B):** Paraffin-embedded sections stained with cytokeratin 19 (CK19) and hepatocyte antigen showed presence of human cells (brown) in all cell-transplanted groups. Fresh frozen sections were used for double staining. Scale bar = 50 μm. **(C):** The presence of double-positive anti-human CK8 (red) and Ki-67 (green) cells was shown in all cell-transplanted groups but was highest in groups 6 and 7, which were injected with chemokines indicating proliferation of human cells in mouse liver. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; blue). Magnification, ×40. **(D):** Double-positive human nuclei antigen (red) and albumin (green) cells were observed in all groups but with group 6, injected with chemokines, showing the highest numbers. Nuclei were counterstained with DAPI (blue). Magnification, ×40.

Table 2. Human albumin concentrations in transplanted mice

Group	Human albumin (ng/ml)
1	ND
2	ND
3	ND
4	20.5 ± 0.61^{a}
5	$70.9 \pm 0.70^{a,b,c,d}$
6	$74.8 \pm 0.91^{a,d}$
7	20.1 ± 0.11^{a}

Statistical analysis was done using the Student's *t* test. Results are shown as mean ± SD ($n = 4$ per group used in duplicate).
[a]Compared with groups 2 and 3 ($p < .001$).
[b]Compared with group 4 ($p < .05$).
[c]Compared with group 6 ($p < .001$).
[d]Compared with group 7 ($p < .001$).
Abbreviation: ND, not detectable.

as demonstrated by the presence of several Ki-67$^+$ human cells even in the hostile environment of chemically damaged livers.

Generally, chemokines attract inflammatory cells [31]. Partial hepatectomy, which stimulates release of growth factors, results in infiltration of inflammatory cells in the liver [22]. Consequently, one would expect increased damage or destruction of livers after additional injection of exogenous chemokine ligands. Although we did not encounter this problem in the present study due to use of nude mice, we believe that the small volumes of injected chemokine ligands quickly disperse into the parenchyma, thereby preventing formation of a gradient and leading to uncontrolled and prolonged action that may be potentially harmful [22]. Furthermore, because the chemokine ligands quickly disperse into the liver lobes, it is important that the cells are transplanted immediately after injection of the chemokines to maximize the effects of these protein molecules.

Adult hepatocyte transplantation is emerging as an alternative "bridge" support for patients waiting for a donor organ [32, 33]. Despite clinical improvements in patients, significant problems have arisen because of inefficient engraftment, death or ectopic distribution of cells that did not engraft in the target tissue, emboli formation, immunological rejection, and transient effects of transplanted cells [34–36]. A number of preconditioning regimens including irradiation or chemicals have been tried but discontinued for various reasons such as senescence, radiation hazards, or an increased risk of cancer [37, 38].

Several animal hepatocyte transplantation models have shown low engraftment efficiency of cells. In rats, transplantation of 2×10^7 hepatocytes results in engraftment of approximately 0.5% of the total recipient liver cells [39], and this hepatocyte loss occurs during the early stages of transplantation [40, 41]. Usually major loss of transplanted cells is thought to be due to failure in entering sinusoids and crossing the sinusoidal-endothelial barrier [40]. In a nonhuman primate model, liver repopulation with transplanted

