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Hepatic lineages isolated from developing rat liver show different ways of maturation☆

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Abstract

Immunocytochemical analysis revealed that different hepatic cell types exist during liver development: (i) cells co-expressing the stem-cell marker Thy1 and the hepatic lineage marker CK-18 and (ii) cells only expressing CK-18 (hepatoblasts). In this study we separated the different hepatic cells and analyzed gene-expression and phenotype. Fetal rat livers were digested by collagenase solution. OX43- and OX44-positive hematopoietic cells were depleted and Thy1-positive cells were enriched using Magnetic cell sorting. The different cell compartments were analyzed by RT-PCR and immunocytochemistry for Thy1, CK-18, AFP, and albumin. Hepatoblasts expressed albumin at all times and AFP in the early stages. Thy1-enriched cells expressed CK-18 at all times, albumin in the early, and AFP in the late stages. Thy1-positive cells from fetal livers express liver specific genes. The data suggest that Thy1-positive hepatic cells develop towards hepatic stem cells, and hepatoblasts develop towards mature hepatocytes of the adult liver.

Keywords: Hepatic stem cells; Hepatoblasts; Hepatic gene expression; Fetal liver development; Cell sorting

In the adult liver a distinct hierarchy of hepatic cell compartments exists. They possess different proliferative and differentiation capacities and all can contribute to mass reconstitution after liver damage [1]. One of these compartments is presented by tissue residing hepatic stem cells (Oval Cells), as demonstrated in several convincing animal studies. They have a bipotential differentiation capacity towards hepatocytes or bile duct epithelial cells [2]; they are highly proliferative under certain conditions, and are clonogenic [3,4]. Oval cells are only involved in liver regeneration under certain conditions, e.g., after combined liver damages or after activation by specific liver injury models [5,6]. In recent studies the origin of Oval cells from extrahepatic stem cells in the bone marrow was shown [7]. Another compartment of hepatic cells consist of "small hepatocytes," which were found to be activated after defined liver damages: e.g., after treatment of animals with the pyrrolizidine alkaloid retrorsine and partial hepatectomy [8,9]. These cells were shown to form colonies in vitro, and to express both hepatic and biliary markers [10,11]. Parenchymal hepatocytes form a third compartment; they also possess an enormous proliferative potential and contribute to mass reconstitution during most forms of hepatic regeneration (unicommitted stem-cell concept) [12,13].

Recently, several types of hepatic progenitors were identified during fetal liver development. In fetal mouse livers hepatic progenitors were isolated by using fluorescence activated cell sorting (FACS) that exhibited typical features of hepatic stem cells: they showed clonogenic potential in vitro, and had a bipotential differentiation capacity towards liver or biliary cells [14,15]. The phenotypic marker expression of these cells (c-kit-, CD49f+, CD29+, CD45-, and TER119-) rather resembled that of more immature stem cells than that of the "hepatopoietic" fraction of the fetal liver [16]. At least,

☆ *Abbreviations:* CK, cytokeratin; ED, embryonic and fetal day of gestation; MACS, magnetic cell sorting.

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they do not share common markers of well-characterized cellular compartments of the adult liver. Another cell type identified in fetal rat livers was the "fetal liver epithelial progenitor cell" (FLEC) [17]. These cells showed bipotential differentiation and a highly proliferative activity after transplantation into adult rat livers. The marker expression of these cells showed typical features of immature hepatic cells (CK-19 and AFP), however expression of stem-cell markers remained unknown. Thus, little is known about the existence and relationship of hepatic lineages during fetal liver development.

In the past the marker expression of different hepatic cell compartments has been studied thoroughly in the adult liver. Different hepatic cell compartments can be distinguished by their characteristic phenotypical marker expression [5,18]. Hepatic stem cells of the adult liver show a typical phenotypic marker pattern by coexpressing well-known hematopoietic stem-cell markers (e.g., CD34, Thy1, and c-kit) and hepatocytic lineage markers (e.g., CK-18, albumin, and AFP) [18–21].

In a recent study, we identified different hepatic-cell compartments that were already present during fetal liver development by immunocytochemical analysis: coexisting in fetal liver cell isolates we found (i) hepatic cells expressing CK-18 and (ii) hepatic cells simultanously expressing CK-18 and Thy1 [22]. This suggests that a cellular hierarchy already exists in the fetal liver.

To further characterize the role of these cell types during fetal organogenesis, we developed a method to separate the different hepatic cell compartments and analyzed gene expression and phenotypic marker pattern in these compartments.

