11. Mouta Carreira, C. *et al.* LYVE-1 is not restricted to the lymph vessels: expression in normal liver blood sinusoids and down-regulation in human liver cancer and cirrhosis. *Cancer Res.* **61**, 8079–8084 (2001).
12. Hansen, B. *et al.* Stabilin-1 and stabilin-2 are both directed into the early endocytic pathway in hepatic sinusoidal endothelium via interactions with clathrin/AP-2, independent of ligand binding. *Exp. Cell Res.* **303**, 160–173 (2005).
13. Saegusa, S., Isaji, S. & Kawarada, Y. Changes in serum hyaluronic acid levels and expression of CD44 and CD44 mRNA in hepatic sinusoidal endothelial cells after major hepatectomy in cirrhotic rats. *World J. Surg.* **26**, 694–699 (2002).
14. Mitaka, T., Sattler, G.L. & Pitot, H.C. Amino acid-rich medium (Leibovitz L-15) enhances and prolongs proliferation of primary cultured rat hepatocytes in the absence of serum. *J. Cell. Physiol.* **147**, 495–504 (1991).
15. Kreamer, B.L. *et al.* Use of a low-speed, isodensity percoll centrifugation method to increase the viability of isolated rat hepatocyte preparations. *In Vitro Cell. Dev. Biol.* **22**, 201–211 (1986).
16. Miyamoto, S., Hirata, K., Sugimoto, S., Harada, K. & Mitaka, T. Expression of cytochrome P450 enzymes in hepatic organoid reconstructed by rat small hepatocytes. *J. Gastroenterol. Hepatol.* **20**, 865–872 (2005).
17. Ikeda, S. *et al.* Proliferation of rat small hepatocytes after long-term cryopreservation. *J. Hepatol.* **37**, 7–14 (2002).
18. Ooe, H. *et al.* Cytochrome P450 expression of cultured rat small hepatocytes after long-term cryopreservation. *Drug Metab. Dispos.* **34**, 1667–1671 (2006).
19. Mitaka, T., Norioka, K., Sattler, G.L., Pitot, H.C. & Mochizuki, Y. Effect of age on the formation of small-cell colonies in cultures of primary rat hepatocytes. *Cancer Res.* **53**, 3145–3148 (1993).

JCB07-0257.R2(21601)

Journal of Cellular Biochemistry 9999:1–15 (2007)

Functional Expression of Organic Anion Transporters in Hepatic Organoids Reconstructed by Rat Small Hepatocytes

Hideki Oshima,[1,2] Junko Kon,[1] Hidekazu Ooe,[1] Koichi Hirata,[2] and Toshihiro Mitaka[1]*

[1]Department of Pathophysiology, Cancer Research Institute, Sapporo Medical University School of Medicine, S-1, W-17, Chuo-Ku, Sapporo 060-8556, Japan

[2]First Department of Surgery, Sapporo Medical University School of Medicine, S-1, W-17, Chuo-Ku, Sapporo 060-8556, Japan

Abstract Small hepatocytes (SHs) are hepatic progenitor cells with hepatic characteristics. They can proliferate to form colonies in culture and change their morphology from flat to rising/piled-up with bile canaliculi (BC), which results in maturation. In this study, we examined whether SHs could express hepatic transporters with polarity, whether the transporters could transport organic anion substrates into BC, and whether the secreted substances could be recovered from BC. Immunocytochemistry and RT-PCR were carried out. [^{3}H]-labeled estrogen derivatives were used to measure the functions of the transporters in SHs isolated from normal and multidrug resistance-associated protein (Mrp) 2-deficient rats. The results showed that organic anion-transporting proteins (Oatps) 1 and 2, Na^+-dependent taurocholate co-transporting polypeptide (Ntcp), Mrp2, and bile-salt export pump (Bsep) were well expressed in rising/piled-up cells and that their expression was correlated to that of hepatocyte nuclear factor 4α. Although small SHs expressed not Oatps and Mrp2 but Mrp3, rising/piled-up SHs expressed Oatp1 and 2 and Mrp2 proteins in the sinusoidal and BC membranes, respectively. On the other hand, breast cancer resistant protein (Bcrp) and Mrp3 expression decreased as SHs matured. The substrate transported via Oatps and Mrp2 was secreted into BC and it accumulated in both BC and cyst-like structures. The secreted substrate could be efficiently recovered from BC reconstructed by SHs derived from a normal rat, but not from an Mrp2-deficient rat. In conclusion, SHs can reconstitute hepatic organoids expressing functional organic anion transporters in culture. This culture system may be useful to analyze the metabolism and excretion mechanisms of drugs. J. Cell. Biochem. 9999: 1–15, 2007.

Key words: organic anion transporter; small hepatocyte; hepatic organoid; maturation; biliary excretion

Abbreviations used: Asc2P, ascorbic acid 2-phosphate; BC, bile canaliculi; Bcrp, breast cancer resistant protein; Bsep, bile-salt export pump; CAR, constitutive androstene receptor; DAB, 3′-diaminobenzidine; DAPI, 6-diamino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; EHBR, Eizai hyperbilirubinemic rat; FD, fluorescein diacetate; FXR, farnesoid X activated receptor; G3PDH, glycerol 3-phosphate dehydrogenase; HNF, hepatocyte nuclear factor; Mdr, multidrug resistance; Mrp, multidrug resistance-associated protein; MH, mature hepatocyte; 5NT, 5′-nuclotidase; Ntcp, Na^+-dependent taurocholate co-transporting polypeptide; Oatp, organic anion-transporting protein; PXR, pregnane X receptor; RAR, retinoic acid receptor; RXR, retinoid X receptor; SDR, Sprague-Dawley rat; SH, small hepatocyte.

Grant sponsor: Science and Technology[Q1] Incubation Program in Advanced Region; Grant sponsor: Japan Science and Technology Agency; Grant sponsor: Ministry of Education, Culture, Sports, Science and Technology, Japan; Grant numbers: 14370393, 17390353, 15390403; Grant sponsor: Ministry of Health, Labour and Welfare; Grant sponsor: Health and Labour Sciences Research Grants; Grant sponsor: Research on Advanced Medical Technology.

*Correspondence to: Toshihiro Mitaka, Department of Pathophysiology, Cancer Research Institute, Sapporo Medical University School of Medicine, S-1, W-17, Chuo-Ku, Sapporo 060-8556, Japan. E-mail: tmitaka@sapmed.ac.jp

Received 18 May 2007; Accepted 11 September 2007

DOI 10.1002/jcb.21601

Published online 00 Month 2007 in Wiley InterScience (www.interscience.wiley.com).

The hepatocyte is a highly differentiated cell that has functional transport polarity. Its plasma membrane is divided into three functionally and structurally distinct domains: the sinusoidal domain, the lateral domain, and the bile canalicular (apical) domain. The vectorial transport of bile salts and organic anions from serum to bile canaliculi (BC) is actively performed in hepatocytes. In the normal rat liver, the sinusoidal uptake of bile salts and organic anions is mediated by Na^+-dependent taurocholate co-transporting polypeptide (Ntcp) and

Na^+-independent organic anion-transporting proteins (Oatps), respectively [Keppler and Konig, 2000; Meier and Stieger, 2002]. Conjugated bile salts and organic anions are actively secreted through bile-salt export pump (Bsep) and multidrug resistance-associated protein (Mrp) 2 expressed at the canalicular membrane, respectively. Mrp3, which is a member of the Mrps and ATP-binding cassette family, localizes in the sinusoidal membrane [Belinsky et al., 1998; Kool et al., 1999] and its expression in normal rat hepatocytes is very low. However, increased expression is observed in the liver of cholestasis [Lee and Boyer, 2000], of the naturally occurring Mrp2-deficient Eizai hyperbilirubinemic rat (EHBR) [Buchler et al., 1996; Hirohashi et al., 1998], and of Dubin–Johnson patients [Konig et al., 1999; Ogawa et al., 2000; Scheffer et al., 2002].

Primary hepatocytes isolated from rodent and human livers have been used extensively for investigating the uptake of bile acids, drugs, and toxicants [Meijer, 1987]. However, primary hepatocytes rapidly lose their differentiated functions and, upon isolation by collagenase digestion of the liver, both polarization and the ability to excrete compounds across the cell in a vectorial manner disappear [Maurice et al., 1988]. In conventional culture of rat hepatocytes, expression of Ntcp mRNA decreases within 72 h [Liang et al., 1993]. The rapid loss is observed for both protein and mRNA expression of other transporters such as Oatp1, Oatp2, Bsep, and Mrp2 [Rippin et al., 2001]. Although the expression of excretion transporters is preferentially maintained compared to that of uptake transporters, they have not been maintained for more than a week in conventional culture conditions. Primary rat hepatocytes sandwiched between collagen gels were shown to maintain hepatic differentiated functions for about 10 days [Dunn et al., 1989; Musat et al., 1993; LeCluyse et al., 1994; Berthiaume et al., 1996]. Cells in the collagen-sandwich configuration were shown to form functional BC between the cells and excrete a variety of endogenous and exogenous compounds into the BC space [Liu et al., 1998, 1999a].

