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Studies of Liver Cells : What are "Small Hepatocytes"?

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ABSTRACT

In this review the research we have performed, will be mainly described through my studies. In particular, the study of hepatic progenitor cells, "small hepatocytes (SHs)," which were first found by me, is detailed. Until now, our laboratory has focused its research on the issue of the "liver", i.e., development, regeneration, stem/progenitor cells, and carcinogenesis. I will summarize our research by 8 themes. In each theme I only quote the references that we have reported.

Key words : Small hepatocytes, Hepatic progenitor cells, Hepatic organoid, Regeneration, Maturation

Abbreviations:

AhR, aryl hydrocarbon receptor; BC, bile canaliculus; BD, bile duct; B-CAM, basal cell adhesion molecule; BEC, biliary epithelial cell; BM, basement membrane; BSEP, bile-salt export pump; CAR, constitutive androstane receptor; C/EBP, CCAAT/enhancer binding protein; CYP, cytochrome P450; DPPIV, dipeptidyl peptidase IV; EC, endothelial cell; ECM, extracellular matrix; EGF, epidermal growth factor; FD, fluorescent diacetate; FGF, fibroblast growth factor; Gal, galactosamine; HB, hepatoblast; HGF, hepatocyte growth factor; HNF, hepatocyte nuclear factor; HSC, hepatic stellate cell; LEC, liver epithelial cell; LETF, liver-enriched transcription factor; MH, mature hepatocyte; MRP, multidrug resistant related protein; NTCP, Na^+-dependent taurocholate cotransporting polypeptide; OATP, Na^+-independent organic anion-transporting protein; NPC, nonparenchymal cell; PH, partial hepatectomy; Ret, retrorsine; SH, small hepatocyte; SEC, sinusoidal endothelial cell; PXR, pregnane X receptor; RXR, retinoid X receptor; rER, rough endoplasmic reticulum; SDH, serine dehydratase; TGP, thermoreversible gelation polymer; TO, tryptophan 2,3'-dioxygenase

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The present laboratory (Department of Pathophysiology) originates from the Department of Pathology, the title of which was changed on April 1st, 2002. The Cancer Research Institute was established in 1955 and the Department of Pathology was one of 2 laboratories with which the Institute began. The first professor was Dr. Hideyuki Tsukada, M.D., Ph. D. (1963-1988) and the second was Dr. Yoichi Mochizuki, M.D., Ph.D. (1988-2002). Then, I, Toshihiro Mitaka, M.D., Ph.D. succeeded to this laboratory. In this review the research we have performed, will be mainly described through my studies.

Since I returned from the University of Wisconsin, U.S.A and began to work as an instructor in 1990, I started to investigate the regulation of growth and maturation of rat hepatocytes. In particular, the study of hepatic progenitor cells, "small hepatocytes (SHs)," which were first found by me, has been carried out. Until now, our laboratory has focused its research on the issue of the "liver", i.e., development, regeneration, stem/progenitor cells, and carcinogenesis. I will summarize our research by each theme. As the research concerning the regulation of growth and maturation of rat hepatocytes (mainly in the 1990s) is described in my previous review article[1], I recommend it to researchers who are interested in the culture of primary hepatocytes.

1. Small hepatocytes and hepatic organoid formation

SHs have been identified as proliferating cells with hepatic characteristics. We first found a remarkable increase of small mononucleate cells within primary hepatocytes cultured in medium supplemented with 10 mM nicotinamide and epidermal growth factor (EGF)[2]. One SH could proliferate to form a colony. Most cells grow slowly and 5 to 6 divisions occur within 10 days[3]. The population of SHs in the young adult rat liver is estimated to be 1.5-2.0% of hepatocytes, and the number of the cells decreases with age[4]. Although SHs can continue growing without losing hepatic characteristics for several months, the immortalization of the cells is so difficult that cell lines have not yet been established. In early culture SHs require both nicotinamide and fetal bovine serum for the enlargement of the colonies, and growth factors such as EGF, hepatocyte growth factor (HGF), transforming growth factor (TGF)-α, and fibroblast growth factor (FGF) 1/2 can stimulate their expansion[5]. 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)[3, 5]. Therefore, the purity of SHs is not so high at plating. However, the fact that the isolated cells contain nonparenchymal cells (NPCs) led to an important finding for us. The cells attached on the dish are a mixture of epithelial cells and NPCs such as stellate (Ito) cells, liver epithelial cells (LECs), Kupffer cells, and sinusoidal endothelial cells (SECs) as well as SHs. Some mature hepatocytes (MHs) are also included in the culture. Only 3 to 4% of the plated cells have capability as progenitor cells that can form SH colonies[5].