Methods

Animals. Fetuses of pregnant female Sprague–Dawley (Charles-River, Sulzfeld, Germany) rats (250–400 g) were used as organ donors. The animals were housed at the veterinary care facility of the University of Hamburg Medical Center, submitted to a 12 h day/night cycle and had access to water and standard rat chow ad libitum. All experiments were approved by the animal care committee of the University of Hamburg Medical Center. German regulations for the care and use of laboratory animals were followed at all times.

Fetal hepatocyte isolation. Rat embryos were harvested by section (ED 16, ED 18, and ED 20) after the mother was sacrificed by an overdose of aether-anesthesia, or after spontaneous delivery (ED 22). Fetuses were dissected under the dissection microscope. The abdominal cavity was opened and the fetal liver under the septum transversum was removed. Fetal liver tissue was obtained by incision of the liver capsula. The liver was shaken in ice-cold hanks-buffered saline solution (HBSS) for 1 min. The tissue was then incubated in HBSS containing 0.6% collagenase type IV (Sigma, St. Louis, USA), 20 mM Hepes buffer, 1 mM $CaCl_2 \cdot 2H_2O$, 0.06% DNase type I, pH 7.3, 37 °C, for 5 min. The collagenase step was repeated three times, and digested cells were collected in a falcon tube from the supernatant, and placed on ice. The collagenase digestion was stopped by 10 ml ice-cold HBSS. Cell suspension was filtered through a 40 μm cellfilter. Cells were washed twice in HBSS and a percoll ($\rho = 1.013$ g/ml, Biochrom, Berlin, Germany) gradient centrifugation (450*g* at 20 °C, 17 min) was used to enrich the viable cell fraction. The Percoll concentrations used were between 52% (ED 22) and 76% (ED 16), respectively. Cell suspension was then prepared for MACS.

OX 43/OX 44 Magnetic cell sorting—depletion of hematopoietic fetal cells. After Percoll gradient centrifugation primary isloated cells were diluted in 1000 μl HBSS supplemented with Hepes 20 mM and EGTA 5 mM, and were marked with 15 μl mAb OX-43 (Serotec, Eching, Germany), and 2.6 μl mAb OX-44 (Serotec) at 4 °C for 30 min. Unbound antibodies were removed by centrifugation with 450*g* at 14 °C for 5 min. Secondary marking was done by incubation with goat-anti-mouse IgG bound magnetic microbeads (Miltenyi Biotec, Bergisch-Gladbach, Germany) at 10 °C for 15 min. Positive cells were absorbed in a magnetic field by Vario-MACS columns, the depleted cell fraction was collected in a tube [22]. The sorted cell fraction was washed (450*g* at 14 °C for 15 min) twice.

Thy1 Magnetic cell sorting separation of fetal hepatocytic cells. Thy1 positive or negative cells were separated by a second MACS-step. Depleted cells were diluted in 1000 μl HBSS supplemented with Hepes 20 mM and EGTA 5 mM and were marked with mAb OX-7 (mouse anti-rat Thy1, Pharmingen, Hamburg, Germany) at 4 °C for 30 min. Unbound antibodies were removed by centrifugation with 450*g* at 14 °C for 5 min. Secondary marking was done by incubation with goat-anti-mouse IgG bound magnetic microbeads (Miltenyi Biotec, Bergisch-Gladbach, Germany) at 10 °C for 15 min. Positive cells were absorbed in a magnetic field by Mini-MACS columns. The column with bound Thy1-positive cells was removed from the magnetic field and cells were washed out with MACS-buffer. The sorted cells were collected in a tube and were washed (450*g* at 14 °C for 15 min) twice.

Cell yield, viability, and statistical analysis. Isolated cells were counted using a hemocytometer. Viability was assessed as % using the trypan-blue test (2% trypan blue). Cell fractions were calculated as % from the total numbers of the different compartments. Mean values (MV) and standard deviations (STD) of cell ratios and viability [%] were calculated. Statistical analysis was performed on a MS-Windows system (Windows 98) using "Excel 2000" software (Microsoft, Redmont, WA, USA). Groups were analyzed for statistical significance by employing Student's *t* test. *p*-Values were two tailed and $p < 0.05$ was considered significant.

