Drug Metabolism Reviews focusing on drug transporter interactions in the liver

Characterization of hepatic-organoid cultures

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Key words: hepatic organoid, stem/progenitor cells, small hepatocytes, differentiation/maturation, polarity, extracellular matrix, hepatic nonparenchymal cells, bile canaliculi

Abbreviations:
AhR, aryl hydrocarbon receptor; BC, bile canaliculi; BD, bile ducts; BM, basement membrane; BSEP, bile-salt export pump; CAR, constitutive androstane receptor; C/EBP, CCAAT/enhancer binding protein; ECM, extracellular matrix; EGF, epidermal growth factor; FD, fluorescent diacetate; FXR, farnesoid X-activated receptor; HA, hyaluronic acid; HB, hepatoblast; HGF, hepatocyte growth factor; HNF, hepatocyte nuclear factor; LEC, liver epithelial cell; HSC, hepatic stem/progenitor cell; LETF, liver-enriched transcription factor; MDR2, multidrug resistance 2; MH, mature hepatocyte; MRP2, multidrug resistant related protein 2; NPC, nonparenchymal cell; NTCP, Na^+-dependent taurocholate co-transporting polypeptide; OATP, Na^+-independent organic anion-transporting protein; PXR, pregnane X receptor; RXR, retinoid X receptor; rER, rough endoplasmic reticulum; SEC, sinusoidal endothelial cell; SDH, serine dehydratase; SH, small hepatocyte; TO, tryptophan 2,3'-dioxygenase

Nomenclature and gene symbols of transporters in this manuscript:
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*;

Abstract

Small hepatocytes (SHs) are "committed progenitor cells" that can further differentiate into mature hepatocytes (MHs). SHs can proliferate to form colonies and the maturation of SHs occurs with the alteration of the cell shape from small and flat to large and rising/piled-up. The hepatic organoids consisting of rising/piled-up cells possess highly differentiated functions like those of MHs and anastomosing networks of bile canaliculi (BC) are developed. The cells can make bile, secrete it into BC, and the bile can be carried without leaking. Thus, the organoids consist of polarized hepatocytes and possess biochemical and physiological functions as hepatic tissue.

Introduction

The liver is one of the most, structurally and functionally, complicated organs in mammals. However, the liver is simply considered to be an aggregate of small histologic units, lobules. Each lobule consists of mature hepatocytes (MHs) and sinusoidal lining cells such as sinusoidal endothelial cells (SECs), stellate (Ito) cells, Kupffer cells, and Pit cells. Of these cells, MHs play the most important roles in hepatic functions: 1) metabolism of nutrition, 2) production of plasma proteins, 3) synthesis and secretion of bile, 4) drug metabolism and detoxification, 5) storage of vitamins and iron, and so on. All MHs may possess all those functions. Other cells work to support MHs so that they can perform their functions as efficiently as possible and for immunological protection. The study of primary culture of MHs has revealed that most differentiated functions of hepatocytes are very difficult to maintain and regain. For the purpose of maintaining the differentiated functions, many researchers have used various substances and changed culture conditions through the use of nicotinamide (Inoue, 1989; Mitaka, 1991), dimethylsufoxide (DMSO; Isom, 1985), and coculture with nonparenchymal cells (NPCs; Guguen-Guillouzo, 1986). In addition, various substrata have been used as beds for the cells, e.g., biomatrices (Rojkind, 1980), proteoglycans (Spray, 1987), collagen gel (Michalopoulos, 1976; Dunn, 1992), and Engelbreth-Holm-Swarm sarcoma-derived gel (EHS) gel (Bissell, 1987; Ben-Ze'ev, 1988; Schuetz, 1988; Musat, 1993). Three-dimensional culture of hepatocytes has also been developed to reconstruct functional hepatic tissues in vitro. Although spheroid formations (Landry, 1985; Koide, 1990; Abu-Absi, 2002) and collagen-gel sandwich culture (Dunn, 1989; LeCluyse, 1994; Berthianume, 1996; Liu, 1998) of primary hepatocytes can maintain hepatic differentiated functions for extended periods, the functions are eventually lost and are thereafter never recovered with time in culture.

Rat small hepatocytes (rSHs) were first found as proliferating small cells among primary hepatocytes cultured in medium supplemented with 10 mM nicotinamide and epidermal growth factor (EGF) (Mitaka, 1992). The first division of an SH is observed at days 2-3 after plating and the cells rapidly proliferate to form a colony (Mitaka, 1995). The average number of cells in a colony reaches about 30 at day 10. The colony continues to expand for more than 2 months. SHs are initially mononucleate cells and the morphology is quite similar to that of hepatocytes except that the size is small (Mitaka, 1992b). Ultrastructurally, the cells have characteristic organelles, mitochondria, rough endoplasmic reticulum (rER), and peroxisomes in their cytoplasm. Cell adhesions such as tight junctions, gap junctions, and desmosomes are also observed and bile canalicular-like structures are formed between the cells. Although SHs have characteristics of hepatocytes, the cells are less differentiated than MHs (Mitaka, 1998a, 1998b). Therefore, we consider that SHs are "committed progenitor cells" that can further differentiate into MHs.

