A humanized mouse model of liver fibrosis following expansion of transplanted hepatic stellate cells

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Abstract

Hepatic stellate cells (HSCs) are major contributors to liver fibrosis, as hepatic injuries may cause their transdifferentiation into myofibroblast-like cells capable of producing excessive extracellular matrix proteins. Also, HSCs can modulate engraftment of transplanted hepatocytes and contribute to liver regeneration. Therefore, understanding the biology of human HSCs (hHSCs) is important, but effective methods have not been available to address their fate *in vivo*. To investigate whether HSCs could engraft and repopulate the liver, we transplanted GFP-transduced immortalized hHSCs into immunodeficient NOD/SCID mice. Biodistribution analysis with radiolabeled hHSCs showed that after intrasplenic injection, the majority of transplanted cells rapidly translocated to the liver. GFP-immunohistochemistry demonstrated that transplanted hHSCs engrafted alongside hepatic sinusoids. Prior permeabilization of the sinusoidal endothelial layer with monocrotaline enhanced engraftment of hHSCs. Transplanted hHSCs remained engrafted without relevant proliferation in the healthy liver. However, after CCl₄ or bile duct ligation-induced liver damage, transplanted hHSCs expanded and contributed to extracellular matrix production, formation of bridging cell-septae and cirrhosis-like hepatic pseudolobules. CCl₄-induced injury recruited hHSCs mainly to zone 3, whereas after bile duct ligation, hHSCs were mainly in zone 1 of the liver lobule. Transplanted hHSCs neither transdifferentiated into other cell types nor formed tumors in these settings. In conclusion, a humanized mouse model was generated by transplanting hHSCs, which proliferated during hepatic injury and inflammation, and contributed to liver fibrosis. The ability to repopulate the liver with transplanted hHSCs will be particularly significant for mechanistic studies of cell-cell interactions and fibrogenesis within the liver.

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Introduction

Repopulation of the liver with transplanted cells is of much interest for biological studies and for therapeutic applications. Experimental transplantation of mature hepatocytes and liver sinusoidal endothelial cells (LSECs) has improved the understanding of how hepatic and endothelial cell compartments could be reconstituted, including for disease corrections [1, 2]. Other studies demonstrated the roles of cell-cell interactions, e.g., signaling from LSECs was found to be critical in liver regeneration [3]. Similarly, hepatic stellate cells (HSCs) may contribute to liver regeneration [4], although the mechanisms—especially within the intact organ *in vivo*—are incompletely defined. Therefore, availability of cell transplantation models, particularly in animals with human cells, may be helpful for translational studies. This requires consideration of the complexities involved in repopulation of the liver by transplanted cells. For

instance, immediately after cell transplantation in the liver, transplanted hepatocytes cause hepatic ischemia with deleterious activation of inflammatory cells [5], which needs to be controlled for improving cell engraftment. Similarly, prior disruption of the endothelial barrier advanced entry of transplanted hepatocytes into the space of Disse, which was necessary for their subsequent integration in liver parenchyma [2]. Also, transplanted cell engraftment required hepatotrophic factors and matrix remodeling, which directly involved HSCs [6]. This role of HSCs in promoting engraftment of transplanted cells seemed distinct from their ability to transdifferentiate into profibrogenic myofibroblast-like cells expressing α -smooth muscle actin (α -SMA) with secretion of cytokines, chemokines or receptors, as well as extracellular matrix (ECM) components [7–11]. Nonetheless, mechanisms driving hepatic fibrogenesis are complex, with interactions between HSCs, other non-parenchymal cells, and hepatocytes through cell-cell contacts and soluble factors [12–15], which have been difficult to extrapolate from studies in vitro.

There is general agreement that HSCs are the major contributor to fibrogenesis in the liver. Following liver injury, HSCs migrate to sites of damage and undergo activation with excessive synthesis of ECM components. Even though the important role of HSCs in hepatic fibrogenesis is well documented, specific antifibrotic, HSC-directed therapies have yet to be established. One reason for this difficulty is that experimental modulation of HSCs in vivo is extremely challenging; until recently there was no established HSC-specific Cre-transgenic mouse model to study this cell compartment [16]. Therefore, we hypothesized that generation of animal models with transplantation of human HSCs (hHSCs) will be valuable for studying the contributions of HSCs in liver injury and fibrosis.

