Small hepatocytes in culture develop polarized transporter expression and differentiation

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Summary

Rat small hepatocytes have been shown to proliferate in culture and to form organoids with differentiated hepatocytes in vitro. To evaluate the degree of polarized transporter differentiation of rat small hepatocytes during 9 weeks of culturing, we studied the time-dependent expression and subcellular localization of the major bile salt and organic anion transport systems of hepatocytes [i.e. the basolateral sodium-taurocholate co-transporting protein (Ntcp), organic-anion-transporting polypeptide 1b2 (Oatp1b2), the canalicular bile-salt export pump (Bsep) and multidrug-resistance-associated protein 2 (Mrp2)]. Small hepatocytes proliferated and differentiated in culture and formed sharply demarcated colonies as assessed by morphology, α-fetoprotein, albumin and Mrp1 expression. Polarized surface transporter expression was evident after 5 weeks of culturing for Ntcp, Oatp1b2 and Mrp2, and after 7 weeks for Bsep. After 9 weeks in culture, the vast majority of matured hepatocytes expressed Ntcp/Oatp1b2 at the basolateral and Bsep/Mrp2 at the canalicular plasma-membrane domains. This polarized transporter expression was accompanied by canalicular secretion of fluorescein-diacetate and cholyglycyl-fluorescein. Furthermore, an anastomizing three-dimensional network of bile canaliculi developed within piling-up colonies. These data demonstrate that cultured rat small hepatocytes acquire a fully differentiated transporter expression phenotype during their development into hepatic ‘organoid-like’ clusters of mature hepatocytes. Thereby, the time-dependent sequence of transporter expression mirrored the ontogenesis of transporter expression in developing rat liver, supporting the concept that small hepatocytes correspond to the hepatocyte lineage derived from embryonic hepatoblasts and/or from a different pool of ‘committed hepatocyte progenitor cells’.

Key words: Rat liver, Progenitor, Cell differentiation, Small hepatocyte

Introduction

Transcellular bile secretion from sinusoidal blood plasma into bile canaliculi is an important function of differentiated hepatocytes in all mammalian species. This vectorial secretory process is driven by the polarized expression of distinct transport systems at the sinusoidal (basolateral) and canalicular (apical) surface domains of hepatocytes. In rat liver, basolateral uptake of bile salts and organic anions is mediated by the Na+-dependent taurocholate co-transporting polypeptide (Ntcp; gene symbol Slc10a1) and the sodium-independent organic-anion-transporting proteins Oatp1a1 [Slco1a1; previously called Oatp1, Scl21a1 (Hagenbuch and Meier, 2004)], Oatp1a4 [Slco1a4; previously called Oatp2, Scl21a5] and Oatp1b2 [Slco1b2; previously called Oatp4, Scl21a10] (Meier and Stieger, 2002). Among hepatic Oatps, Oatp1b2 is most abundantly expressed at the basolateral membrane of hepatocytes (Kakyo et al., 1999; Cattori et al., 2000; Choudhuri et al., 2001; Li et al., 2002). Because hepatic Oatps exhibit broad and partially overlapping substrate specificities, they play an important role for the hepatic clearance of a wide range of endogenous and exogenous amphipathic albumin-bound organic compounds (Cattori et al., 2001; Hagenbuch and Meier, 2003; Meier and Stieger, 2002; Suzuki and Sugiyama, 2000). At the canalicular membrane, conjugated bile salts and organic anions are actively secreted/excreted against the prevailing electrochemical gradient by the bile-salt export pump (Bsep; Abcb11) and the multidrug resistance protein 2 (Mrp2; Abcc2), respectively (Keppler and König, 2000; Meier and Stieger, 2002). These members of the ATP-binding cassette (ABC) transporter superfamily provide the necessary driving force for the generation of bile-salt-dependent and -independent bile flow within the bile canaliculi.