In this review we will focus on the formation and function of hepatic organoids reconstructed by SHs and hepatic NPCs.

Small hepatocytes and hepatic organoid formation

To isolate SHs, we only use a simple technique of low-speed centrifugation by changing the gravity (50 and 150 x *g*) and duration (1 and 5 min) (Mitaka, 1995; 1999). Therefore, the purity of SHs is not so high at plating. The cells in the dish initially consist of NPCs such as stellate (Ito) cells, liver epithelial cells (LECs), Kupffer cells, and SECs as well as SHs. MHs are also contained in the culture. Only 3 to 4% of the plated cells can form SH colonies (Mitaka, 1999). Most SECs disappear within a week and the proliferation of MHs is limited. On the other hand, LECs and stellate cells, as well as SHs, actively proliferate in the culture. The proliferating SH colony initially consists of small mononucleate cells that compactly gather. At around 10 days after plating, the shapes of some SHs start changing. Cells with large cytoplasm, which are sometimes binucleate, appear in the colony and their number increases with time in culture. Thereafter, rising/piling-up cells appear in the colony (**Figure 1**).

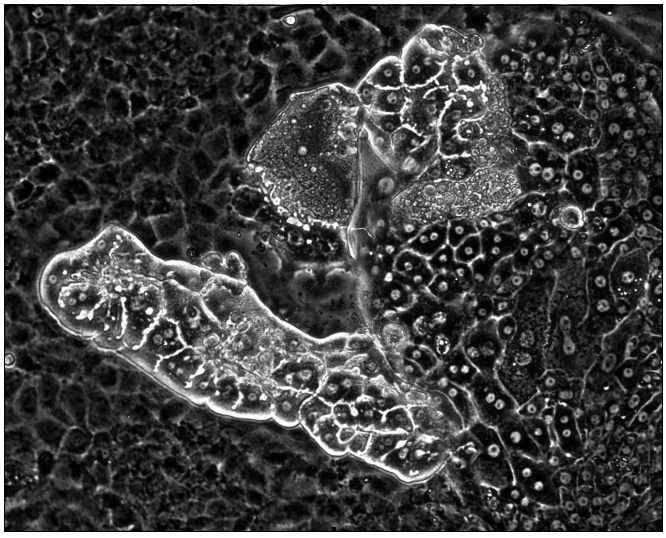

Figure 1 Phase-contrast photo of a colony derived from rat small hepatocytes.
SHs were cultured in DME medium supplemented with 10 mM nicotinamide, 10% FBS, 1 mM ascorbic acid 2-phosphate, 10 ng/ml EGF, 0.5 mg/l insulin, 10^{-7} M dexamethasone, and antibiotics. The colony consists of SHs, large cells, and rising/piled-up cells. Piled-up cells protrude from the colony, which exhibits a liver plate-like structure.

Rising/piling-up cells always appear in a region of the colony surrounded by both stellate cells and LECs. Other colonies that are not surrounded by NPCs expand faster than the colonies surrounded by them and maintain a monolayer. The rising/piling-up cells gradually grow and cover most parts of the colony. With time in culture rising/piling-up cells on the colony rearrange and form trabecular structures that are 2-3 cells thick and similar to liver plates. Immunocytochemical and ultrastructural analyses indicate that desmin-positive stellate cells and vimentin-positive LECs invade under the colony (Mitaka, 1999). In addition, the colony with rising/piling-up cells consists of multilayered cells with large cytoplasm that are rich in mitochondria, rER, peroxisomes, and glycogen granules. The cells are morphologically typical MHs. Some cells possess two nuclei, whereas neither cells with more nuclei nor giant cells, which are often observed in primary hepatocytes stimulated by EGF, appear. In the space between the multilayered cells and NPCs, an extracellular matrix (ECM) accumulates and forms a basement membrane (BM)-like structure, which may be reconstituted with ECM produced by NPCs (Mitaka, 1999). The structure is comprised of laminin, type IV collagen, and fibronectin, but type I collagen is sparse. Accumulation of ECM may result in morphological changes and the maturation of SHs. The fact that BM

