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REVIEW ARTICLE

Human parenchymal and non-parenchymal liver cell isolation, culture and characterization

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Abstract Many reports describing parenchymal liver cell isolation have been published so far. However, recent evidence has clearly demonstrated that non-parenchymal liver cells play an important role in many pathophysiologies of the liver, such as drug-induced liver diseases, inflammation, and the development of liver fibrosis and cirrhosis. In this study, we present an overview of the current methods for isolating and characterizing parenchymal and non-parenchymal liver cells.

Keywords Human hepatocytes · Liver cell isolation · Parenchymal liver cells · Non-parenchymal liver cells · Liver cell culture

List of abbreviations

DMEM	Dulbecco's modified Eagle's medium
EDTA	Ethylenediameinetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
GFAP	Glial fibriallary acidic protein
HEPES	(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HSCs	Hepatic stellate cells
KCs	Kupffer cells
LDL	Low density lipoprotein
LSECs	Liver sinusoidal endothelial cells
NPCs	Non-parenchymal cells
PAS	Periodic acid-Schiff
PBS	Phosphate-buffered saline
SECs	Sinusoidal endothelial cells

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Introduction

In recent years, the use of isolated human liver cells in research and development has garnered increasing interest. Their application possibilities encompass identifications of new biochemical pathways in liver diseases, drug development, safety issues, and new therapeutic strategies for their direct clinical translation, such as for liver support. The isolation of human liver cells requires a well-organized network of surgeons, biologists and technicians to obtain cells in high quantity and quality.

The liver cell populations are divided into two groups: parenchymal and non-parenchymal cell (NPCs) types. Parenchymal cells encompass hepatocytes, which represent 60–70 % of total liver cells, or 90 % of the total liver mass. The smaller NPC fraction comprises numerous different cell types. Prevalent populations of NPCs are cholangiocytes (2–3 %), liver sinusoidal epithelial cells (2.5 %), Kupffer cells (2 %) and hepatic stellate cells (1.4 %) [1].

To study liver cell functions, a sufficient number of liver-specific cells with a high degree of purity and viability is necessary. Therefore, liver cell culture development, tissue engineering and isolation techniques need to be optimized. The heterogeneity of the liver cell populations and their overlapping physical properties, such as size, volume and density, require the usage of different cell isolation techniques to gain highly enriched cell suspensions for each specific cell type. Most published data about the physical properties of liver cells are based on experiments with rat liver tissue [1, 2]. The central liver cell characteristics harnessed for cell separation are size, density and the expression of surface proteins. Many different methods for centrifugation, selective attachment and immunoaffinity techniques are available for cell separation. These techniques will be discussed later in reference to the different liver cell types.

We present an overview of primary liver cell isolation from human liver tissue and introduce the possible pitfalls and limitations of hepatocytes and most of the NPCs isolated from liver resections. The present protocol for the isolation of NPCs is the result of comprehensive literature research as well as ongoing optimization and adaptation to human liver resections of the methods originally developed for isolations from rat livers.

Isolation of human primary hepatocytes

The isolation of primary human hepatocytes was first performed over 40 years ago [3–6]. The application of the two-step isolation procedure with collagenase by Seglen et al. [7] was a significant step toward the primary liver cell isolation that has been commonly used over the past 40 years [8–11].

The resected liver must be transferred rapidly to the laboratory to avoid long-term ischemia. Once a liver resection has been recommended, the laboratory team prepares the isolation procedure as described [9].

A sterile tubing setup is placed into a peristaltic pump. The tubing is connected to buttoned cannulae placed in several vessels of the resection site and fixed with tissue glue (BBraun, Melsungen, Germany). Depending on the size of the tissue, the liver resections can be perfused with up to eight cannulae (Fig. 1). The initial perfusion step is necessary to wash out the remaining blood cells from the liver tissue, warm up the tissue and deplete Ca^{2+} ions through EGTA. This depletion causes a breakdown of desmosomes and consequently a loosening of cell-cell contacts. The initial perfusion should be performed for at least 20 min or until the whole blood has been washed out.

Collagenase perfusion is carried out for 10–20 min depending on the tissue conditions (size, remaining blood, fibrosis, etc.). Then, ongoing digestion by the remaining collagenase is prevented by a stop solution [100 ml fetal calf serum (FCS) in 500 ml sterile PBS] added to the cell suspension released from the digested tissue.