When rat hepatocytes are isolated and cultured under standard (serum-containing) conditions, they rapidly dedifferentiate and lose, at least in part, their characteristic surface-transport polarity. For example, in previous studies, we have shown that primary rat hepatocytes cultured for 72 hours in the presence of insulin, dexamethasone and 10% fetal calf serum (FCS) acquire a cholestatic transporter phenotype with downregulation of Ntcp, Oatp1a1, Oatp1a4 and, albeit to a lesser degree, Bsep and Mrp2 (Liang et al., 1993; Rippin et al., 2001). Similar alterations of transporter expression have been observed in vivo during cholestasis and during liver regeneration after partial hepatectomy (Lee and Boyer, 2000). Obviously long-term preservation or regain of a fully differentiated and functional transport polarity would be important for the successful use of primary cultured hepatocytes for physiological transport studies in vitro and/or
for the development and use of transport-competent hepatocytes in artificial liver devices. Because the latter goal requires large numbers of hepatocytes, culturing conditions are required that promote, first, the proliferation and expansion of the cell pool, and, second, the differentiation of the proliferated cells into mature transport-competent hepatocytes. In this context, two especially promising procedures have recently been reported. First, Mitaka and co-workers elaborated conditions that permitted the reconstruction of (differentiated) hepatic organoids from proliferating small hepatocytes, which might have been derived from the pool of ‘committed progenitor cells’ in rat liver (Mitaka et al., 1999). Second, Michalopoulos and co-workers developed a new chemically defined medium that, when supplemented with both hepatocyte growth factor (HGF) and epidermal growth factor (EGF), promoted proliferation and clonal growth of hepatocytes with final acquisition of a mature phenotype in the presence of an adequate extracellular matrix support (Matrigel) (Block et al., 1996; Michalopoulos et al., 1999). Although these studies are very indicative for final differentiation of the expanded hepatocyte populations, the acquired degree of polarized transporter expression has not been investigated.

In the present study, we adopted the ‘Mitaka’ procedure and investigated the time-dependent expression of Ntcp, Oatp1b2, Bsep and Mrp2 in proliferating small hepatocytes. The data confirm that cultured rat small hepatocytes proliferate within sharply delineated colonies and develop into ‘organoid-like’ clusters of mature hepatocytes during extended culturing periods. After 9 weeks of culturing, the vast majority of hepatocytes within colonies exhibited polarized expression of Ntcp/Oatp1b2 at the basolateral membrane and of Bsep/Mrp2 at the canalicular membrane, indicating the acquisition of a fully differentiated transporter expression phenotype.

Materials and Methods

Chemicals

Collagenase type 2 CLS was from Worthington Biochemical (Freehold, NJ). Dulbecco’s modified Eagle’s medium (DMEM) (with 580 mg l–1 L-glutamine, 1000 mg l–1 D-glucose, 25 mM HEPES), Leibovitz’s L-15 medium (with 300 mg l–1 L-glutamine), penicillin, streptomycin, glutamine, TRIZOL LS reagent and FCS were from Gibco BRL Life Technologies (Gaithersburg, MD). Ascorbic acid 2-phosphate, bovine serum albumin (BSA), dimethylsulfoxide, insulin and dexamethasone were from Sigma (St Louis, MO). William’s medium E (WME) without phenol red and L-glutamine was and dexamethasone were from Sigma (St Louis, MO). William’s Leibovitz’s L-15 medium (with 300 mg l–1 L-glutamine), penicillin, streptomycin, glutamine, TRIZOL LS reagent and FCS were from Gibco BRL Life Technologies (Gaithersburg, MD). Ascorbic acid 2-phosphate, bovine serum albumin (BSA), dimethylsulfoxide, insulin and dexamethasone were from Sigma (St Louis, MO). William’s medium E (WME) without phenol red and L-glutamine was purchased from Amidel (Allschwil, Switzerland). Collagen R was from Serva Electrophoresis (Heidelberg, Germany), and nicotinic acid purchased from Amimed (Allschwil, Switzerland). Collagen R was medium E (WME) without phenol red and L-glutamine was and dexamethasone were from Sigma (St Louis, MO). William’s phosphate, bovine serum albumin (BSA), dimethylsulfoxide, insulin and dexamethasone were from Sigma (St Louis, MO). William’s Leibovitz’s L-15 medium (with 300 mg l–1 L-glutamine), penicillin, streptomycin, glutamine, TRIZOL LS reagent and FCS were from Gibco BRL Life Technologies (Gaithersburg, MD). Ascorbic acid 2-phosphate, bovine serum albumin (BSA), dimethylsulfoxide, insulin and dexamethasone were from Sigma (St Louis, MO). William’s.
from total RNA with random primers using the Promega® Reverse Transcription System (Madison, WI). The reaction mixture was incubated at 42°C for 1 hour. Samples were heated at 94°C for 4 minutes followed by incubation at 4°C for 5 minutes (Hybaid, Catalys, Wallisellen, Switzerland). cDNA was stored at –20°C until use. mRNAs of AFP, Bsep, Mrp1 and Mrp2, were quantified using TaqMan real-time PCR with an ABI PRISM 7700 detector according to the manufacturer’s guidelines (AB Applied Biosystems, Rotkreuz, Switzerland). Primers and probes (Table 1) were designed to produce amplicons spanning an intron-exon boundary (Primer Express 1.0, Sequence Detection System Software; AB Applied Biosystems, Rotkreuz, Switzerland). All probes were labeled with FAM (reporter dye at the 5’ end of the probe) and TAMRA (quencher dye at the 3’ end of the probe), with the exception of the 18S rRNA probe, which was labeled using VIC (reporter dye at the 5’ end of the probe) labeled. The PCR reactions were carried out in 96-well plates (MicroAmp Optical 96-well reaction plate and optical caps; AB Applied Biosystems, Rotkreuz, Switzerland). One PCR reaction per well contained 12.5 µl 2x TaqMan Universal PCR Mastermix (UMM; AB Applied Biosystems, Rotkreuz, Switzerland), 25 ng cDNA, 900 nM probe and 200 nM forward and reverse primers adjusted to 25 µl. real-time PCR for 18S rRNA was carried out with 12.5 µM UMM, 1.25 µl TaqMan ribosomal RNA control reagent and 25 ng cDNA adjusted to 25 µl. The reaction conditions were 50°C for 2 minutes, followed by 40 cycles of the amplification step (95°C for 15 seconds to activate the Taq DNA polymerase and 60°C for 1 minute to anneal/extend). Quantitation of mRNAs for Bsep, Mrp1 and Mrp2 was performed according to the comparative Ct method of AB Applied Biosystems. Ct values were normalized to 18S rRNA and 1 week with x=2–ΔΔCt, where ΔΔCt=(Ctcarrier – Ct18s rRNA at 5, 7 or 9 weeks) – (Ctcarrier – Ct18s rRNA at 1 week). mRNA for AFP was quantitatively analysed using the relative standard curve method described by AB Applied Biosystems. The relative standard curve was constructed using total RNA of embryonic rat liver (embryonic day 15). The Ct values obtained were also normalized to 18S rRNA and 1 week using the comparative Ct method.