While most SECs disappear within one week and the proliferation of MHs is limited,

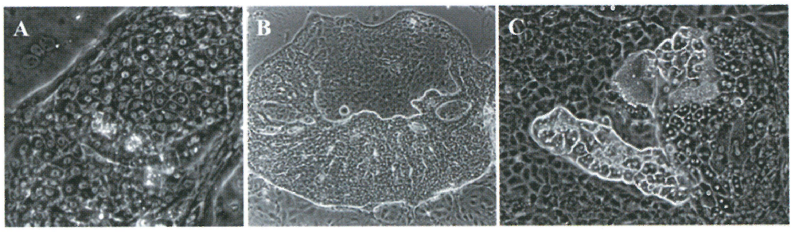

Figure 1. Phase-contrast photographs of small hepatocytes. (A) At day 13 after plating. Small-sized hepatocytes are proliferating and becoming flat and compact. (B) Large-sized hepatocytes are rising on a colony at day 42. (C) A hepatic plate-like structure is formed from a colony. Bile canaliculi (white lucent lines) are observed between cells.

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 (Fig. 1A). At around 2 weeks after plating, the shape of some SHs in a colony alters. Cells with large cytoplasm, which are sometimes binucleate, appear and they occupy a large area of the colony with time in culture. Thereafter, the large-sized SHs three-dimensionally rise and pile up on the colony (Fig. 1B). The morphological changes of cells always begin in a region of the colony surrounded by both stellate cells and LECs. Other SHs that are not surrounded by NPCs maintain their cell shape and a monolayer. The rising/piling-up cells grow slowly and gradually 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 immature liver plates (Fig. 1C). We named the structures "hepatic organoids." Immunocytochemical and ultrastructural analyses of the structures reveal that the colony with rising/piling-up cells consists of multilayered cells with large cytoplasm that are rich in mitochondria, rough endoplasmic reticulum, peroxisomes, and glycogen granules, and show the typical morphology of MHs[5]. In addition, both desmin-positive stellate cells and vimentin-positive LECs invade under the colony and, in the space between the multilayered cells and NPCs, an extracellular matrix (ECM) accumulates to form a basement membrane (BM)-like structure, which may be reconstituted with ECM produced by NPCs. The BM-like 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 those of BM, is overlaid on SH colonies, rapid morphological changes occur in the colonies: flattened compact cells become rising/piling-up cells within a week and then rising/piling-up cells slowly proliferate to form liver-plate-like structures[6]. However, morphological alteration of the colonies is not induced by either the application of individual components such as laminin, type IV collagen, and collagen gel, or by the addition of various growth factors such as FGF, platelet-derived growth factor, nerve growth factor, or TGF-β. In addition, when primary cells including SHs are plated on Matrigel®, the SHs cannot proliferate to form a colony. As Matrigel® is well known to inhibit DNA synthesis of primary hepatocytes and induce spheroid formation of them, the formation of a liver-plate-like structure by Matrigel® has never been observed in other cells, including hepatic stem cells and hepatocytes. This phenomenon is a quite unique property of SHs. Recently, we succeeded in purifying SHs by using hyaluronic acid (HA) and culture for a long time[7]. In the culture the attachment and proliferation of NPCs are inhibited so that no rising/piling-up cells appear. 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. We recently succeeded in culturing hSHs in serum-free medium[8]. For the purpose of clonal expansion of hSHs without serum, HA and HGF, as well as EGF, are necessary. hSHs could proliferate for more than 3 weeks and the average number of SHs in a colony was about 100 cells at day 21. They expressed not only genes related to hepatic differentiated functions but also α-fetoprotein, CD44, D6.1A, and BRI3. In addition, hSHs can also reconstruct hepatic organoids on a dish cooperating with hepatic NPCs (our unpublished data).

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 grow separately. (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 ECM component 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 "piling-up" cells cover the colony to reconstruct hepatic organoids. The structure consists of 2 to 3 layers of cells, and between the cells BC elongate 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, the colonies continue to expand. The horizontally expanded rising/piling-up cells may be rearranged to form plate-like structures that are similar to *in vivo* liver plates and are 2 -3 cells thick.

2. Hepatic differentiated functions of SHs

As described above, the morphological alteration of cells is correlated with the maturation of SHs. It is well known that cell shape is a key factor to regulate the growth, differentiation, and survival of hepatocytes. In the culture of hepatic cells their degree of differentiation may be judged by acquisition of functions of MHs. 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. The genes related to those liver-specific functions are mainly regulated by liver-enriched transcription factors (LETFs) such as CCAAT/enhancer binding proteins (C/EBP) α and β, as well as hepatocyte nuclear factor (HNF) 1α, HNF3, HNF4α, and HNF6. When SHs remain small, HNF4 is expressed in all SHs but neither C/EBPα nor HNF6 is expressed[6].