This project aimed to evaluate whether hHSCs could be successfully transplanted into the liver for studying their fate along with activation and migration to sites of liver injury. To avoid potential variables related to donor-specific differences in the properties of primary hHSCs, we utilized human HSCs that had been immortalized by the catalytic subunit of human telomerase reverse transcriptase (hTERT) and retained most aspects of primary HSCs, including typical morphology and gene expression profiles [17]. To avoid rejection, we transplanted hHSCs into xenograft-tolerant mice lacking T and B cells with non-obese diabetic-severe combined immunodeficiency (NOD/SCID). Marking of hHSCs with radiolabels or a lentivirally-introduced transgene to express green fluorescent protein (GFP) allowed tracking of transplanted cells over short- and long-term, respectively. This led to successful studies of the biodistributions, engraftment, proliferation and fate of transplanted hHSCs with or without fibrogenic damage in the liver.

Materials and methods

Animals

NOD.CB17-Prkdc^{scid}/J mice were from Jackson Laboratories (Bar Harbor, ME), or from the Special Animal Core Facility, Hamburg University Medical Center. Animal Care and Use Committees at Albert Einstein College of Medicine and Hamburg University approved animal use in conformity with National Research Council's Guide for the Care and Use of Laboratory Animals (United States Public Health Service Publication, revised 1996) and German regulations.

Cells

hTERT-hHSCs were from Dr. David Brenner and generation of these cells was described previously [17]. These hHSCs were cultured in 5% CO₂ atmosphere on plastic dishes in Dulbecco's minimum essential medium (DMEM) with antibiotics and 10% fetal bovine serum (FBS) (Atlanta Biologicals, Norcross, GA). Cells were split 1:3 every 4 to 5 d. The morphology of cells was evaluated by light microscopy. Staining for α -SMA was as described previously [6].

Lentiviral vector (LV) transduction of hHSCs with GFP

Vesicular stomatitis virus-pseudotyped LV stocks were obtained by pCCL.PPT.hPGK.GFP.Wpre transfer construct with packaging constructs, pMDLg/pRRE and pRSV-REV, and pMD2.G envelope construct, which were transfected into 293T cells to produce LV, as described previously [18]. For LV transduction, 4×10^6 hHSCs were incubated with vector for 90 min at 37 °C in 1 ml final volume, and transduced cells were then expanded in culture.

Cell transplantation

Mice were anesthetized with i.p. 90 mg/kg ketamine plus 8 mg/kg xylazine. Spleen was isolated by laparotomy and 1×10^6 hHSCs in DMEM with 2% FBS were injected into splenic pulp. Control animals received vehicle (DMEM/FBS) alone.

Radiolabeling of cells and biodistribution analysis

For radiolabeling, $3\text{--}6 \times 10^6$ hHSCs were incubated for 30 min at 37 °C in normal saline with 18.5 MBq Indium-111 (¹¹¹In)-oxine or 370 MBq technetium-99m (^{99m}Tc) pertechnetate (UltraTag[®] kit, Mallinckrodt Inc, Hazelwood, MO) or 370 MBq ^{99m}Tc-exametazime (Ceretek[™], Amersham Biosciences, Piscataway, NJ). Cells were washed twice with

Fig. 1 Experimental design for elucidating proliferation and fate of transplanted hHSCs. After allowing 10 d for intrasplenically transplanted hHSCs to engraft, mice were given CCl_4 injections twice weekly or subjected to BDL with analysis at various time points as indicated

PBS and pelleted under 300 $\times$ g for 5 min. For labeling efficiency, radioactivity was measured in cell pellets and supernatants. Cell viability after radiolabeling was confirmed by trypan blue dye exclusion. For organ biodistributions, 1×10^6 radiolabeled hHSCs were transplanted into NOD/SCID mice followed 1 h later by gamma imaging (Argus; ADAC Laboratories, Milpitas, CA). Energy discrimination used a 15% window centered on 174- and 247-keV peaks of ^{111}In . Dorsal images were acquired for 5 min on $64 \times 64 \times 16$ matrix. After imaging, ^{111}In activity was counted in excised spleen, liver, lungs, and kidneys (Cobra II counter; Packard Instrument Co., Meriden, CT), and expressed as fraction of total radioactivity in these organs.

