**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-ribose) polymerase; NAD, nicotinamide adenine dinucleotide; DMEM, Dulbecco's modified Eagle's medium; OCT, ornithine carbamoyl transferase; Asc2P, L-ascorbic acid 2-phosphate; AFP, $\alpha$-fetoprotein; CK, cytokeratin; EHS, Engelbreth-Holm-Swarm; SDH, serine dehydratase; C/EBP, CCAAT/enhancer binding protein; HNF, Hepatocyte nuclear factor; $\beta$-gal, $\beta$-galactosidase;

**Introduction**

Hepatocytes are the predominant cell type in the liver, constituting over 80% of the liver mass under nonpathological conditions (Fawcett 1994). In the adult liver most hepatocytes are quiescent and few cells divide. Nonetheless, the liver retains 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* after 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 advances in molecular technology make it possible to use several experimental models for analyzing liver regeneration and there is increasing evidence that hepatocytes possess tremendous (perhaps unlimited) replication potential (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 epidermal growth factor (EGF) could induce DNA synthesis in primary hepatocytes. Thereafter, many researchers have tried 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." Following his classification system, the factors are summarized in **Table 1**. Much effort has been devoted to the resolution of the mechanisms by which the mitogens can transduce growth signals to hepatocytes; molecular and biochemical features of the mitogens have been investigated by many laboratories, and their roles in liver regeneration have been clarified. Many excellent reviews are now available (reviews see Fausto *et al.* 1995; Kay & Fausto 1997; Koch *et al.* 1990; Michalopoulos1990; 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 the fact that numerous works concerning the proliferation of primary rat hepatocytes have been published, most reported findings of increased DNA synthesis. Only few papers showed that primary hepatocytes really increased the cell number. Until the 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 maintained differentiated qualities tended to display very limited replication. Therefore, most researchers believed that differentiated hepatocytes could not proliferate in culture dishes.

Since the late 1980s I have been working to establish a culture system in which hepatocytes possessing differentiated characteristics can proliferate for a long time. In this review I introduce recent advances in primary culture, especially of rat hepatocytes.

## Table 1  Hepatic growth factors in primary rat hepatocytes

| Mitogens | | Authors | Year |
|---|---|---|---|
| **Complete Hepatocyte Mitogens** | Substances that are able by themselves, in chemically defined media and in the absence of serum, to stimulate hepatocyte DNA synthesis and mitosis in otherwise quiescent hepatocyte proliferation. | | |
| Epidermal Growth Factor (EGF) | | Richman et al. | 1976 |
| Transforming Growth Factor-α (TGF-α) | | Mead & Fausto | 1989 |
| | | Brenner et al. | 1989 |
| Heparin-Binding EGF-like Growth Factor (HB-EGF) | | Ito et al. | 1994 |
| Hepatocyte Growth Factors (HGF) | | Michalopoulos et al. | 1983 |
| | | Nakamura et al. | 1984b |
| | | Gohda et al. | 1988 |
| acidic Fibroblast Growth Factor (aFGF) | | Kan et al. | 1989 |
| | | Houck et al. | 1990 |
| **Comitogenic Growth Factors:** | Substances that affect hepatocyte growth in a positive direction but in an indirect manner. 1) Enhancement of the mitogenic effect of a growth factor. 2) Decrease of the inhibitory effect of growth inhibitors. 3) Absence of direct mitogenic effects of their own in serum-free medium | | |
| Insulin | | Richman et al. | 1976 |
| Glucagon | | McGowan et al. | 1981 |
| cyclic-AMP | | McGowan et al. | 1981 |
| IGF-I and II | | Koch et al. | 1982 |
| Norepinephrine | | Cruise et al. | 1985 |
| Vasopressin | | Russell & Bucher | 1983 |
| Angiotensin II and III | | Houck & Michalopoulos | 1989 |
| Vasoactive intestinal polypeptide (VIP) | | Kar et al. | 1996 |
| Neurotensin | | Hasegawa et al. | 1994 |
| Prostaglandin Fs | | Skouteris et al. | 1988 |
| Estrogens | | Shi & Yager | 1989 |
| Tumor Necrosis Factor-α (TNF) | | Sato & Yamazaki | 1992 |
| Hepatic stimulatory substance (HSS) | | LaBrecque & Pesch | 1987 |
| | | Francavilla et al. | 1987 |
| | | Fleig & Hoss | 1989 |
| Ethanolamine | | Sasaki et al. | 1997 |

## Multiplication of hepatocytes *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, there are two major methods for the multiplication of hepatocytes *in vitro*: one is the use of nicotinamide, the other is the use of a nutrient-rich medium.  In this section the materials that can modulate the DNA synthesis of hepatocytes in medium are introduced and the mechanisms of their promoting effects are discussed.