In the cells, expression of tryptophan 2,3'-dioxygenase (TO) and serine dehydratase (SDH), which are expressed in highly differentiated hepatocytes, is quite low and not induced, respectively. However, when SHs change from small and flat to large and rising/piling-up, C/EBPα, HNF6, and HNF4α are expressed and both TO and SDH can be induced by appropriate hormones. Furthermore, when SH colonies are treated with Matrigel®, the expression of HNF4 α, C/EBPα, C/EBPβ, and HNF6 is also induced[6]. In addition, the expression of carbamoylphosphate synthetase I and glutamine synthetase, key enzymes of ammonium metabolism, is dramatically induced[9]. These results were also confirmed by microarray analysis[10]. 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. It is known that cytochrome P450s (CYPs) are expressed in highly differentiated hepatocytes and that it is hard for cultured hepatocytes to maintain the expression even for a few days. Recent studies on gene promoter and enhancer sequences have revealed that the expression of CYPs is also regulated by several different LETFs, including HNF1α, HNF1β, HNF3, HNF4, C/EBPα, and C/EBPβ. In SHs, although as much CYP2E1 is expressed as in MHs, low expression of CYP1A1/2, 2B1, 3A2, and 4A1 is observed[11]. However, when SHs are treated with Matrigel®, the ex-

pression of CYP1A1/2, 2B1, 3A2, and 4A1 proteins increases and is further induced in rising/piling-up cells by the appropriate agent. Enzymatic activities of CYP1A, 2B, and 3A also increase after Matrigel® treatment. In addition, we recently reported that aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR), pregnane X receptor (PXR) and retinoid X receptor (RXR) α were expressed in SHs to the same degree as in MHs[12]. 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 it. The expression and inducibility of CYP can also be maintained in cryopreserved SHs[13]. 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

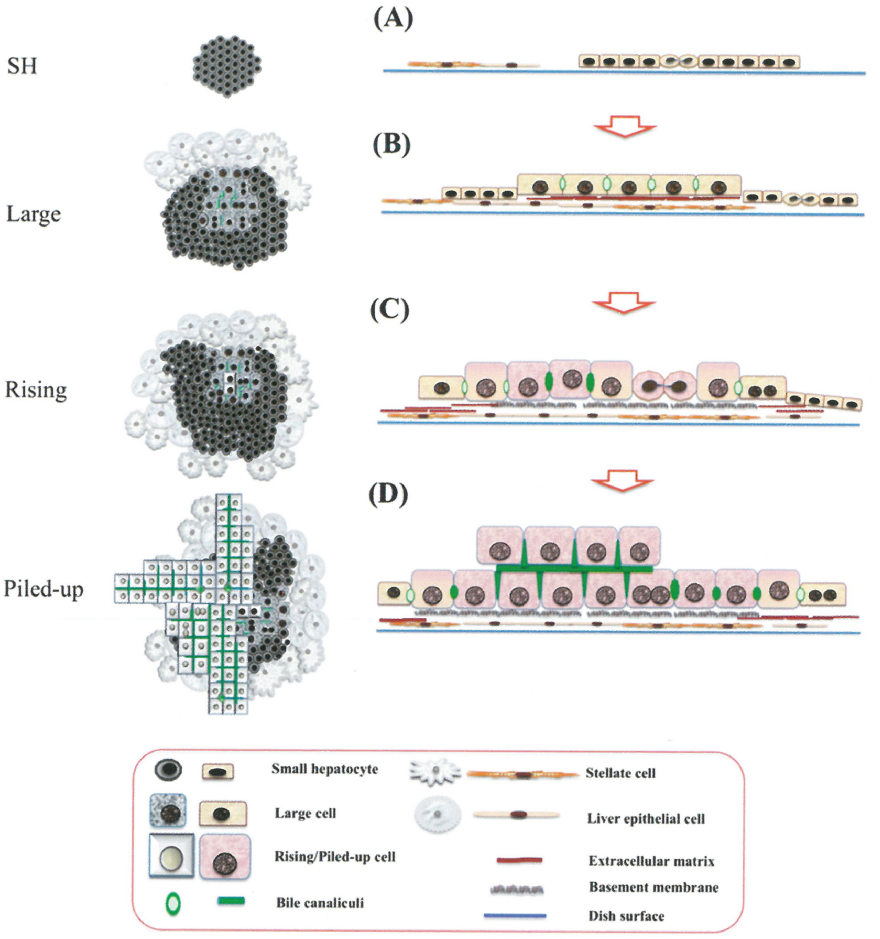

Figure 2. Illustration of SH maturation and hepatic organoid formation.
(A) A single SH proliferates to form a colony, while NPCs such as stellate cells and LECs separately grow. (B) An SH colony and NPCs accidentally attach to each other and then NPCs invade under the colony. ECM produced by cells accumulates 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. (Ref: 10)

rapidly lose hepatic differentiated functions[13,14]. 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[13].