The yield and quality of hepatocyte isolation depend on the degree of initial liver damage (normal, steatotic, cholestatic or fibrotic tissue), the patient's drug pretreatment and the length of warm ischemia time upon surgery. If the initial viability of the hepatocyte isolate is below 70 %, a Percoll density gradient centrifugation (25 % Percoll in PBS for 20 min at $\sim 1{,}200 \times g$) is recommended to obtain a higher degree of cell purity to improve the function and metabolic capacity of isolated hepatocytes. The cells have to be washed and suspended in specific hepatocyte culture medium, such as Williams Medium E supplemented with 10 % fetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 15 mM HEPES, 1 mM sodium pyruvate, 1 mM human insulin and 1 % non-essential amino acids before being plated onto culture dishes [9].

The quality of isolated hepatocytes strongly depends on the quality of the liver tissue used for isolation. Numerous methods can be used for cell characterization: The choice of the methods for quality testing depends on the intended use of the hepatocytes. If they are to be used for drug metabolism studies, the expression of phase I and II drug metabolizing enzymes plays a key role. Therefore, we recommend stimulating the hepatocytes with known inducers, such as rifampicin, 3-metylcholantrene or phenobarbital, that are

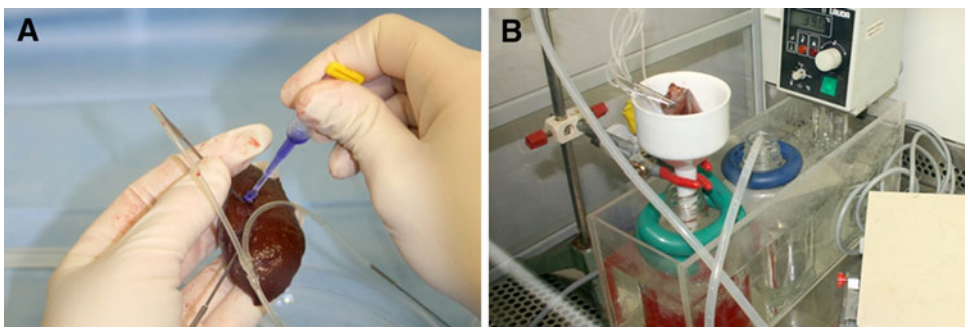

Fig. 1 Cannulation (**A**) and perfusion of human liver tissue (**B**)

accepted by pharmaceutical companies since certain CYP450 isoforms (CYP1A1/2 and CYP3A4) can be regulated in vitro. If hepatocytes are used for cell transplantation studies, an efficient detoxification capacity is necessary. Therefore, urea and glucose metabolism as well as albumin production should be analyzed. The following in vitro assays are used to determine the quality of isolated hepatocytes: measurement of release of transaminases, phase I and II enzyme activities, and glycogen storage by performing periodic acid-Schiff (PAS) staining, Glucose-6-phosphatase (G6Pase) immunostaining and red oil staining visualize cellular lipid droplets [9].

Isolation of human non-parenchymal liver cells (NPCs)