### Use of nicotinamide

Inoue *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 the potential to express tryptophan 2,3-dioxygenase (TO) mRNA, which is thought to be a differentiated function of mature hepatocytes, responding to hormonal induction for more than 2 weeks.  Thereafter, we reported that primary rat hepatocytes cultured in a serum-free chemically defined medium could repeatedly replicate their DNAs and that more than 20% of parenchymal cells went through a second round of cell division by day 4 (Mitaka *et al*. 1991a).  This was the first report to prove that many hepatocytes maintaining hepatic differentiated functions and structures can complete the cell cycle more than two times.  Thereafter, other investigators used nicotinamide in the medium to improve the survival of hepatocytes (Block

*et al*. 1996; Tateno & Yoshizato 1996a; Wu *et al*. 1994). Although they use different media with various additives, 5 to 10 mM nicotinamide is essential to maintain the capability of cell growth. For the proliferation of hepatocytes, 10 mM nicotinamide is the most effective concentration (Inoue *et al*. 1989; Mitaka *et al*. 1991a) and this concentration is known to almost completely inhibit poly(ADP-ribose) polymerase (PARP) activity (Uchigata *et al*. 1982). Nicotinamide, which is classified as an aqueous vitamin for animals, is converted in the cell to NAD and NADP, both of which are abundant cellular coenzymes necessary for a number of biochemical reactions. Consequently, it is reasonable to assume that decreases in the levels of 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 nicotinamide supplementation (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-ribosyl 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 carcinogens (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). In contrast, rapidly proliferating cells exhibit relatively higher activity of poly (ADP-ribosyl)ation than quiescent cells, as seen in the precancerous state of the hamster liver (Romaschin *et al*. 1981) and SV-40-transformed cells (Miwa *et al*. 1977). On the other hand, a decrease in poly (ADP-ribosyl)ation 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 expression 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. The 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 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 an 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 stimulation of protein synthesis as well as 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 the intact rat, interestingly, a protein-free diet or protein deprivation for 3 days followed by an amino acid meal stimulates DNA synthesis of the liver (Bucher *et al*. 1978; Short *et al*. 1974). This indicates that amino acids can be a trigger of hepatic DNA synthesis in the starved rat.

The original formulation of Leibovitz 15 (L-15) was shown to be 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 a 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 these conditions to an appreciable extent (Sawada *et al*., 1987). Recently, we found that 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 cells doubled within 5 days of culture, whereas the number of hepatocytes cultured in DMEM supplemented with EGF increased 1.3-fold. In addition, as the concentration of essential amino acids in the medium after addition of concentrated essential amino acid solution was close to that of essential amino acids in L-15 medium, DNA synthesis of the hepatocytes increased (Mitaka *et al*. 1991b). Although a 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 the 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 to 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 high concentrations of alanine and glutamine inhibited their DNA synthesis (McGowan & Bucher 1983).

To prevent contamination of cultures by nonparenchymal cells, some investigators have removed arginine from the hepatocyte medium (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 of arginine. However, in practice, although we used the modified DMEM (ornithine supplemented arginine-free medium) in our early experiments (Mitaka *et al*. 1992a; 1992b; 1993a; 1993b), the percentage of nonparenchymal cell contamination 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 the urea cycle would have diminished activity or disappear altogether. Recently, we use a commercial medium supplemented with arginine and glucose.

2) *Vitamins*

Nicotinamide is a kind of vitamin. 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 addition of L-ascorbic acid 2-phosphate (Asc2P), a long-acting vitamin C derivative, to 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 time.  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 an ECM such as collagen 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 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 DNA synthesis in primary culture, their intragastric administration stimulated that of hepatocytes *in vivo*.