3. Reconstruction of bile canaliculi 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 BC domain. The BC 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 the BC and pours into bile ducts (BDs). Between rising/piling-up cells BC-like structures are formed and develop into anastomosing networks with time in culture (Fig. 3)[5,16].

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 IV (DPPIV), ectoATPase, 5'-nucleotidase, and multidrug resistant related protein 2 (MRP2) are restrictedly localized in the BC membrane[16]. 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 the 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

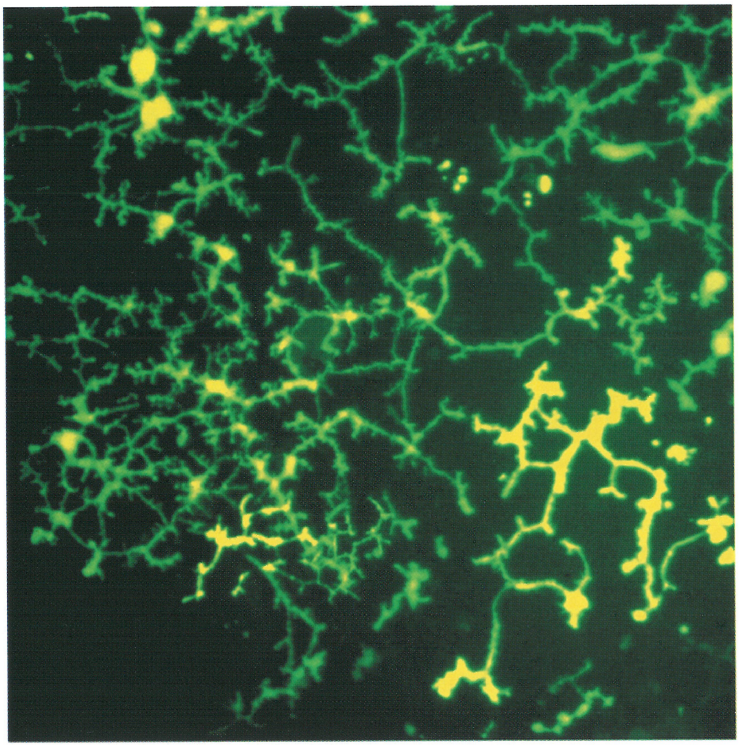

Figure 3. Bile canaliculi in a hepatic organoid. Fluorescent diacetate was added to the culture medium and metabolized fluorescein was secreted into BCs. Beautiful anastomosing networks of bile canaliculi can be observed by fluorescent microscopy.

anions is mediated by Na^+-dependent taurocholate cotransporting polypeptide (NTCP) and Na^+-independent organic anion-transporting proteins (OATPs), respectively. Conjugated bile salts and organic anions are actively secreted through the bile-salt export pump (BSEP) and MRP2 expressed at the apical membrane, respectively. In cultured SHs the gene expression of basolateral (Oatp1/2/4 and Ntcp) and BC transporters (Mrp2/multidrug resistance 2/Bsep) is low[16,17]. When SHs maintain the small and flattened shapes, OATP1 and OATP2 proteins are not distributed in the cell membrane, whereas large and rising/piling-up cells restrictedly express both proteins in basolateral membranes and MRP2 localizes on the BC membrane. 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/piling-up cells, the mechanism of the process is not well understood.

We examined whether the rising/piling-up cells with membrane polarity had 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 (Fig. 3). When bilirubin or FD is added to the culture medium, metabolized substances are secreted into BC formed in rising/piling-up cells. Interestingly, cells that possess large cytoplasm and retain bilirubin and fluorescein appear in the process of colony development. This phenomenon means that, although 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/piling-up cells the secreted bilirubin and fluorescein accumulate in tubules and cystic regions for a long time[5,16]. As the structure is tightly sealed, the secreted substances are not released from it. Furthermore, the tubular structures spontaneously and sequentially repeat contraction and dilatation like a peristaltic movement[15,17]. Dye microinjected into rising/piling-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[17]. Thus, the BC-like structure formed in rising/piling-up cells seems to have almost the same morphological characteristics and functional competence as BC *in vivo*.

4. 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/piling-up cells is 1/3-1/4 of all colonies even 30 days after plating[9]. To establish the applicability 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[18,19], the other to use polycarbonate membranes[20].

1) Rat SH colonies consisting of 30～50 cells are first formed in dishes and then separated from the dishes[18]. 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 increases with time in culture and CYP1A1 is expressed. After treatment with FD, fluorescein is excreted into BC and BC net-

works 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[19], 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 are separately cultured on each membrane[20]. 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 BCs are formed between adhering surfaces of the cells.