RNA extraction from isolated cells. RNA was isolated from the cells after rinsing the cells with PBS. Cells (10^5–10^6) were homogenized in 200 μl RNAzol (Wak Chemie Germany; containing guanidinthiocyanate, and mercaptoethanol) and were kept on ice. For extraction, 200 μl chloroform was added and the mixture was incubated for 5 min at −20 °C. After centrifugation at 6000 rpm for 15 min the supernatant was mixed with 500 μl isopropanolol-2 and 2.5 μl glycogene, and the mixture was incubated at −20 °C for 30 min. After centrifugation at 6000 rpm for 30 min the supernatant was removed and 750 μl ethanol was added for precipitation. This step was repeated twice, before the precipitates were vacuum-dried, and were solved in 50–70 μl DEPC-water. The OD_{260}/OD_{280}-ratio was measured using photometry (DyNA Quant 200, Hoefer, Germany) to determine the RNA-content. One microgram of RNA was dissolved in a total volume of 8 μl DEPC-water and was stored for RT at −80 °C.

Reverse transcription and polymerase chain reaction. Reverse transcription (RT) of extracted RNA was performed using the First-strand c-DNA synthesis kit (Amersham Bioscience Europe, Freiburg, Germany) according to the manufacturer's instruction. Briefly, RNA was denaturated for 10 min at 65 °C. Then, bulk mix (containing reverse transcriptase, 10× PCR-buffer, and $MgCl_2$), dTT, and PdN_6−primer were added in a total volume of 15 μl. The RT-reaction was allowed to proceed at 37 °C for 60 min. The cDNA was stored at −20 °C. Polymerase chain reaction (PCR) with cDNA was performed using primers for Thy1 [23], or CK-18, AFP, albumin [24], or GAPDH (Table 1).

Table 1
Primers used for RT-PCR analysis

Primer name	
GAPDH	S: 5′-CCT TCA TTG ACC TCA ACT AC-3′ A: 5′-GGA AGG CCA TGC CAG TGA GC-3′
Thy1	S: 5′-CGC TTT ATC AAG GTC CTT ACT C-3′ A: 5′-GCG TTT TGA GAT ATT TGA AGG T-3′
CK-18	S: 5′-GGA CCT CAG CAA GAT CAT GGC-3′ A: 5′-CCA CGA TCT TAC GGG TAG TTG-3′
AFP I.	S I: 5′-AAC AGC AGA GTG CTG CAA AC-3′ A I: 5′-AGG TTT CGT CCC TCA GAA AG-3′
AFP II. (nested)	S II: 5′-CAC CAT CGA GCT CGC CTA TT-3′ A II: 5′-TGA TGC AGA GCC TCC TGT TG-3′
Albumin	S: 5′-ATA CAC CCA GAA AGC ACC TC-3′ A: 5′-CAC GAA TTG TGC GAA TGT CAC-3′

For AFP a nested PCR was used. For the PCR 7 μl cDNA-template was mixed with 5 μl 10× PCR-buffer, 1 μl 10 mM dNTP's, 1.5 μl 50 mM $MgCl_2$, 1 μl primers (50 ng/μl), and 1 μl polymerase (Ampli-Taq., Gibco) for each probe. PCR was carried out in a programmable Biometra Uno-Thermobloc (Biometra, Göttingen, Germany) with the following conditions: 94 °C for 10 min and then 25 (albumin), or 29 (Thy1), or 30 (GAPDH, CK-18, and AFP II), or 35 (AFP I) cycles each comprising denaturation for 1 min at 94 °C, annealing for 1 min at 52 °C for Thy1, 60 °C for albumin, CK-18, AFP II, and GAPDH, or 55 °C for AFP I, and extension for 1 min at 72 °C. After the PCR was completed reaction tubes were kept for 4 min at 72 °C and then 4 °C. Negative controls routinely used for each set of primers included control without template. Samples were analyzed on 1% agarose gels. The size of the PCR-fragments was estimated using a 100-base-pair ladder (Gibco-BRL).

Cytospins, fixation, and immunocytochemistry. Cytospins were prepared by centrifugation (50*g*, 10 min) of the cell suspension on glass slides. Fixation was done with methanol at −20 °C for 5 min and acetone at 4 °C for 15 s. Slides were either stained directly or stored frozen at −20 °C till staining. Immunocytochemical analysis was performed using mouse monoclonal antibodies (mAb) specific for Thy1 (Pharmingen, Hamburg, Germany), CK-18 (ICN Cappel, Aurora, USA), rabbit mAb for AFP (Dako, Hamburg, Germany), or polyclonal FITC-labeled anti-rat albumin (DPC, Wiesbaden, Germany). Slides were rinsed with TRIS-buffer (tris-buffered saline 0.05 M, pH 7.5) between the incubation steps.