Most investigators use a combination of enzymatic digestion and density gradient centrifugation to isolate NPCs from human liver tissue. However, considerable variation exists between the different protocols. The two following strategies are recommended for the isolation of NPCs: (1) exclusive isolation of NPCs and (2) simultaneous isolation of hepatocytes and NPCs [1, 2]. Isolation of NPCs from liver tissue is performed by enzymatic digestion, by perfusion of liver resections [12] or of an intact organ [13], or by mechanical disruption and enzymatic digestion [14, 15]. For enzymatic digestion exclusively for the isolation of NPCs, collagenase [15–17], pronase [1, 13], dispase [14] or an enzymatic cocktail of these proteases [1, 2, 12–14] is used. Simultaneous isolation of hepatocytes and NPCs within one step of enzymatic digestion is mainly based on isolation of hepatocytes by a two-step EDTA/collagenase tissue perfusion [9–11] followed by centrifugal separation of hepatocytes and NPCs (Fig. 2). This review will focus on the methods for simultaneous isolation of hepatocytes and NPCs. Therefore, the isolation of cholangiocytes (bile duct cells) will not be addressed in detail, because liberation of cholangiocytes from liver tissue needs rougher enzymatic conditions. The isolation of cholangiocytes might be performed after the isolation of hepatocytes (first digestion step) by including an additional digestion step for the resulting tissue or by mechanical disruption of the whole liver tissue (i.e., no perfusion technique exclusively directed to cholangiocytes) followed by an intensive and enlarged digestion step. After densitometric separation and immunomagnetic enrichment, cholangiocytes are seeded on collagen matrix-coated plates for further cultivation [15–17]. In general, the densities of human liver cells are comparable to those determined for rat NPCs (Table 1). Nevertheless, the human liver has a higher variability in tissue quality. Therefore, liver resections are not directly comparable to livers from standardized laboratory animals. For instance, we observed a broader range of variation in human liver cell densities as liver tissue depends on the patient's disease state. Diet and lifestyle can also influence the physical properties of liver cells. Furthermore, inflammation of the liver due to cholangitis and bile afflux may activate Kupffer cells (KCs), which possess altered cell volume and density after having phagocytosed cells, cell debris and particles. Activation of KCs may also influence their viability. It has been reported that KCs isolated from rat and human livers discarded for orthotropic liver transplantation (diseased liver) had a lifetime of 14 days in culture [2, 18]. When we isolated KCs from human liver resections, we observed that their viability correlates with their activation status (underlying liver disease) and therefore depends strongly on the tissue quality and donor conditions.

Moreover, we discovered that inflammation leads to an invasion of systemic immune cells and consequently to a contamination of the NPC fraction. High consumption of vitamin A or its precursors can lead to an accumulation of lipid droplets in hepatic stellate cells (HSCs) and therefore to altered cell densities. It has been reported that isolation of HSCs from livers of older rodents demonstrated that a controlled diet enriched with vitamin A resulted in higher cell yields because of a sufficient amount of lipid droplets and the resulting lower density of HSCs [19]. The opposite can be observed in liver injury, which leads to a decrease in lipid droplets and in the density of HSCs [20]. Therefore, investigators should bear in mind that the physical properties for separation of human NPCs can be donor-dependent and therefore exhibit drastic variations.

Kupffer cells (KCs)

Kupffer cells were first described by Karl Wilhelm von Kupffer in 1876 [21]. These liver-specific macrophages are located in the periportal region that borders the fenestrated sinusoids and has a close contact to stellate cells and hepatocytes [1]. KCs develop from mature monocytes derived from bone marrow stem cells to a phenotype with lamellipodia, membrane ruffling and an oval nucleus. One major function is the phagocytosis of bacteria, bacterial endotoxins, viruses, and endogenous or foreign proteins. KCs produce a variety of chemotactic, inflammatory, growth-mediated and vasoactive molecules, such as TNF-α and IL-6. Therefore, they play an important role in hepatic tissue damage and liver regeneration, and in innate and adaptive immunological actions of the liver [22].

Simplified isolation procedures for rat parenchymal cells and KCs were described by Page and coworkers [23]. Separation of KCs from cell suspensions rich in NPCs is commonly performed with density-based methods, such as