To investigate the roles of hepatic stellate cells (HSCs) in sinusoids, we established a triculture model of SHs, HSCs, and endothelial cells (ECs)[21]. Cells of the SH fraction isolated from a normal rat liver were plated on a microporous membrane and ECs were cultured on the opposite site of the membrane. With a specific pore size, HSCs were intercalated between layers of hepatocytes and ECs, owing to the limitation of HSC behavior. When only cytoplasmic processes of quiescent HSCs were adjacent to ECs, and the HSC bodies remained on the side of the hepatocytes, the ECs changed morphologically and were capable of long-term survival. We confirmed that HSCs mediated the communication between hepatocytes and ECs in terms of EC morphogenesis.

5. Studies on small hepatocytes as hepatic progenitor cells

Investigation of specific markers of SHs has been carried out. GeneChip analysis revealed that CD44, D6.1A, and BRI3 were specifically expressed in SHs[22]. CD44 plays a role in adhesion of cells to an ECM such as HA, collagen or fibronectin. SHs have been shown to express both CD44 standard and variant 6 forms and the expression disappears with the maturation of SHs. Although CD44 is expressed in cultured SHs, no $CD44^+$ hepatocytes are found in the normal liver. When the rat liver is severely injured by hepatotoxins such as galactosamine (GalN) and 2-acetylaminoflorene, $CD44^+$ hepatocytes transiently appear in the periportal regions of the liver lobules[22]. $CD44^+$ cells isolated from the GalN-treated rat liver possess the characteristics of SHs. When the $CD44^+$ cells isolated from $DPPIV^+$ rat livers are transplanted into the rat liver ($DPPIV^-$) treated with retrorsine and 2/3 partial hepatectomy (Ret/PH) through the spleen, they can integrate into hepatic plates and proliferate to form $DPPIV^+$ foci[23]. Transplanted cells can repopulate the recipient liver and some of the foci survive for more than one year (manuscript submitted for publication).

Stem or progenitor cells have been considered as candidate cell sources of transplantation because they can expand *in vitro* and be cryopreserved for a long term. The cells derived from adult and fetal livers and other organs have been shown to differentiate into cells with hepatic characteristics *in vitro*. In addition, most of the cells can differentiate into hepatocytes in the recipient livers of genetically altered mice, and in toxic injury models. On the other hand, it is also known that hepatic stem/progenitor cells in adult livers are activated when the proliferation of MHs is inhibited by

hepatotoxins. Among them, oval cells and SHs are well recognized as stem and progenitor cells, respectively. Oval cells, named for their possession of ovoid nuclei, are known to express markers for cell membrane proteins such as CD 34, c-kit, and Thy1, shared hematopoietic stem cell markers. Recently, we reported that $Thy1^+$ cells isolated from the liver injured by GalN could differentiate into hepatocytes through $CD 44^+$ SHs[23]. Table 1 shows the phenotypes of $Thy1^+$, $CD44^+$, cultured SHs, and MHs. Both transplanted $Thy1^+$ and $CD44^+$ cells sorted from GalN-treated rat livers can survive, proliferate, and differentiate into hepatocytes in Ret/PH treated livers (Fig. 4). Therefore, when the stem/progenitor cells are transplanted into injured livers, rapid growth and expanded repopulation of donor cells can be expected. We are continuing our experiments to clarify the capacity of SHs.

6. Studies on biliary epithelial cells

Bile is produced by hepatocytes, secreted into BC, and then drained through bile ducts that consist of biliary epithelial cells (BECs). To reconstruct hepatic organoids with bile drainage systems, formation of bile ducts in culture is essential. We succeeded in developing the formation of bile ductular networks by using a primary culture of BECs isolated from a normal rat liver[24]. Isolated BECs were plated on collagen gel and cultured in the medium used for SH culture, in which HGF and transferrin were added, for 4 days. Then, the cells were overlaid

Table 1 Phenotypes of the cells related to hepatic lineage

Markers	$Thy1^+$ cells	$CD44^+$ cells	Small hepatocytes	Mature hepatocytes
Stem/Progenitor cells				
Thy1	++	−	−	−
CD44	+	+++	+++	−
AFP	−	++	−	−
EpCAM	−	++	++	−
c-Kit	++	+	−	−
Dlk	+	++	++	−
Hepatocytes				
Albumin	−	++	+++	+++
CK8	−	++	+++	+
HNF4	−	++	++	+++
HNF6	−	++	+++	+++
C/EBPα	−		−	+++
Biliary epithelial cells				
CK7	−	−	+++	−
CK19	−	−	+++	−
Cx43	+	+	++	−
GGT	−	−	++	−

Dlk, delta-like kinase ; CK, cytokeratin ; Cx, connexin ; GGT, γ-glutamyltranspeptidase

with collagen gel and 1% dimethylsulfoxide was added to the medium from 7 days after plating.