Thy1 and CK-18 staining was performed using the alkaline phosphatase–antialkaline phosphatase (APAAP) technique. Incubation period with primary antibodies was 30 min, antibodies were differently diluted (1:50 or 1:10, respectively). Secondary marking was done with rabbit–anti-mouse IgG mAb diluted 1:50 for 30 min. Slides were then incubated with mouse–APAAP-complex for 30 min. The alkaline phosphate substrate, New Fuchsin, was prepared as described elsewhere [22] and the enzymatic reaction was allowed to proceed for 30 min. Specimens for AFP-staining were incubated after blocking endogenous peroxidase (3% H_2O_2) with primary antibody (1:500 diluted) for 30 min, for secondary marking HRP-conjugated swine-anti-rabbit IgG antibody (1:50) was added. For albumin staining, slides were incubated after blocking endogenous peroxidase (3% H_2O_2) with primary antibody (1:500) for 30 min, and then marked with HRP-conjugated rabbit-anti-FITC (Dako) antibody (1:100) for 30 min. Staining reaction was done with Diamino-benzidine (DAB-plus, DAKO) for 20 min. After rinsing in distilled water, slides were counterstained with hematoxylin.

Results

Cell yield and viability after primary isolation, MACS-depletion, and combined MACS

Cell yield of collagenase-DNase digestion increased significantly ($p < 0.05$) from 34.9×10^6 cells per liver at ED 16 to 205.3×10^6 cells per liver at ED 22 (Fig. 1). After Percoll-purification cell yield was increasing depending on the gestational age from 13.7×10^6 cells per liver at ED 16 to 24.0×10^6 per liver at ED 22. Viability of cells after Percoll ranged from 94.6% to 97.3% viable cells. After MACS depletion of OX43/OX44-positive cells the yield was between 4.4×10^6 cells per liver at ED 16 and 13.1×10^6 cells per liver at ED 22 (Fig. 1) with a viability between 78.4% and 90.6%. By combination of OX43/OX44-depletion and Thy1-positive sorting using MACS 0.27×10^6 cells per liver at ED 16 to 0.55×10^6 cells per liver at ED 22 were obtained. Cell viability after combined MACS ranged from 85.5% (ED 22) to 96.5% (ED 20).

Quantitative analysis of cell-relations of the fetal liver cell compartments

The percentage of OX43/OX44 negative cells from primary cell isolates decreased significantly ($p < 0.001$) from $12.8 \pm 1.9\%$ at ED 16 to $6.5 \pm 0.94\%$ at ED 22 (Fig. 2A), whereas the percentage of OX43/OX44 negative from percoll-purified cells was increasing significantly ($p < 0.01$) from $32.7 \pm 5.3\%$ at ED 16 towards $50.8 \pm 6.1\%$ at ED 22 (Fig. 2B). The percentage of Thy1 positive sorted cells from OX43/OX44 negative cells decreased significantly ($p < 0.001$) from $7.7 \pm 0.16\%$ at ED 16 to $4.1 \pm 1.24\%$ at ED 22 (Fig. 2C).

RT-PCR analysis for Thy1

RT-PCR analysis for gene expression of hematopoietic stem-cell marker Thy1 showed a signal of fetal

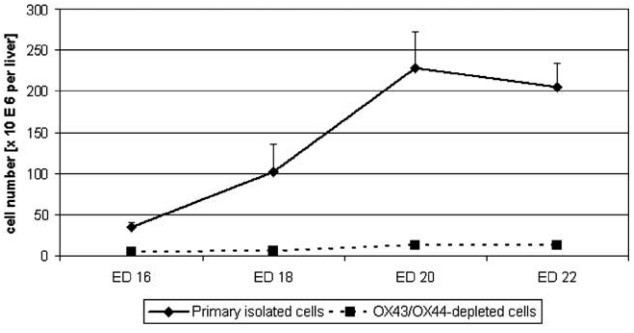

Fig. 1. Total cell numbers (10× cells per liver) of primary fetal liver cell isolates and isolates after OX43/OX44-MACS depletion of hematopoietic cells from ED 16 to ED 22. Increase of cell yield per liver of primary isolated cells was statistically significant ($p < 0.05$).