Determination of albumin secretion

After the indicated culture periods, 1 ml of culture medium of cultured small hepatocytes was collected and stored at –20°C until use. Albumin secretion into the supernatant was quantitated (µg ml–1 every 24 hours) by a sandwich enzyme-linked immunosorbent assay (ELISA) adapted from (Holzman et al., 1993). Ninety-six-well micro plates (MaxiSorb; Nalge Nunc International, Roskilde, Denmark) were coated with 1 µg per well rabbit anti-rat albumin diluted in PBS and incubated for 2 hours at room temperature. Then the non-specific binding sites were blocked with PBS containing 3% (wt/vol) BSA (4°C, overnight). Thereafter, probes in appropriate dilutions were added and plates were incubated for 2 hours at room temperature. Following washing, the second antibody [peroxidase-conjugated rabbit anti-rat albumin diluted at appropriate concentration in PBS with 3% (wt/vol) BSA] was added and plates were incubated for 2 hours at room temperature. After washing again, freshly prepared substrate (4 mg ABTS and 6 µl H2O2 in 100 mM sodium acetate, 50 mM NaH2PO4, pH 4.2) was added to each well. The plates were developed in the dark and were analysed with a Ultramicro Plate Reader (BioRad Laboratories, Hercules, CA) at 415 nm.

Immuno-fluorescence microscopy

At the indicated culture times, small hepatocytes were fixed directly in the culture dishes at room temperature for 1 hour with 4% (wt/vol) paraformaldehyde in PBS with appropriate Triton X-100 concentrations for the respective antibodies and processed as described (Stieger et al., 1994a). Polyclonal antibodies against Ntcp were raised in rabbits against: (1) a fusion protein containing at least 56 C-terminal amino acids of Ntcp (Stieger et al., 1994a); (2) a synthetic peptide consisting of 14 C-terminal amino acids of Oatplb2 (Cattori et al., 2001); (3) an oligopeptide containing 13 C-terminal amino acids of Bsep (Gerloff et al., 1998); and (4) an oligopeptide spanning 11 C-terminal amino acids of Mrp2 (Madon et al., 2000). Fixed cells were probed with these antibodies as described (Stieger et al., 1994a). Hybridomas producing antibodies against the canalicular marker enzyme dipeptidylpeptidase IV (DDP IV) (Petell et al., 1990) were a generous gift from A. Quaroni (Cornell University, Ithaka, NY). The generation of monoclonal antibody 1-18 against a basolateral marker protein has been previously described (Stieger et al., 1994b). Double staining experiments were made using: anti-albumin and anti-AFP antibodies; the basolateral membrane marker 1-18 with the canalicular transporters (1-18/Bsep and 1-18/Mrp2); and the canalicular membrane marker DPP IV with the basolateral transporters (DPP IV/Ntcp and DPP IV/Oatp1b2). Immunofluorescence was analysed by confocal laser-scanning microscopy using a Leica TCS 4D microscope (Leica, Wetzlar, Germany). For visualization of the canalicular network, surface rendering on image stacks was performed with the IsoSurface module of the Imaris software package (Bitplane, Zurich, Switzerland).