After collagen gel overlay, BECs gather to form small ductules and large BD structures are gradually reconstructed. The large BD consists of 7 to 10 BECs with inner diameters of from 20 to 50 μm, and forms interconnected networks of continuous lumina. The cells can respond to secretin, and transporter proteins such as AE2 and CFTR localize in the apical membrane. Therefore, the structures may be functionally and morphologically similar to BDs *in vivo*.

We also found that hepatic stem cells existed in biliary ductules and that the activation might occur even in regeneration of the normal liver[25]. When a thermoreversible gelation polymer (TGP) was applied to a focal defect of the rat liver, complete recovery of hepatic tissues was observed without granulation. Ductular reactions appeared around the wound and ductules elongated from BD in Glisson's sheath to the injured area. The cells in the ductules show the phenotype of oval cells (AFP^+/$albumin^+$/$CK19^+$/$c\text{-}Kit^+$/$Thy1^+$) and then lose it (AFP^-/$albumin^+$/$CK19^+$/$c\text{-}Kit^-$/$Thy1^-$). Finally, the cells lose the characteristics of BECs ($CK19^-$) and differentiate into hepatocytes. The isolated and cultured ductular cells can differentiate into hepatocytes after the cells are covered with TGP.

7. The roles of laminin in the development and regeneration of organs

Laminins are a diverse group of α/β/γ heterotrimers formed from five α, three β and three γ chains; they are major components of all basal laminae. Among the 3 types of laminin chains, α chains play pivotal roles in laminin-mediated cellular functions. We have focused on the roles of laminin α chains and their receptors in hepatic regeneration and hepatocellular carcinoma, as well as in cell adhesion.

1) To investigate the roles of laminins in normal and regenerating livers, their spatiotemporal depositions were characterized by immunohistochemistry[26]. Hepatic laminin chains are variously distributed in Glisson's sheath, sinusoids, central veins, and mesothelium. Of the laminin chains, we found that laminin α1 was transiently expressed in sinusoids during hepatic regeneration. *In vitro* studies also suggest that transient expression of laminin α1 is associated with reorganization of liver lobules.

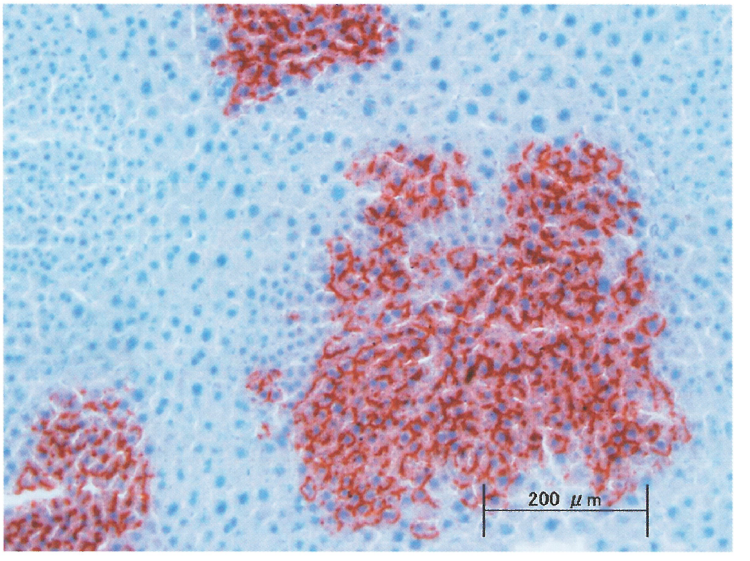

Figure 4. Transplantation of SHs into an injured rat liver. $CD44^+$ cells isolated from a galactosamine-treated rat liver ($DPPIV^+$ rat) were transplanted into a retrorsine-treated rat liver ($DPPIV^-$ rat). At 60 days after transplantation, a frozen section is enzyme-histochemically stained with DPPIV.

2) The Lutheran blood group glycoprotein (Lu), also known as basal cell adhesion molecule (B-CAM), is an immunoglobulin superfamily transmembrane receptor for laminin α5. To examine cell adhesion to laminin α5 via Lu/B-CAM, the binding site of Lu on α5 was characterized[27]. Lu /B-CAM binding to laminin α5 requires the α5 LG1-3 tandem, as do integrin α3β1 and α6β1 binding to laminin. Our results have also shown that Lu/B-CAM and α3β1/α6β1 integrins competitively bind laminin α5.

3) Laminin α5 is distributed as a major component in human hepatocellular carcinoma (HCC)[28]. We also found that Lu/B-CAM and integrin α3 β1/α6β1, receptors for laminin α5, were expressed in HCC. In vitro studies have also suggested that the deposited laminins containing the α5 chain interact with HCC cells through these receptors.