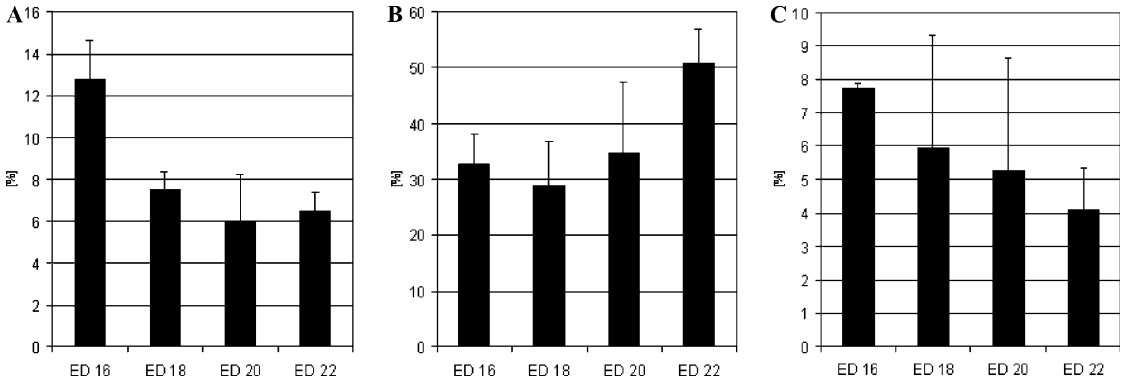

Fig. 2. (A–C) Percent cell fraction [%; means ± SD] of MACS-depleted cells from primary fetal liver cell isolate (A), of MACS-depleted cells from Percoll-purified cells (B), or Thy1-positive sorted cells from MACS-depleted cells (C) at the gestational ages from ED 16 to ED 22 ($N = 8$ each).

livercell isolates at all times with an increasing intensity towards the end of the fetal period (Fig. 3A). Thy1 signal intensity was similar after depletion of OX43/OX44-positive cells. An enrichment of Thy1-gene expression was seen in the fraction of OX43/OX44 depleted and then Thy1-positive MACS sorted cells.

