Isolation, characterization, and cultivation of human hepatocytes and non-parenchymal liver cells

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Abstract

Primary human hepatocytes (PHH) are considered to be the gold standard for *in vitro* testing of xenobiotic metabolism and hepatotoxicity. However, PHH cultivation in 2D mono-cultures leads to dedifferentiation and a loss of function. It is well known that hepatic non-parenchymal cells (NPC), such as Kupffer cells (KC), liver endothelial cells (LEC), and hepatic stellate cells (HSC), play a central role in the maintenance of PHH functions. The aims of the present study were to establish a protocol for the simultaneous isolation of human PHH and NPC from the same tissue specimen and to test their suitability for *in vitro* co-culture. Human PHH and NPC were isolated from tissue obtained by partial liver resection by a two-step EDTA/collagenase perfusion technique. The obtained cell fractions were purified by Percoll density gradient centrifugation. KC, LEC, and HSC contained in the NPC fraction were separated using specific adherence properties and magnetic activated cell sorting (MACS®). Identified NPC revealed a yield of 1.9×10^6 KC, 2.7×10^5 LEC and 4.7×10^5 HSC per gram liver tissue, showing viabilities $>90\%$. Characterization of these NPC showed that all populations went through an activation process, which influenced the cell fate. The activation of KC strongly depended on the tissue quality and donor anamnesis. KC became activated in culture in association with a loss of viability within 4–5 days. LEC lost specific features during culture, while HSC went through a transformation process into myofibroblasts. The testing of different culture conditions for HSC demonstrated that they can attenuate, but not prevent dedifferentiation *in vitro*. In conclusion, the method described allows the isolation and separation of PHH and NPC in high quality and quantity from the same donor.

Keywords: Liver, primary human hepatocytes, non-parenchymal liver cells, primary cell isolation, liver *in vitro* model, liver tissue engineering

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Introduction

The human liver is characterized by a complex structure of different cell populations. The parenchymal hepatocytes are responsible for most of the liver functions, such as, e.g., energy metabolism, bile acid synthesis, and biotransformation of xenobiotics.[1] The non-parenchymal cell (NPC) fraction contains cell types of different origin, including Kupffer cells (KC), liver endothelial cells (LEC), and the hepatic stellate cells (HSC). Previous studies have shown that these cells play a role in physiological liver functions as well as in acute liver damage, such as, e.g., drug-induced liver injury (DILI), hepatitis, as well as in acute inflammation, and in chronic liver diseases, such as liver fibrosis and cirrhosis.[2]

KC are hepatic resident macrophages of monocytic origin.[3] They represent approximately 15% of total liver cells,[1] and with the content of 35% of NPC, KC form the majority of hepatic NPC.[4] KC can be activated by various signals released from the processing of phagocytized particles or by stimulated surface receptors.[5] They produce a variety of pro- and anti-inflammatory cytokines, which influence local cells, but also cells of the systemic immune system.[6] Additionally, in case of defense reactions, KC are capable to produce reactive oxygen intermediates (ROI) that cause injury to parenchymal cells and to NPC. Therefore, KC play a key role in hepatic tissue damage and in numerous liver pathophysiologies, but they also have a central part in liver regeneration and tolerance reactions.[7]

LEC form the inner lining of vessels in the liver. LEC are of mesenchymal origin and can vary in their phenotype depending on their localization.[8] The sinusoidal endothelial cells (LSEC) constitute a physiological barrier between the hepatocytes and the blood.[9] They are characterized morphologically by numerous fenestrations, which are arranged in sieve plates and enable an extensive exchange of substances between the bloodstream and the hepatocytes.[10] Additionally, LEC are very active in receptor-mediated pinocytosis of soluble macromolecules and of colloids.[11] Therefore, besides KC, LEC are part of the systemic scavenger system.[12]

HSC, which are also known as fat-storing cells or Ito cells, are pericytes of mesenchymal origin. They are located in the perisinusoidal space (space of Disse).[13] HSC dispose a different amount of lipid droplets, due to storage of retinol and other fat-soluble molecules.[14] Following liver injury, HSC get activated by cytokines, in particular by TGF-β, and are transformed into a myofibroblast-like cell type.[15] Activated HSC lose their retinol storage capacity, start to express contractile fibers, and secrete extra-cellular matrix (ECM) proteins, which are considered as a key process in the development of liver fibrosis and later cirrhosis.[16,17]

PHH mono-cultures are considered to be the gold standard for the investigation of hepatic metabolism and toxicity of xenobiotics.[18] However, detailed morphological and functional studies have demonstrated that these models are limited due to hepatocyte dedifferentiation and loss of functions within few days.[2] Additionally, mono-hepatocyte cultures have only limited abilities for the *in vitro* reproduction of hepatotoxic effects observed *in vivo*. To compensate these limitations, 3D cultivation protocols have been established successfully.[2,19] Nonetheless, these models are still not able to reproduce some mode of action (MOA) of hepatotoxicity. The lack of cell–cell communication between the different liver cells has been suggested as a main deficiency of current hepatocyte models.[20] In recent years, NPC have become increasingly relevant for the development of human liver co-culture models and in tissue engineering.[21,22]

In order to create standardized co-culture models comprising different liver cell types at a defined proportion, methods for isolation and purification of hepatocytes and NPC fractions are needed. The isolation of human NPC is still debated controversially and most data published on their isolation, culture and characterization refer to non-human cells.[23,24] Only a few protocols dealing with the isolation of human NPC are available[25–27] (reviewed).[28]

The use of human PHH and NPC populations from the same donor is a new approach to liver cell isolation. We have established a protocol for an uncomplicated isolation of PHH, KC, LEC, and HSC from the same donor tissue. All cell types were successfully identified and characterized. Next, the influence of the donor anamnesis, tissue quality and culture conditions on the cell viability and quality of the different cell types was investigated. Furthermore, cell-specific properties of cultivated KC, LEC and HSC were studied in mono-cultures.

Materials and methods

Chemicals

Williams Medium E with Glutamax (WME), supplements (fetal calf serum FCS), Pen/Strep, HEPES, MEM NEAA, Pyruvat, L-glutamine) and PBS were purchased from Gibco (Paisley, UK). RPMI and DMEM media were purchased by GE Healthcare (Pasching, Austria). Dexamethasone was obtained from Merck (Fortecortin®, Darmstadt, Germany). Human insulin was purchased from Sanofi Aventis (Frankfurt am Main, Germany). Percoll, Trypan Blue and HBSS were provided by Biochrom (Berlin, Germany). All other chemicals were purchased from Sigma (Munich, Germany), if not stated differently. Rat tail collagen was prepared in our laboratory according to the protocol established by Rajan et al.[29] The hepatocyte culture medium was based on William's Medium E with GlutaMAX™, supplemented with 10% FCS, 32 mU/mL insulin, 15 mM HEPES, 0.1 mM MEM NEAA (100 ×), 1 mM Pyruvate and 1 mg/L Fortecortin. KC seeding medium was based on RPMI low glucose supplemented with 1% L-glutamine. KC culture medium was based on RPMI low glucose supplemented with 10% FCS, 1% L-glutamine and 6.3 mM N-acetyl-L-cysteine. LEC/HSC separation medium was based on DMEM low glucose supplemented with 1% L-glutamine. LEC/HSC cultivation medium was based on DMEM low glucose supplemented with 10% FCS and 1% L-glutamine. All culture media were supplemented with 100 U/100 μM Pen/Strep prior to use.