Transport assays with cholyglycyl-fluorescein and fluorescein-diacetate

After 7 weeks and 9 weeks culture time, dishes were rinsed with WME supplemented with 2 mM L-glutamine, 10% (vol/vol) FCS, 0.5 µg ml–1 insulin, 10 µg ml–1 EGF, 10–7 M dexamethasone and 100 U ml–1 penicillin/streptomycin. Cells were then incubated at 37°C for 35 minutes with either 2 ml of 1 µM cholyglycyl-fluorescein or of 5 µg ml–1 fluorescein-diacetate in the above medium in an incubator with 5% (vol/vol) CO2 atmosphere and 37°C. Thereafter, the dishes were rinsed three times with WME at room temperature and fluorescence was immediately recorded by an Axiovert 25 fluorescence microscope equipped with an AxioCAM recorder from Zeiss (Oberkochem, Germany).

Table 1. Primers and probes for Real Time PCR (TaQMan)

| Carrier | Forward primer | Probe | Reverse primer |
|---------|----------------|-------|----------------|
| Bsep (U69487) | CTGGGCTTATTCGGGAGGCTGTAC | GCTTCTCAAGACAAGACAGACTCTGGAGAAGG | CTGGGCGGAATTTGCTGAGG |
| α-fetoprotein (X02361) | CGCAACCGCATGAGG | TCTATGACCGTTCCTCCATCGTAAAG | CGACAGAATTTCTTGGAGAAC |
| Mrp 1 (AJ277881) | CGAATGGTGCTCTGAGATGGAGACGGAGAAGGCTTCTTGGCAAATCCAAGCTCTACACGGCCTGAATGGG | GGAGAAGGAGGCTTCTTGGCAAATCCAAG | GGAGAAGGAGGCTTCTTGGCAAATCCAAG |
| Mrp 2 (L49379) | GGTGACGCTATGGCTGCTCT | GGGGACATCTACACCACTCCAGGGATCCC | GCTGCTAGGGGACATAGGCTG |

Numbers in parentheses are accession numbers of the sequences used to design the primers.
number and size with increasing culture time. After 7-9 weeks, the colonies were sharply delineated and were surrounded by a monolayer of non-parenchymal and liver epithelial cells, which showed a more transparent cytoplasm than small hepatocytes (Fig. 1B,C).

Within the colonies, hepatocytes enlarged and structures resembling canaliculi became apparent, as evidenced by immunofluorescence localization of the canalicular transporter Mrp2 (Fig. 1D). After about 9 weeks of culture, the size of the colonies did not increase further, indicating a decrease in the proliferation rate of small hepatocytes. Thereafter, some colonies died and new colonies appeared, indicating continuous proliferation of small hepatocytes during the entire 9 weeks of culture time. This assumption was confirmed by measuring mRNA expression of the two hepatocyte proliferation markers Mrp1 (Roelofsen et al., 1997) and AFP (Mizejewski, 2001).

As indicated in Fig. 2, expression levels of Mrp1- and AFP-encoding mRNAs increased approximately twofold and 70-120-fold, respectively, during the 5-9 week culture periods. Because a well-defined lateral plasma membrane domain appears to be essential for expression of Mrp1 (Roelofsen et al., 1997), the increased Mrp1 expression (Fig. 2A) indicated that at least some of the proliferating hepatocytes had reached a certain differentiation of their surface membrane domains during the later culturing periods. In contrast to Mrp1, AFP-encoding mRNA expression was virtually absent from freshly isolated cells and at 1 week of culture time. It started to rise towards the end of the culture period (Fig. 2B), suggesting that the proliferating small hepatocytes had matured at least to or beyond the stage of hepatocytes developing in rat liver of embryonic day 12 (Moorman et al., 1990). Because AFP is a neoplastic marker in adult liver as well as a differentiation marker for developing hepatocytes during liver ontogenesis (Nahon, 1987), we also tested for coexpression of AFP and albumin by double labeling of hepatocyte colonies. As illustrated in Fig. 3, AFP and albumin were expressed in the same cells, thus confirming differentiation of small hepatocytes along the hepatocyte lineage.