formation is important for the induction of maturation of SHs is proved by the following experiment. When EHS gel (Matrigel®), the components of which are quite similar to BM, is overlaid on SH colonies, rapid morphological changes occur in the colonies: flattened compact cells become rising/piled-up cells within a week and then rising/piled-up cells slowly proliferate to form liver-plate-like structures (Sugimoto, 2002). However, alteration of the colonies is not induced by either the application of individual components such as laminin or type IV collagen, a mixture of them, and collagen gel, or by the addition of various growth factors such as fibroblast growth factor, platelet-derived growth factor, nerve growth factor, or transforming growth factor (TGF)-β. In addition, when primary cells including SHs are plated on Matrigel®, SHs cannot proliferate to form a colony. Matrigel® is well known to inhibit DNA synthesis of primary hepatocytes and induce spheroid formation of them (Bissell, 1987; Koide, 1989; Schuetz, 1988; Hubbell, 1995). Therefore, the formation of a liver-plate-like structure by Matrigel® has been observed in neither primary hepatocytes nor differentiated hepatoma cell lines. Recently, we succeeded in purifying SHs and culture for a long time (Chen, 2007). In the culture, although SHs actively grew to form a colony, no rising/piled-up cells appeared. These results suggest that BM formation is important for the morphological changes of SHs and that proliferation of SHs and the following accumulation and assembly of ECM produced by NPCs are necessary for hepatic organoid formation.

The existence of SHs is speculated in the human liver and the isolation of human SHs (hSHs) has been attempted. Hino et al. (Hino, 1999) reported that hSHs separated from normal liver tissue could proliferate in medium supplemented with nicotinamide, human serum, EGF, and so on. Although they showed that either coculture with Swiss 3T3 cells or their conditioned medium was required for hSHs to grow, we recently succeeded in culturing hSHs in serum-free medium for more than 3 weeks (Sasaki, 2008). For the purpose of clonal expansion of hSHs, hyaluronic acid (HA) and hepatocyte growth factor (HGF), as well as EGF, are necessary. In addition, hSHs can also reconstruct hepatic organoids on a dish cooperating with hepatic NPCs (our unpublished data).

The formation of plate-like structures has been reported in other experiments. Fetal rat hepatocytes can form three-dimensionally elongated, cord-like structures when they are cultured in medium supplemented with TGFβ and EGF (Sánchez, 1998). Furthermore, Michalopoulos et al. (1999) showed that MHs isolated from an adult rat liver could form plate-like structures in Matrigel® after they were cultured on collagen-coated polystyrene beads in a roller bottle. In their experiment the epithelial cells on beads had characteristics of SHs and, after the cells on beads were implanted in Matrigel®, the elongation of plate-like protrusions was observed. These results suggest that hepatic cells with high growth ability and BM-like ECM are critical for the formation of hepatic organoids in culture. To develop and expand hepatic organoids ex vivo by using hepatic stem/progenitor cells (HSCs), coculture of hepatic NPCs that can grow in coordination with HSCs may be necessary because interaction between NPCs and HSCs is important to assemble the secreted ECM for the formation of BM.

The process of SH maturation and the formation of hepatic organoids are illustrated in **Figure 2**. (A) A single SH proliferates to form a colony, while NPCs such as stellate cells and LECs separately grow. (B) The colony of SHs and NPCs accidentally attach to each other and then NPCs invade under the colony. ECM accumulates under the colony. ECM may induce the alteration of SHs from small and flat to large and thick. With the change of cell morphology, they obtain some hepatic differentiated functions. (C) Although the detailed mechanism is not known, each component of ECM may be assembled to make BM. BM formation is necessary to induce the enlargement of the cells. On the other hand, at this time the colony is surrounded by NPCs and its expansion is restricted. Therefore, the increased volume may make the cells go upward and the phenomenon of “rising” is observed

in the colony. With the increase of the volume, the maturation of the cells progresses and the polarity of cell membranes may be established, and the apical domain of the membrane forms bile canaliculi (BC) with adjacent cells. (D) Although BM formation may repress cell division, the rising cells slowly proliferate to expand in the colony and the "piled-up" cells cover the colony to reconstruct hepatic organoids. The structure consists of 2 to 3 layers of cells, and between the cells BC elongates to form long tubules. Anastomoses of BC to each other and their networks are reconstructed in the colony. BC can synchronize their contractions to make bile flow in a certain direction. In addition, as some SHs remain in most colonies, colonies continue to expand. The horizontally expanded rising/piled-up cells may be rearranged to form plate-like structures that are similar to in vivo liver plates and are 2-3 cells thick.