Human liver cell isolation

Tissue samples. Liver cells were isolated from macroscopically healthy tissue that remained from resected human liver of patients with primary or secondary liver tumors or benign local liver diseases. Informed consent of the patients for the use of tissue for research purposes was obtained according to the ethical guidelines of the Charité – Universitätsmedizin Berlin.

Isolation of primary human hepatocytes. PHH and NPC were isolated by a two-step EDTA/collagenase perfusion technique as described elsewhere,[30] with the following modification: In order to minimize proteolytic enzyme activities in the collagenase solution, the perfusion solution II consisted of 100 mg collagenase in 100 mL perfusion solution II supplemented with 10% FCS. For separation of PHH from the NPC-containing fraction, the cell suspension was centrifuged at 50 × *g*, 5 min, 4°C. The cell pellet contained PHH. The supernatant was used later for the NPC isolation (see section on Isolation of NPC). The PHH fraction was subjected to a 25% Percoll density gradient centrifugation at 1250 × *g*, 20 min, 4°C without brake, to remove non-viable cells. The resulting PHH fraction was washed with PBS and re-suspended in PHH culture medium. Cells were counted in a Neubauer counting chamber by Trypan blue staining and seeded on collagen-coated cell culture vessels. PHH were cultured in PHH culture medium at 37°C, 5% CO_2 in a humidified incubator.

Isolation of NPC. The supernatant from the initial centrifugation step, which contains the NPC fraction, was centrifuged at 72 ×*g*, 5 min, 4°C in order to eliminate the remaining erythrocytes. For the sedimentation of the NPC fraction, the supernatant was subjected to a dual centrifugation step using HBSS: (1) 300 ×*g*, 5 min, 4°C for the sedimentation of LEC, HSC and partly KC, (2) 650 ×*g*, 7 min, 4°C for the sedimentation of the majority of KC. Pellets from both centrifugation steps were pooled and re-suspended in HBSS. The cell suspension was transferred on a two-layer (25%/50%) Percoll density gradient and centrifuged at 1800 ×*g*, 20 min, 4°C without brake. NPC were enriched in the interphase between the 25% and the 50% Percoll layer. Dead cells and cell debris on the uppermost layer as well as unwanted cells in the pellet were discarded. The NPC-containing fraction was washed with HBSS and centrifuged by the dual centrifugation step as described above.

Kupffer cells: KC were separated from the NPC fraction by their ability to adhere on cell culture plastics within a short period of time. Therefore, after counting of KC in the NPC-containing cell fraction, the suspension was centrifuged by the dual centrifugation step as described earlier, re-suspended in KC seeding medium, and seeded on cell culture vessels at a density of 5×10^5 KC/cm^2. After incubation for 20 min at 37°C, 5% CO_2 in a humidified incubator, the supernatant was collected and used later for LEC and HSC separation. The adherent KC were washed with HBSS and cultivated in KC culture medium at 37°C, 5% CO_2 in a humidified incubator.

Liver endothelial cells: The supernatant of the KC separation step contained the cell populations of LEC and of HSC. In order to separate LEC from HSC, the magnetically activated cell sorting system MACS® (Miltenyi Biotech, Bergisch-Gladbach, Germany) was used. The cell suspension was centrifuged at 300 ×*g*, 5 min, 4°C. The cells contained in the pellet were immunolabeled (CD31 MicroBeads) and separated as described in the manufacturer's protocol. The magnetically retained CD31-positive LEC were eluted and suspended in LEC/HSC culture medium. After counting, cells were seeded at a density of 1.25×10^5 LEC/cm^2 in cell culture vessels coated with rat tail collagen and cultured at 37°C, 5% CO_2 in a humidified incubator.

Hepatic stellate cells: HSC were CD31 negative and passed the column during the MACS® separation. The cells were collected and the HSC were seeded at a density of 5×10^4 HSC/cm^2 in LEC/HSC culture medium in cell culture vessels coated with rat tail collagen at 37°C, 5% CO_2 in a humidified incubator.

Identification of NPC

Immunofluorescence staining. The different NPC types were identified by morphological characteristics and immunofluorescence staining of cell type-specific antigens (Table 1). For staining, KC, LEC, HSC, and cholangiocyte (CC) cultures were washed with PBS and fixed with 4% formaldehyde (Herbeta, Berlin, Germany) for 10 min. After washing the cells three times with PBS, they were permeabilized with 0.5% Triton X-100 for 2 min. After a further washing step, the cells were incubated with 1% bovine serum albumin for 1 h to block non-specific binding, followed by the incubation with cell type-specific primary antibodies for 1 h (Table 1). Cells incubated without primary antibodies served as a negative control. The immunolabeled cells were washed three times with PBS and finally incubated with the secondary antibodies Alexa Fluor® 488 goat anti-mouse IgG or Alexa Fluor® 594 goat anti-rabbit IgG (both Santa Cruz Biotechnology, Dallas, TX, USA) for 1 h. Cell nuclei were stained with Hoechst 33342 for 15 min. Images were taken with a Keyence microscope BZ-9000 (Neu-Isenburg, Germany).

Functional characterization of NPC

Phagocytosis assay. The ability of KC to phagocytize small particles was investigated in KC cultures 12 h after seeding. KC were incubated over 24 h with KC seeding medium containing 4×10^6 FITC labelled Fluoresbrite™ Plain YG 3.0 Microspheres (Polyscience Inc., Warrington, USA) per mio KC. At the end of incubation, the cells were fixed with 4% formaldehyde and cell nuclei were labelled with Hoechst 33342 for 15 min. The fluorescence of phagocytized latex beads was detected with the Keyence fluorescence microscope.

Pinocytosis assay. The ability of the uptake of acetylated LDL is known to be highly specific for LEC. Therefore, the cultured cells were incubated with LEC/HSC separation medium containing 10 μg/mL of acetylated LDL labeled with 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO-Ac-LDL) (Tebu-bio, Offenbach, Germany) for 4 h at 37°C. After washing the cells several times with probe-free

Table 1 Primary antibodies used for immunofluorescence staining of human parenchymal and non-parenchymal liver cells

Antibody	Type	Species	Reactivity	Manufacturer	Dilution	Marker
α SMA	Monoclonal	Mouse	Human	Sigma, USA	1:100	HSC
CD31	Polyclonal	Rabbit	Human	Thermo Scientific,	1:50	LEC
CD68/ SR-D1	Monoclonal	Mouse	Human	R&D Systems, USA	1:100	KC
CK 18	Monoclonal	Mouse	Human	Santa Cruz, USA	1:100	PHH
CK 19	Polyclonal	Rabbit	Human	Santa Cruz, USA	1:100	CC
GFAP	Monoclonal	Mouse	Human	Sigma, USA	1:100	HSC
Ki67 (H-300)	Polyclonal	Rabbit	Human	Santa Cruz, USA	1:50	Proliferation
Vimentin	Monoclonal	Mouse	Human	Santa Cruz, USA	1:100	HSC LEC

DMEM medium, they were fixed with 4% formaldehyde for 10 min. The cell nuclei were stained with Hoechst 33342 for 15 min and the emission of acetylated LDL was examined with the Keyence fluorescence microscope.