**Albumin secretion**

To further determine the differentiation state and functional competence of the growing small-hepatocyte colonies, we next tested for the secretion of albumin. As illustrated in Fig. 4, virtually no albumin secretion was found after 1 week of

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**Fig. 1.** Development and maturation of small-hepatocyte colonies in culture. (A) A small-hepatocyte colony after 1 week of culture. (B) A small-hepatocyte colony after 7 weeks of culture. Notice the hexagonal shape of most cells, indicating differentiation of small hepatocytes into mature hepatocytes. (C) Phase-contrast microscopy picture showing a clearly demarcated colony of cells appearing as mature hepatocytes. Cells were cultured for 9 weeks and then fixed with 4% paraformaldehyde in the presence of Triton X-100 and subsequently processed for immunofluorescence localization of Mrp2. (D) Immunofluorescence localization by laser-scanning microscopy indicated expression of Mrp2 in structures resembling bile canaliculi. Scale bars, 40 μm.

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**Fig. 2.** Time-dependent mRNA expression of Mrp1 (A) and AFP (B) in cultured rat small hepatocytes. Cells were cultured for up to 9 weeks. Cells were harvested at day 1 and weeks 1, 3, 5 and 9 with Trizol. mRNA of Mrp1 and AFP was quantitatively measured by real-time PCR and normalized to 18S rRNA and mRNA expression of 1-week cultures [Ct value for Mrp1=27.61±0.46; Ct value for 18S=13.9±0.97; the quotient (Ct value for Mrp1 / Ct value for 18S)=13.71±0.8 (n=4, mean±standard deviation); Ct value for AFP=35.41±0.6; the quotient (Ct value for AFP/Ct value for 18S=21.5±1.0 (n=4, mean±SD)]. 0.0023% AFP mRNA was detected in 1-day cultures relative to 1-week cultures.
Transport polarization of small hepatocytes

Thereafter, albumin secretion into the medium increased steadily up to a plateau level of approximately 0.35 mg ml\(^{-1}\) per 24 hours after 6 weeks of culture time. Similar to Mrp1- and AFP-encoding mRNA expression (Fig. 2), the amount of albumin in the medium paralleled the increasing size and number of small-hepatocyte colonies. Although some contribution of contaminating and late-proliferating mature hepatocytes to the increasing albumin secretion cannot be definitely excluded, this possibility is unlikely because, unlike the 'hepatocyte growth medium' supplemented with HGF and EGF growth factors reported by Block et al. (Block et al., 1996), the adopted 'Mitaka' culture conditions do not support the large-scale proliferation of mature hepatocytes. The morphology of the colonies, the expression of proliferation marker mRNAs, the coexpression of AFP and albumin, and the albumin secretion data thus all strongly suggest the expansion and differentiation of small hepatocytes into mature hepatocytes with extended culture time.

Polarized transporter expression

In the following we tested the time-dependent expression of two basolateral (Ntcp, Oatp1b2) and two canalicular (Bsep, Mrp2) transporters in the growing colonies by immunofluorescence microscopy. Ntcp expression is illustrated in Fig. 5A. Although, in 1 week cultures the appearing small colonies were constantly negative for Ntcp (Fig. 5Ab), immunopositive colonies could be detected after 5 weeks of culturing (Fig. 5Ad). However, although initial cell surface staining was observed, significant intracellular immunofluorescence was also present. In 7-week-old colonies, the intracellular staining had virtually disappeared and the plasma-membrane-associated immunopositivity had increased (Fig. 5Af). At 9 weeks, virtually all hepatocytes demonstrated a characteristic basolateral expression of Ntcp (Fig. 5Ah), indicating full maturation of the major sodium-dependent hepatocellular bile-salt uptake system.

Among the basolateral sodium-independent bile-salt and organic-anion transport systems, we focused our analysis on Oatp1b2, because this transporter is predominantly expressed in rodent liver. As illustrated in Fig. 5B, intracellular immunopositivity was already detected in 1-week-old colonies (Fig. 5Bb). With longer culturing times the immunopositivity became increasingly associated with the cellular surface (Fig. 5Bd,f). After 9 weeks, most colony cells demonstrated well-demarcated surface staining, although some heterogeneity in Oatp1b2 expression levels persisted between individual cells (Fig. 5Bh). Nevertheless, the data are compatible with the development of most undifferentiated small hepatocytes into mature hepatocytes with basolateral expression of the major hepatocellular bile-salt and organic-anion transport systems.