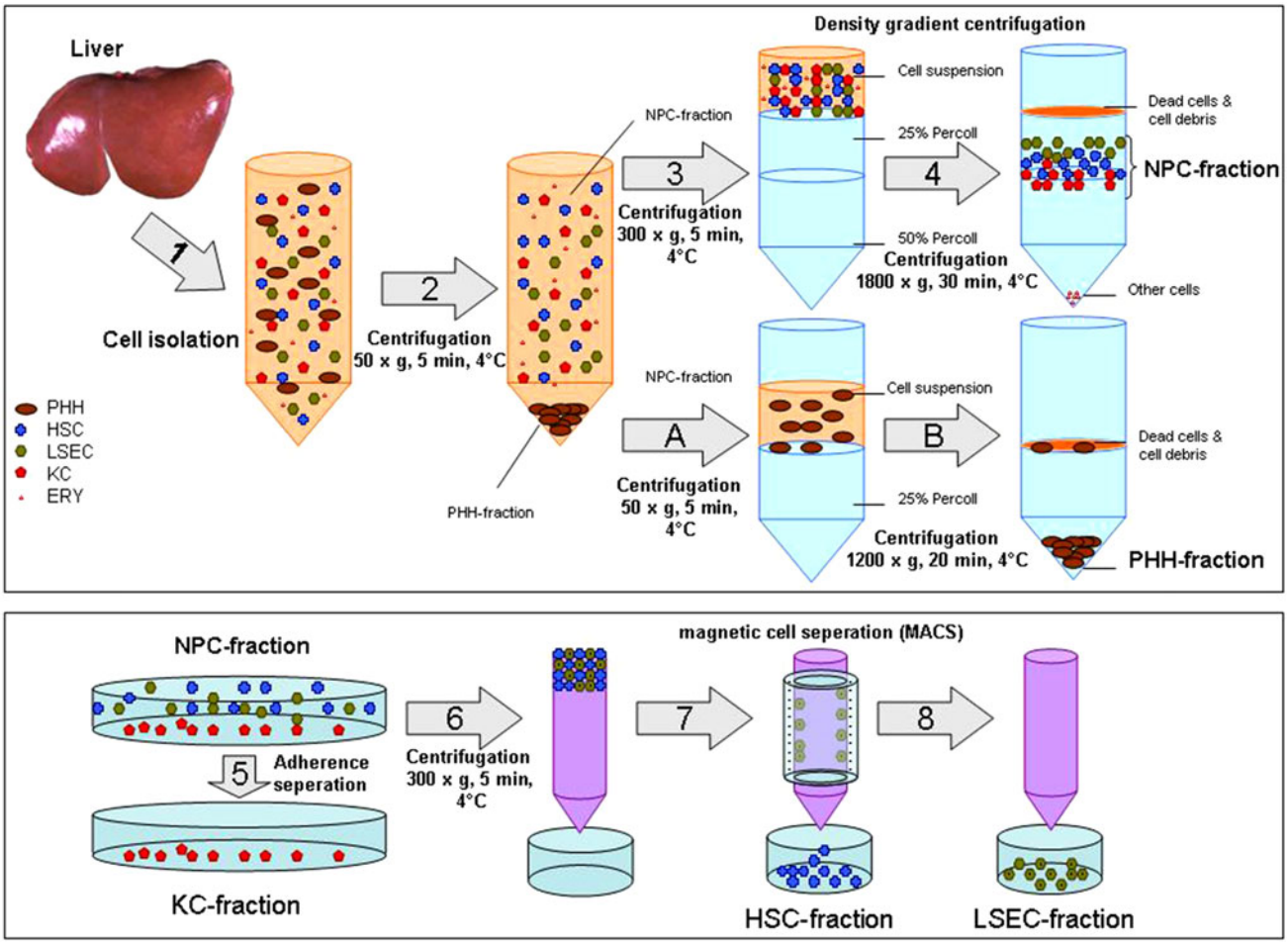

Fig. 2 Schematic overview of PHH and isolation of NPCs from human liver resections. Preparation: Prepare PHH (step 1). Collect supernatants after centrifugation (50×*g*, 5 min, 4 °C; step 2). Perform PHH cleanup in parallel (step **A**–**B**). Centrifuge (300×*g*, 5 min, 4 °C; step 3). Discard the supernatant; pool pellets with HBSS and fill up to 50 ml, then centrifuge again (300×*g*, 5 min, 4 °C);prepare one 25 % and one 50 % Percoll solution for density gradient centrifugation—the number of Percoll Falcon tubes depends on the total cell count. For the density gradient centrifugation, 50 × 10^6 viable cells in a total volume of 4–5 ml should be added at most—stack 20 ml of 50 % and 20 ml of 25 % Percoll solution; carefully overlay the Percoll solution with the cell suspension and centrifuge (1,800×*g*, 30 min, 4 °C; step 4)—cave: turn off the brake!—after centrifugation, two layers are visible; dead cells and cell debris are located on top of the 25 % Percoll solution. The non-parenchymal cell fraction is located at the interphase between the 25 and the 50 % Percoll solution. Erythrocytes and more dense cells are found in the pellet. Collect NPC-rich fractions, fill up each with HBSS to the 50 ml mark and centrifuge the cell suspension (300×*g*, 5 min, 4 °C). Pool the cell pellets with FCS-free media (DMEM), and centrifuge again (300×*g*, 5 min, 4 °C). The yield and viability of the NPC-enriched cell suspension are assessed by using Trypan Blue exclusion as described above. For the separation of KCs: plate cell suspension on required cell culture plastics and incubate (30 min, 5 % CO_2, 37 °C) in a humidified incubator. KCs adhere during the incubation time (step 5). Collect supernatants, wash cells with HBSS and change the media (RPMI supplemented with 10 % fetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2 mM L-glutamine). Separation of HSCs and LSECs: centrifuge non-adherent cell suspension (300×*g*, 5 min, 4 °C; step 6). Perform magnetic cell separation according to the manufacturer's manual, e.g., Miltoni Biotech MACS Beads CD31 for separation of LSECs. Collect eluent cell suspension containing the HSC fraction (step 7). Collect magnetically separated cell suspension that contains the LSEC fraction (step 8). Wash the cells in HBSS, centrifuge at 300×*g*, 5 min, 4 °C, and resuspend in media (DMEM supplemented with 10 % fetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2 mM L-glutamine). Determine the cell number and viability by Trypan Blue exclusion using a Neubauer counting chamber. Seed cells