Enhancement of hHSC engraftment and proliferation

For endothelial injury, 200 mg/kg monocrotaline (MCT) was given i.p. in phosphate buffered saline, pH 7.4 (PBS), 16–24 h before cell transplantation. Control mice received PBS alone. For hepatic injury, the experimental design is shown in Fig. 1. To induce toxin-mediated liver fibrosis, 1 ml/kg carbon tetrachloride (CCl_4) mixed 1:10 (v/v) in corn oil was given i.p. twice weekly for up to 6 weeks, starting 10 d after cell transplantation. Controls received corn oil alone. To induce biliary fibrosis, bile duct ligation (BDL) was performed 10 d after transplantation of hHSCs, as described previously [14].

Polymerase chain reaction (PCR)

Qualitative and quantitative PCR was performed for the human Charcot-Marie-Tooth disease type 1A repeat element (CMT1A) as well as murine c-mos sequence as described previously [19]. Quantitative PCR for human beta-globin gene was performed as described previously [20].

Western blot

Total proteins were from culture medium and extracted from hHSCs, blotted and analyzed for human collagen type I as described previously [21].

Tissue staining and in situ hybridization

For GFP-staining, liver tissue was fixed in 4% paraformaldehyde for 4 h with anti-GFP (1:300 Molecular Probes) staining, essentially as described previously [18]. Sections were incubated with secondary peroxidase-conjugated mouse or rabbit-specific goat IgG (1:300, Dako, Real Envision, HRP Rabbit/Mouse) for 30 min and developed with diaminobenzidine at room temperature. To distinguish GFP+ transplanted cells from other cells or matrix components, tissues were co-stained with anti-GFP plus primary antibodies against following: anti-mouse

Fig. 2 Analysis of hHSC characteristics and biodistribution studies with radiolabeled cells. **a** Immunohistochemistry showing α -SMA was expressed in all hHSCs. Note typical spindle shape or elongated morphology of cells in culture conditions. **b** Western blot of cell lysates and culture supernatants demonstrated collagen expression ($\alpha 1$ and $\alpha 2$ chains) by cultured hHSCs. **c** Lentivirally transduced hHSCs stably expressed GFP in all cultured cells supporting its use as a reporter in cell transplantation studies. **d** Comparison of the size of hHSCs released by trypsin-EDTA from culture dishes indicated these were substantially smaller than mouse hepatocytes (**e**) but were still much larger than the 3–5 μ m diameter of hepatic sinusoids. **f** Gamma imaging of a mouse 1 h after intrasplenic transplantation of ^{111}In labeled hHSCs showing tracer activity in spleen and liver. **g** Organ distributions of transplanted hHSCs as fractions of total ^{111}In activity indicated that most hHSCs were deposited in the liver. Original magnifications A,C: 400x, D,E: 200x

F4/80 (eBioscience, Frankfurt, Germany), anti-human albumin, anti-mouse LSEC (CD146, Miltenyi Biotech, Bergisch-Gladbach, Germany), anti-human collagen type I (Southern Biotech, Birmingham, Alabama), collagen type IV (1:500, Sigma-Aldrich, Munich, Germany), anti-mouse cytokeratin (1:500, Dako, Hamburg, Germany), anti-human laminin (1:50, Dako). Alexa Fluor 488 and Alexa Fluor 555 conjugated secondary antibodies (Invitrogen) were used for fluorescence microscopy. Nuclei were stained with Hoechst 33258 (Invitrogen). Fibrillary tissue collagen was identified and quantitated by picrosirius red staining as described previously [14]. In situ hybridization for primate-specific alphoid satellite sequences was performed as described previously [19].

Statistical methods

Data are shown as mean $\pm$ SD. Differences were analyzed by *t*-tests with SigmaStat 3.1 software (Systat Inc., Point Richmond, CA). *P* < 0.05 was considered significant.

Results

Deposition and engraftment of transplanted hHSCs in mouse liver

In culture, hHSCs proliferated well with myofibroblast-like morphology, and showed expression of α -SMA by