Small hepatocytes (SHs) have been identified as proliferating cells with hepatic characteristics [Mitaka et al., 1992]. A single SH can proliferate to form a colony and cells in colonies change in shape from flat and small to large, and then to rising/piled-up cells with time in culture [Mitaka et al., 1995, 1999]. The morphological changes of SHs are correlated with their maturation. The characteristics of rising/piled-up cells are similar to those of mature hepatocytes (MH). In addition, rising/piled-up cells form BC between the cells, and ectoATPase, 5′-nucleotidase (5NT), and MRP2 are restrictedly expressed in the structure [Sudo et al., 2004]. Furthermore, fluorescein and bilirubin are secreted into the structure and the secreted substances are tightly enclosed within the BC space.

In the present experiment, we examined whether SHs could express hepatic transporters with polarity, whether the transporters could transport organic anion substrates into BC, and whether the secreted substances could be recovered from BC. The results revealed that the expression of Oatp1, Oatp2, Ntcp, Mrp2, and Bsep increased with time in culture. In rising/piled-up cells Oatp1, Oatp2, and Mrp2 localized in the sinusoidal and BC membranes, respectively. On the other hand, breast cancer resistant protein (Bcrp) and Mrp3 expression decreased as SHs matured. The substrate transported via Oatps and Mrp2 was secreted into BC and it accumulated in both BC and cyst-like structures. The secreted substrate could be efficiently recovered from BC constructed by SHs derived from the normal rat, but not from the Mrp2-deficient rat.

MATERIALS AND METHODS

Isolation and Culture of Small Hepatocytes

Male Sprague-Dawley rats (SDR, Shizuoka Laboratory Animal Center, Hamamatsu, Japan) and EHBR (Sankyo Lab Service Co., Tokyo, Japan), which are genetically Mrp2-deficient, weighing 250–350 g, were used. All animals received humane care and the experimental protocol was approved by the Committee of Laboratory Animals according to Sapporo Medical University guidelines. Details of the isolation and culture procedure of cells were previously described [Mitaka et al., 1999]. Finally, 9×10^5 viable cells were seeded on a 100-mm dish (Corning Glass Works, Corning, NY) and cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical Co., St. Louis, MO) supplemented with 20 mmol/L HEPES, 25 mmol/L $NaHCO_3$, 30 mg/L L-proline, 10% fetal bovine serum (FBS, HyClone, Logan, UT), 10 mmol/L nicotinamide (Katayama Chemical Co., Osaka,

Japan), 1 mmol/L ascorbic acid 2-phosphate (Asc2P; Wako Pure Chem., Tokyo, Japan), 10 ng/mL epidermal growth factor (EGF; BD Biosciences, Bedford, MA), 0.5 mg/L insulin, 10^{-7} mol/L dexamethasone, and antibiotics. After 4 days of culture, 1% dimethyl sulfoxide (DMSO; Aldrich Chem. Co., Milwaukee, WI) was added to the medium.

Subculture of SH Colonies

To collect the colonies, they were detached from dishes 12 days after plating. The method used for the subculture of SH colonies was previously reported [Sugimoto et al., 2002]. Colonies ($2–4 \times 10^3$ colonies/35-mm dish) were plated on dishes coated with rat tail collagen. Four hours after replating, the medium was replaced with serum-free DMEM supplemented with 1% DMSO. The experimental schedule is shown in Figure 1A.

Photographs of Cells

Morphological changes of SH colonies were observed and recorded using a phase-contrast microscope equipped with a CCD camera (Olympus Optical Co., Tokyo, Japan).

RT-PCR

Total RNA was extracted from cultured cells using TRIzol reagent (Invitrogen, Carlsbad, CA) as previously described [Sugimoto et al., 2002]. RNA was resuspended in H_2O, incubated for 5 min at 60°C and then stored at −80°C until use. PCR reaction was performed using primers for hepatic transporters and glycerol 3-phosphate dehydrogenase (G3PDH). The primers are listed in Table I.

Immunocytochemistry of Cultured Cells

Cells were fixed with cold absolute ethanol or 4% paraformaldehyde/PBS. For fluorescent immunostaining, we used rabbit anti-rat Oatp1 (Alfa Diagnostic International, San Antonio, TX), goat anti-mouse Oatp2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), mouse anti-human MRP2 (Alexis Biochemicals, San Diego, CA), and rabbit anti-human MRP3 antibodies (Sigma Chem Co.) as primary antibodies and rabbit anti-5NT (a gift from Dr. I. Wada, Fukushima Medical University, Japan). Alexa^{488}-conjugated anti-goat, mouse and rabbit IgG antibodies, and Alexa^{594}-conjugated anti-mouse IgG (Molecular Probe, Eugene, OR) were also used as secondary antibodies. 6-Diamino-2-phenylindole (DAPI; Sigma) was used for the staining of nuclei. A confocal laser microscope (Zeiss, Jena, Germany) was used for observation.

Uptake Studies of Radiolabeled Substances

At 15, 18, 21, 24 days SH colonies were preincubated with warmed transport buffer (116 mmol/L NaCl, 5.3 mmol/L KCl, 1.1 mmol/L

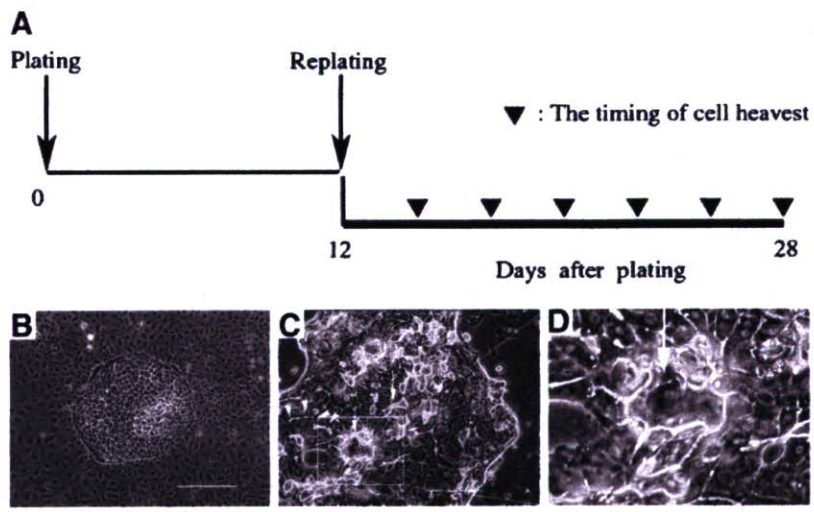


Fig. 1. (**A**) Illustration of experimental schedule. Phase-contrast micrographs of representative small hepatocyte (SH) colonies. (**B**) A flat colony is composed of only small cells and its morphology shows a monolayer at day 12. (**C**) A piled-up colony is composed of rising/piled-up cells. Translucent belts, which construct bile canaliculi (BC)-like structures, are observed between piled-up cells (arrowheads) in the colony at day 28. Typical cyst-like structures are indicated by arrows. (**D**) An enlargement of the cyst-like structure shown in C (white square). Bars, 200 μm.

TABLE I. XXXX[Q2]

Primer name		Sequence (5′-3′)	Annealing tenp. (°C)	Amplicon size (bp)
Oatp1	Sense	AAGCGAAGAAGCTGGAAACA	54	297
	Antisense	CACCACAGGTCTGTGCAGTT		
Oatp2	Sense	TGCACACTTAGCATTCTGGC	54	495
	Antisense	TGCATGTAACCCAACTCCAA		
Oatp4	Sense	GCCCAACCTTCACGATCAAA	56	604
	Antisense	GCCAAGGATTGGTCCAATCAT		
Ntcp	Sense	ATGCCCTTCTCTGGCTTTCT	56	499
	Antisense	GCTCCATGGTTCTGATGGTT		
Mrp1	Sense	TTCTAGTGTTGGACGAGGCT	58	208
	Antisense	TGGCCATGCTATAGAAGACG		
Mrp2	Sense	ACCTTCCACGTAGTGATCCT	56	1085
	Antisense	GATTTCCCAGACCCTACAGT		
Mrp3	Sense	CAGCGACAACGGGTGAGTTT	58	382
	Antisense	TCGGTGTCTGTCAGGTCTGTGT		
Mdr1b	Sense	GAAATAATGCTTATGAATCCCAAAG	54	302
	Antisense	GGTTTCATGGTCGTCGTCTCTTGA		
Mdr2	Sense	AAGAATTTGAAGTTGAGCTAAGTCA	54	1085
	Antisense	TGGTTTCCACATCCAGCCTAT		
Bcrp	Sense	CAGGTAGGCAATTGTGAGGAAGA	56	270
	Antisense	AATCAGGGCATCGATCTGTCA		
Bsep	Sense	GTTCAGTTCCTCCGTTCAAA	54	328
	Antisense	AAGCTGCACTGTCTTTTCAC		
HNF1a	Sense	AGCTGCTCCTCCATCATCAGA	63	118
	Antisense	TGTTCCAAGCATTAAGTTTTCTATTCTAA		
HNF3b	Sense	CCTACTCGTACATCTCGCTCATCA	58	43
	Antisense	CGCTCAGCGTCAGCATCTT		
HNF4	Sense	GTGAACTTCTTCTGGATGAG	57	572
	Antisense	TTGGCAACTATGACATTGGT		
G3PDH	Sense	ACCACAGTCCATGCCATCAC	57	582
	Antisense	TCCACCACCCTGTTGCTGTA		

