The Current Status of Primary Hepatocyte Culture

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Abbreviations used: FBS, fetal bovine serum; TO, tryptophan 2,3-dioxygenase; EGF, epidermal growth factor; HGF, hepatocyte growth factor, TGF, transforming growth factor; PARP, poly (ADP-riose) polymerase; NAD, nicotinamide adenine dinucleotide; DMEM, Dulbecco’s modified Eagle’s medium; OCT, ornithine carbamoyl transferase; Asc2P, L-ascorbic acid 2-phosphate; AFP, α-fetoprotein; CK, cytokeratin; EHS, Engelbreth-Holm-
Swarm; SDH, serine dehydratase; C/EBP, CCAAT/Enhancer Binding Protein; HNF, Hepatocyte nuclear factor; β-gal, β-galactosidase;
Introduction

Hepatocytes are the predominant cell type in the liver, constituting over 80% of the liver mass under nonpathological conditions (Fawcett 1994). In an adult liver most hepatocytes are quiescent and a few cells divide. Nonetheless, the livers retain the capacity for complete and rapid renewal in response to cell loss. Studies on liver regeneration in rodents have provided some clues to the proliferative capacity of the hepatocyte. We can practically see the dramatic proliferation of the cells in vivo by a two-thirds surgical resection of the liver. Once two-thirds of the rat liver is surgically resected, the remnant cells immediately start to proliferate and the original volume of the liver is restored within a week (Higgins & Anderson 1931). To restore the original liver mass, the average hepatocyte theoretically divides about 1.7 times (Fabrikant 1969; Stöcker & Heine 1971). However, the replicative potential of the hepatocyte is not limited to this small number of cell divisions. Recent advance of molecular technology makes us possible to use several experimental models for analyzing liver regeneration and the evidence that hepatocytes possess tremendous (perhaps unlimited) replication potential has piled up (Overturf et al. 1996; 1997; Rhim et al. 1994; Sandgren et al. 1991). In spite of their prolific growth ability in vivo, it takes a long time to establish experimental conditions in which hepatocytes can continue to grow in vitro. Richman et al. (1976) first showed that primary hepatocytes could enter the DNA synthesis induced by epidermal growth factor (EGF). Thereafter, many researchers have been trying to find the essential factors which can trigger hepatic regeneration. Until now, many factors have been reported to induce and/or enhance the replicative DNA synthesis of hepatocytes in vivo and in vitro. Michalopoulos (1990) categorized those factors involved in liver growth as “complete mitogens” and “comitogens”. I summarized the factors following his classification as shown
in Table 1. As much attentions have been paid for the resolution of the mechanisms how the mitogens can transduce the growth signals to hepatocytes, molecular and biochemical features of those mitogens have been investigated by many laboratories and their roles on the liver regeneration have been clarified. Many excellent reviews are now available (reviews see Fausto et al. 1995; Kay & Fausto 1997; Koch et al. 1990; Michalopoulos 1990; Michalopoulos & DeFrances 1997). Thus, in this review I will mainly focus on the growth capacity of hepatocytes and the culture conditions.

Even if these complete mitogens combined with various co-mitogens possess a strong capability to induce DNA synthesis, only about half of rat hepatocytes in primary culture go through one cell cycle of division (McGowan 1986). In spite of that numerous reports concerning the proliferation of primary rat hepatocytes have been published, most are the findings of the increased DNA synthesis. Only few papers showed that primary hepatocytes really increased the cell number. Until 1980s, differentiated hepatocytes, the cells that are isolated from normal adult livers, could be maintained in primary culture for short periods of time, but generally these cells exhibited limited propagability and lifespan in culture (review see McGowan 1986; Strain 1994). In general, replicating cultures of hepatocytes tended to lose most of the characteristics associated with differentiation, and cultured hepatocytes that maintain differentiated qualities tended to display very limited replication. Therefore, most researchers have believed that the differentiated hepatocytes may not proliferate in culture dishes.

From the late 1980s I have been investigating to establish the culture system that hepatocytes possessing the differentiated characteristics can proliferate for a long time. In this review I introduce the recent advances of primary cultures, especially rat hepatocytes.
Multiplication of hepatocytes \textit{in vitro}

Recently, we and other investigators have made significant advances toward the development of culture conditions that promote proliferation of primary hepatocytes isolated from normal adult rats (Block et al. 1996; Cable and Isom 1997; Inoue et al. 1989; Mitaka et al. 1991a; 1991b; Tateno and Yoshizato 1996a). To my knowledge, the methods for the multiplication of the hepatocytes \textit{in vitro} are classified into two: one is the use of nicotinamide, the other is the use of a nutrient-rich medium. In this section the materials, which can modulate the DNA synthesis of hepatocytes, in the medium are introduced and the mechanisms of their promoting effects are discussed.