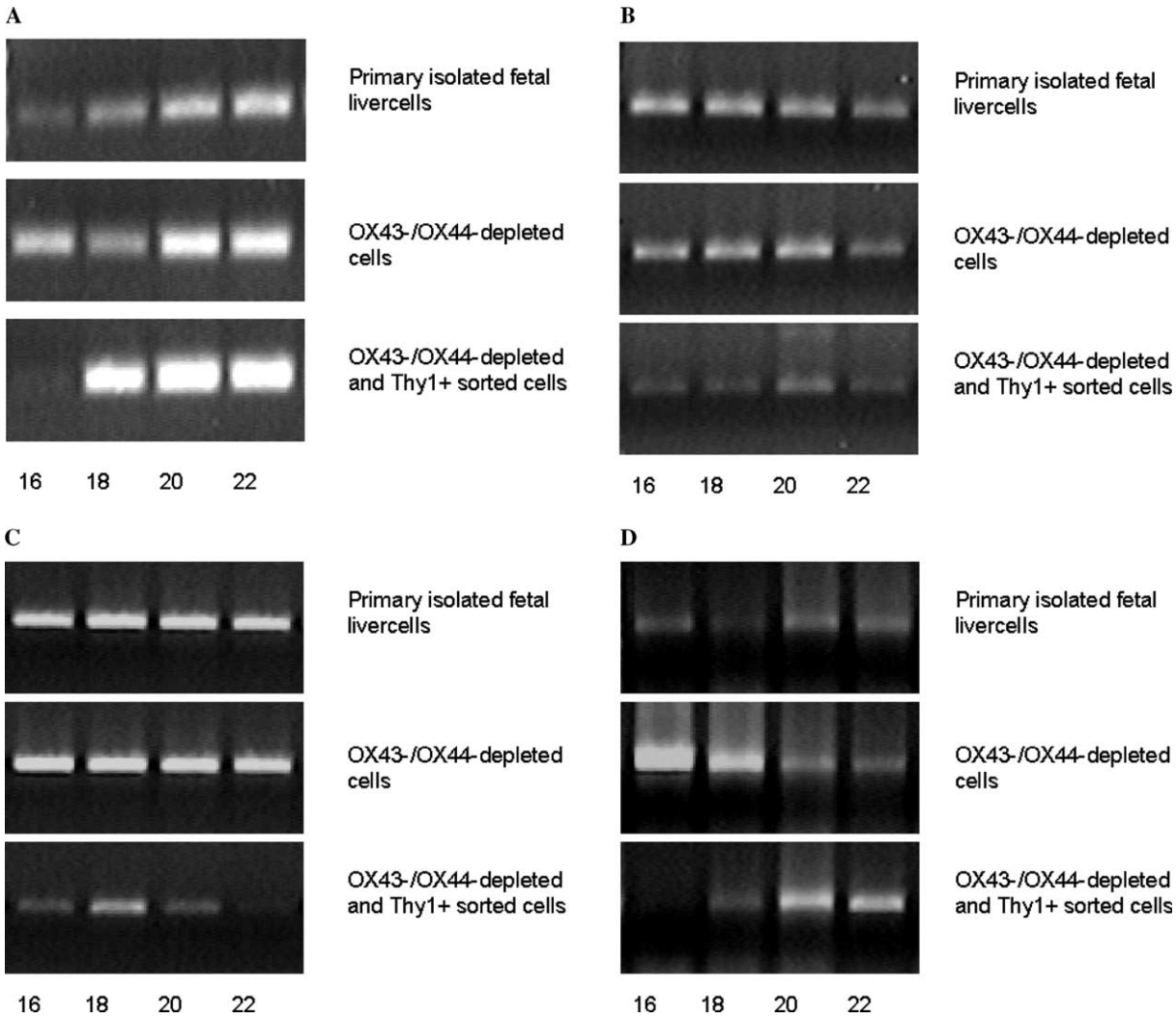

Fig. 3. (A–D) Agarose gel electrophoresis of RT-PCR analysis for Thy1- (A), CK-18- (B), albumin- (C), and AFP- (D) gene expression from primary isolated fetal liver cells, fetal liver cells after MACS-depletion of OX43-/OX44-positive hematopoietic cells, and after separation of Thy1-positive cells by combined MACS at the gestational ages from ED 16 to ED 22.

RT-PCR analysis for hepatic gene expression (CK-18, AFP, and albumin)

RT-PCR analysis for CK-18 showed a constant signal of primary isolated cells over the whole observation period (Fig. 3B). After depletion of OX43/OX44-positive cells a similar signal intensity was found for CK-18 expression. Thy1-positive sorted cells showed initially (ED 16 and ED 18) a weak signal. At later stages (ED 20) a stronger signal was observed. RT-PCR analysis for albumin showed a signal in primary isolated fetal livercells and in OX43/OX44 depleted cells at all gestational ages (Fig. 3C). After Thy1-sorting an obvious signal for albumin gene expression was found at ED 16 and ED 18, and a weak signal was found at ED 20/ED 22. RT-PCR analysis for AFP showed a weak signal in primary isolated cells at all times (Fig. 3D) and an enhanced signal in OX43/OX44 depleted cells in the early gestational ages (ED 16 and ED 18). After Thy1-MACS-enrichment an enhanced signal for AFP gene expression was found in the late gestational age (ED 20 and ED 22).