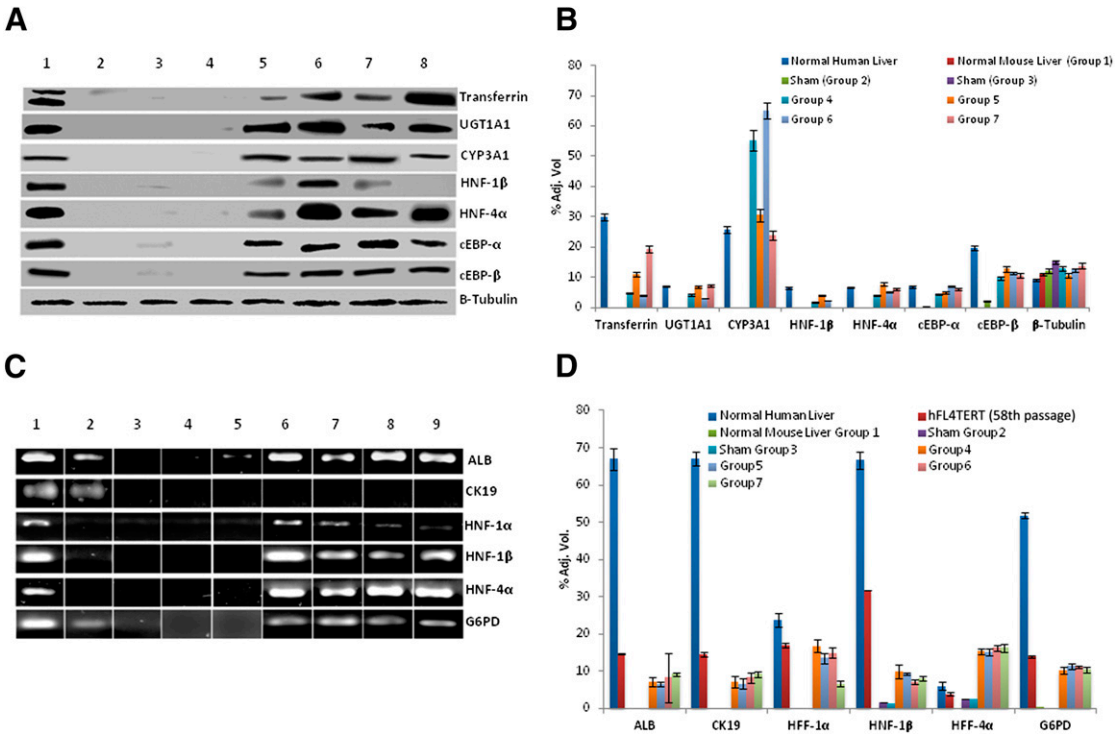

Figure 4. Western blot analysis of mouse livers transplanted with hFL4TERT. **(A):** Mouse livers transplanted with hFL4TERT cells were analyzed to detect important hepatic proteins such as transferrin, UGT1A1, CYP3A1, HNF-1β, HNF-4α, cEBP-α, and cEBP-β using Western blot. Normal human liver was used as positive control (lane 1). Normal mouse liver (lane 2) and sham-transplanted mice (lanes 3 and 4) served as negative controls. Expression of β-tubulin was used as loading control. Expression of human transferrin, UGT1A1, CYP3A1, HNF-1β, HNF-4α, cEBP-α, and cEBP-β was detected in hFL4TERT-transplanted mouse livers (group 4 shown in lane 5, group 5 shown in lane 6, group 6 shown in lane 7, and group 7 shown in lane 8), whereas normal mouse livers and sham transplanted livers did not show any detectable human proteins. Data obtained are expressed as mean $\pm$ SEM. **(B):** Densitometric analysis of the intensity of the bands in the Western blots was performed by ImageQuant 5.1. Values were normalized to β-tubulin. Data obtained (n = 4) are expressed as mean $\pm$ SEM. **(C):** Human hepatocyte-specific gene expression as detected by real-time polymerase chain reaction. Mouse livers transplanted with hFL4TERT cells were also analyzed for presence of human-specific ALB, CK19, HNF-1α, HNF-1β, HNF-4α, CYP3A4, and CYP3A7 mRNA. Normal human liver mRNA was used as positive control (lane 1). hFL4TERT cells (lane 2), normal mouse liver (lane 3), and sham-transplanted mouse liver mRNAs (lanes 4 and 5) were used as negative controls. G6PD was used as a housekeeping gene. Human CYP3A4 and CYP3A7 mRNAs were not detected (data not shown), but human ALB, HNF-1α, HNF-1β, and HNF-4α mRNAs were specifically expressed in hFL4TERT-transplanted livers. Intense hepatocyte nuclear factor (HNF-1α, HNF-1β, and HNF-4α) mRNA expression was observed in group 4 (lane 6), group 5 (lane 7), group 6 (lane 8), and group 7 (lane 9) compared with G6PD expression. **(D):** Densitometric analysis of mRNA in hFL4TERT-transplanted mouse livers was performed by ImageQuant 5.1. Densitometry values were normalized to G6PD. Abbreviations: Adj. Vol., adjusted volume; ALB, albumin; cEBP, CCAAT/enhancer binding protein; CK, cytokeratin; hFL4TERT, human fetal liver cells transformed with human telomerase reverse transcriptase; HNF, hepatocyte nuclear factor.

hepatocytes was approximately 2%–7% [40], whereas in our study, robust engraftment was recorded (34.14%, 37.34%, 45.25%, and 26.16% engraftment in groups 4, 5, 6, and 7, respectively).

Transplantation of stem and progenitor cell populations, rather than mature hepatocytes, has been investigated recently, exploiting their known proliferative potential. Stem/progenitor cells are minimally immunogenic and are readily cryopreserved but are small and engraft with lower efficiency than the larger mature cells [42–45]. Clinical trials with transplantation of fetal liver-derived cells have revealed no evidence of emboli formation and need for immune suppression, improved end-stage liver disease scores, longer survival of seriously ill patients, and improved liver functions in all transplanted patients [45].

Conclusion

The issue of limited engraftment can be overcome using this novel approach. Although we tested the effects of CXCL9, CXCL10, and CXCL11 in the present study, ligands to other receptors expressed on hepatocytes such as SDF-1 can also be tested. We believe that this approach to establish humanized livers in mouse models will be useful for pharmaceutical and virological studies. In addition to laboratory models, it might represent a potential regimen to practice in clinical setup with promising prognosis for the survival and functional proliferation of transplanted hepatocytes. The efficacy and rapid action of these cells and the lack of proinflammatory activity make this approach an attractive strategy with which to augment repopulation of transplanted hepatocytes in vivo.