isopycnic centrifugation through gradient materials, such as Percoll, Nycodenz or Stractan [2, 23–25]. Based on studies with rat and human KCs, most researchers use Percoll gradients between 25 and 50 %, and subsequently the KCs accumulate on the interphase above the 50 % fraction, as demonstrated in Fig. 2 [25]. The resulting Kupffer cell-enriched cell suspension still contains a mixture of other NPCs. The ability of KCs to adhere quickly on plastic is harnessed for further cell purification from other NPCs [1]. However, the detachment of KCs by trypsination

Table 1 Density and size of parenchymal and non-parenchymal human liver cells and their markers [1, 36–39]

Cell type	Density (g/cm^3)	Size (cm)	Part of total liver cells (%)	Marker/antibody
Stellate cells	1.04–1.05	0.0010–0.0012	1.4	GFAP, vimentin, desmin, autofluorescence vitamin A, α-SMA, β-TGF
Endothelial cells	1.05–1.06	0.0008–0.0010	2.5	CD31, vimentin, desmin, SE-1 (CD32b)
Pit cells (NTKs)	1.05–1.06	0.0008–0.0010	Rare	CD8, CD56
Kupffer cells	1.06–1.08	0.0010–0.0012	2	CD68, CD163,
Cholangiocytes	1.08–1.10	0.0012–0.0015	2–3	CK19, HEA125
Hepatocytes	1.12–1.14	0.0020–0.0025	60–70	CK18