KH_2PO_4, 0.8 mmol/L $MgSO_4$, 1.8 mmol/L $CaCl_2$, 11 mmol/L D-glucose and 20 mmol/L HEPES, adjusted to pH 7.4). Thereafter, they were initiated by adding 3 mL of pre-warmed choline-containing transport buffer supplemented with 1.5 μmol/L [^{3}H]-estrone-3-sulfate (E_1S, 250 Ci/mmol, Perkin Elmer Life & Analytical Sciences, Wellesley, MA). One hour later, the transport buffer was aspirated and the cells were immediately rinsed four times with a tracer-free ice-cold transport buffer [Boelsterli et al., 1995]. This procedure could remove more than 99% of the extracellular tracer. One milliliter of 1% Triton X-100/transport buffer was added to the culture plates and the cells were solubilized for 10 min at RT. One milliliter aliquots of the cell lysate were mixed with 10 mL of liquid scintillation cocktail (Aquazol-2, Perkin Elmer Life & Analytical Sciences) and the cell-associated radioactivity was determined using a liquid scintillation counter (LS 6000 L, Beckman Instruments, Inc., Fullerton, CA). Protein contents were determined with a BCA Protein Kit (Pierce, Rockford, IL). This experiment was performed in triplicate using at least three independent preparations.

Excretion Studies

Twenty-five milligrams of fluorescein diacetate (FD; Sigma) was dissolved in 1 mL of DMSO and dissolved FD was added to the culture medium (final FD concentration was 250 μg/ml). The cells were incubated in a 5% CO_2/95% air-incubator at 37°C for 10 min. Then the dish was rinsed three times with pre-warmed Hanks buffer solution. SH colonies were immediately photographed using a phase-contrast microscope equipped with a fluorescence device (Olympus).

SH colonies were incubated in 1.5 mL of Hanks buffer at 37°C for 10 min. The dish was incubated with 1.5 mL of the buffer containing 150 pmol/L [^{3}H]-estradiol-17β-D-glucuronide ($E_2$17βG, 50 Ci/mmol, American Radiolabeled Chem Inc., St. Louis, MO) at 37°C for 3 h. $E_2$17βG is absorbed mainly through Oatps and excreted through Mrp2 [Trauner and Boyer, 2003]. The incubation buffer was then removed and the dish was washed four times with 2 mL of ice-cold standard buffer to quench the transport activity and to remove the extracellular radioactivity. Dishes were incubated with 1 mL of the transport buffer or an excretion buffer (0.025% trypsin and 0.05% EGTA

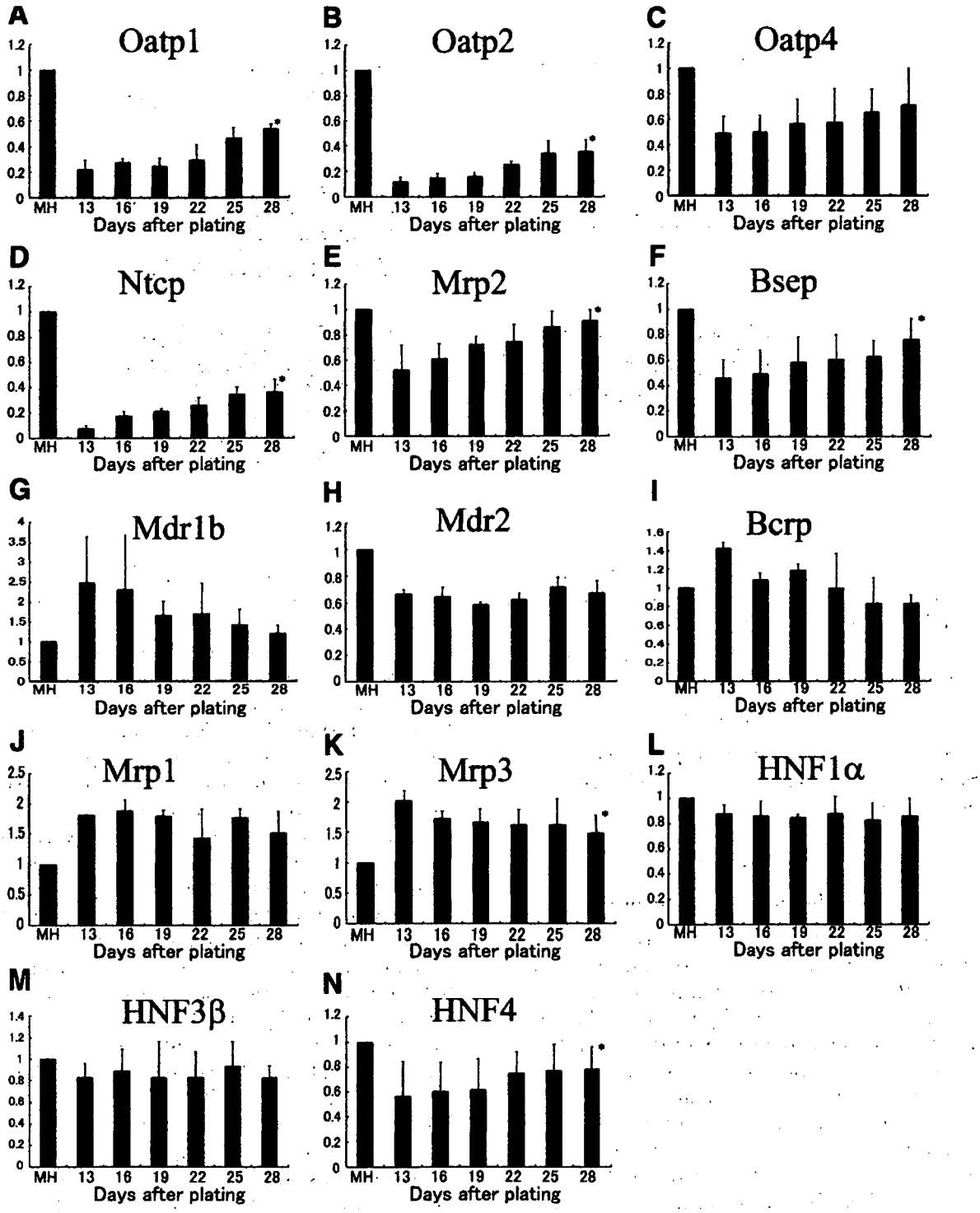


Fig. 2. Expression of hepatic transporter mRNAs in cultured SH. Cells were harvested at days 13, 16, 19, 22, 25, and 28. Each column shows the relative ratio of the mRNA expression in SHs to that in MHs: uptake transporters expressed in the basolateral domain, Oatp1 (**A**), Oatp2 (**B**), Oatp4 (**C**), and Ntcp (**D**); export transporters expressed in the apical domain, Mrp2 (**E**), Bsep (**F**), Mdr1b (**G**), Mdr2 (**H**), and Bcrp (**I**); export transporters expressed in the basolateral domain, Mrp1 (**J**) and Mrp3 (**K**). Expression of liver-enriched transcription factors HNF1α (**L**), HNF3β (**M**), HNF4α (**N**) is also shown. The value of mRNA expression of MH is 1.0. (**O**) Representative data from RT-PCR. The values are the averages of at least three independent experiments and bars show SD. Statistical analysis shows the comparison to the value at day 13; $^{*}P < 0.05$.

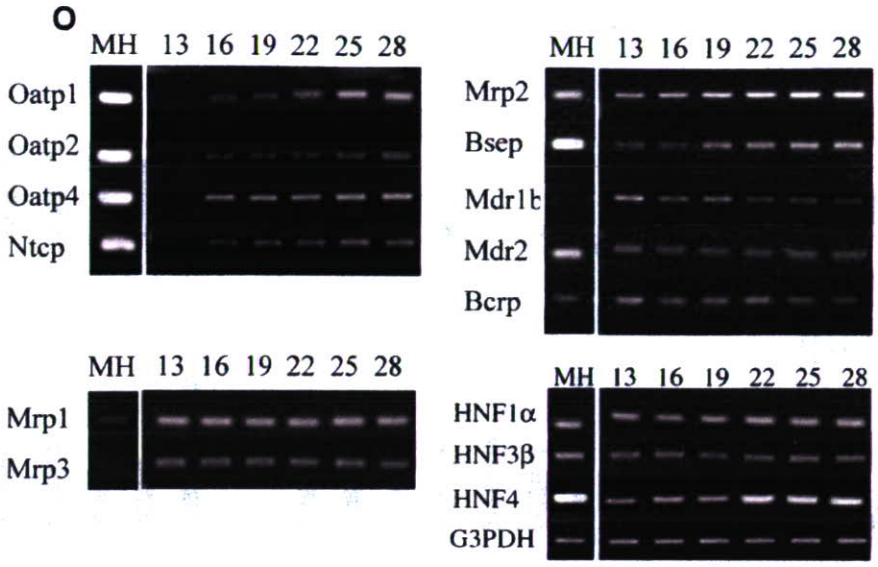


Fig. 2. *Continuted.*

in Ca^{2+}-free transport buffer) for 20 min. One milliliter aliquot of cell lysates was mixed with 10 mL of liquid scintillation cocktail and the cell-associated radioactivity was determined using a liquid scintillation counter.