Fluorescence microscopy of retinol. The specific blue-green autofluorescence of the retinol stored in HSC was excited at a wavelength of 328 nm and visualized by the Zeiss Axio Imager M1 (Jena, Germany).

Cultivation and activation of NPC

Mitochondrial activity. In order to analyze the metabolic activity of the cells, the colorimetric XTT assay (Roche Diagnostics GmbH, Mannheim, Germany) was used. The assay is based on the reduction of the yellow tetrazolium salt XTT to a highly colored formazan dye by dehydrogenase enzymes in metabolically active cells. The assay was performed according to the manufacturer's protocol. After 24 h of incubation, the absorbance was measured at a wavelength of 492 nm by means of a microplate photometer (FLUOstar OPTIMA, BMG Labtech, Ortenberg, Germany).

Proliferative capacity of isolated NPC. The proliferative capacity of the different NPC populations was analyzed on day 3 of cultivation by immunofluorescence staining of the proliferation marker Ki67 (Table 1).

Activation of KC. Intracellular formation of reactive oxygen intermediates (ROI) indicating KC activation was measured by the DCF assay.[31] The assay is based on deacetylation of dichlorodihydrofluorescein diacetate (DCF-DA) by cellular esterases and oxidation by ROI to the fluorogenic dichlorodihydrofluorescein (DCF). KC cultures were incubated with serum- and phenol red-free RPMI 1640 medium (Lonza, Basel, Switzerland) containing 20 μM DCF-DA (Santa Cruz, Dallas, TX, USA). After incubation for 30 min at 37°C, the supernatants were discarded and the cells were incubated with serum-free RPMI medium without phenol red for 1 h. The fluorescence of DCF was measured at an excitation wavelength of 492 nm and an emission wavelength of 520 nm with a fluorescence microplate reader. In order to assess the initial KC activation, ROI were measured directly after the isolation process. Then, the KC were cultivated for five days for the investigation of a time-dependent activation.

Activation of HSC. Activated HSC typically transdifferentiate into myofibroblasts. Transdifferentiated HSC were identified by the detection of the synthesis of extracellular matrix proteins and expression of α-smooth muscle actin (α-SMA). HSC were cultivated on cell culture vessels coated with Matrigel® (BD Biosciences, San Jose, CA, USA), rat tail collagen or not coated for 1, 3 or 5 days for the investigation of coating-dependent activation. For the detection of the activation and transformation of HSC, immunofluorescence staining for α-SMA was performed (Table 1), as described under 2.3.1.

The Sulforhodamine B (SRB) colorimetric assay was used for the detection of intra- and extracellular matrix proteins of the HSC. Cells cultured for 1, 3 or 5 days were fixed with 4% formaldehyde, washed twice with PBS, covered with 0.4% SRB solution, and incubated at room temperature for 30 min in the dark. Unbound SRB was removed from cells by washing the cultures four times with 1% acetic acid solution. For quantification, SRB was resolved in 10 mM TRIS solution for 10 min. The absorbance was measured photometrically at 565 nm using a microplate reader.

Scanning electron microscopy of LEC. For the detection of LEC fenestrations typical for native LEC, scanning electron microscopic observation was performed. Cultured LEC were fixed with 2.5% glutaraldehyde with cacodylate buffer. Samples were dried with hexamethyldisilazan (HDMI). LEC were examined with a DSM 982 Gemini Scanning electron microscope (Zeiss, Jena, Germany).

Statistical analysis

Data was processed with the software GraphPadPrism 5. Each experiment was carried out with cells from at least three donors ($N = 3$), if not stated differently. Results were given as mean ± standard deviation (SD).

Results

Isolation of PHH and NPC populations

PHH and NPC were isolated in parallel from human liver tissue samples. An overview of the isolation procedure is given in Figure 1. We had previously observed that an extended digestion of 10–15 min favors the NPC yield (data not shown). In order to protect the PHH from proteolytic damage caused by the extended digestion, 10% FCS were added to the collagenase solution. By this modification, PHH could be isolated in parallel with high yields of NPC. From the NPC fraction, we successfully isolated KC, LEC and HSC.

Isolated PHH showed a cell diameter ranging between 20 and 30 μm in suspension. They dispose of a large cytoplasmic volume with varying amounts of lipid droplets (Figure 2a). The yield of PHH amounted to $14.8 \times 10^6 \pm 6.6 \times 10^6$ viable PHH/g liver tissue ($N = 12$). The average viability was 76.6 ± 4.2% (Table 2).

KC appeared as a small cell type with a diameter of about 5 μm with a low cytoplasm/nucleus ratio and an irregular granulated cell surface in suspension (Figure 2b). This cell type dominated the NPC fraction and amounted to $1.9 \times 10^6 \pm 0.2 \times 10^6$ viable KC/g liver tissue ($N = 5$). The viability of isolated KC was 92.8 ± 3.5% (Table 2).

The MACS® separation technique for the isolation of CD31-positive cells was used for LEC purification. LEC in suspension are characterized by a diameter of about 10 μm and multiple granules (Figure 2c). The yield of LEC amounted to $2.7 \times 10^5 \pm 0.1 \times 10^5$ viable LEC/g liver ($N = 8$) and the viability reached 95.6 ± 2.8% (Table 2).

The CD31-negative cell fraction was dominated by a cell type with numerous lipid droplets characteristic for HSC. HSC displayed a diameter of about 20 μm. The cells had a granulated appearance with a varying amount of lipid droplets in different sizes (Figure 2d). The yield of isolated HSC