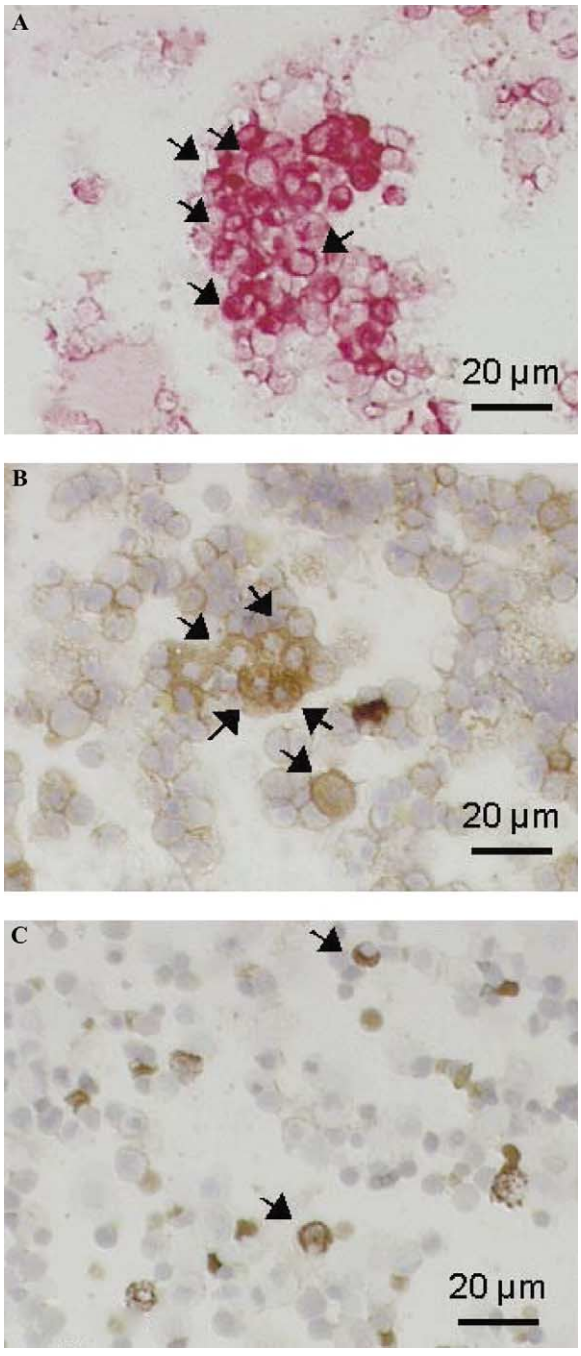

Fig. 4. (A–C) Immunocytochemical staining of double sorted fetal liver cell isolates (OX43/OX44 depleted and Thy1-positive sorted fraction) for CK-18 (A, ED 22), albumin (B, ED 18), and AFP (C, ED 18) showed phenotypical marker expression of hepatic cells at all observed time points (magnification 20×4, *arrows indicate positive cells*).

Immunocytochemical analysis for CK-18, albumin, and AFP

Cell isolates after OX43/OX44 depletion and combined Thy1 positive sorting showed positive staining reaction for hepatic phenotypical markers CK-18 (50–60%), albumin (40–50%), and AFP (5–10%) (Figs. 4A–C). Cell isolates after OX43/OX44 depletion also showed CK-18-positive (50–60%), albumin-positive (40–50%), and AFP-positive (10–20%) cells (data not shown).

Discussion

Enrichment of hematopoiesis depleted and Thy1 positive cells of the fetal liver

In a previous study, we found different hepatic cell types during fetal liver development. One compartment of cells coexpressed CK-18 and hematopoietic stem-cell marker Thy1. In addition we found another cell compartment of CK-18 positive and Thy1 negative cells [22]. In the present study, we isolated fetal liver cells in three steps (Fig. 5): (i) collagenase/DNase digestion and

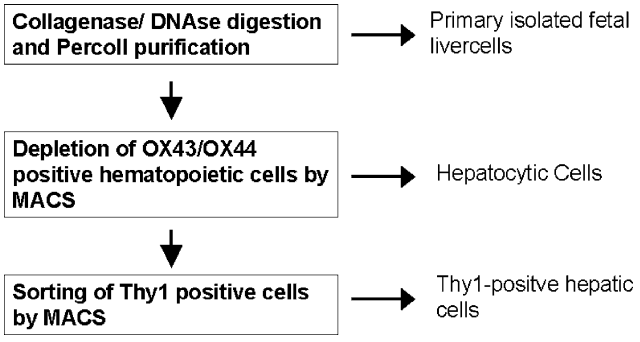

Fig. 5. Scheme of the procedure for isolation of the different hepatic cell compartments from fetal liver.

Percoll-purification, (ii) MACS-depletion of OX43/OX44 positive hematopoietic cells, and (iii) enrichment of Thy1-positive cells by MACS using mAb anti-Thy1. Efficient enrichment of Thy1 positive cells is demonstrated by an obvious enhanced Thy1 gene expression in the Thy1-sorted cell compartment. Thy1 was revealed to be a phenotypic marker of hepatic stem cells in the adult liver, the Oval cells [25]. This study as well as our previous study shows that Thy1 expression is present during the whole period of late fetal liver development.

Quantitatively, we found a significant increase of primary isolated cells during liver development from ED 16 towards ED 22. After depletion of hematopoietic cells by Percoll and OX43/OX44 MACS-depletion, we found similar cell numbers at all observed time points (Fig. 1). The recovery rate after OX43/OX44 depletion increased from 32% at ED 16 to 51% at ED 22. This shows that in the fetal period the majority of cells in the liver are of hematopoietic origin, with an increase of hepatic cells towards the end of the gestation. In contrast to this, the recovery of Thy1-positive, putative stem cells from the hematopoiesis depleted fraction was decreasing significantly from early (ED 16) to late (ED 22) gestation (see Fig. 2 for details). These data indicate that combining MACS depletion of OX43/OX44 positive cells with MACS enrichment of Thy1 positive cells leads to a specific enrichment of Thy1 positive cells in the hematopoiesis depleted cell fraction of the fetal liver.