Statistical Analysis

Statistical analysis was performed using Student's *t*-test. A *P*-value of <0.05 was considered significant.

RESULTS

Morphology of SH Colonies

When many colonies grew to consist of 30–50 cells (Fig. 1B), they were harvested and $2–4 \times 10^3$ colonies were replated on a new dish [Sugimoto et al., 2002; Miyamoto et al., 2005]. The SH colonies attached to the dishes and most attached cells could continue proliferating in a monolayer (flat colony) and form large colonies. About 7 days later, large and rising/piled-up cells appeared in the colonies and their number increased with time in culture. Between the cells BC (Fig. 1C) were formed and cyst-like structures (Fig. 1D, arrows) were sometimes observed in the late culture.

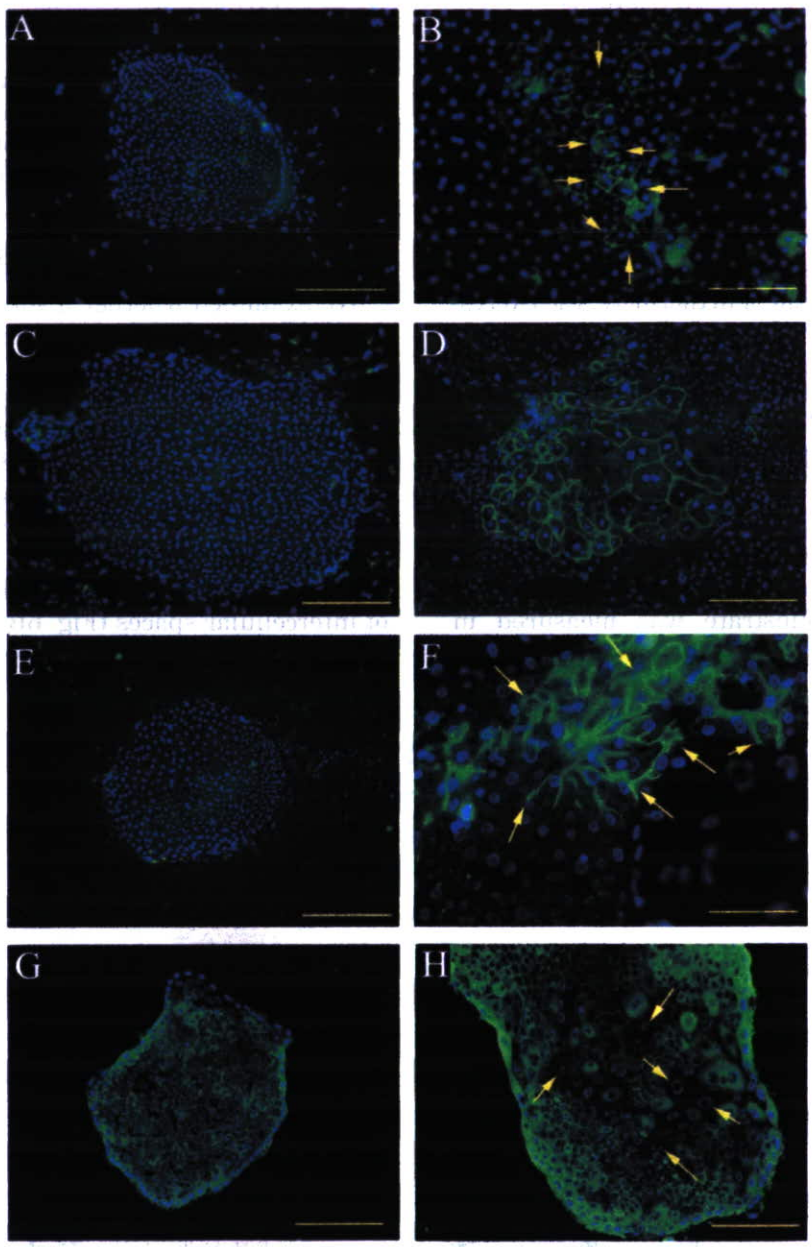
Expression of Hepatic Transporters in the SH Colony

The mRNA expression of hepatic organic anion transporters was examined using RT-PCR. The results are shown in Figure 2. At day 13 (1 day after replating) uptake transporters such as Oatp1 (Fig. 2A), Oatp2 (Fig. 2B), Oatp4 (Fig. 2C), and Ntcp (Fig. 2D) showed low expression but Oatp1, Oatp2, and Ntcp expression gradually increased with time in culture. However, even at day 28, their expression was lower than that of MH. Excretion transporters such as Mrp2 (Fig. 2E) and Bsep (Fig. 2F) located in the canalicular membrane were expressed at day 13 and their expression gradually increased. Although the expression of Bsep was about 60% that of MHs and that of Mrp2 was about 80% at day 28. On the other hand, while the expression of Mdr1b was not observed in MHs, SHs expressed it (Fig. 2G). Conversely, Mdr2 expression in SHs was about 60% of that in MHs and no change was observed during culture (Fig. 2H). Furthermore, Bcrp in SHs was well expressed at day 13 and the intensity gradually decreased nearly to that in MHs at day 28 (Fig. 2I). Both Mrp1 (Fig. 2J) and Mrp3 (Fig. 2K), which are scantly expressed in MHs, were continuously expressed in SHs.

The expression of transporters can be regulated by nuclear transcription factors [Trauner and Boyer, 2003; Kullak-Ublick et al., 2004]. As shown in Figures 2L–N, liver-enriched transcription factors such as HNF1α and 3β were constantly expressed in SHs just as in MHs and HNF4α expression increased with time in culture. This result was consistent with that of our previous report [Sugimoto et al., 2002]. Nuclear hormone receptors such as farnesoid

X activated receptor (FXR), pregnane X receptor (PXR), retinoic acid receptor (RAR), and retinoid X receptor (RXR) were constantly expressed during culture and the degree of their expression in SHs was the same as that in MHs, whereas the expression of constitutive androstane receptor (CAR) in SHs was much less than in MHs. However, expression of CAR gradually increased with time in culture (data not shown).

We immunocytochemically investigated the expression of the transporters. As shown in



COLOR

Fig. 3. Fluorescent immunocytochemistry for organic anion transporters in cultured SHs. A colony consisting of small SHs, which are actively proliferating, shows a flattened shape (**A**,**C**,**E**,**G**). A colony consisting of large (**B**,**D**) and piled-up cells (**F**,**H**). Oatp1 is not expressed in small cells (A), whereas Oatp1 is well expressed in cell membranes of large (B, arrows) and piled-up cells (data not shown). Oatp2 is not expressed in small cells (C), whereas Oatp2 is well expressed in whole cell membranes of large cells (D), which show a honeycomb pattern. Mrp2 is not expressed in small cells (E), whereas Mrp2 is restrictedly expressed in the BC-like structures that are formed between piled-up cells (F, arrows). Mrp3 is well expressed in the cell membranes of small cells (G). Although large cells in the colony strongly express Mrp3, the protein disappears in piled-up cells (H, arrows). All photos are of the same magnification. Hepatic transporters and nuclei are shown in green ($Alexa^{488}$) and blue (DAPI), respectively. Bars, 200 μm.