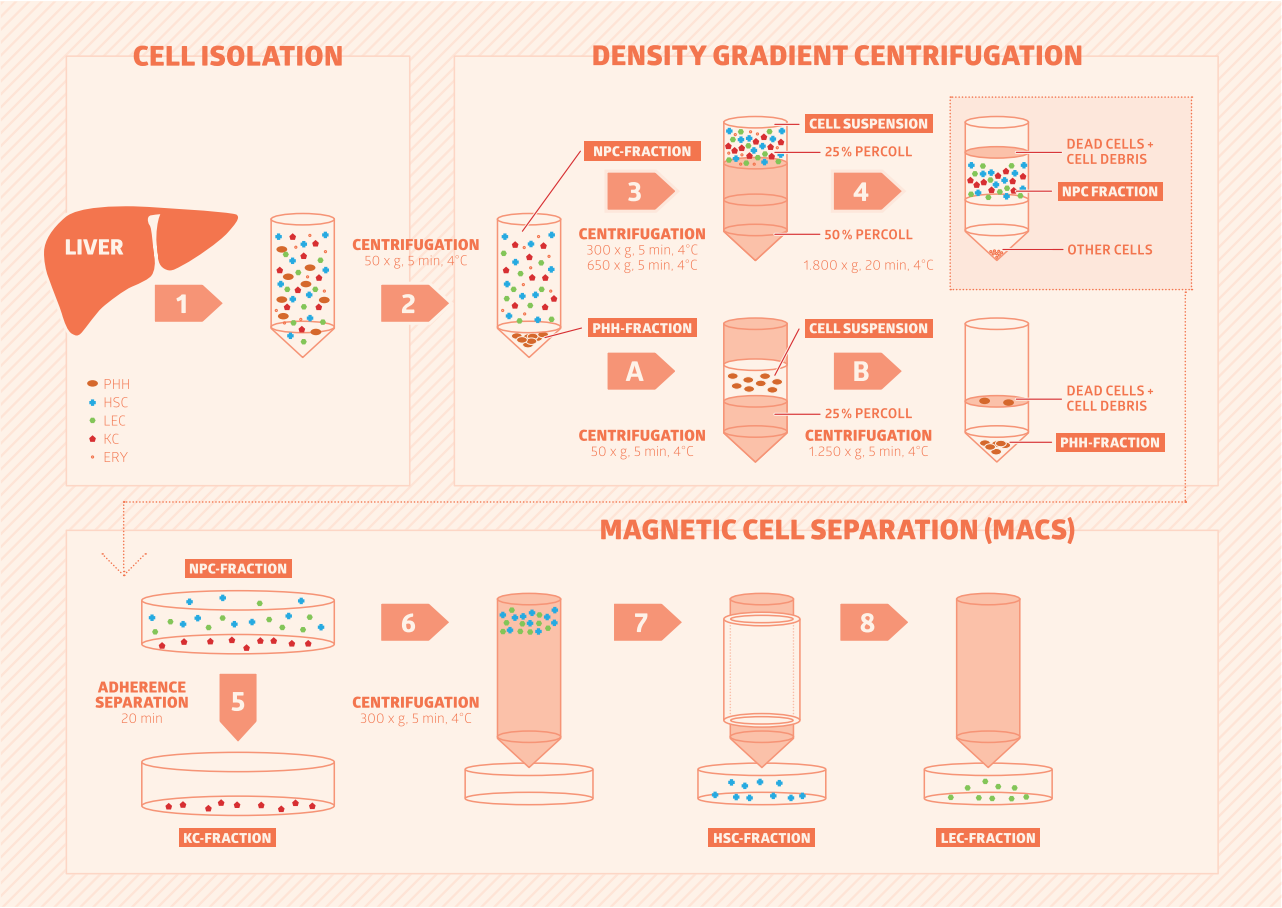

Figure 1 Schematic overview of the isolation of PHH and NPC from human liver resections. Liver cells were isolated by a two-step EGTA/collagenase P perfusion technique (1). The cell suspension was centrifuged initially at 50 ×*g*, 5 min, 4°C (2). PHH (pellet) were separated from the NPC (supernatant). PHH were purified by a Percoll density gradient centrifugation at 1250 ×*g*, 20 min, 4°C (A and B). The supernatant of PHH isolation (2) was centrifuged first at 300 ×*g*, 5 min, 4°C (3). KC were found in the supernatant of the first centrifugation step. Cells were treated with a second centrifugation at 650 ×*g*, 7 min, 4°C. Both cell pellets were pooled and re-suspended in HBSS. Afterwards, the cell suspension was transferred to a two-layer (25% / 50%) Percoll density gradient and centrifuged at 1800 ×*g*, 20 min, 4°C (4). Dead cells on top of the 25% Percoll layer were discarded. NPC were located around the interphase of the 25% and the 50% Percoll layer. KC were separated from the NPC fraction by an adherence separation step (5). LEC and HSC were separated by MACS®. For this purpose, the remaining cells were centrifuged at 300 ×*g*, 5 min, 4°C, and labelled with CD31-conjugated MicroBeads (6). CD31-negative HSC passed the MACS® separation column (7). The column was removed from the magnetic field and the CD31-positive LEC were dissolved from the column (8). (A color version of this figure is available in the online journal.)

amounted to $4.7 \times 10^5 \pm 0.2 \times 10^5$ viable HSC/g liver ($N = 8$) and the calculated viability was 89.6 ± 3.8% (Table 2).

Identification of liver cells

For the identification of PHH as well as the different NPC populations and for the determination of their purity, the isolated cells were cultured and investigated for cell type-specific markers by immunofluorescence staining. Positively stained cells were counted in relation to the total cell number of cells positive for Hoechst. Additionally, the different liver cell populations were investigated for morphological features when cultured in 2D mono-cultures.

PHH were identified by their cubical shape and their polyploidy (Figure 3a). They were positive for the hepatocyte marker CK18 (Figure 3b) and showed a purity of 92.3 ± 3.2% (Table 2).

KC were isolated by their selective adherence to cell culture plastics. They are characterized by their small round shape and by their prominent round cell nucleus (Figure 3c). KC showed immunoreactivity for the macrophage-specific surface protein CD68 (Figure 3d). The purity of fluorescent positive cells amounted to 81.0 ± 5.4% (Table 2).

The LEC were separated from the remaining HSC population with CD31 MicroBeads. They had a spindle-shaped appearance with an oval nucleus (Figure 3e). LEC expressed the mesenchymal cell marker vimentin (Figure 3f). The purity of the fluorescence-positive cells in the LEC fraction amounted to 81.0 ± 1.7% (Table 2).

HSC were characterized by their round cell shape with prominent lipid droplets (Figure 3g). They were further identified by immunofluorescence staining for GFAP (Figure 3h) and the purity as judged by GFAP expression amounted to 93.0 ± 1.7% (Table 2).