\textbf{Use of nicotinamide}

Inoue \textit{et al}. (1989) first showed that the number of hepatic nuclei even at 16 days after plating increased more than 2-fold compared to that of the nuclei at day 1 in William’s Medium E supplemented with 5% calf serum, 10 mM nicotinamide, and 10 ng/ml EGF. The cultured hepatocytes maintained a high level of albumin mRNA expression and had a potential to express tryptophan 2,3-dioxygenase (TO) mRNA, which is thought to be a differentiated function of mature hepatocytes, responding to the hormonal induction for more than 2 weeks. Thereafter, we reported that primary rat hepatocytes cultured in a serum-free chemically defined medium could replicate their DNAs multiply and that more than 20% of parenchymal cells by day 4 went through a second round of cell division (Mitaka \textit{et al}. 1991a). It is the first report to prove that many hepatocytes maintaining hepatic differentiated functions and structures can complete the cell cycle more than two times. Then, other investigators have used nicotinamide in the medium for improving the survival of hepatocytes (Block \textit{et al}. 1996; Tateno & Yoshizato 1996a; Wu \textit{et al}. 1994). Although they
use the different medium and various additives in their medium, 5 to 10 mM nicotinamide is essential to maintain the capability of cell growth. For the proliferation of hepatocytes, 10 mM of nicotinamide was the most effective concentration (Inoue et al. 1989; Mitaka et al. 1991a) and this concentration is known almost completely to inhibit poly(ADP-ribose) polymerase (PARP) activity (Uchigata et al. 1982). Nicotinamide, which is classified as an aqueous vitamin for an animal, is converted in the cell to NAD and NADP, both of which are the most abundant cellular coenzymes necessary for a number of biochemical reactions. Consequently, it is reasonable to assume that a decrease in intracellular NAD and NADP may influence many functions of hepatocytes. In primary culture it is known that hepatocytes rapidly lose their intracellular NAD with time in culture (Paine et al. 1979). This reduction of NAD in the cells can be prevented by a supplementation of nicotinamide (Inoue et al. 1989; Paine et al. 1979). On the other hand, nicotinamide is a known inhibitor of PARP, an enzyme that catalyzes polymerization of the ADP-ribose moiety of NAD (Ueda & Hayaishi 1985). Althaus et al. (1982) reported that the activity of PARP sharply increased after isolated hepatocytes were plated onto plastic dishes, probably as an adaptive change to the in vitro environment. Moreover, they showed that inhibitors of PARP, including nicotinamide, could enhance the DNA repair synthesis of cultured hepatocytes elicited by chemical carcinogen (Althaus et al. 1980; 1982). Inhibitors of PARP are also known to suppress the DNA synthesis of HeLa cells by blocking the G2 phase of the cell cycle (Kidwell et al. 1982). On the contrary, rapidly proliferating cells exhibit relatively higher activity of poly (ADP-ribose)lation than quiescent cells, as seen in the precancerous state of hamster livers (Romaschin et al. 1981) and SV-40 transformed cells (Miwa et al. 1977). On the other hand, a decrease in poly (ADP-ribose)lation during differentiation has been observed in many other
types of cells (Kanai et al. 1982; Morioka et al. 1979; Ohashi et al. 1984; Porteous et al. 1979). The addition of nicotinamide to cultured hepatocytes has been reported to inhibit the loss of cytochrome p450 activity (Paine et al. 1979) and mRNA expressions of albumin and TO (Inoue et al. 1989). Taking these results into consideration, maintenance of the NAD level in cultured hepatocytes may be more important for enhancing the cyclic replication of DNA in cultured hepatocytes than for maintaining the differentiated functions. An inhibitory effect of nicotinamide on PARP may play a separate role, namely that of increasing the survival of cells and the maintenance of hepatocyte-specific functions. Thus, both effects of nicotinamide may be necessary for hepatic growth and differentiation.

**Use of nutrient-rich medium**

Recently, medium compositions have been reexamined for the purpose of improving the growth of hepatocytes with highly differentiated functions. Major attention has been given to the components such as amino acids, vitamins, trace metals, bicarbonate and others.

1) *Amino acids*

Amino acids may be particularly important as liver cell nutrients because they can serve as protein precursors, as energy substrate (Seglen & Solheim 1978), and as natural inhibitors of hepatic protein degradation (Schworer & Mortimore 1979). Seglen et al. (1983) reported that the inclusion of higher concentrations of amino acids and insulin resulted in a stimulation of protein synthesis as well as an inhibition of protein degradation. By using their medium supplemented with insulin and glucocorticoid, they could maintain hepatocytes in a protein-free medium without any detectable protein loss for a week. However, the cells could not proliferate in their medium. In an intact rat an interesting phenomena is known that protein-free diet or protein deprivation
for 3 days followed by an amino acid meal stimulated the DNA synthesis of the liver (Bucher et al. 1978; Short et al. 1974). This result indicates that amino acids can be a trigger of hepatic DNA synthesis in a starved rat.

The original formulation of Leibovitz 15 (L-15) is a medium designed for use in a nonbicarbonate buffer system and the medium is ideal for the growth and maintenance of Hep-2 and stable monkey kidney cells in a diagnostic virus laboratory (Leibovitz 1963). By using high concentrations of amino acids, especially arginine, to support the phosphate buffer and by substituting galactose for glucose, Leibovitz could use this medium in free gas exchange with the atmosphere. Two factors - high amino acid concentrations (about 3-fold compared to Dulbecco’s modified Eagle’s medium [DMEM]) and the lack of need for a CO$_2$ incubator - were important reasons why some investigators chose L-15 medium for primary culture of adult rat hepatocytes. In fact, the medium has been used for many years to study the maintenance or induction or both of several differentiated hepatocyte functions (Michalopoulos et al. 1976; Sawada et al. 1987; Staecker et al. 1988). Although primary hepatocytes can be satisfactorily maintained in L-15 medium in a 100% air incubator, they do not undergo mitosis and divide under this condition to an appreciable extent (Sawada et al., 1987). Recently, we have found that the hepatocytes could proliferate in a modified L-15 medium when supplemented with sodium bicarbonate and incubated in a 5% CO$_2$/95% air incubator (Mitaka et al. 1991b). The number of the cells doubled within 5 days of culture, whereas the number of the hepatocytes cultured in DMEM supplemented with EGF was 1.3-fold increase. In addition, as the concentration of essential amino acids in the medium by adding the concentrated essential amino acid solution into DMEM was near to that of essential amino acids in L-15 medium, DNA synthesis of the hepatocytes increased (Mitaka et al. 1991b). Although mixture of nonessential
amino acids did not have this effect, it is known that proline is necessary to induce DNA synthesis of hepatocytes and 30 µg/ml in a medium is enough for the induction of the maximum DNA synthesis of cultured hepatocytes (Hasegawa et al. 1982; Houck & Michalopoulos, 1985; Nakamura et al. 1984a). Now, we and other researchers routinely add proline into the culture medium. Recently, Hasegawa et al. (1994) reported that a high concentration of glutamic acid enhanced EGF-induced DNA synthesis of primary rat hepatocytes, whereas a high concentration of alanine and glutamine inhibited the DNA synthesis of primary hepatocytes (McGowan & Bucher 1983).