In contrast to basolateral Ntcp and Oatp1b2, canalicular Bsep was hardly visible in 1-week-old and 5-week-old colonies (Fig. 5Bb,d). However, after 7 weeks, a punctate and a characteristic branching pattern of immunofluorescent reactivities were observed, indicating canalicular expression of Bsep (Fig. 5Bf). The latter became even more pronounced after 9 weeks, with clear-cut demarcation of canalicular spaces between hepatocytes (Fig. 6Bh). Hence, within colonies, small hepatocytes had differentiated into mature hepatocytes with a polarized expression of the major bile-salt transport systems.

Finally, we also examined the expression of the canalicular organic-anion transporter Mrp2. As illustrated in Fig. 6B, weak cytoplasmic fluorescence was already observed after 1 week of...
culturing (Fig. 6Bb). At 5 weeks, occasional islands with strong canalicular expression of Mrp2 were seen within some colonies (Fig. 6Bd). Although some intracellular immunofluorescence persisted in 7-week-old colonies (Fig. 6Bf), the canalicular staining became more and more stringent, and culminated in a virtually exclusive canalicular Mrp2 expression at 9 weeks (Fig. 6Bh).

Immunofluorescence signals of Mrp2 were generally more intense than those of Bsep, suggesting that more transporter protein is expressed for Mrp2 than for Bsep (Fig. 6A,B). To substantiate this assumption further, relative mRNA levels were determined by real-time PCR for both transporters. Table 2 shows that Mrp2 mRNA levels were about 80-fold higher at 1 week culture time than Bsep mRNA levels. At 5 weeks, Mrp2 mRNA levels exceeded Bsep mRNA levels over 300-fold and this excess did not change significantly after 7-9 weeks of culture time. Hence, translation of the increased Mrp2 mRNA could well explain the higher Mrp2 than Bsep protein immunofluorescence in Fig. 6.

To investigate more directly the degree of polarization of the mature hepatocytes, double-labeling experiments with hepatocyte surface-domain-specific antigens and transporters were also performed. As illustrated in Fig. 7, expression of the basolateral transporters Ntcp (Fig. 7Aa) and Oatp1b2 (Fig. 7Ad) did not overlap with the expression of the canalicular enzyme DPP IV (Fig. 7Ab,e). Similarly, expression of canalicular Bsep (Fig. 7Ba) and Mrp2 (Fig. 7Bd) did not overlap with the immunofluorescence of the basolateral marker protein 1-18 (Fig. 7Bb,e). These results corroborate the development of polarized transporter expression and the maturation of cultured small hepatocytes into differentiated hepatocytes.

Three-dimensional structures of bile canaliculi
Although primary cultured mature hepatocytes secrete fluorescein into minute bile canalicular spaces (Gebhardt and Jung, 1982), they do not develop an anastomizing network of bile canaliculi as is the case in intact liver. Therefore, we wondered whether bile canaliculi of differentiated small-hepatocyte colonies form a three-dimensional network of interconnected channels. As illustrated in Fig. 8, this was indeed the case, further supporting the suggestion that small hepatocytes colonies develop into differentiated hepatic organoids.

### Table 2. Normalized Ct-values of Canalicular Transport Systems

| Culture time (weeks) | 1   | 5   | 7   | 9   |
|----------------------|-----|-----|-----|-----|
| 18s rRNA             | 13.90±0.95 | 15.06±0.39 | 13.47±0.89 | 15.42±0.89 |
| Mrp2                 | 23.72±0.64 | 24.51±0.46 | 24.57±0.55 | 24.43±0.47 |
| Bsep                 | 30.03±0.57 | 33.20±0.49 | 33.15±0.78 | 32.37±0.55 |
| Mrp2/18 s rRNA       | 9.82±1.15 | 9.45±0.60 | 11.10±1.05 | 9.01±1.01 |
| Bsep/18 s rRNA       | 16.13±1.11 | 18.14±0.63 | 19.68±1.18 | 17.00±1.05 |
| mRNA Mrp2/mRNA Bsep  | 79  | 335 | 383 | 254 |

Rat small hepatocytes were cultured and harvested in Trizol at weeks 1, 5, 7 and 9 to isolate mRNA for real time PCR quantitation as described in Materials and Methods. Data (mean±SD, n=4) are given as Ct values with the exception of the quotient Mrp2/Bsep.
Transport polarization of small hepatocytes

To test the functional competence of polarized transporter expression, 7-week-old small-hepatocyte colonies were incubated with either fluorescein-diacetate or the fluorescent bile salt derivative cholyglycyl-fluorescein. Consistent with previous observations (Mitaka et al., 1999), hepatocyte colonies demonstrating bile canalicular structure showed secretion of fluorescein into the canalicular spaces (Fig. 9A), thus confirming the expression of functionally active Mrp2 at the canalicular membrane. In addition, cholyglycyl-fluorescein was also secreted into the canalicular spaces (Fig. 9B), indicating the expression of functionally competent bile-salt uptake (i.e. Ntcp) and bile-salt secretory (i.e. Bsep) systems at the basolateral and canalicular surface domains, respectively (Holzinger et al., 1998; Holzinger et al., 1997). Hence, the data are fully consistent with the development of a functionally competent transport polarization in long-term cultured small hepatocytes.