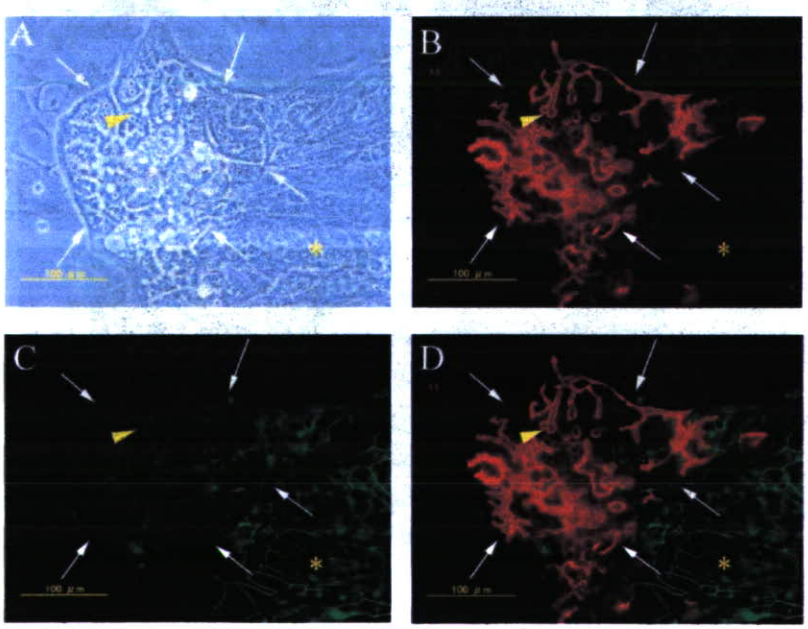
Figure 3, in a flat colony, Oatp1, Oatp2, and Mrp2 were not expressed on the cell membrane, whereas Mrp3 was clearly stained on the membranes of cells in the colony. On the other hand, in rising/piled-up cells Oatp1 and Oatp2 appeared on the membranes. When piled-up cells formed BC between the cells, Mrp2 was restrictedly expressed in the structure (Fig. 3F). The appearance of those proteins was correlated to the expression of their mRNAs. Different from Oatp1, Oatp2, and Mrp2, Mrp3 expression was inversely correlated to the morphology of the cells in a colony (Fig. 3G,H). Figure 4 clearly shows the alteration of Mrp3 expression. In small cells Mrp3 localized on the membrane, whereas in large cells the expression was observed in both the cytoplasm and membrane. Furthermore, the expression disappeared in the cells forming BC, in which Mrp2 was restrictedly expressed.

Uptake of Anion Transporter Substrate

Uptake of [^{3}H]-E_1S, which is passed through Oatps and excreted through Bcrp (Suzuki et al., 2003; Trauner and Boyer, 2003), was measured in the SH culture. MHs cultured for 3 h were used as a control. First, the time-dependent uptake of the substrate was measured in colonies possessing many rising/piled-up cells. As shown in Figure 5A, the amount of E_1S uptake in MHs rapidly increased until 10 min after the addition and then the uptake was saturated. However, the uptake in SHs gradually increased until 60 min. Thereafter, it reached a plateau (data not shown). The total amount of E_1S in SHs was about one-third of that in MHs at 60 min. The amount of the uptake in SHs increased with time in culture and the uptake was near the maximum at day 21 (Fig. 5B). Thereafter, a significant increase was not observed. The ability of E_1S uptake in SH colonies may be correlated to the number of rising/piled-up cells.

Recovery From the Substances From BC

We examined whether a substance excreted into BC could be recovered without impairment of the cells. The duration of the treatment time and the concentrations of chelating agents and/or trypsin were examined (data not shown). Finally, the combination of 0.025% trypsin and 0.05% EGTA in Ca^{2+}-free transport buffer was chosen as an excretion buffer. As shown in Figure 6A,B, treatment with the excretion buffer for 20 min resulted in the enlargement of intercellular spaces (Fig. 6B), but the treatment did not affect the cell viability as



COLOR

Fig. 4. Fluorescent immunocytochemistry for Mrp2 (red) and Mrp3 (green) in SHs. Cells were fixed at day 24. (**A**) A phase-contrast photograph of piled-up cells (white arrows) in a colony. BC-like structures are observed between the cells. (**B**) Mrp2 localizes along the structure (yellow arrowhead) corresponding to the red arrowhead in (A), whereas small cells do not express Mrp2. White arrows show the piled-up cells corresponding to black arrows in (A). (**C**) Mrp3 is expressed in small cells in a colony (asterisk), whereas piled-up cells (white arrows) do not show any positivity. (**D**) Double-fluorescent immunocytochemistry shows no co-expression of Mrp2 and Mrp3 in the colony. All photos are of the same magnification. Bars, 100 μm.

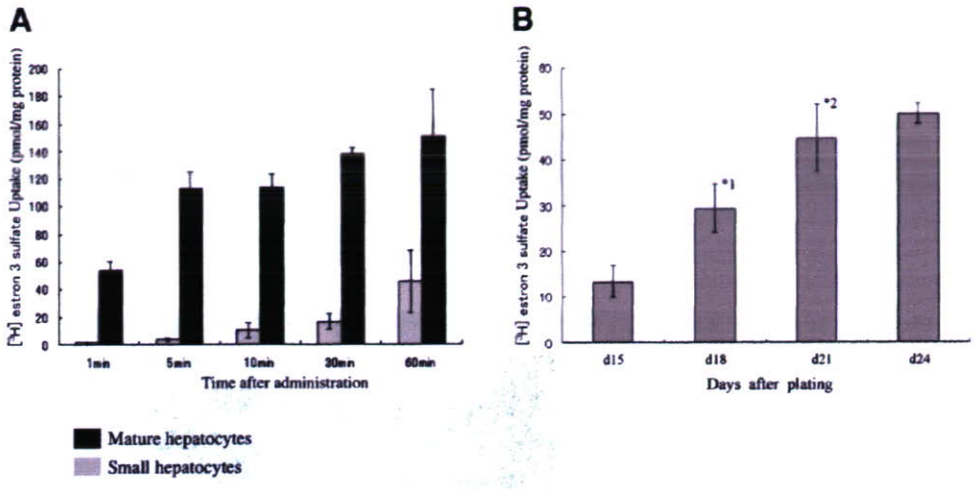


Fig. 5. Uptake and excretion of [^{3}H]-labeled estrogen derivatives of mature SH. (**A**) Uptake of [^{3}H]-estron-3-sulfate by SH at day 24 and MH at 24 h after plating. [^{3}H]-estron-3-sulfate was applied for 1 h. (**B**) Uptake of [^{3}H]-estron-3-sulfate by SH with time in culture. The details of the method are described in Materials and Methods section. Statistical analysis: $P < 0.01$ versus d15,*^{1} $P < 0.05$ versus d18*^{2}.

evidenced by trypan blue exclusion (data not shown). In fact, the cells could form BC after the experiment. To confirm the excretion from BC, FD was added to the cells. It is known that FD is metabolized in hepatocytes and that the fluorescein is transported through Mrp2 into BC. Fluorescein was restrictedly observed in the BC/cyst-like structure (Fig. 6D, arrows) corresponding to the translucent spaces between the piled-up cells (Fig. 6C, arrows). Although the treatment with the control buffer did not affect the fluorescent image of the cells, 20 min after the treatment, fluorescein observed in BC completely disappeared (Fig. 6E, arrows).

Next, we examined whether $E_2$17βG excreted into BC could be efficiently recovered. As shown in Figure 6F, at day 21 the radioactivity of the solution recovered from the cells treated with the excretion buffer was significantly higher than that from the cells treated with the control one. At day 24 the difference was markedly enlarged although the radioactivity of the control increased. This result demonstrated that, whenever SHs matured to form well-developed BC, $E_2$17βG excreted to BC/cyst-like structures could be easily collected.

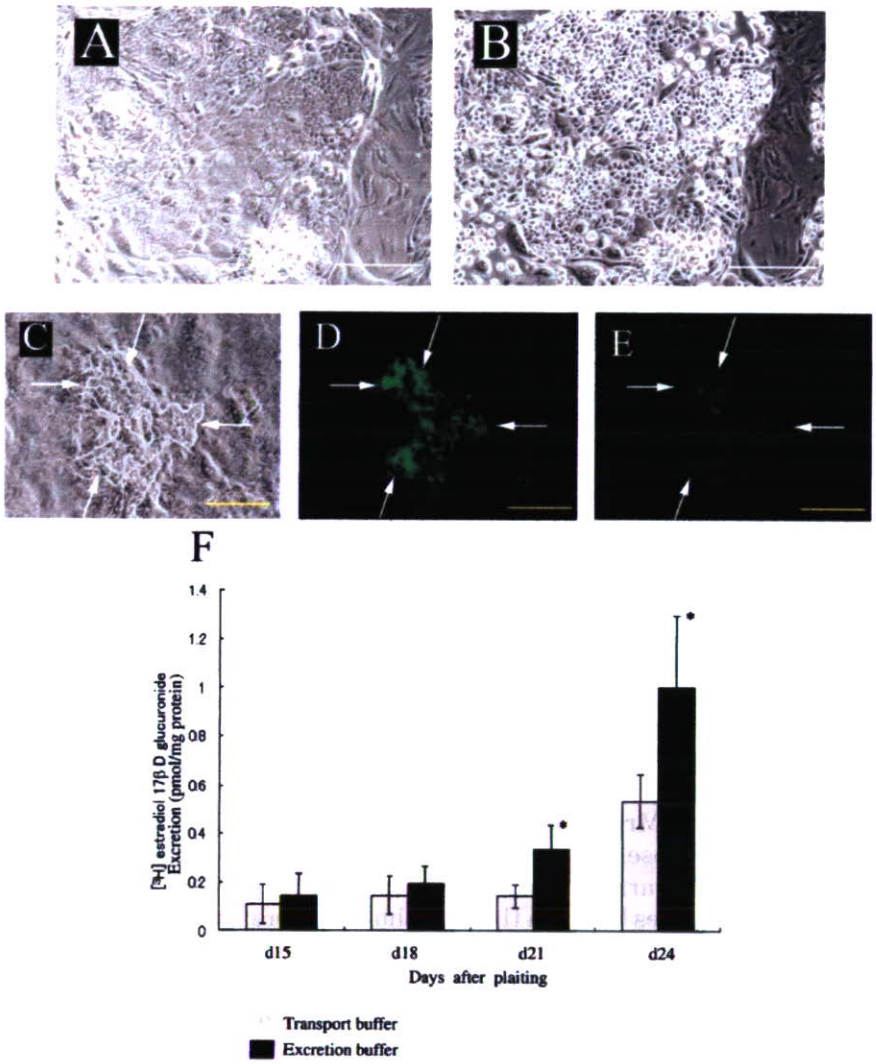
Expression of Organic Anion Transporters in EHBR