The analysis of the presence of other NPC, hepatocytes or cholangiocytes in the different NPC populations showed that all fractions contained small amounts of other NPC types. In contrast, all NPC fractions were negative for the

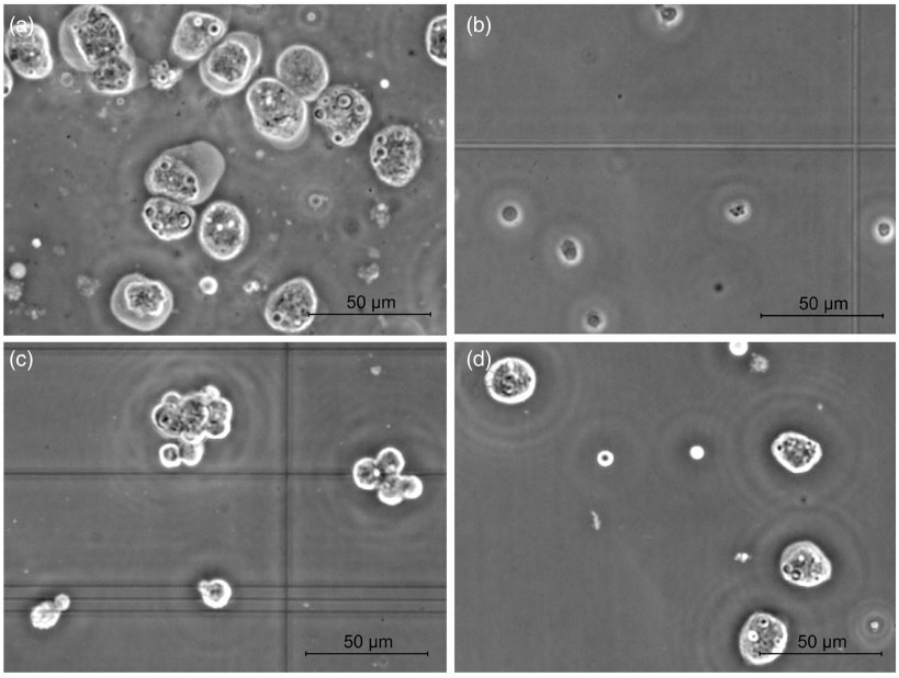

Figure 2 Morphological appearance of human liver cells in suspension. Phase contrast images of isolated liver cell populations directly after the process of isolation and separation. The pictures display PHH (a), KC (b), LEC (c), and HSC (d); magnification: × 400

Table 2 Yields, viability and purity of isolated PHH and NPC: cell numbers and cell viability were determined directly after the isolation process

	PHH	KC	LEC	HSC
Cell count/g liver tissue	$14.2 \times 10^6 \pm 6.6 \times 10^6$	$1.9 \times 10^6 \pm 0.2 \times 10^6$	$2.7 \times 10^5 \pm 0.1 \times 10^5$	$4.7 \times 10^5 \pm 0.2 \times 10^5$
Viability (%)	76.6 ± 4.2	92.8 ± 3.5	95.6 ± 2.8	89.6 ± 3.8
Purity (%)	92.3 ± 3.2	81.0 ± 5.4	81.0 ± 1.7	93.0 ± 1.7

Note: Data are given as means ± SD. The cell purity of individual cell types was determined after IF staining for cell-specific antigens.

hepatocyte marker CK18 and for the cholangiocyte marker CK19 (data not shown).

Functional characterization of isolated NPC

For functional characterization, the different NPC types were investigated with respect to cell type-specific features and differentiated functions.

KC were characterized by their ability for the phagocytosis of fluorescent latex beads. The particles were taken up by the KC and accumulated in the intracellular compartment around the nucleus (Figure 4a). Each KC incorporated multiple particles, which resulted in an increased cellular volume.

LEC were characterized by their capacity of lipoprotein uptake. The treatment with fluorescent LDL particles revealed an accumulation of intact round particles around the plasma membrane, whereas the fluorescent signal in the cytoplasm appeared partially as round particles and partially in a diffuse form (Figure 4b).

A characteristic feature of HSC is their retinol storage capacity, which was analyzed by fluorescence microscopy. The typical specific blue–green autofluorescence of retinol was observed as droplets in the perinuclear region (Figure 4c). These droplets corresponded to the lipid droplets observed by light microscopy (Figure 3g).

Cultivation and activation of isolated NPC

The stability of the different NPC populations was investigated in mono-cultures of KC, LEC, and HSC by analysis of morphological and functional changes of the isolated cells during culture.

Cultivation and activation of KC. The intracellular ROI formation was measured as a marker for KC activity. The measurement of the initial value immediately after the isolation process revealed a broad variety in ROI levels (Figure 5a). In young healthy donors, characterized by benign non-inflammatory liver diseases, very low values for ROI were registered. KC from donors with chronic inflammation, portal vein embolization or chemotherapy showed increased activation levels. Cells isolated from liver tissue containing a surgically caused resection area exhibited the highest ROI levels. The comparison of ROI levels with donor anamnesis suggests that donor conditions

influence the initial KC activation. However, due to the small sample size, further studies are needed to validate these findings.

The analysis of the viability and activation levels of KC during culture over 1–5 days showed that the viability of KC continuously decreased over a culture period of five days. At day 5, the viability of KC was reduced by about 2/3 (Figure 5b). The determination of the activation levels during the cultivation period of five days revealed an increase in the ROI formation until day 2, which was followed by a steady decrease of the ROI levels until day 5 (Figure 5c), corresponding with the observed loss of viability.

Proliferation activities of cultured KC as analyzed by immunofluorescence staining for Ki67 were not detected (see Supplementary Information, Figure 1a).

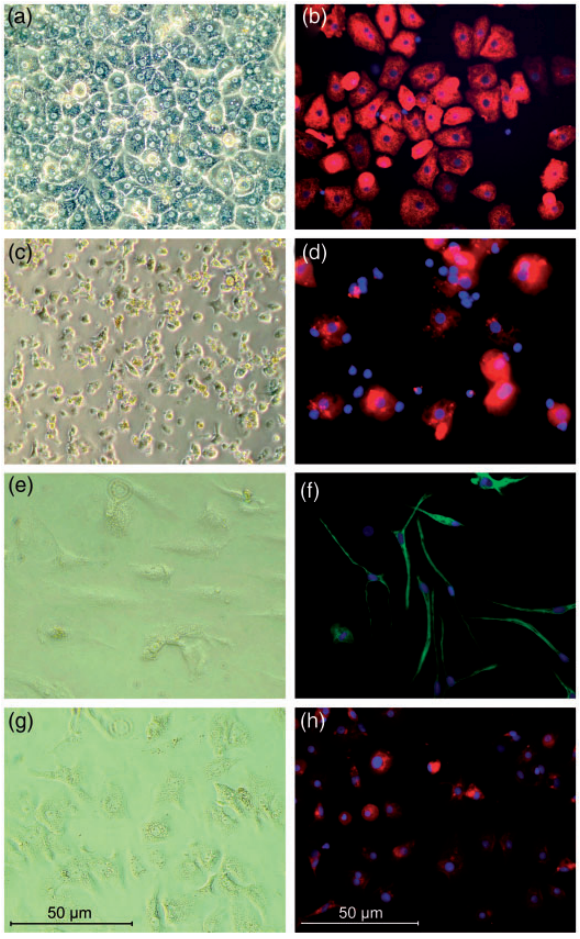

Figure 3 Morphological appearance of adherent liver cells and immunofluorescence staining of cell type-specific antigens. Phase contrast images of isolated PHH (a), KC (c), LEC (e), and HSC (g) cultivated under cell type-specific culture conditions for at least 12 h at 37°C. PHH (a) displayed positive signals for the hepatocyte marker CK18 (b), KC (c) showed positive signals for the marker CD68 (d), LEC (e) were positive for vimentin (f), HSC (g) were positive for GFAP (h). The cell nuclei were stained with Hoechst; magnification: × 400. (A color version of this figure is available in the online journal.)