Discussion

The present study investigated the time course of polar expression of basolateral and canalicular transport proteins in long-term culture of so-called small hepatocytes. The data confirm that rat hepatic cells isolated from the supernatant of an initial low-speed centrifugation (50 g for 1 minute) of a collagenase-digested liver-cell suspension and cultured in a medium supplemented with 10 mM nicotinamide, 1 mM ascorbic acid 2-phosphate, 10 μg l−1 EGF, 1% (vol/vol) DMSO and 10% (vol/vol) FCS results in the proliferation and clonal growth of small hepatocyte-like cells (Mitaka et al., 1999). With increasing culture time the proliferating cells formed sharply delineated aggregates/spheroids (e.g. Fig. 5Ac,Be, Fig. 6Ac,Bc) and eventually acquired a mature hepatocyte phenotype as indicated by the continuously increasing albumin secretion (Fig. 4) and the polar expression of Ntcp (Fig. 5A, Fig. 7Aa) and Oatp1b2 (Fig. 5B, Fig. 7Ad) at the basolateral and of Bsep (Fig. 6A, Fig. 7Ba) and Mrp2 (Fig. 6B, Fig. 7Bd) at the canalicular membrane domains. Hence, the data demonstrate that, under the adopted culturing conditions, clonally proliferating small hepatocytes develop into mature hepatocytes that exhibit a physiologically polarized and functionally competent expression of the major hepatocellular bile-salt and organic-anion transport systems.

Small-hepatocyte colonies have been extensively characterized previously with respect to both morphology and expression of differentiated hepatocyte marker proteins such as albumin, transferrin and cytokeratins 8 and 18 (Mitaka et al., 1992a; Mitaka et al., 1992b; Mitaka, 1998). Furthermore, clonally grown small hepatocytes have been indicated to form bile canaliculi and to exhibit canalicular secretion of fluorescein (Mitaka et al., 1999). Our studies extend these earlier studies and demonstrate the continuously increasing polarized expression of functionally active hepatobiliary bile-salt and organic-anion transporters at the perspective plasma-membrane domains of maturing small hepatocytes. However, it should be realized that the hepatic origin of the small...
hepatocytes has not been further investigated in this study. Cultures from the initial 50 g supernatant are enriched in small hepatocytes as well as in hepatic nonparenchymal cells including so-called ‘liver epithelial cells’ (Mitaka et al., 1998). Although small-hepatocyte colonies could be easily differentiated from contaminating surrounding cells by their typical morphology (e.g. Fig. 1A,B, Fig. 5Aa,c) (Mitaka, 1998), close interactions with surrounding nonparenchymal and liver epithelial cells at the border of colonies are obviously important for continuous clonal growth of the small hepatocytes (e.g. Fig. 1B,C, Fig. 5Ba,c). These observations are very similar to the ones previously reported by Mitaka et al. (Mitaka et al., 1999). Because the high concentration of nicotinamide within the culture medium preferentially stimulates the proliferation of small hepatocytes (Mitaka, 1998; Tateno and Yoshizato, 1996), the observed increases in the expression of the hepatocellular markers Mrp1 and AFP (Fig. 2), and in albumin secretion (Fig. 4) are consistent with the hepatocyte characteristics of proliferating small hepatocytes. Furthermore, the initial very low expression level of AFP-encoding mRNA is consistent with the reported AFP immunonegativity of early proliferating small hepatocytes (Mitaka et al., 1999). Hence, although our results are consistent with the concept that small hepatocytes derive from a pool of ‘committed progenitor cells’ that can further differentiate into mature hepatocytes, their exact relationships, if any, to ‘liver epithelial cells’ (Mitaka, 1998), ductular or periductular stem (oval) cells (Sell, 2001), and/or bipotential embryonic hepatoblasts (Rogler, 1997) remains unknown.