In order to prevent contamination of cultures by nonparenchymal cells, some investigators have removed arginine from hepatocyte media (Sato et al. 1960; Koch & Leffert. 1974; 1980). The basis for the selectivity of arginine-free medium is the liver’s unique capacity to synthesize arginine from ornithine via the urea cycle. Hepatocytes, and, to a lesser extent, the epithelial cells of the intestinal mucosa contain the mitochondrial enzyme, ornithine carbamoyl transferase (OCT), which converts ornithine to citrulline (Raijman 1974). Therefore, replacing arginine with ornithine in the basal medium inhibits the survival of cells that do not contain the enzyme, OCT, since these cells will be starved for arginine. However, in practice, although we used the modified DMEM (ornithine supplemented arginine-free medium) in our early experiment (Mitaka et al. 1992a; 1992b; 1993a; 1993b), a percentage of contaminated nonparenchymal cells in cultures did not decrease. As Block et al. (1996) pointed out, the absence of arginine might be rate limiting for the growth of the proliferating hepatocytes. Because, as the proliferating hepatocytes lose specific hepatocyte related functions, it is likely that the pathways generating arginine through urea cycle would diminish their activity or disappear altogether. Recently, we are using a commercial medium supplemented with arginine and
glucose.

2) **Vitamins**

Nicotinamide is a kind of vitamins. Its importance for both proliferation and differentiation of primary hepatocytes was described above. Vitamin C is known to stimulate the collagen synthesis of hepatocytes (Hata *et al.* 1985), and the DNA and collagen synthesis of fibroblasts (Hata & Senoo 1989). The importance of collagen synthesis for the induction of DNA synthesis in primary hepatocytes was reported by Nakamura *et al.* (1984a). They showed that specific inhibitors of collagen synthesis such as cis-4-hydroxy-L-proline strongly inhibited DNA synthesis of hepatocytes. Recently, it was reported that an addition of L-ascorbic acid 2-phosphate (Asc2P), a long-acting vitamin C derivative, in the medium promoted the growth of small hepatocytes (Mitaka *et al.* 1995; Tateno and Yoshizato 1996a; 1996b). On the other hand, Senoo *et al.* (1989) showed that the addition of Asc2P improved the life span of cultured hepatocytes and made it possible to maintain their functional activity for a long term. Thus, although no direct effect on the hepatocytes is clarified, vitamin C is important for the proliferation and differentiation of primary hepatocytes. In other words, production of ECM such as collagens may regulate the growth and/or differentiation of hepatocytes.

Retinoic acid is reported to induce differentiation and inhibit proliferation of malignant cells such as malignant melanoma and leukemia cells (Britman *et al.* 1980; Lotan & Lotan. 1981). Ikeda & Fujiwara (1993) showed that it could inhibit the mitogen-induced DNA synthesis in primary culture of rat hepatocytes. Although Ohmura *et al.* (1996) reported that all trans retinoic acid and 9-cis retinoic acid could not induce the DNA synthesis in primary culture, their intragastric administration stimulated that of hepatocytes *in vivo.*

3) **Trace metals**
Selenium have been mentioned as factors that support in some manner the survival of replicating cell lines maintained in culture in serum-free medium (Ham & McKeehan 1978). Although $1 \times 10^{-5}$ M Na$_2$SeO$_3$ in serum-free medium inhibited mammary cell cultures, $5 \times 10^{-8}$ M Na$_2$SeO$_3$ stimulated the growth of the cells (Medina & Oborn 1981). In primary culture of rat hepatocytes it was reported that the content of intracellular selenium was lost with time in culture (Newman & Guzelian 1982). However, the supplementation of selenium in the culture medium could prevent the loss and maintain the synthesis of P450 responding to phenobarbital. Although no one show the direct evidence that selenium can enhance the proliferation of cultured hepatocytes, we and other researchers usually add the metal into the medium when hepatocytes are cultured in the chemically defined medium for a long time (Block et al. 1996; Cable & Isom 1997; Mitaka et al. 1991a; 1991b; 1992a).

Recently, Cable & Isom (1997) reported that all three trace metals of copper, iron, and zinc are necessary for primary hepatocytes cultured in chemically defined medium to proliferate for a long time. They explain why those metals are required for the hepatocyte growth: Iron is a cofactor for ribonucleotide reductase. Because the prolonged metal deprivation of copper, iron, and zinc resulted in decreased the activity of the enzyme in leukemic lymphocytes (Oblender & Carpentieri. 1991a; 1991b). The role of zinc in the proliferation of hepatocytes is less clear but may involve altered gene transcription associated with proliferation. Especially, zinc plays a critical role in the family of zinc finger transcription factors (reviews see Berg 1990; Pieler & Bellefroid; 1994). Although copper has no biochemically-defined role in DNA synthesis or gene transcription, the ability of pancreatic acinar cells to transdifferentiate into hepatocytes was reported in the copper depletion rats (Rao et al. 1986).
Relating to iron source, the importance of transferrin has been pointed out in the chemically-defined medium (Block et al. 1996). They showed that iron saturated diferric transferrin promoted the growth of cultured hepatocytes much more than unsaturated one combined with elemental iron did. On the contrary, Cable & Isom (1997) reported that either the combination of apo-transferrin and ferrous sulfate or holo-transferrin (saturated) could serve as an iron source in their culture system. Somehow, both transferrin and iron are necessary for hepatocytes to proliferate in a serum-free cultures for a long time.