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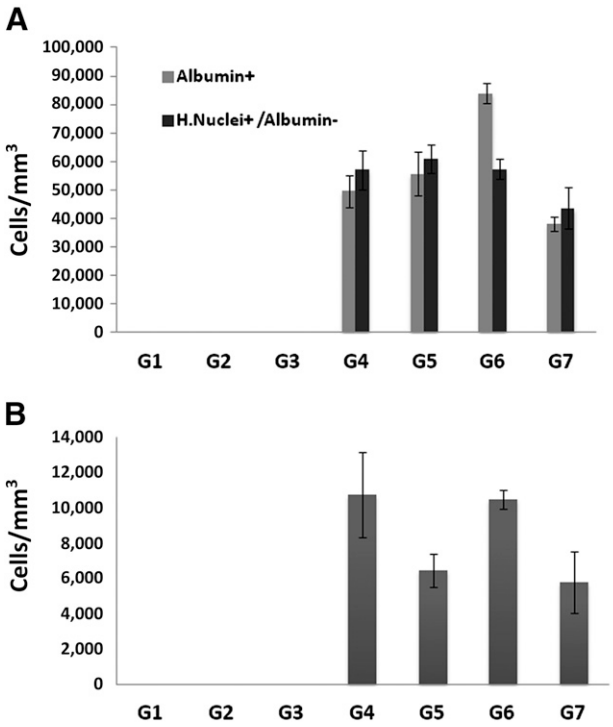

C

Groups	Cells/mm^3 ± SEM (n = number of sections viewed)		
	Albumin$^+$	H. Nuclei$^+$ /Albumin$^-$	Ki-67$^+$/CK8$^+$
1	ND	ND	ND
2	ND	ND	ND
3	ND	ND	ND
4 ($n = 4$)	49596.78 ± 5631.20** ($n = 172$)	57078.38 ± 6888.94 ($n = 40$)	10740.71 ± 2416.32 ($n = 43$)
5 ($n = 4$)	55633.66 ± 7729.35* ($n = 140$)	61052.47 ± 4907.22 ($n = 22$)	6488.89 ± 951.66 ($n = 23$)
6 ($n = 4$)	83971.54 ± 3710.06*** ($n = 210$)	57415.34 ± 3523.64 ($n = 33$)	10508.76 ± 559.93 ($n = 30$)
7 ($n = 4$)	38104.72 ± 2337.26 ($n = 220$)	43634.26 ± 7356.85 ($n = 19$)	5781.25 ± 1744.74 ($n = 19$)

Figure 5. Quantification of transplanted human fetal liver cells transformed with human telomerase reverse transcriptase (hFL4TERT) in mouse livers. A total of 500 serial liver sections were analyzed. Engrafted cells in all groups were manually counted in three to five different areas of each liver section, and the results are presented as cells per mm^3. CK8$^+$/Ki-67$^+$, human nuclei$^+$/albumin$^+$ (double-positive) cells and human nuclei$^+$/albumin$^-$ (single-positive) cells were enumerated. **(A):** Single-positive (human nuclei$^+$/albumin$^-$) cells were highest in group 5. The numbers of double-positive (human nuclei$^+$/albumin$^+$) cells were highest in group 6, followed by groups 5, 4, and 7 ($p = .0003$). **(B):** CK8$^+$/Ki-67$^+$ cells were highest in group 5. **(C):** Quantification of transplanted human cells in livers of nude mice with acute liver injury. Results are expressed as mean ± SEM. *, $p < .05$, **, $p < .001$, ***, $p < .0001$. Abbreviations: +, positive; −, negative; CK, cytokeratin; G, group; H., human; ND, not detectable.

Author Contributions

M.J.: collection and assembly of data, data analysis and interpretation, manuscript writing; M. Oltean: collection and assembly of data, data analysis and interpretation; P.B.P.: collection and assembly of data, manuscript writing; D.H.: collection of data; M.K.: data analysis and interpretation; J.H. and M. Olausson: data analysis and interpretation, manuscript writing; S.S.-H.: conception and design, financial support, data analysis and interpretation, final approval of manuscript.

Disclosure of Potential Conflicts of Interest

J.H. is the compensated chair of the board of directors of NovaHep AB, is an uncompensated inventor on intellectual property involving cells used in this study, and has an uncompensated ownership interest in NovaHep AB, the company owning intellectual property on cells used in this study. S.S.-H. has an uncompensated patent in NovaHep AB, is an uncompensated shareholder and board member of NovaHep AB, and has compensated royalties from Alenex AB. The other authors indicated no potential conflicts of interest.

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Chemokine-Mediated Robust Augmentation of Liver Engraftment: A Novel Approach

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