Thy1 positive sorted fetal liver cells show hepatocytic differentiation

RT-PCR analysis of Thy1 gene expression confirmed the immunocytochemical data of our previous study during fetal liver development: in general we observed an increase in Thy1 gene expression from early towards late gestational ages before and after MACS depletion of hematopoietic cells. RT-PCR analysis and immunocytochemical staining showed liver specific gene expression in double sorted Thy1-positive cells: in this compartment, we observed a constant CK-18-RNA expression, a decreasing albumin-RNA expression, and an increasing of AFP-RNA expression in the late fetal period (for details see Figs. 3B–D). Immunocytochemical staining confirmed the results of the PCR analysis on a protein-level: cells in the Thy1-sorted fraction were positive for the phenotypical hepatic markers CK-18, AFP, and albumin (see Figs. 4A–C). This strengthens the data of our previous work that Thy1 positive cells of the fetal liver exhibit hepatocyte specific gene expression and phenotype. Stem cells in the adult liver show a distinct marker expression pattern with typical co-expression of hematopoietic stem-cell markers (e.g., CD34, Thy1, c-kit, and others) [25–27] and hepatic lineage markers (e.g., CK-18, Albumin, and others) [18–21]. By PCR analysis and immunostain we found a similar phenotypical profile of Thy1 positive cells in the fetal liver. After isolation of this cell type, in vitro experiments have to reveal the stem cell like properties of these cells in respect to clonal growth and bipotential differentiative capacity.

Different degrees of hepatic maturation were found in the cell compartments

RT-PCR analysis showed that all the different cell compartments of the fetal liver obtained by MACS expressed the hepatic lineage marker CK-18. Furthermore, in the different compartments, other liver markers such as AFP and albumin were also expressed. While albumin gene expression was similar at all time points in the compartment of OX43/OX44 depleted cells, we observed that signals were weaker in the Thy1 enriched compartment with a peak in the earlier fetal period. AFP gene expression demonstrated another pattern in the different compartments: in the OX43/OX44 depleted compartment, a decrease of gene expression was observed from early towards late gestational ages, whereas in the Thy1 enriched compartment, in general, a weaker AFP expression was found at ED 18 with an increase towards the end of gestation. This indicates that in the obtained compartments different degrees of hepatic maturation are present.

In several studies, the maturation of fetal liver cells and their corresponding phenotypical marker expression was investigated extensively: analysis of marker profiles of CK-7, CK-8, CK-18, CK-19, GGT, albumin, and AFP during rat liver development suggested that ''hepatoblasts'' undergo a gradual maturation throughout liver development towards mature hepatocytes, or intra- and extrahepatic bile ducts [28–30]. The described hepatoblasts expressed markers which were similar to stem cells observed during carcinogenesis [30,31]. As a consequence it was postulated that hepatoblasts can mature to Oval cells of the adult liver. In another study, the differentiative potential of isolated immature hepatocytes from embryonic/fetal livers at ED 12 and ED 16 was studied. It could be demonstrated that these cells were bipotential (differentiated towards hepatic or biliary lineage) depending on the culture conditions. Thus, the authors felt that these immature hepatocytes with their highly proliferative capacity could be precursors of adult hepatic stem cells [32]. Both studies suggested that in the early liver development hepatoblasts/immature hepatocytes appear which are stem cell like. While the vast majority of these cells differentiate into mature hepatocytes/bile ductular cells at birth, some may retain their stem cell like capacities and give rise to adult hepatic stem cells observed during carcinogenetic models. Sigal et al. were the first, who found hints for the existence of different hepatic fetal cell types in the sense of a cellular hierarchy. In isolated fetal rat livers depleted of

OX43/OX44 positive cells by immunopanning [33] they found either OC.3- (Oval cell antigen) positive or OC.3-negative cells. These cells were described as larger granular cells which expressed AFP and GGT. The hypothesis from this was that OC.3 positive cells were committed progenitors of bile duct cells [34]. In our previous study we also observed hepatic cells expressing an adult Oval cell marker, with Thy1 [25] instead of OC.3. In contrast to the findings of Sigal and Brill, in this study, we identified Thy1-positive cells expressing hepatocytic markers: we found albumin expression in this cell fraction in earlier fetal stages and AFP expression in the late fetal period. This is contrary to the gene expression profile found in the OX43/OX44 negative compartment. We assume, that this is a result of different degrees of maturation in the compartments, with a more immature state in the Thy1-positive compartment at birth. This means that OX43/OX44 depleted cells exhibited different maturation patterns modulated by their Thy1-gene expression: Thy1-enriched cells showed patterns of differentiation towards the end of the fetal period, while hematopoiesis depleted cells developed towards mature hepatocytes. This indicates the existence of cellular hierarchy during fetal liver development as it is observed in adult livers, with hepatoblasts instead of hepatocytes. At least, our data indicate, that at all observed gestational ages different hepatocellular compartments are present during fetal liver development. Thy1-positive and Thy1-negative hepatocytic cells might be early progenitors during fetal liver development of the different hepatic lineages in the adult liver. Further studies have to evaluate the biological properties of these different fetal hepatic lineages and to reveal the relationship of these compartments with the adult hepatic cell types.

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