5) Bicarbonate and others

L-15 medium is designed for an air-only incubator, under such conditions, primary rat hepatocytes are reported not to enter DNA synthesis even if any mitogens are added. However, when L-15 medium is adjusted to be used in a CO₂-incubator by adding NaHCO₃, a dramatic increase of DNA synthesis are observed in the primary hepatocytes (Mitaka et al. 1991b; 1991c). The DNA synthesis increased paralleled to the increase of NaHCO₃ concentration in the medium and 20 mM NaHCO₃ in L-15 medium was best. Nevertheless the importance of CO₂/bicarbonate for the stimulation of growth has already been pointed out in various cell lines (Ehman & Misfeldt. 1983; Itagaki & Kimura 1974), the concentration of NaHCO₃ has never been reexamined in the primary culture of hepatocytes. Without paying attention to the concentration of NaHCO₃ in medium, it is made in most laboratories by the manufacture’s instruction. However, the suitable concentration for the maximum stimulus to DNA synthesis exists in each medium (Mitaka et al. 1991c). For example, DMEM/F12 is 25 mM instead of 14 mM, Williams Medium E is 30 mM, and DMEM is 25 mM instead of 45 mM.

In early studies with serum-free medium, a high concentration of pyruvate (Hasegawa & Koga 1981; McGowan & Bucher 1983) and lactate
(McGowan & Bucher 1983) was reported to stimulate the replicative DNA synthesis of rat hepatocytes with or without EGF. Oxaloacetate was also shown to have a potential to induce DNA synthesis of rat hepatocytes (Li et al. 1993). On the other hand, a co-mitogenic effect of putrescine, a kind of polyamines, to rat hepatocytes was also reported by Nagoshi & Fujiwara (1994). Recently, Sasaki et al. (1997) reported that ethanolamine could enhance the DNA synthesis of rat hepatocytes stimulated by EGF.

**Growth of Small Hepatocytes**

Small hepatocytes have been identified as proliferating cells with hepatic characteristics. We first found a remarkable increase of small mononucleate cells within primary hepatocytes cultured in serum-free medium supplemented with 10 mM nicotinamide and 10 ng/ml EGF (Mitaka et al. 1991a). Thereafter, other researchers have reported the growth of the cells in the medium supplemented with nicotinamide (Block et al. 1996, Tateno & Yoshizato. 1996a; 1996b; Wu et al. 1994). On the other hand, we also observed the appearance of small hepatocytes in the L-15 medium supplemented with EGF in a CO₂-incubator (Mitaka et al. 1991b) although the frequency of the cells was less than that of cells in the nicotinamide-supplemented medium (Mitaka T. unpublished data). Recently, Cable and Isom (1997) reported the growth of small hepatocytes in a chemically defined RPMI based medium supplemented with EGF and 2% DMSO. These results may indicate that nicotinamide is not essential for the proliferation of small hepatocytes. Namely, whenever the isolated hepatocytes are cultured in the medium in which they can really proliferate, small hepatocytes may be given an environment to start their growth.

The small hepatocytes can rapidly proliferate in the medium supplemented with nicotinamide and growth factors such as EGF, HGF, or TGF-
α, and have a tendency to form a colony (Fig. 1A) (Mitaka et al. 1992a; 1993b).
Although the size of these cells is about 1/2 to 1/3 that of typical hepatocytes
(Mitaka et al. 1992a; 1995; Tateno & Yoshizato. 1996b) and they have a less-
differentiated appearance, the cells possess typical characteristics of
hepatocytes: they are immunocytochemically positive for albumin (Fig. 1C),
transferrin, cytokeratin 8 and 18, and electron-microscopically show abundant
mitochondria, peroxisomes with crystalline nucleoids, and bile canalicular
structures (Mitaka et al. 1992a and 1992b). Therefore, we name the cell a small
hepatocyte. On the other hand, this small size results in the dilemma that small
hepatocytes also appear from the nonparenchymal cell-rich fraction (the
supernatant after 50 x g centrifugation for 1 min) (Mitaka et al. 1995; Tateno &
Yoshizato. 1996b), which is well known to be rich in hepatic epithelial cells
(Furukawa et al. 1987; Williams et al. 1977). In primary cultures of rat
hepatocytes major contaminants of nonparenchymal cells are the epithelial cells.
The cells have been studied for a long time and are known to clonally
proliferate in primary culture (Grisham 1980; Grisham & Thorgeirsson. 1997;
Williams. 1976; Yang et al. 1993). In addition, the cells are considered as a
candidate for a hepatic stem cells because they can differentiate into
hepatocytes and/or bile duct epithelial cells when they are transplanted into
appropriate sites in vivo (Coleman et al. 1993; Sirica et al. 1990). The hepatic
epithelial cells have been thought to be derived from hepatic parenchymal cells
but, now, hepatocytes in collagenase-dispersed cell suspensions are consider not
to be the obligate precursors of hepatic epithelial cells (Grisham et al. 1993).
Because identifiable hepatocytes never proliferated to form epithelial colonies
under their culture conditions. Grisham et al. (1993) speculate that those cells
may arise from small nondescript epithelial cells, which are morphologically
distinct from hepatocytes. Thus, the addition of a high concentration of
nicotinamide in the medium is the only difference whether small hepatocytes can proliferate or not. As shown in Fig. 1B, the morphology of both cells is quite different and easy to identify. However, after a long cultivation, the transitional cells sometimes appear in the peripheral zone of small hepatocyte colony, in which we can not distinguish small hepatocytes from epithelial cells (Mitaka T. unpublished observation).