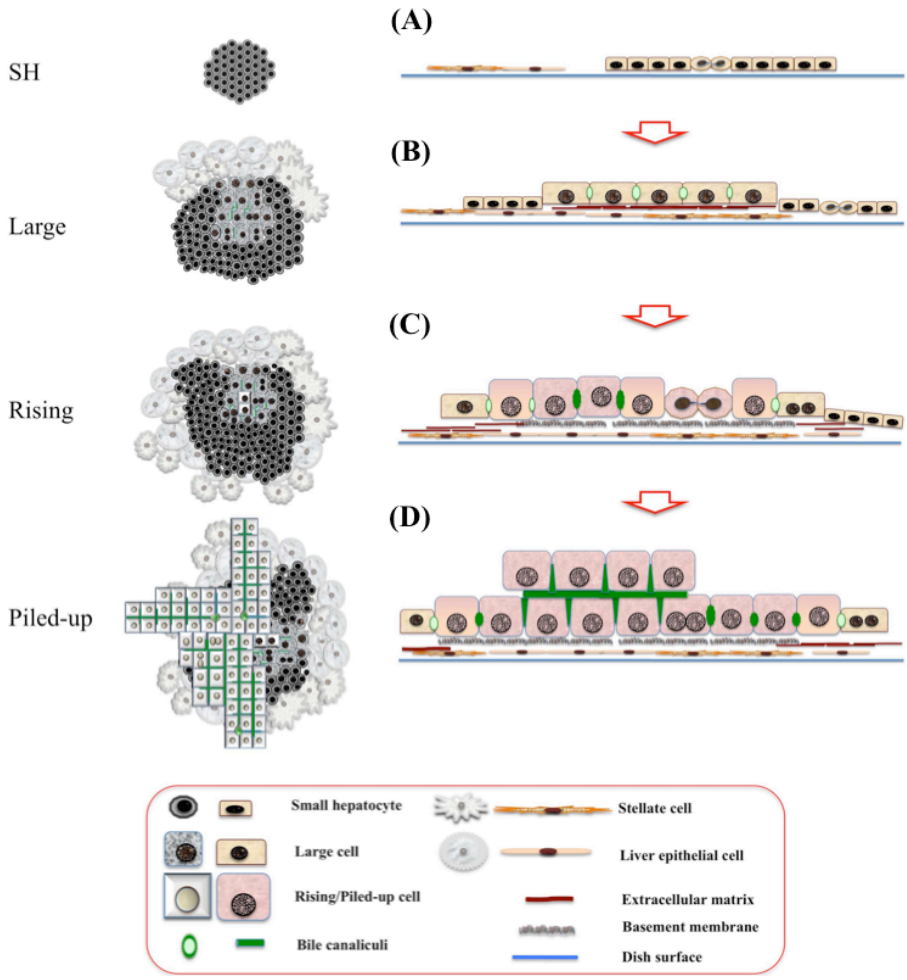

Figure 2 Illustration of SH maturation and hepatic organoid formation.
(A) A single SH proliferates to form a colony, while NPCs such as stellate cells and LECs separately grow. (B) An SH colony and NPCs accidentally attach to each other and then NPCs invade under the colony. ECM produced by cells accumulates under the SH colony. ECM may induce the alteration of the SH shape from small and flat to large and thick. (C) ECM is assembled to make BM. Rising cells appear in the colony. With the increase of the cell volume, the maturation of the cells progresses and the polarity of cell membranes may be established, and the apical domain of the membrane forms BC with adjacent cells. (D) Rising cells slowly proliferate to expand on the colony and the "piled-up" cells cover the colony to reconstruct hepatic organoids. The structure consists of 2 to 3 layers of cells, and between the cells BC horizontally elongate to form networks.

Hepatic functions of cells in organoids

As described above, the morphological alteration of cells is correlated with the maturation of SHs. It has been emphasized that cell shape is a key factor to regulate the growth, differentiation, and survival of hepatocytes (Walt, 1986; Maher, 1988). In the culture of hepatic cells the degree of their differentiation may be judged by how similarly to MHs the cells can function. Liver-specific functions of the cells are generally evaluated by the expression of mRNAs and/or proteins such as serum proteins, metabolizing enzymes of nutrients, drug metabolizing enzymes, and various membrane transporters. Many genes of those liver-specific molecules are mainly regulated by liver-enriched transcription factors (LETFs) such as CCAAT/enhancer binding proteins (C/EBPs) α and β, as well as hepatocyte nuclear factors (HNFs) 1, HNF3, HNF4α, and HNF6 (Tian, 1991; Kuo, 1992; Cereghini, 1996; Uzma, 1996). HNF4 is expressed in all SHs, whereas neither C/EBPα nor HNF6 is expressed (Sugimoto, 2002). In the cells, tryptophan 2, 3'-dioxygenase (TO) expression is quite low and serine dehydratase (SDH) expression is not induced. On the other hand, C/EBPα and HNF6 proteins, as well as HNF4α, are expressed in the nuclei of both large and rising/piled-up cells. When SH colonies are treated with Matrigel®, the expression of HNF4α, C/EBPα, C/EBPβ, and HNF6 mRNAs is induced (Sugimoto, 2002). Although the expression of HNF3α is not affected, that of HNF1α is suppressed. In addition, as the expression of LETFs increases, TO is well expressed and SDH can be induced by treatment with both glucagon and dexamethasone. Furthermore, carbamoylphosphate synthetase I and glutamine synthetase, key enzymes of ammonium metabolism, are dramatically induced in rising/piled-up cells that appear in extended culture with Matrigel® (Mitaka, 2001). These results were also confirmed by the result of microarray analysis. As shown in **Figure 3**, GeneChip® analysis of cells reveals that, compared to hepatoblasts (HBs) and SHs, the cells treated with Matrigel® show high expression of many LETFs and nuclear receptors as well as genes related to hepatic differentiated functions, though the expression is less than that of MHs.