Cultivation of LEC. The measurement of cell viability in LEC cultures maintained over 5 days by means of the XTT assay showed a constant increase in the mitochondrial activity (Figure 6a). No morphological signs of cell injury were observed during the entire culture period. A morphological feature of LEC is the occurrence of fenestrations on the surface of LSEC. These fenestrations allowed for a better substance transport between blood and hepatocytes. LEC were investigated using scanning electron microscopy during the first two days of cultivation. Larger gaps were observed between the individual cells. These gaps were formed between cell–cell contacts of LEC (Figure 6b). Additionally, we observed diffuse punctuated structures, which could not be determined clearly as fenestrations or as sieve plates (Figure 6c). Proliferation of LEC was demonstrated by the immunofluorescence staining of the proliferation marker Ki67 (see Supplementary Information, Figure 1b).

Cultivation of HSC. HSC cultured for five days showed an increase in purity of the cell population from 93% on day 1 up to 98% on day 5 (see Supplementary Information, Figure 2a). The measurement of cell viability indicated an increase of the mitochondrial activity (see Supplementary Information, Figure 2b). The microscopic examination confirmed that the cells were healthy during the observed culture period.

The morphological investigation of the HSC cultures revealed that they underwent an activation process within increasing cultivation time and were transformed into a proliferating myofibroblast-like cell type. The proliferation of HSC was confirmed by a positive immunofluorescence staining for the proliferation marker Ki67 (see Supplementary Information, Figure 1c). In addition, an increased protein content was measured on day 5, indicating the production of extracellular matrix proteins (see Supplementary Information, Figure 2c). In order to

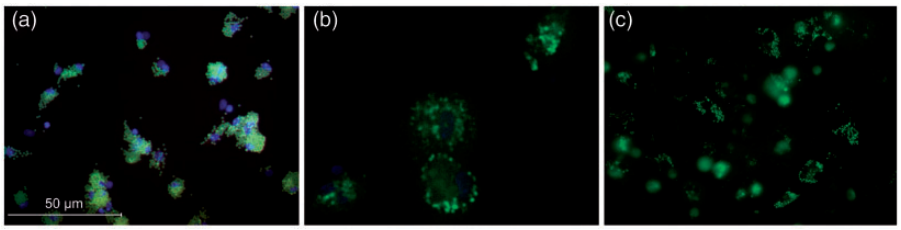

Figure 4 Functional characterization of NPC. Phagocytic activity of KC was demonstrated by IF staining of incorporated latex beads (a). Pinocytic activity of LEC was demonstrated by IF staining detecting the uptake of acetylated LDL particles (b). HSC were characterized by specific autofluorescence of retinol storage at 328 nm (c); magnification: × 400. (A color version of this figure is available in the online journal.)

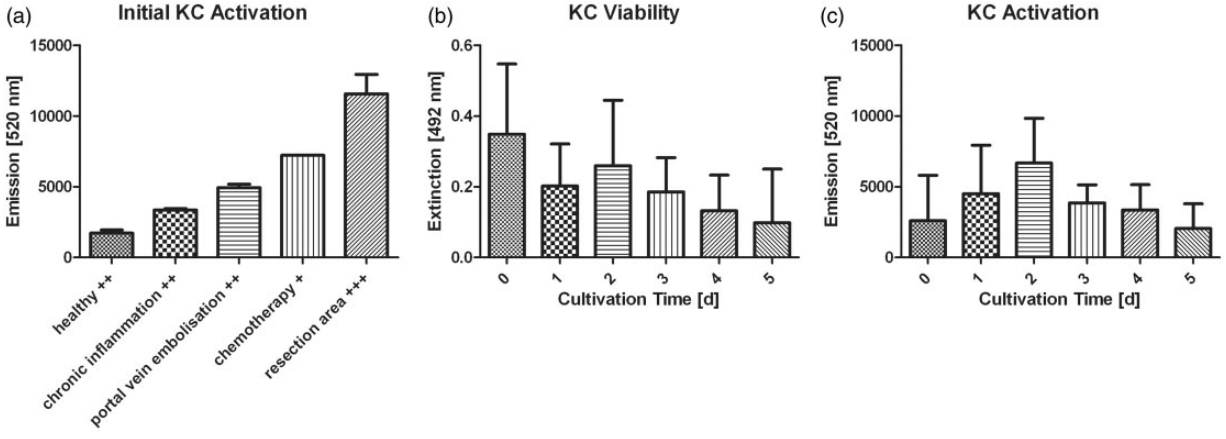

Figure 5 Activation of KC in mono-culture. The initial KC activation levels were determined by the detection of the intracellular ROI measured by the DCF assay (emission at 520 nm). The test was performed directly after the adherence of the isolated KC. The ROI levels of cultures from donors with different anamnesis and tissue quality are shown in a, with (+) $N = 1$; (++) $N = 2$; (+++) $N = 3$. KC viability (b) was determined over a cultivation period of five days by measurement of the mitochondrial activity using the XTT assay (extinction at 492 nm). KC activation (c) was determined over a cultivation period of five days by ROI measurement. Data represent means ± SD of three independent experiments

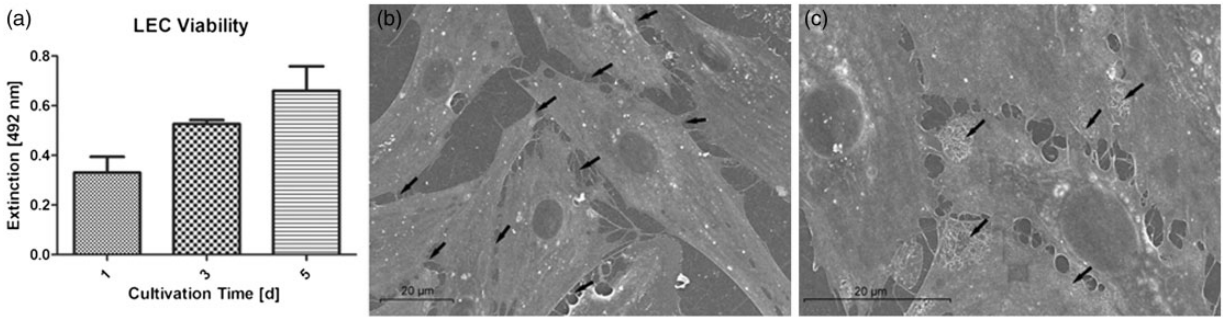

Figure 6 Viability and activation of LEC in mono-culture. The viability of LEC was determined over a cultivation period of five days by the measurement of the mitochondrial activity with the XTT assay (extinction at 492 nm) (a). Fenestrations and sieve plates were detected in LEC cultures using scanning electron microscopy on day 2 of cultivation. (b) Spindle-shaped LEC with distinct cell–cell connections (black arrows), magnification: × 1000. (c) Irregular surface structures (black arrows) probably arising from previous fenestrations; magnification: × 2000

investigate a potential effect on the transformation process by the coating material used, HSC were seeded in culture vessels coated with collagen, Matrigel® or not coated. HSC activation was determined by immunofluorescence staining for α-SMA at days 1, 3, and 5 of culture. The results showed that HSC adhered best on collagen-coated tissue plates, while the number of adherent cells was lower for the Matrigel® and on the surfaces without coating. All cultivated HSC displayed changes in their morphology and showed an increased α-SMA expression from day 3 of HSC culture, indicating HSC transformation into myofibroblasts (Figure 7).