The first cell division of small hepatocytes is observed from day 2 to day 3, after which rapid growth and colony formation are found. The average number of the cells in a colony reached about 27 at day 10 (Mitaka et al. 1995). Tateno & Yoshizato (1996b) also reported colonies that consisted of more than 100 cells at day 10. These results indicate that a small hepatocyte can divide 5 to 7 times within 10 days. As the cells do not show division in the first 2 to 3 days, it is calculated that the division of the fastest proliferating cells may occur every 24 h after the latent time. Twenty-two to twenty-four h cell cycles of mature hepatocytes were also shown in primary culture (Mitaka et al. 1991a; 1991b). On the other hand, Tateno and Yoshizato estimated that the doubling time of small hepatocytes during first 10 days was 51 ± 3 hours and increased to 753 ± 203 hours in the following 20 days. There are three interpretations why the delay of the doubling time after 10 days culture are observed in small hepatocytes: a) most small hepatocytes in a colony show the slowing doubling time after 5 to 7 divisions. b) part of small hepatocytes may differentiate into mature hepatocytes of which the cell division is suppressed. c) the materials like growth-inhibiting cytokines and/or ECM produced by nonparenchymal cells may hide the real prolific capacity of small hepatocytes. In our experiments many colonies are surrounded by nonparenchymal cells 10 days after plating (Fig. 1B). In addition, the hepatocytes that have a large cytoplasm and that are sometimes binucleate may often appear after day 10 (Fig. 1D).
Therefore, now, I guess that b) and c) may be feasible to explain the reason why the delay of the doubling time after 10 days culture are observed in small hepatocytes. At present, we do not have exact data whether small hepatocytes can unlimitedly divide or not. Recently, the experiments that the regenerative capacity of hepatocytes is examined in vivo have been performed by using transforming mouse models: albumin-urokinase transgenic mice (Rhim et al. 1994; Sandgren et al. 1991) and fumarylacetoacetate hydrolase deficient mice (Overturf et al. 1996; 1997). Their studies show that the upper limit of cell doublings is at least 69 or 7.3 x 10^{20}-fold expansion (Overturf et al. 1997). On the other hand, Tateno & Yoshizato (1996b) reported that a single small cell divided up to 1400 cells. This number equal to about 10 cell doublings. These results suggest that small hepatocytes may have a potential to divide unlimitedly if the cells are settled in the improved culture conditions.

**Small hepatocytes in liver growth.**

It is well known that a major feature of postnatal liver growth is the marked slowing of the proliferation of hepatocytes; in rats, the high pulse labeling rate of 5-25% at birth declines steadily to about 0.1% in the adult, modulated only by a transient spurt of liver growth and hepatocyte proliferation that occurs about the time of weaning in rats (Grisham 1969; Wright & Alison 1984). As described above, after two-thirds partial hepatectomy of an adult rat, a dramatic growth of the remnant liver is observed to restore the original liver mass. Theoretically, if all hepatocytes can equally participate the growth, the average hepatocyte divides about 1.7 times (Fabrikant 1969; Stöcker & Heine 1971). However, it is known that the number of hepatocytes which can participate in the regeneration is different among the age of rats (Stöcker & Heine. 1971). The in vivo experiment of continuous administration of $^{3}$H-
thymidine to rats showed that 99% of hepatocytes in a weaning rat, 93% of cells in a young adult rat, and only 77% of cells in a 2.5-year-old rat synthesized their DNA during regeneration after two-thirds partial hepatectomy. On the other hand, we reported that small hepatocytes got mixed among mature ones and that the small hepatocytes appeared to form colonies with time in culture (Fig. 1A). The frequency of the colony formation is varied and decrease with ages of rats: about 6% of hepatocytes isolated from 4-week old rats, about 2.5% of those from 8-week old rats, about 1.5 to 2.0% of those from adult rats were small hepatocytes (Mitaka et al. 1993a). In the aged rats (more than 2-year old) the frequency was less than 0.5%. Furthermore, the cells isolated from younger than 3-week-old rat were very small, and more than 90% of the cells were mononucleate. Taking these results into consideration, we have proposed that hepatocytes may be classified into three types of cells with respect to their ability to divide: (a) cells that have a high potential to proliferate and form colonies and, therefore, continue to supply daughter cells (Type I cells, small hepatocytes); (b) cells for which the number of possible cell divisions is limited to several divisions; these cells normally express the fully differentiated functions (Type II cells); and (c) cells that lose the ability to divide and reach the final differentiated state (Type III cells). As Type II and Type III cells are functionally enough differentiated, they are considered mature hepatocytes. On the basis of this classification of hepatocytes, we can interpret postnatal liver growth and the proliferation of parenchymal cells as shown in Fig. 2.