Cytochrome P450 (CYP) constitutes a superfamily of mono-oxygenases that participate in the metabolism of endogenous substrates and play a key role in the detoxification as well as in the metabolic activation of xenobiotics (Gonzalez, 1994; Guengerich, 1990; Wrighton, 1992). The CYP isozymes involved in xenobiotic metabolism are most highly expressed in the liver. In culture of hepatocytes and hepatocyte-like cells, CYP expression has been used as an indicator of highly differentiated functions of hepatocytes. However, the cells rapidly lose CYP expression in vitro. Therefore, various trials for maintaining and inducing the enzyme have been performed in many laboratories. Recent studies on gene promoter and enhancer sequences have revealed that several different LETFs, including HNF1α, HNF1β, HNF3, HNF4, C/EBPα, and C/EBPβ, may regulate constitutive and inducible CYP expression. In most cases, two or more factors are involved in liver-specific gene expression (Gonzalez, 1996). Nuclear receptors such as aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR), retinoid X receptor (RXR), and pregnane X receptor (PXR) are also involved in the transcription of CYP and other enzyme genes (Honkakoski, 1998; Ma, 2001; Kliewer, 2003). AhR and PXR regulate expression of CYP1A1 and CYP3A, respectively, in a ligand-specific manner (Kliewer, 2003; Ma, 2001). CAR and RXR play a central role in constitutive expression and the induction of CYP2B (Honkakoski, 1998). Regulation of the CYP2 family has been most extensively studied. Of the CYP2 family, 2E1 is the only isozyme whose regulation of gene expression is dependent on HNF1α, and no effects are observed with HNF4, C/EBPα, or C/EBPβ (Liu, 1995).

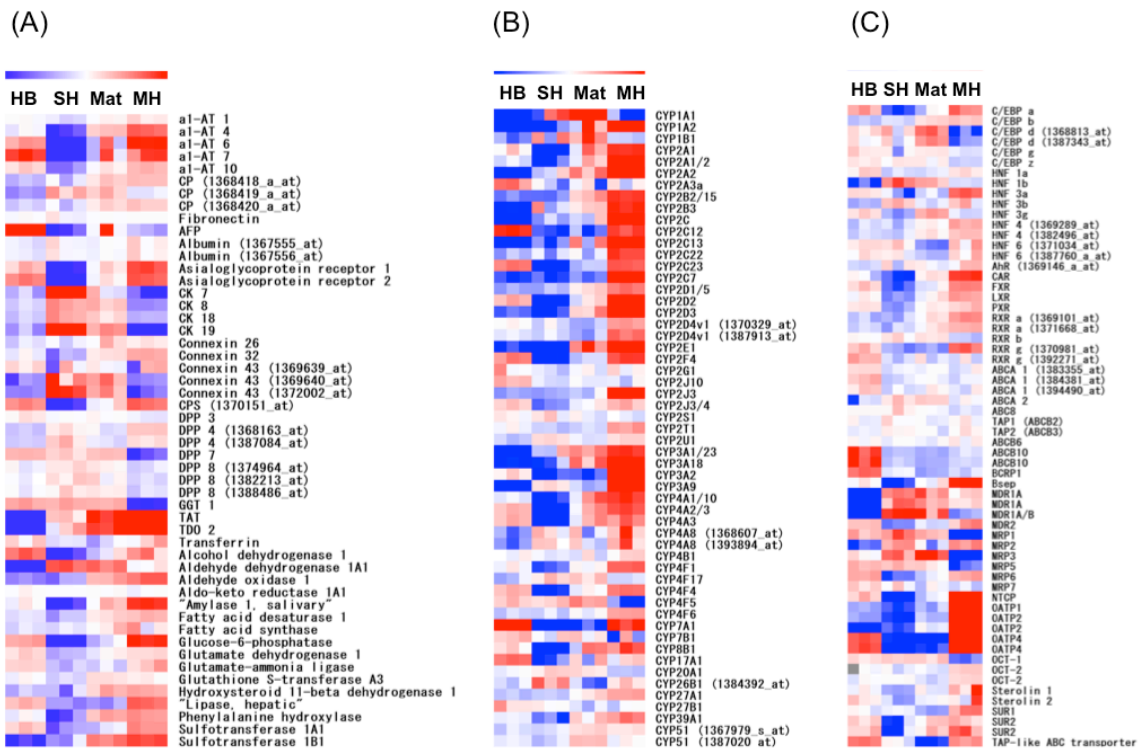

Figure 3 Gene expression patterns of HBs, SHs, mature SHs, and MHs.

Gene expression of cells was analyzed by using GeneChip® (Affimetryx). RNA of SHs was isolated from SH colonies that were cultured for 10 days and that of mature SHs was isolated from SH colonies treated with Matrigel® for 4 days. RNA of MHs was purified from hepatocytes soon after their isolation from a normal rat liver. RNA of HBs was purified from E20 rat livers. Three independent experiments were performed. Gene expression patterns of (A) typical serum proteins, structural proteins, and metabolizing enzymes, (B) cytochrome P450 isozymes, and (C) transcription factors and nuclear receptors are shown.