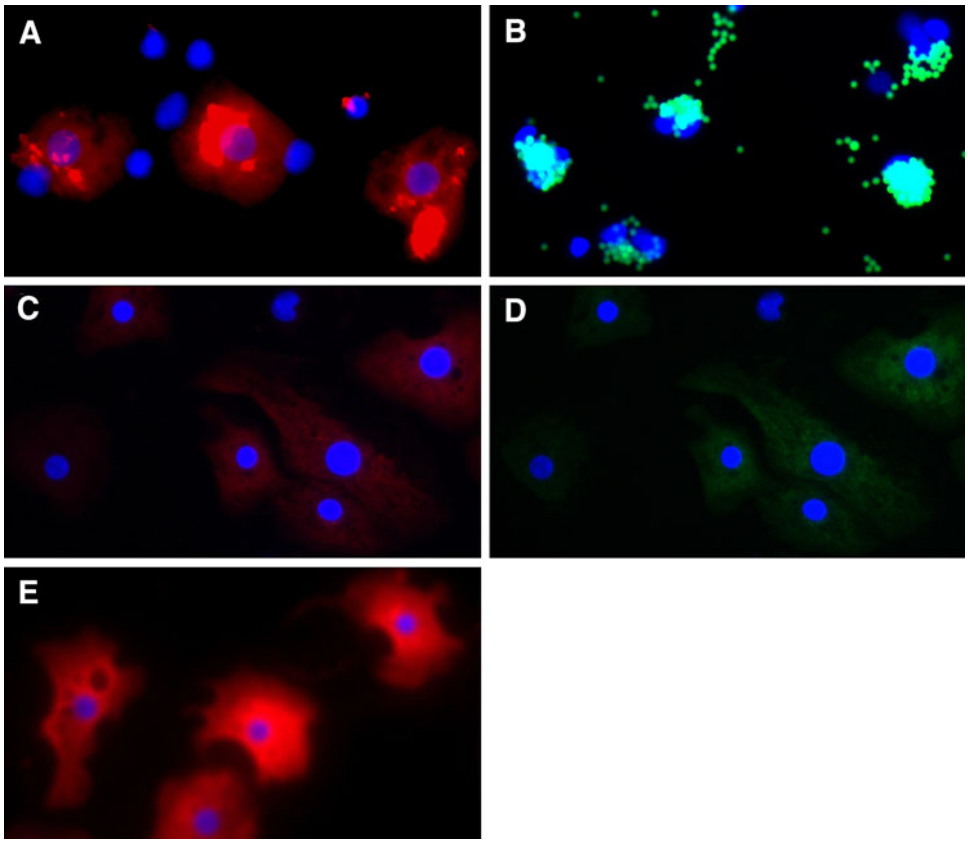

Fig. 3 Fluorescence images of primary human non-parenchymal liver cells. Kupffer cells in culture were characterized by immunostaining with antibodies to CD68 (**A**) and after phagocytosis of fluorescent latex beads (**B**). Human hepatic stellate cells in culture were immunostained with antibodies to GFAP (**C**) and vimentin (**D**). Liver sinusoidal endothelial cells in culture were immunostained with antibodies to CD31 (**E**). Cell nuclei were identified by using Hoechst staining. The original magnification was ×400

leads to a dramatic cell loss due to the high affinity of these cells to plastic after adhesion. The purity of KCs is determined by FACS or immunofluorescent staining beforehand.

Freshly isolated KCs are cultured under standard culture conditions, such as RPMI-Media containing 10 % FCS, L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin directly on plastic or glass surfaces. KCs can be identified by their morphological characteristics (Table 1, Fig. 3), surface markers and their affinity to phagocytose-labeled materials, such as latex beads (Table 1, Fig. 3). The initial activation level is measured by the endogenous peroxidase activity [24].

Hepatic stellate cells (HSCs)

Hepatic stellate cells are located in the space of Disse between parenchymal cells and sinusoidal endothelial cells with direct contact to the neighboring cell types. They have a spindle-shaped body with oval nuclei [20]. HSCs are the major cell type for vitamin A (retinol) storage in humans and therefore a key player in retinol metabolism [26]. Under physiological conditions, HSCs stay in a quiescent phase. They are activated by tissue damage, such as surgical interventions and drug- or alcohol-mediated apoptosis or necrosis. They evolve to myofibroblast-like cells with a high proliferation rate and lose their vitamin A storage

ability. During this phase, they start to synthesize extracellular matrix components, such as collagen and glycoproteins [27]. Therefore, HSCs play an important role in tissue regeneration. Due to their chronic activation under pathophysiological conditions, they are involved in the formation of liver fibrosis [27].

The isolation of rat liver NPCs, including HSCs, was first described by Knook et al. [28]. First, the fractionation of the cell suspension rich in NPCs is necessary to obtain a high amount of isolated HSCs (Fig. 2). Density gradient centrifugation with commercially available reagents, such as Percoll or Nycodenz, constitutes an effective and frequently used method [1, 2]. The densities of HSCs are slightly different from those of other liver cells depending on the number of cellular lipid droplets (see above, NPCs). Beside the conventional isolation of HSCs, FACS protocols for high-purity isolation of HSCs using autofluorescence based on vitamin A storage have been developed recently [19].

Hepatic stellate cells can be characterized by their morphology or by their expression of specific markers, i.e., immunostaining for intermediate filaments, such as the glial fibriallary acidic protein (GFAP), desmin or vimentin. Furthermore, the cytoskeletal protein alpha-smooth muscle actin (α-SMA) is a marker for activated HSCs [27]. Additionally, HSCs can be identified by blue-green autofluorescence of their cytoplasmatic vitamin A droplets [20].

Hepatic stellate cells can be maintained in vitro on a variety of extracellular matrices, which might lead to the up- or downregulation of their activation [20]. Culturing freshly isolated cells on glass or non-coated tissue culture plastic causes progressive cellular activation. HSCs cultured on a basement membrane-like matrix (Matrigel) remain in a quiescent state. Activated HSCs placed on Matrigel become inactive and lose their myofibroblastic characteristics [20, 29]. They can be cultured in standard culture conditions, such as Dulbecco's Modified Eagle's Medium (DMEM) containing 10 % FCS, L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin [26] for several weeks by splitting and subcultivation.