Discussion

In order to establish functional and reproducible human liver cell co-cultures and tissue-engineered *in vitro* liver models, the availability of parenchymal and non-parenchymal liver cells at a defined quality and quantity is indispensable. In the present study, we have developed a protocol for the isolation and separation of human PHH and different NPC populations, including KC, LEC, and HSC, from the tissue of surgical liver resections. PHH and NPC were simultaneously isolated in a high purity and quality, and the NPC were successfully cultured in mono-cultures. The cell types were clearly identified through the analysis of specific morphological cell characteristics and immunofluorescence staining of cell type-specific antigens. Cell-specific features and their *in vitro* fate were examined during a defined cultivation time to assess the suitability of the cells for functional co-culture systems. Most available publications deal with the isolation of a single NPC population from non-human species and therefore they cannot be directly transferred to humans due to species-specific differences.[10,32,33] Data on human primary NPC isolations are scarce in the literature. The present protocol is based on a previously described PHH isolation procedure.[30] The addition of 10% FCS to the collagenase-containing perfusion solution allowed for mild digestion of the tissue over prolonged digestion periods. By this modification, PHH of good quality and quantity could be isolated in parallel with high amounts of NPC.

The yield of KC was about 1.9×10^6 viable KC/g liver tissue with an average viability of about 93%, which is

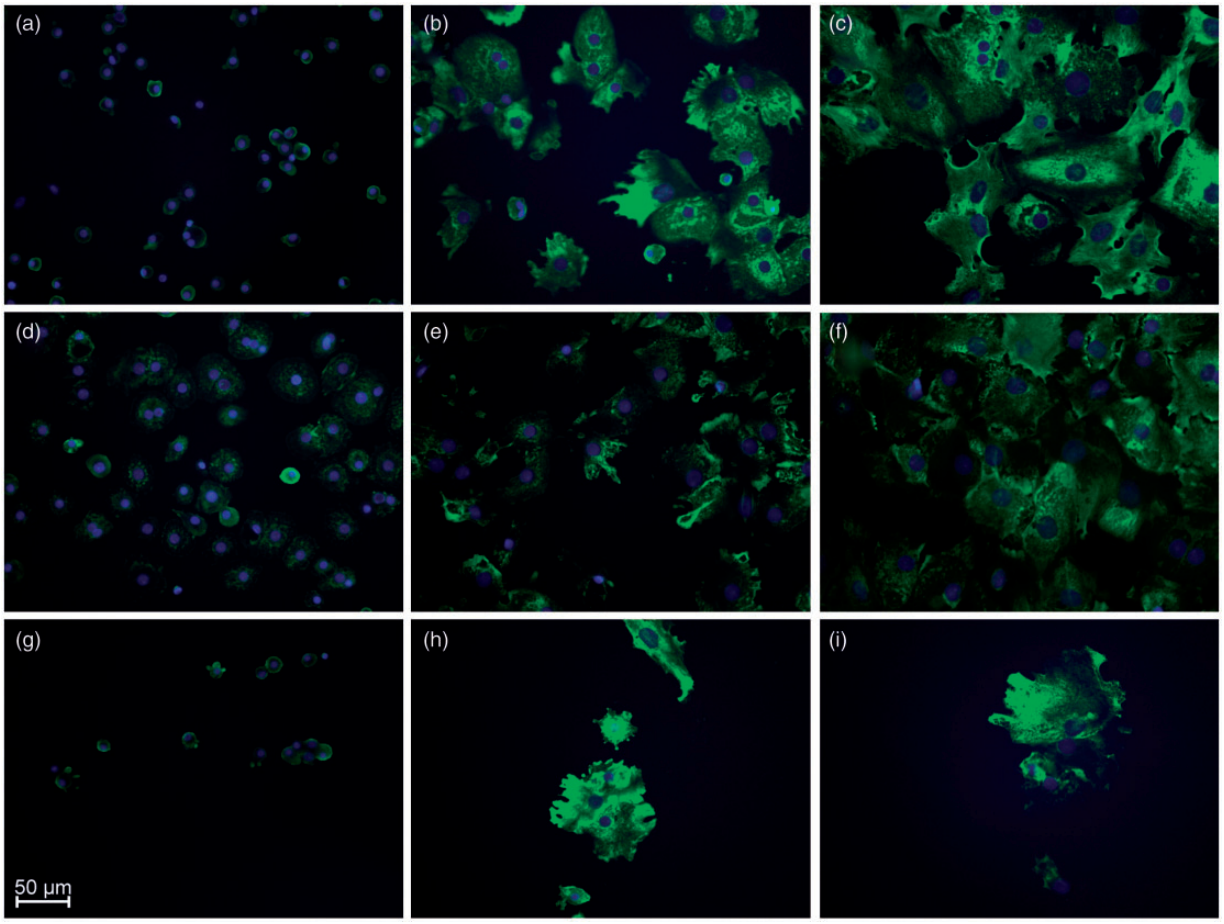

Figure 7 Activation of HSC in mono-culture. Isolated HSC were cultivated under cell type specific conditions for five days on uncoated plastic (a, b, c), collagen (d, e, f) or matrigel® (g, h, i). Immunofluorescence staining with α-SMA as a marker for HSC activation after one day (a, d, g), three days (b, e, h), and five days (c, f, i); magnification: × 400. (A color version of this figure is available in the online journal.)

comparable to the KC yield obtained by Alabraba et al. (2007), who isolated about 2.3×10^6 KC/g liver with a viability and purity of about 98%.[25] Cultivated KC were identified by their ability for the phagocytosis of fluorescent latex beads. Furthermore, KC expressed the macrophage-specific antigen CD68.[34] The counting of CD68 and phagocytosis positive cells revealed a KC purity of about 81%. Further characterization included the evaluation of the KC activation levels. Activated KC produce reactive ROI as part of the NF-κB signaling pathway. These ROI have a high potential for being used as a marker for the determination of the KC activation.[6] High initial levels of ROI were detected in KC from liver tissue with a surgically caused resection border. This could be mediated by the release of amplified signals from the freshly damaged and destroyed liver tissue. KC can be activated by various endogenous sources, such as e.g. cytokines, cell debris from necrosis, and apoptotic bodies.[4] Cells from other donors showed also high initial activation levels, while healthy liver tissue from young donors with benign tumours and no secondary diseases or interventions showed the lowest KC activation levels. Thus, the results indicate that diseases and interventions leading to hepatic tissue damage results in an increase in KC activation. Prolonged cultivation of KC in mono-culture revealed an increase in ROI production in association with a decrease in cell viability until day 5. These findings suggest that initial cell activation and progressive cell death lead to an activation of the remaining KC.