3) *Trace metals*

Selenium has been mentioned as a factor that in some manner supports the survival of replicating cell lines maintained in culture in serum-free medium (Ham & McKeehan 1978).  Although $1x10^{-5}$ M $Na_2SeO_3$ in serum-free medium inhibited mammary cell cultures, $5x10^{-8}$ M $Na_2SeO_3$ 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 culture medium with selenium could prevent the loss and maintain the synthesis of P450 responding to phenobarbital.  Although no one has presented direct evidence that selenium can enhance the proliferation of cultured hepatocytes, we and other researchers usually add the metal to chemically defined medium when hepatocytes are cultured in for a long time (Block *et al*. 1996; Cable & Isom 1997; Mitaka *et al*. 1991a; 1991b; 1992a).

Recently, Cable & Isom (1997) reported that the trace metals copper, iron, and zinc are all necessary for primary hepatocytes cultured in chemically defined medium to proliferate for a long time.  They explained why these metals are required for 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.  Zinc, in particular, 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 copper-depleted rats (Rao *et al*. 1986).

As for the iron source, the importance of transferrin has been pointed out in chemically defined medium by Block *et al*. (1996).  They showed that iron-saturated diferric transferrin promoted the growth of cultured hepatocytes much more than unsaturated transferrin combined with elemental iron.  Contrastingly, 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 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 mitogens are added.  However, when L-15 medium is adjusted for use in a $CO_2$-incubator by adding $NaHCO_3$, a dramatic

increase of DNA synthesis is observed in primary hepatocytes (Mitaka *et al*. 1991b; 1991c). The DNA synthesis increases parallel to the increase of the $NaHCO_3$ concentration in the medium and 20 mM $NaHCO_3$ in L-15 medium is best. Though the importance of $CO_2$/bicarbonate for the stimulation of growth has already been pointed out in various cell lines (Ehman & Misfeldt. 1983; Itagaki & Kimura 1974), the concentration of $NaHCO_3$ has never been reexamined in primary culture of hepatocytes. Without paying attention to the concentration of $NaHCO_3$ in medium, it is made in most laboratories by following the manufacturer's instructions. However, a suitable concentration for maximum stimulus of DNA synthesis exists in each medium (Mitaka *et al*. 1991c). For example, for DMEM/F12 it is 25 mM instead of 14 mM, for Williams Medium E it is 30 mM, and for DMEM it is 25 mM instead of 45 mM.

In early studies with serum-free medium, high concentrations of pyruvate (Hasegawa & Koga 1981; McGowan & Bucher 1983) and lactate (McGowan & Bucher 1983) were reported to stimulate the replicative DNA synthesis of rat hepatocytes with or without EGF. Oxaloacetate was also shown to have the 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 polyamine, on 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 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 L-15 medium supplemented with EGF in a $CO_2$-incubator (Mitaka *et al*. 1991b) although the frequency of the cells was less than that of cells in 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. Thus, perhaps whenever isolated hepatocytes are cultured in a medium in which they can really proliferate, small hepatocytes may be given an environment to start their growth.