Interestingly, the time-dependent expression of individual transporters in the growing small-hepatocyte colonies showed some striking similarities with the ontogenesis of the hepatocellular bile-salt and organic-anion transport polarity. Thus, basolateral expression of Ntcp was more pronounced and more uniform in most cells of 5- and 7-week-old colonies than was the basolateral labeling of Oatp1b2 (Fig. 5Ad,f,Bd,f). This latter finding is reminiscent to the sequence of expression in developing rat liver, where expression of Ntcp is confined to the plasma membrane and occurs shortly before birth, whereas

Fig. 7. Double labeling of cultured rat small hepatocytes with canalicular transporters and plasma-membrane-domain-specific proteins. Cells were cultured for 7 weeks. They were fixed with 4% paraformaldehyde in the presence of Triton X-100 and subsequently processed for immunofluorescence localization of the various proteins. (A) Basolateral expression of Ntcp (a) and Oatp1b2 (d) did not overlap with expression of canalicular DPP IV (b,e). Arrows indicate basolateral membrane, which stained positive for Ntcp (a) and Oatp1b2 (d) but was negative for DPP IV (b,e). Arrowheads indicate bile canaliculi, which stained positive for DPP IV (b,e) but remained negative for Ntcp (a) and Oatp1b2 (d). (c,f) Corresponding phase-contrast microscopy pictures. (B) Canalicular expression of Bsep (a) and Mrp2 (d) did not overlap with basolateral expression of the 1-18 antigen (b,e). Arrows indicate the canalicular membrane, which stained positive for Bsep (a) and Mrp2 (d) but was negative for the 1-18 antigen (b,e). Arrowheads indicate the basolateral plasma membrane, which was positive for the 1-18 antigen (b,e) but remained negative for Bsep (a) and Mrp2 (d). (c,f) Corresponding phase-contrast pictures. Scale bar, 20 μm.
expression of Oatp1b2 starts with intense intracellular expression before basolateral localization and is delayed predominantly to the postnatal period (Boyer et al., 1993; Li et al., 2002; Gao et al., unpublished). Similarly, the earlier and more intense canalicular expression of Mrp2 in 1- and 5-week-old colonies compared with later expression of Bsep (i.e. 5-7-week-old colonies) (Fig. 6Bb,d,Ad,f) is consistent with the early prenatal expression of Mrp2 and the expression of Bsep around birth in developing rat liver (Zinchuk et al., 2002; Gao et al., unpublished). Hence, long-term cultures of small hepatocytes appear to recapitulate the developmental expression pattern of polarized hepatocellular bile-salt and organic-anion transport proteins, and thus could correspond to the hepatocyte lineage derived from hepatoblasts, which represents the major parenchymal cells forming hepatic cords between days 10 and 17 in rat liver development (Shiojiri et al., 1991).

Having shown that small hepatocytes proliferate and acquire a mature polarized transporter phenotype in culture, we next tried to subculture and expand the mature hepatocyte population. Unfortunately, this was not possible despite the addition of HGF and EGF, and the switch to a chemically defined medium specifically adapted to promote proliferation of mature hepatocytes (Block et al., 1996; Michalopoulos et al., 1999). Hence, the proliferative potential of mature hepatocytes originating from small hepatocytes seems to be limited in vitro, indicating the requirement of alternative strategies for the generation of high numbers and possible in vitro mass production of bile-salt- and organic-anion-transport-competent hepatocytes. Such alternative procedures might include: (1) culturing primary hepatocytes under proliferating conditions (Block et al., 1996; Michalopoulos et al., 1999); (2) further development of partially polarized hepatoma derived cell lines such as Wif-B (Bender et al., 1999; Bravo et al., 1998) or H4IIEC3 (Ng et al., 2000) cells; and (3) isolation, expansion and differentiation of bone-marrow-derived stem cells (Avital et al., 2001; Schwartz et al., 2002). The last of these procedures is especially attractive because a subpopulation of bone-marrow-derived stem cells can acquire differentiated hepatocellular metabolic functions (Avital et al., 2001; Schwartz et al., 2002), has the capability to repopulate regenerating liver in vivo (Avital et al., 2001) and can restore certain biochemical functions of diseased liver (Lagasse et al., 2000).

In conclusion, this study demonstrates that cultured rat small hepatocytes differentiate into mature hepatocytes displaying polarized surface expression of the major hepatocellular bile-salt and organic-anion transport systems of intact liver. This in vitro development of the hepatocellular transport polarity mirrors to a great extent the chronology of transporter expression during liver ontogenesis. The findings support the concept that isolated small hepatocytes derive from a pool of ‘committed hepatocyte progenitor cells’, the exact nature of which remains to be determined. Although small-hepatocyte
colonies represent the first primary liver cell culture system that develops a fully polarized bile-salt- and organic-anion-transporter phenotype in vitro, new strategies are required to produce sufficiently high numbers of differentiated hepatocytes in vitro for use in artificial liver devices and/or hepatocyte transplantation.

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