Although as much CYP2E1 is expressed in SHs as in MHs, low expression of CYP1A1/2, 2B1, 3A2, and 4A1 is observed in SHs (Miyamoto, 2005). However, when SHs are treated with Matrigel®, the expression of CYP1A1/2, 2B1, 3A2, and 4A1 proteins increases and is further induced in rising/piled-up cells by an appropriate agent. Enzymatic activities of CYP1A, 2B, and 3A also increase after Matrigel® treatment. In addition, we recently reported that as much AhR, CAR, PXR and RXRα were expressed in SHs as in MHs (Ooe, 2009). Expression of CYP1A1/2, 2B1, and 3A2 in SHs depends on the expression of AhR, CAR, PXR and RXRα. On the other hand, CYP2E1 expression decreases with time in culture and the cells treated with Matrigel® dramatically lose the expression. This may result from the fact that the expression of HNF1α is suppressed in the cells with Matrigel® (Ma, 2001). Comprehensive analysis of gene expression shows that many Cyp genes are up-regulated in SHs treated with Matrigel® (**Fig. 3B)**.

The expression and inducibility of CYP can also be maintained in cryopreserved SHs (Ooe, 2006). Even after cryopreservation for more than a year, thawed SHs can proliferate to form a colony and maintain hepatic functions, though MHs cannot grow and rapidly lose hepatic differentiated functions (Ikeda, 2002; Ooe, 2006). In addition, after treatment with Matrigel®, it has been confirmed that mature SHs possess CYP activity and testosterone can be sequentially metabolized as effectively as by MHs (Ooe, 2006).

Bile canalicular formation in hepatic organoids

In the normal mammalian liver 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 apical (BC) domain. The apical domain of paired hepatocytes is separated from the lateral domain by tight junctions and forms BC. BC are rich in microvilli, and components of bile, which are produced and metabolized in the cells, are secreted into the structure. The secreted bile passes through BC and pours into bile ducts (BDs). Between rising/piled-up cells BC-like structures are formed and develop into anastomosing networks with time in culture (Mitaka, 1999; Sudo, 2004). In order to show that the BC-like structure has the same physiological functions as in vivo BC do, it is important to examine whether the plasma membranes forming the structure have polarity. Immunocytochemical analysis has indicated that membrane proteins of BC such as dipeptidyl peptidase 4, ectoATPase, 5'-nucleotidase, and multidrug resistant related protein 2 (MRP2) are restrictedly localized in the BC membrane (Sudo, 2004). Actin filaments are assembled under the membrane and tight junctional protein ZO1 is stained along the tubular structure. Ultrastructurally, microvilli are well developed in the lumen of the structure and tight junctions are observed in the end of lateral membrane close to the BC-like structure.

The vectorial transport of materials such as bilirubin, bile salts, and organic anions from serum to BC is actively performed in hepatocytes. 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, 2000; Meier, 2002). Conjugated bile salts and organic anions are actively secreted through the bile-salt export pump (BSEP) and MRP2 expressed at the apical membrane, respectively. Although the gene expression of basolateral (Oatp1/2/4 and Ntcp) and BC transporters (Mrp2/multidrug resistance 2 [Mdr2]/Bsep) is low in SHs, their expression increases with time in culture (Oshima, 2008). In SHs, OATP1 and OATP2 proteins are not distributed in the cell membrane, whereas in large and rising/piled-up cells these proteins are restrictedly expressed in basolateral membranes. On the other hand, MRP2 localizes on the BC membrane. Pfäundler et al. (2004) also showed that NTCP and OATP1b2 proteins appeared in the basolateral membrane of SHs with time in culture and that BSEP and MRP2 localized in the apical membranes of rising/piled-up cells. Mrp3 is a member of the Mrps and ATP-binding cassette family and localizes in the sinusoidal membrane (Belinsky, 1998). Although its expression in the normal rat hepatocyte is very low, increased expression is observed in the liver of cholestasis (Lee and Boyer, 2000), those of the Mrp2-deficient Eizai hyperbilirubinemic rat (Buchler, 1996) and patients with Dubin-Johnson syndrome (Konig, 1999; Scheffer, 2002). In culture, Mrp3 is expressed in SHs and large cells in colonies, whereas the protein quickly disappears in rising/piled-up cells (Oshima, 2008).

It is well known that expression of most hepatic transporters is regulated by LETFs (Trauner, 2003). 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 farnesoid X-activated receptor (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. GeneChip® analysis shows that the expression of many LETFs and nuclear receptors, especially HNF3γ, HNF4, C/EBPα /β, CAR, FXR, LXR, PXR, and RXR, is clearly up-regulated in SHs treated with Matrigel® (**Fig. 3C**). The increase of the gene expression may induce the production of many proteins related to not only highly differentiated hepatic functions but also cell polarity. The production of the membrane proteins may not be sufficient for the maturation of SHs. Sorting them toward the proper site in the cell membrane is necessary to generate membrane polarity. Therefore, the acquisition of membrane polarity is required for SHs to complete their maturation. Although membrane polarity is formed in rising/piled-up cells, the

mechanism of the process is not well understood.