Small hepatocytes can rapidly proliferate in 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 named them small hepatocytes. 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, hepatic epithelial cells are the major contaminants derived from nonparenchymal cells. The cells have been studied for a long time and are known to clonally proliferate in primary culture (Grisham 1980; Grisham &
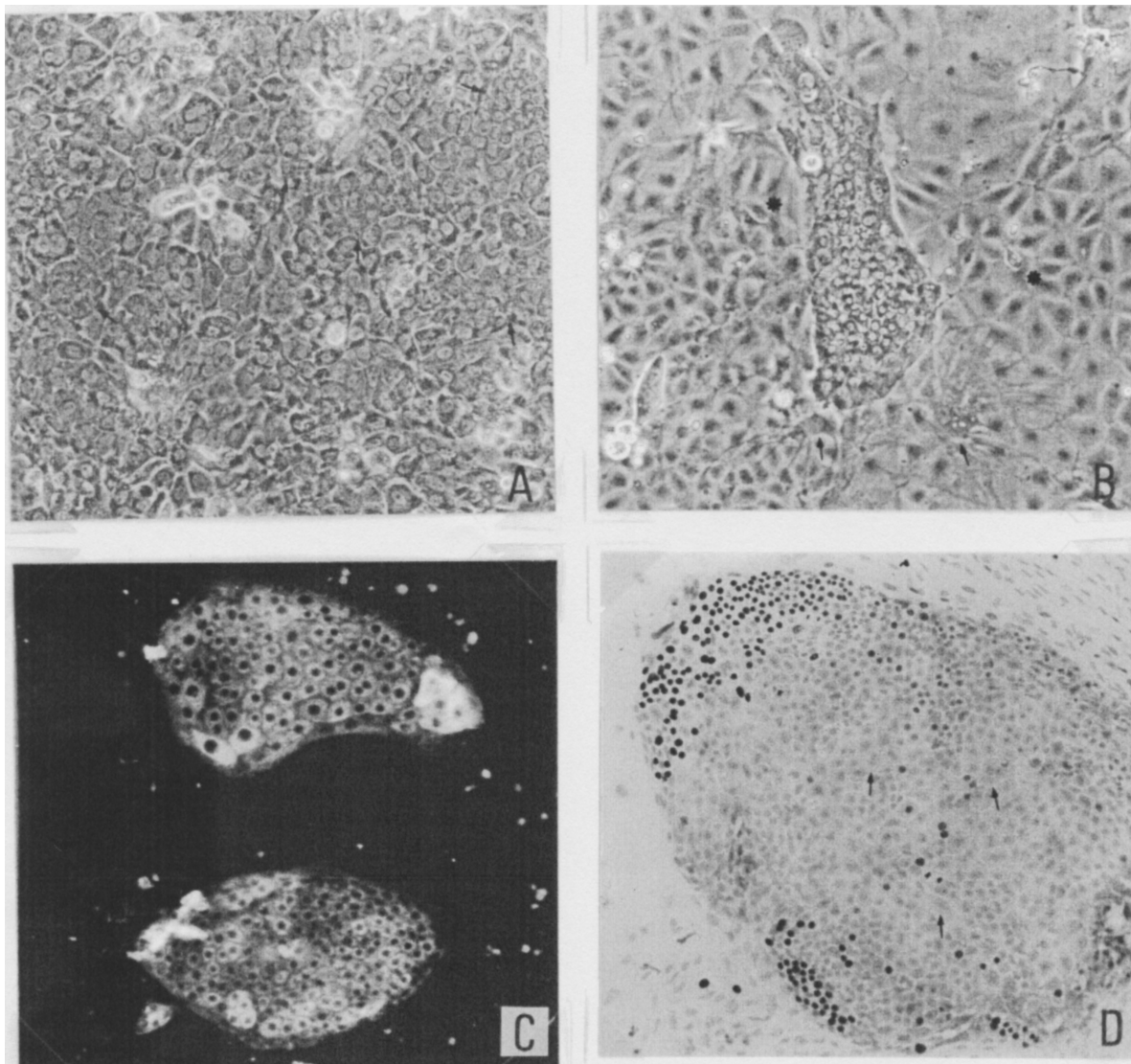
**Figure 1** (A) Phase-contrast photographs of primary rat hepatocytes cultured in serum-free modified DMEM supplemented with 10 mM nicotinamide and 10 ng/ml EGF at day 10. 1% DMSO was added to the medium from day 4. Arrows show the small hepatocyte colony. (x 118) (B) Phase-contrast photographs of a 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 1min. 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 1min. 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 1min. 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)