Fig. 3 Engraftment of hHSCs in mouse liver. **a** PCR of livers from transplanted mice for human specific CMT1 indicated presence of hHSCs in recipients. **b** Immunohistochemistry for GFP reporter showing survival and distribution of transplanted cells in the liver after 7 d. **c** In situ hybridization for human aliphoid sequences 1 h after transplantation demonstrated hHSCs in the space of Disse or within hepatic sinusoids (arrows). Combined staining for GFP (green) and anti-mouse LSEC (CD146) (**d**) or F4/80 (**e**) after 7 d indicated that transplanted hHSC were within proximity to other non-parenchymal cells in the liver but were distinct from those other cell types. Original magnifications: B, D, E: 200x, C: 400x

immunostaining (Fig. 2a) and collagen by western blotting (Fig. 2b), which are characteristics of HSCs. Also, virtually all LV-transduced hHSCs expressed GFP (Fig. 2c). Consistent with an activated phenotype, hHSCs displayed neither vitamin A autofluorescence nor lipid droplets (not shown). The mean diameter of trypsinized hHSCs ($11.1 \pm 3.5 \mu\text{m}$) was greater than that of distal hepatic sinusoids ($3\text{--}5 \mu\text{m}$) but less than that of adult hepatocytes ($20\text{--}35 \mu\text{m}$) (Figs. 2d, e), suggesting that transplanted hHSCs would be entrapped within the liver after deposition into the portal circulation. To confirm this possibility, we radiolabeled hHSCs for biodistribution studies. The hHSCs incorporated ^{111}In -oxine (to $62 \pm 8\%$ efficiency) but neither $^{99\text{m}}\text{Tc}$ -exametazime nor $^{99\text{m}}\text{Tc}$ -pertechnetate. Gamma imaging of mice receiving ^{111}In -labeled-hHSCs showed transplanted cells translocated rapidly from the spleen to the liver without entering extrahepatic organs, e.g., lungs (Fig. 2f), thereby confirming their entrapment in the liver. Measurement of ^{111}In activity in excised organs established that the majority of transplanted hHSCs were in the liver (Fig. 2g). Some transplanted hHSCs remained in the spleen. Entry of hHSCs in lungs was negligible. Therefore, intrasplenic

injection of hHSCs was successful in depositing cells to liver sinusoids, as was desired.

We then studied engraftment of GFP-labeled hHSCs in mouse liver. PCR for human-specific CMT1 element identified transplanted cells in every mouse ($n = 3$ per time-point) (Fig. 3a). Transplanted cells were detected throughout a 7 days period, although the abundance of CMT1 PCR products decreased over time. Persistence of transplanted hHSCs in the liver was directly established by GFP staining, as well as by in situ hybridization for primate-specific aliphoid satellite sequences identifying every centromere (Figs. 3b, c). hHSCs were localized in sinusoids or portal vein radicles immediately after transplantation. The abundance of transplanted hHSCs decreased after 7 days, which was in agreement with PCR results. It was noteworthy that transplanted hHSCs were distinct from sinusoidal liver cells, such as LSECs and hepatic macrophages, as shown by double immunofluorescence for GFP + transplanted cells and endothelial or F4/80 macrophage markers, respectively (Figs. 3d, e). Similarly, hHSCs were distinct from hepatocytes expressing albumin or cytokeratin (CK)-18 (not shown).

Fig. 4 Induction of proliferation in transplanted hHSCs. Immunohistochemistry for GFP reporter 6 weeks after cell transplantation showing little proliferation of hHSCs in liver of otherwise untreated mouse. Transplanted cells mostly formed small clusters (a). By contrast, sinusoidal endothelial disruption with MCT prior to cell transplantation

produced superior engraftment and proliferation of hHSCs (b). The greatest extent of hHSC expansion was observed in mice pretreated with MCT followed by CCl₄ twice weekly (c). This greater proliferation of transplanted hHSCs was confirmed by qRT-PCR for human beta-globin (d). Original magnification A: 200x, B, C: 100x

Induction of hHSC proliferation in the mouse liver

We determined whether fibrogenic liver injury induced by CCl₄ would promote proliferation of transplanted hHSCs. For this purpose, we analyzed proliferation of transplanted cells over 6 weeks in groups of mice given vehicle alone, MCT pretreatment alone, or MCT pretreatment plus CCl₄ twice weekly post-transplant ($n = 6$ each) (Fig. 4). GFP-immunostaining identified few transplanted hHSCs in mouse livers without either MCT or CCl₄. In these mice, hHSCs were observed in small groups, most likely representing accumulations in areas of ischemic necrosis induced by cell transplantation (Fig. 4a). In mice pretreated with MCT alone, hHSCs engrafted better with greater abundances (Fig. 4b). However, transplanted hHSCs expanded most in mice given MCT followed by repeated CCl₄ (Fig. 4c). Transplanted hHSCs accumulated along fibrotic bands encircling liver nodules in this condition. To quantitate these differences in engraftment and proliferation of transplanted hHSCs we performed qRT-PCR for human beta-globin in mouse livers. The extent of liver repopulation with hHSCs approached $1.0 \pm 2.5\%$ in mice receiving MCT alone ($n = 4$ with 2 liver lobes per mouse) vs. $8.7 \pm 4.9\%$ in mice receiving MCT plus CCl₄ ($n = 6$ with 2 lobes per mouse), $p < 0.05$ (Fig. 4d).