Liver sinusoidal endothelial cells (LSECs)

Sinusoidal endothelial cells forming blood vessel walls within an organ are found in the spleen, bone marrow and liver. They possess a minimal basement membrane and lack classical tight junctions. In the liver, the gap between SECs and hepatocytes forms the space of Disse. LSECs differ from SECs in other organs by their discontinuous nature, as they are interspersed with KCs, and by their specialized morphology, called fenestrae, within the cytoplasm [30]. Fenestration is characterized by small holes within the cells that act as dynamic filters and provide controlled access to the parenchymal cells and HSCs in the space of Disse to macromolecules, chylomicrons and chylomicron remnants in the blood [31]. The number of fenestrations per endothelial cell (animal studies revealed up to 40 % of the cell) depends on the occurrence of diseases and decreases after a viral infection, with age or during cirrhosis. SECs have a high endocytotic capacity [31] as they possess several scavenger receptors on their surface. Small particles and macromolecules, such as extracellular material like glycosaminoglycans, are taken up by SECs, whereas large particles and cellular debris are phagocytosed by KCs. Furthermore, SECs express some markers that are common to all endothelial cells and may therefore be used to isolate and identify a cell of endothelial lineage.

Protocols for the isolation of SECs start with a fractionation step of the NPCs, as described above. NPCs are discriminated via differential centrifugation techniques, including counterflow elutriation, and continuous or discontinuous density gradients with metrizamide [12] or Percoll [14]. The underlying principles of these protocols are the different volumes of parenchymal cells and the lower cell density of HSCs compared to other NPCs. Nevertheless, resulting NPC fractions, consisting mainly of SECs and KCs, may be further fractionated by choosing specific culture conditions with SEC growth media and collagen- or fibronectin-coated culture dishes [12, 14, 32]. A more effective procedure to obtain pure SECs of high quality is the immunomagnetic selection of endothelial cells from the NPC fraction using an antibody against CD31 after density gradient centrifugation [12]. In rats, several other markers have been described for the positive selection of LSECs by FACS and MACS such as SE-1 (CD32b) and Stab 2 [33, 34]. SECs are cultivated on matrix component-coated dishes and can be propagated by trypsin splitting and subcultivation. Conflicting results have reported loss of SEC-specific features, such as scavenger function and fenestration, after 2 days in culture, or toxic effects of serum on proliferating cells [30]. In vitro, SECs can be characterized by the expression of endothelial-specific markers, such as CD31, SE-1, von Willebrand factor, E-selectin and endoglin (CD105). Furthermore, SECs may be defined by the ability to bind Ulex lectin and the uptake of acetylated LDL [30, 32].

Numerous methods are available for the characterization of NPCs. The choice of the methods for quality testing depends on the intended use of the single population of NPCs. We recommend judging the quality and purity by immunofluorescent staining (Fig. 3).

Cell isolation for clinical applications

To use hepatocytes for clinical applications, particular attention shall be paid to GMP requirements. All potential

donors must be tested for HIV, various forms of viral hepatitis, underlying malignant diseases, and bacterial or parasitic infections before the organ harvest.

Although hepatocyte transplantation is an established method in the clinic, it is facing the major challenge of restricted availability of organs with sufficiently high cell quality. Hepatocyte transplantation competes for organs with orthotopic liver transplantation. The current sources of liver cells for cell transplantation are mainly from donor organs refused for organ transplantation because of their long ischemic time or because the donors had a cardiac arrest, were very old, had advanced steatosis, or poor liver function or fibrosis. Since hepatocytes from non-marginal donors show a much better metabolic profile, it is necessary to improve the isolation techniques of marginal organs and possibly consider alternative technologies, such as the use of stem cells, stem cell-derived liver-like cells or fetal livers, as recently discussed [35].

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Conflicts of Interest Georg Damm, Elisa Pfeiffer, Britta Burkhardt, Jan Vermehren, Andreas K. Nüssler and Thomas S. Weiss declare that they have no conflicts of interest.

Compliance with Ethical Requirements In the studies with human subjects carried out by the authors that are mentioned in this review, all procedures met the ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008. Informed consent was obtained from all patients for inclusion in the study. Concerning the studies with animals carried out by the authors that are mentioned in this review, all institutional and national guidelines for the care and use of laboratory animals were followed.

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