Thorgeirsson. 1997; Williams. 1976; Yang *et al*. 1993). In addition, the cells are considered to be candidates for 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 considered not to be 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) speculated that those cells might arise from small nondescript epithelial cells, which are morphologically distinct from hepatocytes. Thus, the addition of a high concentration of nicotinamide to the conventional medium is the only determinant of whether small hepatocytes can proliferate. As shown in **Fig. 1B**, the morphology of both cells is quite different and easy to identify. However, after long cultivation, the transitional cells sometimes appear in the peripheral zone of the small hepatocyte colony, in which small hepatocytes can not distinguished from hepatic 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 cells in a colony reaches about 27 at day 10 (Mitaka *et al*. 1995). Tateno & Yoshizato (1996b) 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 the first 10 days was $51 \pm 3$ hours and increased to $753 \pm 203$ hours in the following 20 days. There are three interpretations of why a delay of the doubling time after 10 days of culture is observed in small hepatocytes: a) most small hepatocytes in a colony show the slowing doubling time after 5 to 7 divisions, b) some small hepatocytes may differentiate into mature hepatocytes, the cell division of which is suppressed, and c) factors 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, b) and c) may be feasible to explain the reason why a delay of the doubling time after 10 days of culture is observed in small hepatocytes. At present, we do not have exact data concerning whether small hepatocytes can unlimitedly divide or not. Recently, experiments in which the regenerative capacity of hepatocytes is examined *in vivo* have been performed by using transgenic mouse models: albumin-urokinase transgenic mice (Rhim *et al*. 1994; Sandgren *et al*. 1991) and fumarylacetoacetate hydrolase deficient mice (Overturf *et al*. 1996; 1997). These studies show that the upper limit of cell doubling is at least 69 or $7.3 \times 10^{20}$-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 equals about 10 cell doublings. These results suggest that small hepatocytes may have the 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 at about the time of weanling 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 in 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 regeneration is different according to the

age of the rats (Stöcker & Heine. 1971).   An *in vivo* experiment employing continuous administration of $^3$H-thymidine to rats showed that 99% of hepatocytes in a weanling rat, 93% of cells in a young adult rat, and only 77% of cells in a 2.5-year-old rat synthesized DNA during regeneration after two-thirds partial hepatectomy.  On the other hand, we reported that small hepatocytes were 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 varied and decrease with the age of the rat: about 6% of hepatocytes isolated from 4-week old rats, about 2.5% of those from 8-week old rats, and about 1.5 to 2.0% of those from adult rats were small hepatocytes (Mitaka *et al*. 1993a).  In aged rats (more than 2 years old) the frequency was less than 0.5%.  Furthermore, the cells isolated from rats younger than 3 weeks old 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 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 sufficiently 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**.
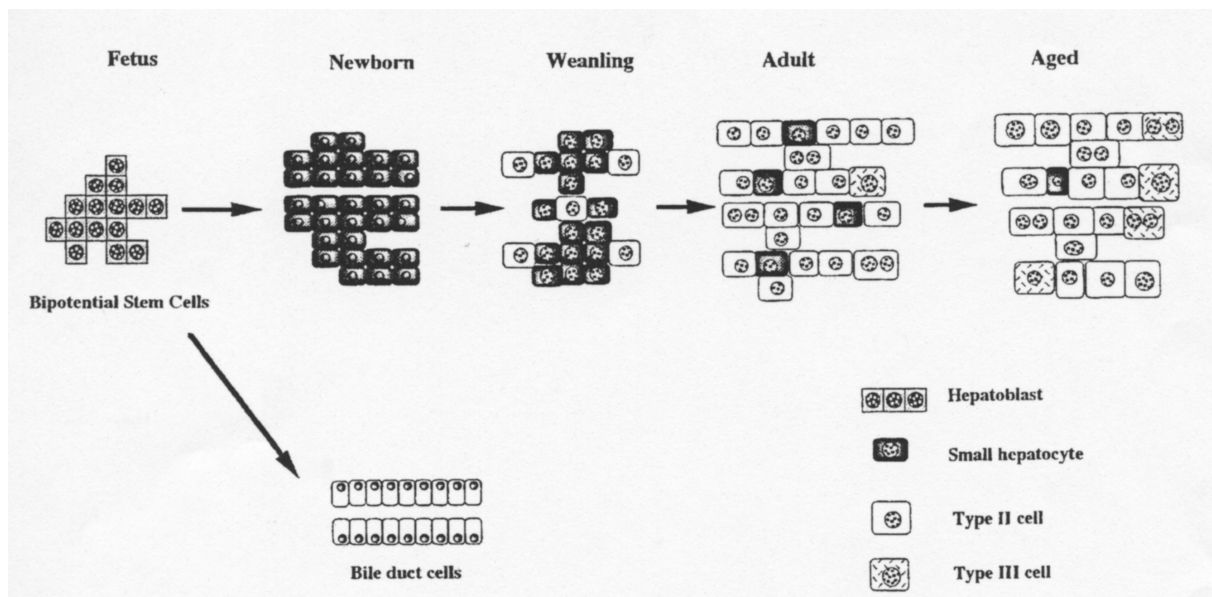