The liver develops from a deverticulum of the ventral floor of the foregut (Du Bois 1963). The founder cells invade the mesenchyma of the septum transversum containing the vitelline veins. Endodermal cells eventually generate hepatoblasts and nonparenchymal epithelial cells, while the mesenchyma gives rise to sinusoidal lining cells. Differentiation of already
committed hepatic endodermal cells into hepatoblasts depends on interactions with the hepatic mesoderm (Houssaint 1980; Fukuda-Taira 1981; Le Bouarin 1975). Parenchymal cells forming hepatic cords from days 10 to 17 in rat liver development are generally referred to as hepatoblasts (Shiojiri et al. 1991). These cells have a broader developmental potential than parenchymal cells at 18 days of development (immature hepatocytes) and share few characteristics in common with mature hepatocytes of adult livers. Hepatoblasts can have three different fates in liver development: (i) differentiation into hepatocytes; (ii) generation of intrahepatic biliary ducts; and (iii) formation of portions of the extrahepatic ducts. The process of development of primitive intrahepatic bile ducts from hepatoblasts is of particular interest for the stem cell question. In rats, mice and most likely also in humans, AFP+, albumin+ hepatoblasts located near large vascular spaces close to the hilus give rise to primitive intrahepatic bile ducts (Shiojiri 1984; Shiojiri et al. 1991; Van Eyken et al. 1988a; 1988b). These structures contain cells that express AFP and albumin as well as cytokeratins (CK) 7, 8, 18, and 19. In the rat, the pattern of CK expression is a particularly good marker for these cells as the surrounding hepatoblasts contain only CK8 and CK18 and expression of CK7 and CK19 is present exclusively in the newly formed ducts (Shiojiri et al. 1991; Van Eyken et al. 1988a). In adult livers of rodents and humans, hepatocytes express only CK-8 and CK-18 while bile ducts express CK7, 8, 18, and 19 (Van Eyken et al. 1987). Furthermore, Germain et al. (1988) were able to isolate day 12 fetal liver cells and plate them in culture on fibronectin-coated dishes. At the start of the cultures the cells expressed AFP, albumin, CK8, and an antigen recognized by MAb BPC5. Depending on culture conditions, the cells could be induced to undergo differentiation into the hepatocyte or bile duct lineages. Hepatocyte differentiation was promoted by addition of DMSO, TGFβ, and IGF-II while
sodium butyrate induced ductular differentiation. The bipotential progenitor capacity of these cells decreased progressively during development until differentiation became restricted only to the hepatocyte lineage. The proportion of parenchymal cells capable of differentiating into both lineages dropped from 70% at day 12 to 20% and 5%, respectively, at day 18 and 5 days after birth (Germain et al. 1988). On the other hand, intrahepatic bile duct production from hepatocytes may continue by 1-2 weeks after birth in the mouse and rat liver. As we and others reported (Mitaka et al. 1992b; 1995; Tateno & Yoshizato 1996b), small hepatocytes have the expressions of albumin⁺, AFP⁻, CK7⁻, CK8⁺, CK18⁺, and CK19⁻. Therefore, small hepatocytes may be the hepatocyte lineage derived from hepatoblasts. As shown in Fig. 2, soon after birth (newborn), liver plates may consist of small hepatocytes although periportal hepatocytes may have the bipotential progenitor capacity. Because the periportal cells are positively stained by antibodies against calf keratin and human CK, which react exclusively with bile duct cells in adult liver (Shiojiri et al. 1991; 1994; Van Eyken et al. 1988a). During weaning, some small hepatocytes in liver plates differentiate into Type II cells. At present we do not know the possible mechanisms what induce the differentiation of small hepatocytes. However, we observe that small hepatocytes can continue to proliferate without their differentiation (maturation) when the growth of nonparenchymal cells is inhibited in the co-culture (unpublished observation). The direct effects of and/or the products such as cytokines and/or ECM by the nonparenchymal cells may regulate the differentiation of small hepatocytes into mature ones. With growth, many of small hepatocytes become Type II cells, thus decreasing the number of small hepatocytes. In this period both small hepatocytes and Type II cells divide, and this activity contributes to the rapid increase in the total number of parenchymal cells. Consequently, small
hepatocytes randomly distribute in the liver acinus. Recently, the growth pattern of hepatocytes was three-dimensionally analyzed using the mosaic liver of \textit{spf}^{ash} mouse (Shiojiri \textit{et al.} 1997). They showed that, although hepatocytes proliferate and migrate extensively during development, they might allocate their daughter cells contiguously and that the orientation of their allocation might be random, leading to the formation of three-dimensionally large contiguous quasiclones of hepatocytes. Iannaccone and his associates (Khokha \textit{et al.} 1994; Ng & Iannaccone 1992) also reported that hepatocytes located anywhere in the parenchyma could proliferate and that the progeny were located contiguous to the parent but randomly oriented directionally. On the other hand, to study the proliferative potential of differentiated hepatocytes, Kennedy \textit{et al.} (1995) used a transgenic mouse line in which the human $\alpha_1$-antitrypsin promoter directs expression of $\beta$-galactosidase ($\beta$-gal) in a liver cell. In neonatal mice, a single or doublets of $\beta$-gal-positive hepatocytes are randomly scattered throughout the liver. With aging, the positive cells are forming larger clusters. In addition, the $\beta$-gal expressing cells show no predilection for the periportal or pericentral region. Furthermore, Bralet \textit{et al.} (1994) showed the fate of normal hepatocytes of adult rats by the genetic labeling method. They perfused the retroviral vectors carrying $\beta$-gal gene direct into the liver after 2/3 partial hepatectomy. The labeled cells formed clusters with age but the their distributions in liver lobules did not change throughout the life although the number of the labeled cells had a tendency to locate near periportal regions (zone 1 and 2 by Rappaport. [1976]). They also estimated the life span of hepatocytes to be between 150 and 450 days. The values are similar to previous estimation (MacDonald 1961). These results support my speculation of random distributions of small hepatocytes and stand against the “hepatocyte streaming theory” (Arber \textit{et al.} 1988; Sell 1994; Sigal \textit{et al.} 1994; Zajicek \textit{et al.} 1985). It
is a theory that periportal stem cells give rise to daughter cells that stream toward the pericentral region and undergo apoptosis after approximately one year.