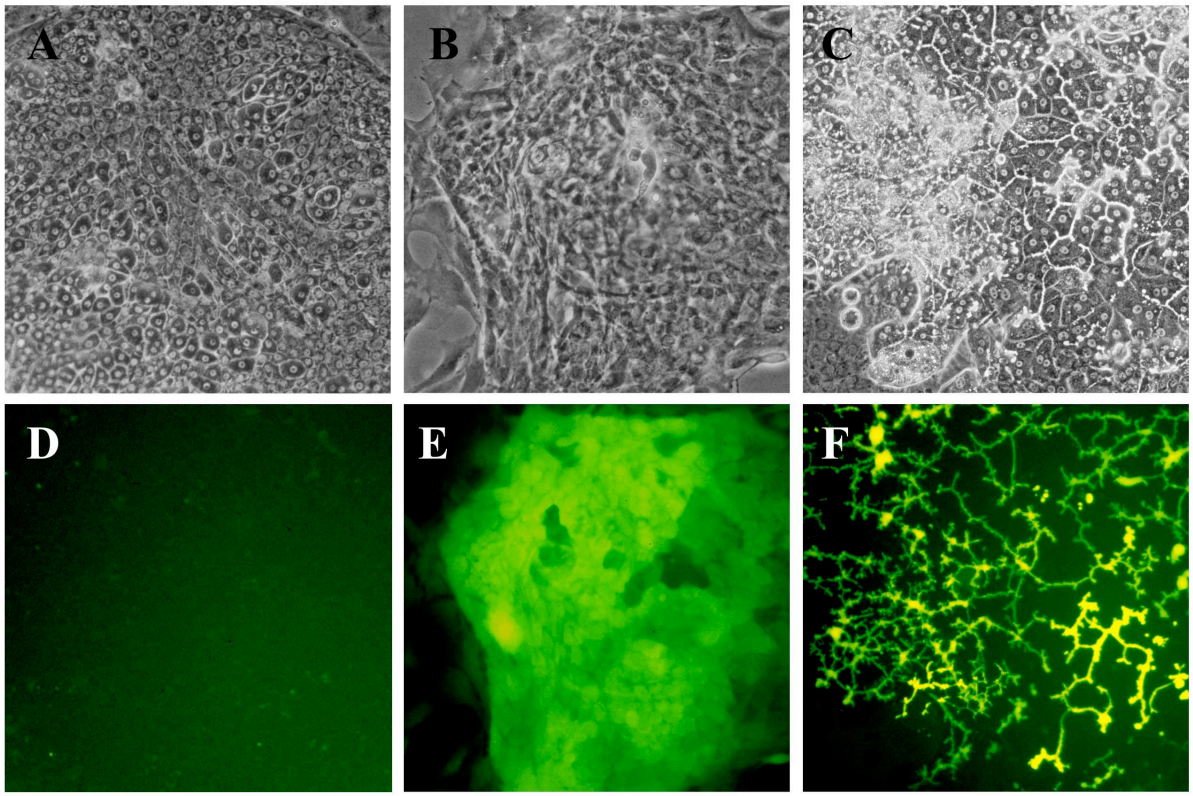

Figure 4 Physiological abilities of SHs, large cells and rising/piled-up cells.
Corresponding phase-contrast (A, SHs; B, large cells; C, rising/piled-up cells) and fluorescein images (D-F) are shown. (D) SHs cannot metabolize FD in the cytoplasm and FD may exude from cells. (E) Large cells in colony can decompose FD into acetate and fluorescein. As functional BC are not formed between cells, fluorescein is retained in the cytoplasm for a short time and may be excreted from cells through MRP3. (F) Fluorescein is secreted into BC through MRP2 and is retained for a long time. Fluorescent BC are clearly shown to form anastomosing networks on the colony.

It is important to prove whether the rising/piled-up cells with membrane polarity have vectorial transport of substances from medium to BC. Bilirubin is taken up by OATP2, glucuronized in the cytoplasm, and then secreted into BC via MRP2. Fluorescent diacetate (FD) is taken up by passive diffusion, and then decomposed by esterase into acetate and fluorescein. The fluorescein is secreted into BC via MRP2. When bilirubin or FD is added to the culture medium, metabolized substances are secreted into BC formed in rising/piled-up cells. Interestingly, cells that possess large cytoplasm and retain bilirubin and fluorescein appear in the process of colony development (**Fig. 4**). This phenomenon means that, although the esterase and MRP2 are expressed in the cells, MRP2 is not sorted to the apical membrane of the cells. In other words, large cells not forming functional BC are not able to distribute MRP2 proteins to their BC membranes. In rising/piled-up cells the secreted bilirubin and fluorescein accumulate in tubules and cystic regions for a long time (Mitaka, 1999; Pfäundler, 2004; Sudo, 2004). As the structure is tightly sealed, the secreted substances are not released from it. On the other hand, the functional competence of polarized transporters is confirmed by using other substances. After addition of cholylglycyl-fluorescein, which is taken up through NTCP and secreted through BSEP, fluorescein accumulates in the BC (Pfäundler, 2004). In addition, radioactive estrone-3-sulfate (E_1S) and estradiol-17β-D-glucuronide (E_2G) are absorbed mainly through