Functional activity of transplanted hHSCs in mouse liver

We examined the ability of transplanted hHSCs to produce ECM components. In MCT plus CCl₄-treated mice, immunostaining studies showed presence of substantial human collagen I in hHSC recipient livers (Fig. 5a). This was not the case in control mouse livers (Fig. 5b). Double staining for GFP and human collagen I demonstrated transplanted hHSCs were in close proximity to human collagen deposits, suggesting that this ECM component originated from transplanted hHSCs (Fig. 5c). Similarly, transplanted cells co-localized with collagen IV and laminin (Figs. 5d, e), which was also in agreement with the origin of these ECM components from transplanted hHSCs—a caveat being that the antibodies used to identify these components were not human-specific. Next, to determine the extent of liver fibrosis that could be assigned to transplanted hHSCs, we studied additional NOD/SCID mice with or without hHSC transplantation followed by CCl₄ for six consecutive weeks. Quantitative analysis of Sirius Red-stained areas, as described previously [22], demonstrated that CCl₄ caused greater fibrosis in mice with hHSCs vs. control mice without hHSC transplantation (Figs. 5f, g). The fractions of Sirius Red-stained areas constituted $7.2 \pm$

Fig. 5 Transplanted hHSCs produced ECM components in mouse liver. (a, b) Immunohistochemistry showed presence of human collagen I in hHSC-transplanted (a) vs. untransplanted mouse livers (b) after 6 weeks of CCl₄. Co-staining for GFP reporter (green) in transplanted hHSC and collagen I (c), collagen IV (d) and laminin (e)

(red) showed ECM components within proximity to transplanted hHSCs. Sirius Red staining of vehicle control (f) and hHSC recipient livers (g) allowed quantification of fibrillary collagen (h) irrespective of its source in transplanted hHSCs or liver resident murine HSCs. Original magnifications A-G: 200x

3.5% in hHSC transplant group ($n = 10$) vs. $1.0 \pm 0.5\%$ in controls with CCl₄ alone ($n = 8$), $p < 0.01$ (Fig. 5h).

Injury-related distributions of hHSCs in mouse liver

Transplantation of hHSCs in MCT-pretreated mice followed 10 d later by either hepatocellular injury with CCl₄ or cholestatic injury with BDL demonstrated whether or not transplanted hHSCs would migrate to sites of injury. Control mice received vehicle alone or underwent sham surgery. After CCl₄ injury, transplanted hHSCs accumulated in fibrotic septae with predominantly pericentral and also porto-central distribution, which was typical for liver fibrosis induced by CCl₄. By contrast, BDL resulted in predominantly periportal accumulation of transplanted hHSCs (Figs. 6a, b). This distribution of hHSCs was confirmed by double staining for GFP and pan-cytokeratin as a marker of bile duct cells (Figs. 6c, d).

To visualize migration of hHSCs within the injured liver, transplanted hHSCs were localized at various time-points. Transplanted hHSCs were present throughout the liver lobule after 10 d (Figs. 7a, b). However, 1 and 2 weeks after

CCl₄ hHSCs were predominantly in pericentrally located necrotic areas (Figs. 7c, d). During further CCl₄-induced liver damage, hHSCs were in fibrotic septae joining adjacent central veins and occasionally extending from portal to central areas, indicating transplanted cells had migrated during fibrogenesis (Figs. 7e, f). In vehicle-treated controls, this pattern of hHSC distribution in the liver lobule was not observed at time points up to 4 weeks (Figs. 7g, h).

To additionally evaluate whether Kupffer cells (KCs) may have contributed in recruitment of hHSCs through cell-cell interactions activated by tissue inflammation, we analyzed whether these cell types preceded one another or were simultaneously engaged at sites of injury. We found KCs and transplanted hHSCs co-localized in both, the CCl₄ and BDL injury model, although this was more pronounced in CCl₄-treated mice (Figs. 8a, b). In controls with MCT pretreatment only, we did not observe distinct co-localization of KCs and hHSCs (Fig. 8c). No GFP-positive cells were observed in mice without hHSC transplantation (Fig. 8d). Therefore, this hHSC transplantation model allowed identification of additional cell-cell interactions within the liver.