LEC were separated from the remaining NPC fraction by using CD31 Micro Beads as part of the magnetic cell sorting system MACS®. LEC were isolated with a yield of about 2.7×10^5 viable LEC per g/liver tissue with a viability of about 96% and a purity of 81%. The human liver contains endothelial cells of different origins, including LEC from the venous and arterial vessels of the liver as well as the LSEC. The latter represent about 2.5% of lobular parenchyma.[1] Concerning the isolation of human LEC, cell yields between 10^3 and 10^6 cells/organ with a purity of about 90% have been reported.[27,35] The isolated LEC population was positive for the endothelial cell marker CD31 and the mesenchymal cell marker vimentin, consistent with the findings by others.[36] In addition, the uptake of acetylated LDL was demonstrated, which is characteristic for LEC.[12] The ultrastructural examination using scanning electron microscopy provided no clear evidence for the presence of fenestrations during the first two days of cultivation, which is a reliable marker of LSEC. This could be caused either by

a loss of this hallmark feature during culture or by the fact that fenestrations are missing completely. The presence of fenestrations on CD31 positive cells is controversially debated.[11] While it is unclear whether LSEC are CD31 positive, the existence of an intermediate cell type has been suggested, which is CD31 positive and has less fenestrations than pure LSEC.[37] It was possible to cultivate the isolated LEC under cell type-specific conditions for several days. Proliferation of cultured LEC was shown using the proliferation marker Ki67. Due to the possibility of their long-term cultivation and proliferation *in vitro*, the LEC are well suited for being used in *in vitro* co-culture models.

After separation of KC and LEC, we obtained a HSC fraction with a cell number of 4.7×10^5 viable HSC per g/liver, a viability of about 90% and a purity of about 93%, as determined by GFAP staining of HSC cultures. In 1992, Friedman et al. published data on the isolation for human HSC. Cell yields of about 2.3×10^5 HSC/g liver with a purity of 91% were shown, which is by half lower than our values.[26] The isolated HSC could also be identified by the specific blue–green autofluorescence of intracellularly stored retinol and positive signals for α-SMA, which is a marker for HSC.[14] HSC could be cultivated under cell type specific conditions for several days. The cultured HSC showed a proliferation activity as detected by the proliferation marker Ki67. During the cultivation time, we observed a morphological and physiological transformation process connected to the synthesis of extracellular matrix components and an increased expression of α-SMA. The latter is described as a reliable marker for HSC activation.[17] Matrix protein formation and α-SMA expression are in concordance with investigations made by various other work groups.[13,17,38] Possible reasons for HSC activation could be the cell isolation process, but also the artificial culture conditions used in HSC mono-cultures, which may trigger a transformation process as a signal of tissue damage. Therefore, we tested the influence on HSC activation of different coating matrices. The results showed that HSC adhered on non-coated, rat collagen- or Matrigel®-coated tissue plates at varying densities. While the HSC showed a high confluence on non-coated and collagen-coated plastic surfaces, they adhered only poorly on Matrigel®. For Matrigel® deactivating properties on HSC transdifferentiation have been reported.[39] In contrast to those results, we did not observe any deactivation processes for Matrigel® as determined by α-SMA expression. The α-SMA signals as well as the increase in metabolic activity, which was measured by the XTT assay, indicate a general activation level after day 3 on all matrices. Additionally, we observed a high number of proliferating cells on day 3 in the cultures. Taken together, the testing of different culture conditions for HSC showed that the tested culture conditions can attenuate, but not prevent cell activation *in vitro*.

In general, protocols for human non-parenchymal liver cell isolation, characterization and culture are rare and, in majority, not definite. To date, no isolation procedures have been described, which make it possible to isolate, separate and investigate the entirety of human liver cells from the same tissue. The isolation method presented here allows for gaining human parenchymal cells and non-parenchymal liver cells, namely KC, LEC, and HSC separately in a high purity and quality. Liver tissue was obtained from patients with different liver diseases and health states, which may influence the quality, quantity and activation state of the isolated cells and cause inter-individual variances in the isolation and culture outcome of parenchymal cells and NPCs. The studies on cultivation of the different NPC types also showed that the work window for these cells is limited to 3–4 days depending on cell quality. Currently, many efforts are being undertaken to improve *in vitro* liver models. PHH cultivation in 2D mono-cultures leads to dedifferentiation and loss of functions.[40] Reconstruction of the *in vivo* tissue architecture consisting of a 3D environment of ECM and NPC is a promising approach to solve some of these problems. First investigations on a commercially available 3D liver co-culture system, which uses rat hepatocytes and NPC, have shown that liver functions, including cytochrome P450 inducibility, can be maintained over several months in comparison to 2D mono-cultures.[21] A similar approach is the generation of a human liver spheroid model by the formation of a microtissue using PHH, KC and endothelial cells in a hanging drop culture platform.[22] Both models have been used successfully for the detection of hepatotoxic effects at a higher sensitivity compared to classic liver models. While those systems represent static models characterized by discontinuous medium supply, open dynamic culture systems reflect more closely the physiological environment of the cells by enabling continuous medium exchange and oxygenation of the cultured cells. PHH cocultured with NPC in a perfused 3D multicompartment bioreactor showed tissue-like cell reorganization and stable maintenance of metabolic activities.[41] However, the analysis of functionality of NPC as well as donor-specific information are missing in these studies. Co-culture systems and *in vitro* liver tissues consisting of defined cell numbers of PHH and NPC from the same donor are a new promising approach for liver tissue engineering. The long-term survival, functionality and influence of NPC on co-culture and on engineered liver tissues have to be investigated.

In conclusion, we present a new method for simultaneous extraction of human PHH and different NPC populations from the same donor tissue. The cells were clearly identified and characterized on the basis of morphological properties, specific marker expression and functional analysis. For the use of NPC in functional co-culture models and tissue engineering, a monitoring of the activation and of the cell fate of NPC is recommended.

Authors' contributions: EP designed, performed and established the cell separation protocols and cell culture methods; developed and established methods for cell characterization; evaluated and analyzed experimental data; and wrote the manuscript. VK established functional assays for cell characterization and evaluated experimental data; designed, coordinated, and performed the Kupffer cell isolations, cell culture, and cell characterization. KZ designed and coordinated the research on liver endothelial cells and cell isolation method development and also

contributed to writing the manuscript and provided useful discussions. JGH analyzed and evaluated data, contributed to writing the manuscript and provided useful discussions. AKN; designed, coordinated, and performed the research and cell isolation method development; analyzed and evaluated data; contributed to the development of cell isolation protocols; contributed to writing the manuscript, and provided useful discussions. DS performed the liver surgery, provided liver tissue samples, contributed to writing the manuscript and provided useful discussions. GD designed, coordinated, and performed the research and cell isolation method development; developed methods for cell characterization and evaluated experimental data; analyzed data; and wrote the manuscript.

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