With increasing age of rats, some Type II cells complete their role of proliferation and become Type III cells. As described above, one-forth of hepatocytes in liver of more than 2.5 year-old rats loses the responsiveness to the growth stimulation after 2/3 partial hepatectomy (Stöcker & Heine 1971). Furthermore, many hepatocytes isolated from aged rats did not enter S-phase in spite of EGF-stimulation (Sawada et al. 1989). After completing their role as differentiated hepatocytes, Type III cells will die (apoptosis). Thus, in normal adult rat liver, to compensate for the eliminated cells by death or other reasons, Type II cells as well as small hepatocytes may produce new cells contiguous to the eliminated ones. In addition, during liver regeneration after two-thirds partial hepatectomy, divisions of Type II cells may be enough to restore the original mass of the liver although small hepatocytes possibly participate in the event.

Until now, although we have carried out experiments to identify the origin and the location of small hepatocytes in liver lobules, we have not succeeded yet. The major reason why we can not distinguish small hepatocytes from mature ones seems to be due to the similarity of both cells. This also indicates that small hepatocytes may get mixed in hepatic plates. If this speculation is true, it is explainable that their clonal expansions are observed in the cultures of the cells that are isolated from both parenchymal (pellet) and nonparenchymal fractions (supernatant) after 50 x g centrifugation for 1 min. Although Block et al. (1996) speculated from their results that mature hepatocytes can function as or be a source of bipotential facultative hepatic stem cells (hepatoblasts), there is a possibility that a population of small hepatocytes
may mix in the isolated cells and that small hepatocytes may clonally grow in their culture conditions.

**Are small hepatocytes hepatic stem cells?**

The existence of hepatic stem cells or progenitor cells is still being argued and the definition of these cells is now confusing. At present, as described by Fausto (1990), if the stem cells are defined as undifferentiated, multipotent cells, and the progenitor cells as being non-hepatocyte epithelial cells, capable of dual-lineage generation (biliary and hepatocytic) and both of these lineages express hepatocyte markers, no pure population of either type of cells has been isolated from normal, injured or preneoplastic adult livers. Although oval cells, which appear during hepatocarcinogenesis and chemically induced severe liver injury (Grisham. 1980; Sell 1990; Sigal *et al.* 1994; Sirica *et al.* 1990; Thorgeirsson & Evart 1992), are one possible candidate for progenitor cells, identification of the progenitor cells in the normal adult liver has not been achieved and they do not expand initially in regeneration or after most injuries (Farber 1984). The oval cells are oval in shape with nuclei that are oblong and are invaginated with heterochromatin condensed along the nuclear membrane (Grisham & Hartroft. 1960). Furthermore, no in vitro systems have yet been developed where full morphological and functional differentiation of the progenitor cells into hepatocytes occurs. Different from oval cells, small hepatocytes are round in shape and had round nuclei with chromatin composition identical to that observed in the larger cells. In addition, small hepatocytes appear morphologically and functionally to be true hepatocytes as described above. Thus, small hepatocytes seem to fit into neither the stem cell nor the progenitor cell classification. We also observed that small hepatocytes sometimes expressed the characteristics of bile duct cells such as
CK7 and 19, and Cx43, which were also shown in the small hepatocytes by other researchers (Block et al. 1996; Cable & Isom 1997; Tateno & Yoshizato 1996b). However, the expressions are restricted to some of the cells in a colony, and we have observed neither the typical mature bile-duct cells nor the formation of bile ducts derived from small hepatocytes.

Mature and small hepatocytes in vivo can undergo transdifferentiation to form ductular structures under specific pathological conditions (Betto et al. 1996; De Voice & Desmet. 1992; Popper 1990) and they can be induced to form bile ductular structures in vitro when exposed to HGF (Block et al. 1996; Nishikawa et al. 1996). Hepatocyte-derived cell lines can also be induced to form ductular structures in culture (Talbot et al. 1996; Tee et al. 1996). The converse also seems to be true. Bile duct cells in the liver can be induced to form hepatocytes following bile duct ligation and treatment with chemicals, such as furan or carbon tetrachloride (Elmore & Sirica 1991; Sirica et al. 1994). These studies suggest that hepatocytes and bile duct cells retain the capacity to transdifferentiate to each other when the liver is confronted with severe pathological insults. As the situation of small hepatocytes in in vitro may be different from that of the cells in in vivo, it is, therefore, feasible to express some characteristics of bile duct cells. Thus, it is practicable to think that small hepatocytes may be "committed progenitor cells" that can further differentiate into mature hepatocytes.

**Maturation (Differentiation) of cultured hepatocytes**

An important goal for the researchers using primary hepatocytes has been to culture differentiated cells that can proliferate. The proliferating hepatocytes must preserve various functions such as secreting proteins, metabolizing nutrients, a bile production, detoxifying exo-and endogenous substances as
much as the cells in vivo do. Now, we can handle the proliferating hepatocytes, small hepatocytes as well as typical hepatocytes. But these hepatocytes are shown to possess various levels of differentiated functions of hepatocytes. Until now, many researchers including us have been using not only albumin, tyrosine aminotransferase (TAT), cytochrome P450, TO, serine dehydratase (SDH), Cxs 32 and 26, etc. as the biochemical markers but also bile-canaliculus formation, gap junctions, peroxisomes with crystalline nucleoids, many mitochondria, etc, as the morphological markers of the “differentiation.”