To confirm whether $E_2$17βG could be transported into BC via Mrp2 and recovered from the structure, SHs isolated from an EHBR (EHBR-SH) were used to form BC. EHBR-SHs could proliferate to form a colony similar to those derived from SDR. As shown in Figure 7A, neither MHs nor SHs derived from EHBR expressed Mrp2. Although SDR-MHs did not express Mrp3, EHBR-MHs clearly expressed it. No difference was observed in the expression of Oatp1, Oatp2, and Oatp4 between SDR and EHBR. EHBR-SHs could form BC in the colony, and 5NT (Fig. 7B-b) and ectoATPase (data not shown) were immunocytochemically stained along the structure as with SDR-SHs. Immunostaining also showed that Mrp2 was not expressed in the BC formed by the piled-up cells from EHBR (Fig. 7B-a), but that it was intensely stained in both the membrane and cytoplasm (Fig. 7B-c). In contrast, Mrp3 expression disappeared in the piled-up cells from SDR (Fig. 4).

$E_2$17βG was added to EHBR cells, which developed BC well, and they were treated with the excretion buffer for 20 min. The radioactivity of $E_2$17βG in the collected solution was not different between the excretion and control buffers (Fig. 7C). This result showed that $E_2$17βG might be not excreted into BC and that alternative transporters might make no or only a minor contribution to the excretion of $E_2$17βG into BC in Mrp2-deficient hepatocytes.

DISCUSSION

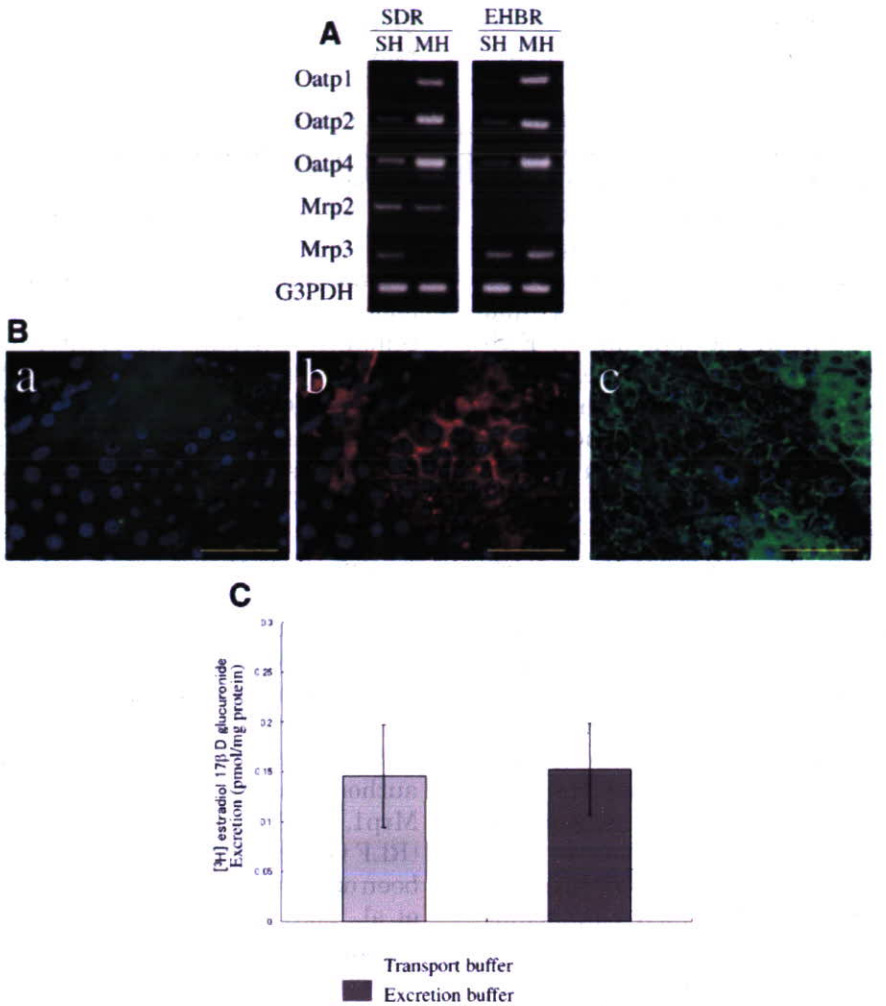
In the present experiment we showed that, although the expression levels were lower than those of MHs, basolateral (Oatp1/2/4 and



COLOR

Fig. 6. Effects of the exclusion buffer on the cells forming BC-like structures of an SH colony. Phase-contrast photos show the morphological changes of the cells before (**A**) and after (**B**) treatment with exclusion buffer. (**C**) A phase-contrast photograph of piled-up cells forming a BC-like structure in a colony (arrows). (**D**,**E**) Fluorescent photographs of the piled-up cells in the colony show the colony corresponding to that in (C). FD was added to the culture medium. (D) Fluorescein secreted into a BC-like structure is observed in the piled-up lesion of the colony (arrows). (**E**) Twenty minutes after the treatment with the exclusion buffer, accumulated fluorescein is excluded from the structures. All photos are of the same magnification. Bars, 200 μm. (**F**) Excretion of accumulated [^{3}H]-estradiol-17β-D-glucuronide from the BC-like structures formed in piled-up cells in SH colonies. The details of the method are described in Materials and Methods section. Statistical analysis: $^*P < 0.01$ versus control buffer.

Ntcp), and canalicular transporters (Mrp2/Mdr2/Bsep) were expressed in SHs even after about 1 month of culture. The expression patterns of individual transporters were different. The expression of Oatp1, Oatp2, Ntcp, and Bsep mRNAs was initially very low and gradually increased, correlated to the increase in the number of mature SHs. It is well known that expression of most hepatic transporters is regulated by transcription factors [Trauner and Boyer, 2003]. For example, HNF1α is related to the expression of Oatp1/2/4 and Ntcp, HNF3β to that of Ntcp/Mdr2, and HNF4α to that of Oatp1/Ntcp/Bsep/Mdr2. On the other hand, nuclear receptors such as RAR and FXR regulate the expression of Ntcp/Mrp2 and Bsep/Mrp2, respectively. In addition, PXR and CAR are involved in the regulation of Oatp2/Mrp2 and Mrp2, respectively. In the present experiment the expression of HNF4α and CAR in SHs increased with time in culture although the intensity of their expression was lower than that of the expression in MHs. In contrast, the expression of HNF1α, HNF3β, RAR, FXR, PXR,



COLOR

Fig. 7. Expression of hepatic transporters in Eisai hyperbilirubinemic rat (EHBR) and Sprague-Dawley rats (SDR). (**A**) Expression of organic anion transporter mRNAs in SH and MH isolated from an EHBR and an SD rat. (**B**) Fluorescent immunocytochemistry for Mrp2, Mrp3, and 5NT in SHs isolated from an EHBR at day 28. BC-like structures formed in an EHBR-derived SH colony are stained with an anti-5NT antibody (red, b), whereas the corresponding structure is not stained with an anti-Mrp2 antibody (green, a). Mrp3 is expressed in SHs isolated from an EHBR rat (c). (**C**) Excretion of [^{3}H]-estradiol-17β-D-glucuronide from BC-like structures formed in an EHBR-derived SH colony at day 28. No difference in the excretion of radioactive substances is observed between the excretion and control buffer. Bars, 100 μm.

and RXR was not changed for the whole culture period and the intensity of the expression in SHs was similar to that of the expression in MHs. In this study, although the details of the regulation of these transporters were not well investigated, the upregulation of HNF4α and CAR with SH maturation may play a crucial role in the increased expression of Oatp1, Ntcp, and Mrp2.