8. Cadherin mediated cell-cell adhesion system

Classic cadherins are major cell-cell adhesion molecules involved in the development, maintenance and function of most tissues. In addition, cadherins play important roles in cell signaling, proliferation, recognition and differentiation. The loss of E-cadherin expression in late stage tumors leads to the promotion of invasion and metastasis. Current issues being investigated are the molecular mechanisms underlying the regulation of cadherin's adhesive function, the cadherin cytoplasmic region, the actin cytoskeleton, and how intracellular signaling molecules control the state of the adhesive bond at the cell surface. In previous studies, we established chemical cross-linking analysis to examine the presence of the lateral dimer form of E-cadherin at the cell surface. As a result, we confirmed that E-cadherin formed a lateral dimer, termed the "cis-dimer", *in vivo*. Based on these findings, we propose that the "cis-dimer" form is a "functional unit" in regulation of cadherin-based cell-cell adhesion. In addition, the formation of the "cis-dimer" of E-cadherin is unaffected by cell-cell adhesion and cytoskeletal organization[29].

Perspectives

Since I was promoted to professor, 8 years have passed. During that period, we first tried to identify specific markers for "small hepatocytes" and found 3 candidates, CD44, D6.1A, and BRI3. CD44 is the most specific marker among them. Proliferating SHs express CD44, while the expression disappears with the maturation of the cells. Using HA, which is a ligand for CD 44, we succeeded in selectively isolating SHs and culturing them in a serum-free medium. For the clinical application of the stem/progenitor cells, the expansion of the cells must be performed in serum-free conditions. In addition, until now, the differentiation/maturation of stem cells to specific cells, especially those originating from endoderm such as digestive tissues, is still difficult to induce in culture. Although various hepatocyte-like cells induced from human ES and iPS cells have been reported by many laboratories, the cells do not possess full hepatic differentiated functions. Most cells have only some hepatic characteristics, whereas SHs can maintain many highly differentiated functions *in vitro* for a long time even after long-term cryopreservation. Recently, we succeeded in separating human SHs from the liver of an aged person. We believe that SHs have great potential to be a cell source for transplantation. We are now studying the development and functions of BECs as well as SHs. To reconstruct liver tissues, a combination of hepatocytes and the biliary system is necessary. In the near future, by establishing an effective method for cell expansion and reconstructing a large-sized hepatic tissue *in vitro*, we would like to make transplantable liver tissue. We hope that our research will result in helping patients suffering from hepatic diseases.

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REFERENCES

1. Mitaka T. The current status of primary hepatocyte culture. Int J Exp Pathol 1998; 79: 393-409.
2. Mitaka T, Mikami M, Sattler GL, Pitot HC, Mochizuki Y. Small cell colonies appear in the primary culture of adult rat hepatocytes in the presence of nicotinamide and epidermal growth factor. Hepatology 1992; 16: 440-447.
3. Mitaka T, Kojima T, Mizuguchi T, Mochizuki Y. Growth and maturation of small hepatocytes isolated from adult rat liver. Biochem Biophys Res Commun 1995; 214: 310-317.
4. Mitaka T, Norioka K, Sattler GL, Pitot HC, Mochizuki Y. Effect of age on the formation of small-cell colonies in cultures of primary rat hepatocytes. Cancer Res 1993; 53: 3145-3148.
5. Mitaka T, Sato F, Mizuguchi T, Yokono T, Mochizuki Y. Reconstruction of hepatic organoid by rat small hepatocytes and hepatic nonparenchymal cells. Hepatology 1999; 29: 111-125.
6. Sugimoto S, Mitaka T, Ikeda S, Harada K, Ikai I, Yamaoka Y, Mochizuki Y. Morphological changes induced by extracellular matrix are correlated with maturation of rat small hepatocytes. J Cell Biochem 2002; 87: 16-28.
7. Chen Q, Kon J, Ooe H, Sasaki K, Mitaka T. Selective proliferation of rat hepatocyte progenitor cells in serum-free culture. Nat Protoc 2007; 2: 1197-1205.
8. Sasaki K, Kon J, Mizuguchi T, Chen Q, Ooe H, Oshima H, Hirata K, Mitaka T. Proliferation of hepatocyte progenitor cells isolated from adult human livers in serum-free medium. Cell Transplant 2008; 17: 1221-1230.
9. Mitaka T, Sato F, Ikeda S, Sugimoto S, Higaki N, Hirata K, Lamers WH, Mochizuki Y. Expression of carbamoylphosphate synthetase I and glutamine synthetase in hepatic organoids reconstructed by rat small hepatocytes and hepatic nonparenchymal cells. Cell Tissue Res 2001; 306: 467-471.
10. Mitaka T, Ooe H. Characterization of hepatic-organoid cultures. Drug Metab Rev 2010; 42: 472-481.
11. Miyamoto S, Hirata K, Sugimoto S, Harada K, Mitaka T. Expression of cytochrome P 450 enzymes in hepatic organoid reconstructed by rat small hepatocytes. J Gastroenterol Hepatol 2005; 20: 865-872.
12. Ooe H, Kon J, Oshima H, Mitaka T. Thyroid hormone is necessary for expression of constitutive androstane receptor in rat hepatocytes. Drug Metab Dispos 2009; 37: 1963-1969.
13. Ooe H, Kon J, Miyamoto S, Oozone Y, Ninomiya S, Mitaka T. Cytochrome P450 expression of cultured rat small hepatocytes after long-term cryopreservation. Drug Metab Dispos 2006; 34: 1667-1671.
14. Ikeda S, Mitaka T, Harada K, Sugimoto S, Mochizuki Y. Proliferation of rat small hepatocytes after long-term cryopreservation. J Hepatol 2002; 37: 7-14.
15. Sudo R, Ikeda S, Sugimoto S, Harada K, Hirata K, Tanishita K, Mochizuki Y, Mitaka T. Bile canalicular formation in hepatic organoid reconstructed by rat small hepatocytes and nonparenchymal cells. J Cell Physiol 2004; 199: 252-261.
16. Oshima H, Kon J, Ooe H, Hirata K, Mitaka T. Functional expression of organic anion transporters in hepatic organoids reconstructed by rat small hepatocytes. J Cell Biochem 2008; 104: 68-81.
17. Sudo R, Kohara H, Mitaka T, Ikeda M, Tanishita K. Coordinated movement of bile canalicular networks reconstructed by rat small hepatocytes. Ann Biomed Eng 2005; 33: 696-708.
18. Harada K, Mitaka T, Miyamoto S, Sugimoto S, Ikeda S, Takeda H, Mochizuki Y, Hirata K. Rapid formation of hepatic organoid in collagen sponge by rat small hepatocytes and hepatic nonparenchymal cells. J Hepa-