Fig. 6 Injury-related distributions of transplanted hHSCs in mouse liver. Immunohistochemistry for GFP (a–b) as well as double-immunofluorescence for GFP (green) and biliary marker pan-cytokeratin (red) (c–d) showed pericentral distribution of transplanted hHSCs after CCl₄-induced liver injury (a and c) and periportal accumulation after BDL (b and d). CV central vein, PF portal field, BD bile duct. Original magnifications 200x

Discussion

In the liver, HSCs represent the major fibrogenic cell-type and are deemed crucial for progression of cirrhosis and related complications, such as portal hypertension. Therefore, understanding HSC pathophysiology will be important for therapeutic strategies aimed at delaying or reversing fibrosis in chronic liver diseases. Much current knowledge of HSC biology has been derived from studies with HSCs in vitro. However, we and others previously established that the widely used model of HSC activation by culturing isolated HSCs on a plastic surface does not accurately reflect changes of activated HSCs in vivo [23]. Obviously, interaction with the hepatic tissue environment is required for physiological HSC activation. This is in line with our finding that presence of liver macrophages during HSC culture shifted the gene expression pattern of in vitro activated HSCs towards the pattern observed in vivo [24]. Similarly, culture activation of primary human HSCs does not accurately reflect the biological phenotype of activated HSCs isolated from cirrhotic livers [25]. Whereas several existing mouse models of liver fibrosis provided valuable insight into fibrogenesis, the species-specific differences between murine and human HSCs do restrict their value as models for human fibrosis. Here, we successfully established a mouse model that allowed targeting of human HSCs to the liver along with analysis of their biological behavior and fate during hepatic fibrogenesis in vivo. Specifically, this study provided insights into how the liver may be repopulated with transplanted hHSCs. First, we found hHSCs were

efficiently targeted to the liver via the intrasplenic route. Second, transplanted hHSCs engrafted in the liver, especially when the hepatic endothelium was injured beforehand to open up the space of Disse where HSCs are normally located. Third, transplanted hHSCs proliferated and migrated to the sites of liver damage and interacted with other cell types serving roles in hepatic injury and repair. Fourth, transplanted hHSCs displayed fibrogenic potential by generating myofibroblast-like cells and producing ECM components. In these ways, transplantation of hHSCs was effective for generating this humanized mouse model of liver fibrosis.

Radiolabeling of hHSCs was convenient for cell biodistribution analysis and this approach should similarly be useful in future transplantation studies, e.g., of primary hHSCs. The incorporation of ¹¹¹In but not of ^{99m}Tc in hHSCs was recapitulated in primary mouse HSCs (Bente et al., unpublished data), indicating this was specific to this cell type. Previously, we showed that LSECs as well as KCs incorporated ¹¹¹In and not ^{99m}Tc [18, 26], whereas hepatocytes incorporated both of these tracers [27], highlighting cell type-specific differences in radioligand uptake or transport mechanisms in parenchymal vs. non-parenchymal liver cells.

The restriction of transplanted hHSCs to the liver indicated that for achieving superior cell engraftment mechanical factors should be less important than cellular alterations, such as endothelial disruption by MCT. This was in accordance with previous studies demonstrating that endothelial damage improved engraftment of transplanted hepatocytes as well as LSECs [1, 18, 28]. Similarly, prior

Fig. 7 Migration of transplanted hHSCs to sites of liver injury. Immunohistochemistry for GFP visualized time-dependent migration of transplanted hHSCs to the site of CCl₄-induced injury (a–f). In untreated control mice, hHSC distribution was without such a specific pattern (g–h). Original magnifications: 200x

damage in native KCs with gadolinium chloride facilitated engraftment of transplanted KCs in the liver [26].