Primary rat and human hepatocytes cultured in the collagen-sandwich configuration have been extensively characterized for use in estimating the biliary clearance of drugs in vitro [Liu et al., 1999a,b; Hoffmaster et al., 2004; Lengyel et al., 2005; Turncliff et al., 2006]. However, short-term maintenance of some hepatic transporters was reported in sandwich-cultured primary hepatocytes [Liu et al., 1999b; Luttringer et al., 2002; Hoffmaster et al., 2004]. In sandwiched hepatocytes Oatp2 expression decreased and that of Oatp1 was maintained, whereas the expression of excretion transporters such as Mdrs and Mrp2 increased with time in culture [Hoffmaster et al., 2004]. Furthermore, the taurocholate uptake decreased with time in culture, which might be correlated to lesser expression of Ntcp [Liu et al., 1999a], and the uptake of opioid peptide, which is a substrate for Oatps, decreased by half at 5 days of culture [Hoffmaster et al., 2004]. In contrast, the expression of Oatp1/2/4, Ntcp,

Mrp2, Mdr2, and Bsep in SHs could be expressed and maintained for more than 1 month. During the culture, the distribution of Oatp2 and Mrp2 proteins dramatically changed without an apparent alteration of gene expressions; Oatp2 appeared in the basolateral membranes of both large and rising/piled-up cells and Mrp2 in the BC membranes of rising/piled-up cells. Increased activity of the transporters was also found in the experiments with [^{3}H]-E_1S and [^{3}H]-$E_2$17βG treatment; the amounts of E_1S- and $E_2$17βG-uptake and of $E_2$17βG excretion into BC increased with the maturation of SHs. Therefore, compared to the sandwiched cells, mature SHs may maintain expression of most hepatic transporters for a much longer period and reflect the state of the uptake and excretion of organic anions in in vivo hepatocytes more exactly than collagen-sandwiched cells.

It is of interest that Mrp3 expression disappeared from the basolateral domain in rising/piled-up cells in this culture. Although Mrp3 expression is quite low in the normal rat liver, it is dramatically upregulated in cholestasis [Ogawa et al., 2000; Donner and Keppler, 2001]. Mrp3 expression increases after common bile duct ligation in the rat and in hepatocytes in patients with Dubin–Johnson syndrome and the TR-/GY/EHBR rat in which canalicular expression of Mrp2 is genetically absent [Hirohashi et al., 1998; Konig et al., 1999; Donner and Keppler, 2001]. In the present experiment, Mrp3 was expressed in small and large cells in colonies, whereas the protein dramatically disappeared in piled-up cells. On the other hand, Mrp3 was highly expressed in the membranes of rising/piled-up cells from EHBR, and collagen-sandwiched hepatocytes also expressed it for the entire culture period [Tian et al., 2004]. These phenomena may indicate that, when excretion through Mrp2 is inhibited for some reason, accumulation of the substrates for Mrp2 generates pressure to excrete them from cells by inducing Mrp3. One explanation for the disappearance of Mrp3 may be the formation of BC/cyst-like structures in SH colonies and the distribution of Mrp2 in BC membranes creating a route for excretion and releasing the pressure. Although the BC network in the culture is a closed system and the excreted substances accumulate within the canal, cystic structures formed in a colony gradually become large. Considering the fact that the upregulation of Mrp3 is often observed in cholestatic conditions, the cyst formation may release the pressure of accumulating bile in closed BC and create a flow directly to the cyst.

Pfändler et al. [2004] also demonstrated that time-dependent expression of individual transporters correlating with the maturation of SHs in growing colonies had some striking similarities with the ontogenesis of the hepatocellular bile-salt and organic-anion transport polarity. We have demonstrated that the SH is a committed hepatic progenitor cell that can further differentiate into an MH [Mitaka et al., 1999; Kon et al., 2006]. The degree of 'differentiation of SHs' may be more than for hepatoblasts and less than for MHs [Mitaka et al., 1999; Mitaka, 2001]. Under conditions in which the proliferation of MHs is inhibited by treatment with hepatotoxins, liver damage results in the activation of hepatic progenitor cells. Ros et al. [2003] reported that, compared to MHs, hepatic progenitor cells (oval cells) possessed high expression of ABC transporters such as Mrp1 and Mrp3 and low expression of Mdr2, Bsep, and Mrp2. Furthermore, the authors also observed high expression of Mdr1b, Mrp1, and Mrp3 in a hepatic progenitor cell line (RLF Ø13 cells). Recently, Bcrp expression has been observed in hematopoietic stem cells [Zhou et al., 2001; Alison, 2003] and stem cells (side population cells) from other tissues such as skeletal muscle [Tamaki et al., 2002], pancreas islets [Lechner et al., 2002], and heart [Martin et al., 2004]. Although Shimano et al. [2003] reported the expression of Bcrp in oval cells, SHs had high expression of Mrp1 and Mrp3, not Bcrp. The high expression of those transporters in SHs may be very similar to the expression pattern seen in hepatic progenitor cells reported by Ros et al. [2003]. In the hepatic progenitor cells, instead of Brcp, Mrp1, and Mrp3 may have an important role in removing both exogenous and endogenous toxic drugs/metabolites from cells, which may enable them to survive in conditions associated with excessive metabolic stress and serve as a proliferative reservoir.

In conclusion, the present study shows that rat SHs mature and reconstruct a hepatic organoid consisting of rising/pile-up cells, which develops complicated BC-networks. Major hepatocellular bile salt and organic-anion transport systems may be established in this hepatic organoid and maintained for more than 1 month. The vectorial transport of substrates from medium to BC is functionally active and

the excreted substrates can be efficiently recovered from BC. This culture system may be useful for estimation of the biliary clearance of substances. Further experiments and new strategies may be necessary to produce sufficiently large numbers of mature SHs in vitro. In addition, the establishment of a method for the isolation and culture of human SHs is urgently needed for future use in artificial liver devices and as a source for liver transplantation.

NOMENCLATURE AND GENE SYMBOLS OF TRANSPORTERS

Oatp1	Oatp1a1/*Slco1a1*
Oatp2	Oatp1a4/*Slco1a4*
Oatp4	Oatp1b2/*Slco1b2*
Ntcp	Slc10a1
Bsep	Abcb11
Mrp1	Abcc1
Mrp2	Abcc2
Mrp3	Abcc3
Mdr1b	Abcb1;
Mdr2	Abcb4
Bcrp	Abcg2

ACKNOWLEDGMENTS

We thank Dr. Yamato Kikkawa (Tokyo University of Pharmacy and Life Science, Japan) and Dr. Kazunori Sasaki for suggestive discussions, and Ms. Minako Kuwano for technical assistance. We also thank Mr. K. Barrymore for help with the manuscript. This study was supported by grants of the Science and Technology Incubation Program in Advanced Region, the Japan Science and Technology Agency and of the Ministry of Education, Culture, Sports, Science and Technology, Japan (14370393 and 17390353 for T. Mitaka; 15390403 for K. Hirata), and Ministry of Health, Labour and Welfare, Health and Labour Sciences Research Grants, Research on Advanced Medical Technology for T. Mitaka.

REFERENCES

Alison MR. 2003. Tissue-based stem cells: ABC transporter proteins take centre stage. J Pathol 200:547–550.

Belinsky MG, Bain LJ, Balsara BB, Testa JR, Kruh GD. 1998. Characterization of MOAT-C and MOAT-D, new members of the MRP/cMOAT subfamily of transporter proteins. J Natl Cancer Inst 90:1735–1741.

Berthiaume F, Moghe PV, Toner M, Yarmush M. 1996. Effect of extracellular matrix topology on cell structure, function, and physiological responsiveness: Hepatocytes cultured in a sandwich configuration. FASEB J 10:1471–1484.

Boelsterli UA, Zimmerli B, Meier PJ. 1995. Identification and characterization of a basolateral dicarboxylate/cholate antiport system in rat hepatocytes. Am J Physiol 268:G797–G805.

Buchler M, Konig J, Brom M, Kartenbeck J, Spring H, Horie T, et al[Q3]. 1996. cDNA cloning of the hepatocyte canalicular isoform of the multidrug resistance protein, cMrp, reveals a novel conjugate export pump deficient in hyperbilirubinemic mutant rats. J Biol Chem 271: 15091–15098.

Bunting[Q4] KD. 2002. ABC transporters as phenotypic markers and functional regulators of stem cells. Stem Cells 20:11–20.

Donner MG, Keppler D. 2001. Up-regulation of basolateral multidrug resistance protein 3 (Mrp3) in cholestatic rat liver. Hepatology 34:351–359.

Dunn JCY, Yarmush ML, Koebe HG, Thompkins RG. 1989. Hepatocyte function and extracellular matrix geometry: Long-term culture in a sandwich configuration. FASEB J 3:174–177.

Hirohashi T, Suzuki H, Ito K, Ogawa K, Kume K, Shimizu T, et al[Q5]. 1998. Hepatic expression of multidrug resistance-associated protein-like proteins maintained in Eizai hyperbilirubinemic rats. Mol Pharmacol 53:1068–1075.