It has been reported that culture of freshly isolated hepatocytes under conventional conditions, which involves plating cells on a substratum of dried rat tail collagen in the presence of EGF and insulin, is accompanied by an extensive and fairly rapid loss of the liver-specific gene transcription (Bissell et al. 1987; Clayton & Darnell 1983). Therefore, for the purpose of maintaining the differentiated functions, investigators have used various substances and changed culture conditions through the use of phenobarbital (Miyazaki et al. 1985), extracellular matrix including Engelbreth-Holm-Swarm (EHS) gel (Matrigel®) (Ben-Ze’ev et al. 1988; Bissell et al. 1987; Dunn et al. 1992; Musat et al. 1993; Rojkind et al. 1980; Schuetz et al. 1988; Spray et al. 1987), coculture with nonparenchymal cells (Guguen-Guillozo 1986), spheroid formation (Koide et al. 1989), DMSO (Baribault & Marceau 1986; Isom et al. 1985; Kost & Michalopoulos 1991; McGowan 1988; Mitaka et al. 1993c) as well as nicotinamide (Inoue et al. 1989; Mitaka et al. 1991a). Recently, we established a culture system in which, by adding 2% DMSO to the culture medium after hepatocytes proliferate, the cells are able to recover differentiated functions such as albumin and transferrin secretion (Mitaka et al. 1993c; Mizuguchi et al. 1996). In our culture system, although expressions of Cx 32, Cx 26, TO, and SDH, thought to a highly differentiated function of mature
hepatocytes, have never been maintained or re-induced in primary hepatocytes cultured for a long period of time by using the conventional culture methods, their mRNA expressions were gradually restored with time after the addition of 2% DMSO and maintained for a month (Kojima et al. 1995a; 1995b; Mizuguchi et al. 1996). These liver-specific genes encoding proteins are generally known to be regulated by liver-enriched transcription factors such as CCAAT/enhancer binding protein (C/EBP), hepatocyte nuclear factor (HNF) 1, 3 and 4 (reviews see Cereghini 1996; Chojkier 1995; Pietrangelo & Shafritz. 1994; Zaret 1994). Therefore, the transcription factors play a crucial role in both induction and maintenance of the differentiated state of hepatocytes as well as liver development. In addition, it is generally accepted that hepatocyte differentiation is controlled primarily at the level of gene transcription (Zaret 1994). We recently reported that hepatic differentiation requires not only inhibition of DNA synthesis but also induction of appropriate transcription factors. Expression of HNF3γ, C/EBPα, and C/EBPβ may be necessary for hepatocytes to acquire highly differentiated functions (Mizuguchi et al. 1998).

In our culture system (L-15 + EGF + 2% DMSO) differing from conventional conditions, primary hepatocytes are plated on dishes at subconfluent density and can proliferate to reach about twice the initial density by day 6 (Mitaka et al. 1993c). In addition, a thick coating of rat tail collagen is necessary for the hepatocytes to differentiate and to be maintained on the dishes after 2% DMSO treatment. Both high density and support by ECM result in the alteration of cell shape. In fact, the shape of hepatocytes become cuboidal and the height of the basolateral surface of the cells increases with time in culture after addition of 2% DMSO (Kojima et al. 1997). The enlargement of attached areas between cells may result in the development of the structures of cell-cell adhesions such as desmosomes, gap junctions and bile canaliculus-like
structures (Mitaka et al. 1993c; Kojima et al. 1995a; 1995b; 1997). That the cell shape is a key factor to regulate the growth, differentiation, and survive of cells has been emphasized (Ingber 1994; Mayer 1988; Ruoslahti 1997; Watt 1986). Ben-Ze’ev et al. (1988) suggested that cell shape, which they found to be controlled by cell-cell contact and cell-matrix interactions, might be a primary regulator of tissue-specific gene expression and that cytoskeletal components might interact directly with the nuclear matrix to affect gene transcriptional rates. Recently, by the use of a method for the fabrication of a rubber stamp and the creation of patterned substrata, Singhvi et al. (1994) showed that limiting the degree of hepatocyte extension provided control over the growth of the cell and albumin secretion. Furthermore, DiPersio et al. (1991) have suggested that matrix-stimulated activation of specific albumin-regulating factors will only occur when the matrix on which cell is grown promotes a cuboidal differentiated cell morphology.

**Perspectives**

In this review I described the proliferation and differentiation (maturation) of primary rodent hepatocytes. Recent advance of culture conditions and a discovery of proliferating hepatocytes make a great step forward to the dream that we can freely regulate the proliferation and differentiation of true hepatocytes. In near future, the cells will be used for artificial liver, gene therapy, biosensor, etc, and finally, a reconstruction of real liver.

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Figure Legends

Figure 1. (A) Phase-contrast photographs of primary rat hepatocytes cultured in serum-free modified DMEM supplemented with 10 mM nicotinamide and 10 ng/ml EGF at day 10. 1% DMSO was added to the medium from day 4. Arrows show the small hepatocyte colony. (x 118) (B) Phase-contrast photographs of a small hepatocyte colony cultured in DMEM supplemented with 10% FBS, 10 mM nicotinamide, 1 mM Asc2P, and 10 ng/ml EGF at day 9. The cells were isolated from the supernatant after 50 x g centrifugation for 1 min. 1% DMSO was added to the medium from day 4. Asterisks show hepatic epithelial cells and arrows show stellate (Ito) cells. (x124) (C) Immunofluorescence for albumin of small hepatocyte colony at day 20. The cells were isolated from the supernatant after 50 x g centrifugation for 1 min. 1% DMSO was added to the medium from day 4. (x272) (D) Immunocytochemistry for BrdU at day 35. The cells were isolated from the supernatant after 50 x g centrifugation for 1 min. 1% DMSO was added to the medium from day 4 and 40 µM BrdU was added to the medium 24 h before fixation. Darkened nuclei are those stained with BrdU. Arrows show the mature hepatocytes with a large cytoplasm and binucleus. (x82)

Figure 2 Model of Liver Lineage.