Remarkably, extensive proliferation of transplanted hHSCs required profibrogenic stimuli *in vivo*, since repeated CCl₄ administration produced 8-fold greater liver repopulation with these cells. Transplanted hHSCs themselves were not cleared by this progressive liver injury. On the other hand, as hHSCs accumulated during CCl₄-induced liver injury in fibrotic septae bridging pericentral or portovenous areas, this model identified for the first time injury-related migration of intrahepatic HSC-derived

myofibroblasts. Although this migration of HSCs is considered to be central in fibrogenesis [29–31], it has not been possible previously to directly analyze this mechanism *in vivo*. Recently, Mederacke et al. generated a Cre-transgenic mouse for unequivocal identification of almost all endogenous HSCs *in vivo*, thereby allowing substantiation of these cells as major contributors in toxic, metabolic or biliary injuries in the liver [16]. Nonetheless, immunohistochemical identification of HSCs can be unreliable for tracking HSC migration in tissues since expression of cellular markers used for this purpose, e.g., α -SMA,

Fig. 8 Co-localization of transplanted hHSCs and KCs. Combined immunofluorescence for GFP reporter (green) in transplanted hHSCs and F4/80 marker (red) of monocytes/macrophages showed close proximity of transplanted hHSCs and KCs. This was most pronounced in mice with CCl₄-induced liver injury (a) and was also observed in mice after BDL (b). In mice with MCT pretreatment only, KCs and hHSCs were restricted to necrotic areas (c) Controls without transplantation of hHSCs were negative for GFP staining (d) Original magnifications: 200x

desmin, glial fibrillary acidic protein, etc., may be regulated variably based on the functional state of the cells [30]. Moreover, lineage tracing for demonstrating migration of HSCs into areas of liver damage may not be possible due to difficulties in separating cells arising locally via transdifferentiation or changes induced by epithelial-mesenchymal transitions, cell proliferation, etc. By contrast, use of genetically marked transplanted hHSCs permitted identification of their recruitment to sites of injury and also functionality, i.e., production of ECM components, including collagens and laminin, in response to profibrogenic stimuli.

Co-localization of transplanted hHSCs and KCs after CCl₄ and BDL-induced liver injury demonstrated these cell types were in close proximity. This was in agreement with our previous findings indicating that functional interactions between HSCs and KCs are significant in hepatic fibrogenesis [24]. As model systems have been successfully developed to identify the fate of transplanted KCs and their roles in regulating inflammatory events and processes through cytokine expression [26], exciting experimental studies to address interactions between hHSCs and KCs could now be launched. The biological behavior of transplanted hHSCs reproduced that of resident HSCs in vivo, and allowed for the first time delineation of how HSCs may migrate toward sites of injury, which has again been hypothesized to be a key mechanism in liver fibrogenesis [32]. In contrast with previous difficulties in determining whether HSCs originate locally or migrate to sites of liver injury, our study unequivocally established the ability of HSCs to migrate and to accumulate during liver injury. This finding should allow experimental studies of HSC-specific

interventions, e.g., ex vivo genetic modification of HSCs prior to transplantation for demonstrating potential of candidate genes in fibrogenesis. Indeed, HSCs were readily and efficiently transduced by LV demonstrating the feasibility of introducing genes for interventions, such as gene constructs able to limit fibrogenesis and alter other processes ranging from inflammation to immunomodulation in the liver.

We found transplanted hHSCs did not transdifferentiate into alternative cell types. Whether HSCs possess stem/progenitor cell potential to generate additional cell types has been controversial [33–35]. However, we observed neither albumin nor CK-18 expression as hepatocyte markers in transplanted hHSCs, including after CCl₄-induced hepatic injury. Although it may be that immortalized hHSCs studied here were no longer able to reproduce properties of primary hHSCs and species-specific barriers may have affected transdifferentiation capacity of these cells in mice, it should be noted that fetal human hepatocytes immortalized by hTERT retained their transdifferentiation potential, including after transplantation in immunodeficient mice [36].

We previously showed that hepatocyte transplantation activated native HSCs, which contributed in turn to transplanted cell engraftment in the liver [6]. In other experimental settings, co-transplantation of HSCs with pancreatic islet cells under the renal capsule improved parenchymal cell survival via immunoregulatory events and HSC-mediated generation of myeloid-derived suppressor cells [37, 38]. Since human hepatocytes tend not to survive and proliferate well in the mouse liver, our model could allow studies to determine whether co-transplantation of hHSCs

would benefit engraftment and function of these or other human liver cell types. This should accelerate progress in other relevant translational research areas.

In summary, we demonstrated that immortalized hHSCs were successfully transplanted to generate a humanized mouse liver model of liver fibrosis and HSC migration *in vivo*. This animal model may offer new opportunities to study human fibrogenesis and to modulate liver fibrosis in a hHSC-specific manner *in vivo*.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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