tol 2003; 39: 716-723.
19. Sugimoto S, Harada K, Shiotani T, Ikeda S, Katsura N, Ikai I, Mizuguchi T, Hirata K, Yamaoka Y, Mitaka T. Hepatic organoid formation in collagen sponge of cells isolated from human liver tissues. Tissue Eng 2005; 11: 626-633.
20. Sudo R, Mitaka T, Ikeda M, Tanishita K. Reconstruction of 3D stacked-up structures by rat small hepatocytes on microporous membranes. FASEB J 2005; 19: 1695-1697.
21. Kasuya J, Sudo R, Mitaka T, Ikeda M, Tanishita K. Hepatic stellate cell-mediated 3D tri-culture model of hepatocytes and endothelial cells. Tissue Eng A 2011; 17: 361-370.
22. Kon J, Ooe H, Oshima H, Kikkawa Y, Mitaka T. Expression of CD44 in rat hepatic progenitor cells. J Hepatol 2006; 45: 90-98.
23. Kon J, Ichinohe N, Ooe H, Chen Q, Sasaki K, Mitaka T. Thy1-positive cells have bipotential ability to differentiate into hepatocytes and biliary epithelial cells in galactosamine-induced rat liver regeneration. Am J Pathol 2009; 175: 2362-2371.
24. Hashimoto W, Sudo R, Fukasawa K, Ikeda M, Mitaka T, Tanishita K. Ductular network formation by rat biliary epithelial cells in the dynamical culture with collagen gel and dimethylsulfoxide stimulation. Am J Pathol 2008; 173: 494-506.
25. Nagaya M, Kubota S, Suzuki N, Akashi K, Mitaka T. Thermoreversible gelation polymer induces the emergence of hepatic stem cells in the partially injured rat liver. Hepatology 2006; 43: 1053-1062.
26. Kikkawa Y, Mochizuki Y, Miner JH, Mitaka T. Transient expression of laminin α1 chain in regenerating murine liver: Restricted localization of laminin chains and nidogen-1. Exp Cell Res 2005; 305: 99-109.
27. Kikkawa Y, Sasaki T, Nguyen MT, Nomizu M, Mitaka T, Miner JH. The LG1-3 tandem of laminin α5 harbors the binding sites of Lutheran/basal cell adhesion molecule and α3β1/α6β1 integrins. J Biol Chem 2007; 282: 14853-14860.
28. Kikkawa Y, Sudo R, Kon J, Mizuguchi T, Nomizu M, Hirata K, Mitaka T. Laminin alpha 5 mediates ectopic adhesion of hepatocellular carcinoma through integrins and/or Lutheran/basal cell adhesion molecule. Exp Cell Res 2008; 314: 2579-2590.
29. Takeda H. cis-Dimer formation of E-cadherin is independent of cell-cell adhesion assembly in vivo. Biochem Biophys Res Commun 2004; 316: 822-826.

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