Hoffmaster KA, Turncliff RZ, LeCluyse EL, Kim RB, Meier PJ, Brouwer KLR. 2004. P-glycoprotein expression, localization, and function in sandwich-cultured primary rat and human hepatocytes: Relevance to the hepatobiliary disposition of a model opioid peptide. Pharm Res 21:1294–1302.

Keppler D, Konig J. 2000. Hepatic secretion of conjugated drugs and endogenous substances. Semin Liver Dis 20:265–272.

Kon J, Ooe H, Oshima H, Kikkawa Y, Mitaka T. 2006. Expression of CD44 in rat hepatic progenitor cells. J Hepatology 40:90–98.

Konig J, Rost D, Cui Y, Keppler D. 1999. Characterization of the human multidrug resistance protein isoform MRP3 localized to the basolateral hepatocyte membrane. Hepatology 29:1156–1163.

Kool M, van der Linden M, de Haas M, Scheffer GL, de Vree JM, Smith AJ, et al[Q6]. 1999. MRP3, an organic anion transporter able to transport anti-cancer drugs. Proc Natl Acad Sci USA 96:6914–6919.

Kullak-Ublick GA, Stieger B, Meier PJ. 2004. Enterohepatic bile salt transporters in normal physiology and liver disease. Gastroenterology 126:322–342.

Lechner A, Leech CA, Abraham EJ, Nolan AL, Habener JF. 2002. Nestin-positive progenitor cells derived from adult human pancreatic islets of Langerhans contain side population (SP) cells defined by expression of the ABCG2 (BCRP1) ATP-binding casset transporter. Biochem Biophys Res Commun 293:670–674.

LeCluyse EL, Audus KL, Hochman JH. 1994. Formation of extensive canalicular networks by rat hepatocytes cultured in collagen-sandwich configuration. Am J Physiol 266:C1764–C1774.

Lee J, Boyer JL. 2000. Molecular alterations in hepatocyte transport mechanisms in acquired cholestatic liver disorders. Semin Liver Dis 20:373–384.

Lengyel G, Veres Z, Szabó P, Vereczkey L, Jemnitz K. 2005. Canalicular and sinusoidal disposition of bilirubin mono- and diglucuronides in sandwich-cultured human and rat primary hepatocytes. Drug Metab Dispos 33:1355–1360.

Liang D, Hagenbuch B, Stieger B, Meier PJ. 1993. Parallel decrease of Na^+-taurocholate cotransporter and its encoding mRNA in primary cultures of rat hepatocytes. Hepatology 18:1162–1166.

Liu X, Brouwer KLR, Gan L-SL, Brouwer KR, Stieger B, Meier PJ, et al[Q7]. 1998. Partial maintenance of taurocholate uptake by adult rat hepatocytes cultured in a collagen sandwich configuration. Pharm Res 15:1533–1539.

Liu X, Chism JP, LeCluyse EL, Brouwer KR, Brouwer KLR. 1999a. Correlation of biliary excretion in sandwich-cultured rat hepatocytes and in vivo in rats. Drug Metab Dispos 27:637–644.

Liu X, LeCluyse EL, Brouwer KR, Lightfoot RM, Lee JI, Brouwer KLR. 1999b. Use of $Ca2^+$ modulation to evaluate biliary excretion in sandwich-cultured rat hepatocytes. Am J Physiol Gastrointest Liver Physiol 277:G12–G21.

Luttringer O, Theil FP, Lave T, Wernli-Kuratli K, Guentert TW, de Saizieu A. 2002. Influence of isolation procedure, extracellular matrix and dexamethasone on the regulation of membrane transporters gene expression in rat hepatocytes. Biochem Pharmacol 64:1637–1650.

Martin CM, Meeson AP, Robertson SM, Hawke TJ, Richardson JA, Bates S, et al[Q8]. 2004. Persistent expression of the ATP-binding cassette transporter, Abcg2, identifies cardiac SP cells in the developing and adult heart. Dev Biol 265:262–275.

Maurice M, Rogier E, Cassio D, Feldmann G. 1988. Formation of plasma membrane domains in rat hepatocytes and hepatoma cell lines in culture. J Cell Sci 90:79–92.

Meier PJ, Stieger B. 2002. Bile salt transporters. Annu Rev Physiol 64:635–661.

Meijer DK. 1987. Current concepts on hepatic transport of drugs. J Hepatol 4:259–268.

Mitaka T. 2001. Hepatic stem cells: From bone marrow cells to hepatocytes. Biochem Biophys Res Commun 281:1–5.

Mitaka T, Mikami M, Sattler GL, Pitot HC, Mochizuki Y. 1992. Small cell colonies appear in the primary culture of adult rat hepatocytes in the presence of nicotinamide and epidermal growth factor. Hepatology 16:440–447.

Mitaka T, Kojima T, Mizuguchi T, Mochizuki Y. 1995. Growth and maturation of small hepatocytes isolated from adult rat liver. Biochem Biophys Res Commun 214:310–317.

Mitaka T, Sato F, Mizuguchi T, Yokono T, Mochizuki Y. 1999. Reconstruction of hepatic organoid by rat small hepatocytes and hepatic nonparenchymal cells. Hepatology 29:111–125.

Miyamoto S, Hirata K, Sugimoto S, Harada K, Takeda H, Mitaka T. 2005. Expression of cytochrome P450 enzymes in hepatic organoid reconstructed by rat small hepatocytes. J Gastroenterol Hepatol 20:865–872.

Musat AI, Sattler CA, Sattler GL, Pitot HC. 1993. Reestablishment of cell polarity of rat hepatocytes in primary culture. Hepatology 18:198–205.

Ogawa K, Suzuki H, Hirohashi T, Ishikawa T, Meier PJ, Hirose K, et al[Q9]. 2000. Characterization of inducible nature of MRP3 in rat liver. Am J Physiol Gastrointest Liver Physiol 278:G438–G446.

Pfändler MAS, Höchli M, Inderbitzin D, Meier PJ, Stieger B. 2004. Small hepatocytes in culture develop polarized transporter expression and differentiation. J Cell Sci 117:4077–4087.

Rippin SJ, Hagenbuch B, Meier PJ, Stieger B. 2001. Cholestatic expression pattern of sinusoidal and canalicular organic anion transport systems in primary cultured rat hepatocytes. Hepatology 33:776–782.

Ros JE, Roskams TAD, Geuken M, Havinga R, Splinter PL, Petersen BE, et al[Q10]. 2003. ATP binding cassette transporter gene expression in rat liver progenitor cells. Gut 52:1060–1067.

Scheffer GL, Kool M, de Haas M, de Vree JM, Pijnenborg AC, Bosman DK, et al[Q11]. 2002. Tissue distribution and induction of human multidrug resistant protein 3. Lab Invest 82:193–201.

Shimano K, Satake M, Okaya A, Kitanaka J, Kitanaka N, Takemura M, et al[Q12]. 2003. Hepatic oval cells have the side population phenotype defined by expression of ATP-binding cassette transporter ABCG2/BC RP1. Am J Pathol 163:3–9.

Sudo R, Ikeda S, Sugimoto S, Harada K, Hirata K, Tanishita K, et al[Q13]. 2004. Bile canalicular formation in hepatic organoid reconstructed by rat small hepatocytes and nonparenchymal cells. J Cell Physiol 199:252–261.

Sugimoto S, Mitaka T, Ikeda S, Harada K, Ikai I, Yamaoka Y, et al[Q14]. 2002. Morphological changes induced by extracellular matrix are correlated with maturation of rat small hepatocytes. J Cell Biochem 87:16–28.

Suzuki M, Suzuki H, Sugimoto Y, Sugiyama Y. 2003. ABCG2 transports sulfated conjugates of steroids and xenobiotics. J Biol Chem 278:22644–22649.

Tamaki T, Akatsuka A, Ando K, Nakamura Y, Matsuzawa H, Hotta T, et al[Q15]. 2002. Identification of myogenic-endothelial progenitor cells in the interstitial spaces of skeletal muscle. J Cell Biol 157:571–577.

Tian X, Zamek-Gliszczynski MJ, Zhang P, Brouwer KLR. 2004. Modulation of multidrug resistance-associated protein 2 (Mrp2) and Mrp3 expression and function with small interfering RNA in sandwich-cultured rat hepatocytes. Mol Pharmacol 66:1004–1010.

Trauner M, Boyer JL. 2003. Bile salt transporters: Molecular characterization, function, and regulation. Physiol Rev 83:633–671.

Turncliff RZ, Hoffmaster KA, Kalvass JC, Pollack GM, Brouwer KLR. 2006. Hepatobiliary disposition of a drug/metabolite pair: Comprehensive pharmacokinetic modeling in sandwich-cultured rat hepatocytes. J Pharm Exp Ther 318:881–889.

Zhou S, Schuetz JD, Bunting KD, Colapietro AM, Sampath J, Morris JJ, et al[Q16]. 2001. The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. Nat Med 7:1028–1034.

Q1: Please check the grant sponsors.

Q2: Please provide the caption for Table I

Q3: Please provide all the authors name.

Q4: Please cite the reference in the text.