OATPs and excreted through breast cancer resistance protein and MRP2, respectively (Trauner, 2003). When E_2G is administered to SH colonies treated with Matrigel®, the radioactive substance is secreted into BC. The secreted E_2G can be recovered from BC (Oshima, 2008). Furthermore, the tubular structures spontaneously and sequentially repeat contraction and dilatation like a peristaltic movement (Sudo, 2004; 2005a). Dye microinjected into rising/piled-up cells can rapidly flow along BC. Considering the expression of gap junctional protein connexin 32 and the assembly of actin filaments along BC networks, signals may be sequentially transduced through gap junctions into adjacent cells and cause synchronous peristaltic contractions (Sudo, 2005a). Thus, the BC-like structure formed in rising/piled-up cells seems to have almost the same morphological characteristics and functional competence as BC in vivo.

Hepatic organoid formation using scaffolds

When SHs and NPCs are plated on culture dishes and cultured, hepatic organoids with BC can be formed. However, it takes a long time to reconstruct them in a dish and the number of the colonies with rising/piled-up cells is 1/4 of all colonies even 30 days after plating (Mitaka, 2001). To establish the application of the tissues for transplantation and an artificial liver, it is important to develop methods with which a large number of hepatic organoids can be rapidly and efficiently formed. We used the method of "tissue engineering, the combination of cells and a scaffold." Two different approaches have been tried; one is to use a collagen sponge (Harada, 2003; Sugimoto, 2005), the other to use polycarbonate membranes (Sudo, 2005b).

1) Rat SH colonies consisting of 30~50 cells are first formed in dishes and then separated from the dishes (Harada, 2003). Collected colonies are poured onto collagen sponges (Helistat®) and cultured. SH colonies expand toward the inside of the sponge and the maturation of SHs gradually progresses. The secretion of serum proteins and urea increase with time in culture and CYP1A1 is expressed. After treatment with FD, fluorescein is excreted into BC and BC networks are clearly observed inside the sponge. The results mean that SHs may differentiate into MHs in the sponge. BD-like structures are also formed in the sponge, whereas sinusoid and vessel formation are not obvious. Finally, large hepatic organoids with well-developed BC networks are reconstructed in the sponge. On the other hand, when human hepatic cells (including MHs, SHs, and NPCs) isolated from a normal adult liver are directly plated on the sponge and cultured in medium supplemented with nicotinamide, human serum, HGF, and so on (Sugimoto, 2005), about a month later, hepatic organoids with BDs are formed in the sponge and the upper surface of the sponge is covered with biliary epithelial cells. Although the formation of functional BC was not confirmed, their structure was ultrastructurally observed between the cells. Thus, with a collagen sponge, a large number of hepatic organoids may be efficiently reconstructed within a relatively short period. Until now, no connection between BC and BDs in the sponge has been observed to develop in the hepatic organoids.
2) Pairs of polycarbonate membranes are prepared and SHs separately cultured on each membrane (Sudo, 2005b). After SHs expand to form large colonies, one membrane is inverted on top of the other to form an SH bilayer. SHs of the upper and lower layers adhere to one another and form 3D stacked-up structures. Hepatic differentiated functions increase in the cells and functional BC are formed between adhering surfaces of the cells.

Conclusion

The fact that secreted substances make bile and the bile can be carried without leaking means that hepatic basic structures are almost built up and that information (signals) can be rapidly and synchronously transduced between cells in the organoid. These physiological

functions of hepatocytes can be observed only in polarized cells. Even if isolated primary hepatocytes are used, it is quite difficult to reconstitute these organoids. Therefore, the organoid reconstructed by SHs and hepatic NPCs may be the cell aggregate possessing the most hepatic differentiated functions in vitro. Although molecular biological and biochemical aspects of hepatic functions have been evaluated by the degree of hepatic differentiation, now we should add the physiological aspects to the evaluation of the cultured cells. As hSHs can now be isolated from the healthy adult liver, hepatic organoids incorporating hSHs will be made. In addition, as rSH colonies can be cryopreserved for a long time without losing their ability, hSHs may also be cryopreserved, which will lead to the establishment of an hSH bank. Thus, it may not be too long until the human hepatic organoid is used in pharmaceutical experiments as preclinical trials and as a cellular source of an artificial liver device.

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