**Figure 2**  Model of Liver Lineage.

The liver develops from a diverticulum 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 to only 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 until 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 express 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 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 weanling, some small hepatocytes in liver plates differentiate into Type II cells. At present we do not know what the possible mechanisms induce the differentiation of small hepatocytes. However, small hepatocytes can continue to proliferate without differentiation (maturation) when the growth of nonparenchymal cells is inhibited in co-culture (unpublished observation). The direct effects of and/or the products such as cytokines and/or ECM of the nonparenchymal cells may regulate the differentiation of small hepatocytes into mature ones. With growth, many 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 the *spf*$^{ash}$ mouse (Shiojiri *et al*. 1997). This research 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-dimensional, large contiguous quasiclones of hepatocytes. Iannaccone and associates (Khokha *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 *et al*. (1995) used a transgenic mouse line in which the human $\alpha_1$-antitrypsin promoter directs expression of β-galactosidase (β-gal) in the liver cell. In neonatal

mice, single or doublet of β-gal-positive hepatocytes are randomly scattered throughout the liver. With aging, the positive cells form larger clusters. In addition, the β-gal-expressing cells show no predilection for the periportal or pericentral region. Furthemore, Bralet *et al*. (1994) showed the fate of normal hepatocytes of adult rats by a genetic labeling method. They perfused retroviral vectors carrying the β-gal gene directly into the liver after 2/3 partial hepatectomy. The labeled cells formed clusters with age but the their distribution in liver lobules did not change throughout life, although a number of labeled cells had a tendency to locate near periportal regions (zones 1 and 2 of Rappaport. [1976]). They also estimated the life span of hepatocytes to be between 150 and 450 days. These values are similar to a previous estimation (MacDonald 1961). These results support my hypothesis of random distributions of small hepatocytes and stand against the "hepatocyte streaming theory" (Arber *et al*. 1988; Sell 1994; Sigal *et al*. 1994; Zajicek *et al*. 1985), which speculates 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, in rats, some Type II cells complete their role of proliferation and become Type III cells. As described above, one-fourth of hepatocytes in the liver in rats more than 2.5 year-old rats lose responsiveness to growth stimulation after 2/3 partial hepatectomy (Stöcker & Heine 1971). Furthermore, many hepatocytes isolated from aged rats do not enter the 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 the normal adult rat liver, to compensate for cells eliminated by death or for 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, division of Type II cells may be sufficient to restore the original mass of the liver, although small hepatocytes possibly participate in the event.

Although we have carried out experiments to identify the origin and the location of small hepatocytes in liver lobules, we have not yet succeeded. The major reason why we can not distinguish small hepatocytes from mature ones seems to be the similarity of the cells. This also indicates that small hepatocytes may be mixed in hepatic plates. If this speculation is true, it would explain why their clonal expansion is observed in cultures of 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 be mixed with 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 unclear. 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). These 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 and have 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 expression is 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 into each other when the liver is confronted with severe pathological insults. As the situation of small hepatocytes *in vitro* may be different from that of the cells *in vivo*, it is, therefore, feasible that they could 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 researchers using primary hepatocytes has been to culture differentiated cells that can proliferate. The proliferating hepatocytes must preserve various functions such as secretion of proteins, metabolization of nutrients, bile production, and the ability to detoxify exo-and endogenous substances much as cells *in vivo* do. At present, we can handle the proliferating hepatocytes, small hepatocytes as well as typical hepatocytes, but these hepatocytes possess various degrees of the 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 biochemical markers but also bile-canaliculus formation, gap junctions, peroxisomes with crystalline nucleoids, many mitochondria, and so on, as morphological markers of "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 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 hepatocyte 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 expression of Cx 32, Cx 26, TO, and SDH, thought to a highly differentiated function of mature hepatocytes, has never been maintained or re-induced in primary hepatocytes cultured for a long period of time by using the conventional culture methods, their mRNA expression was 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), and hepatocyte nuclear factors (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 HNF3$\gamma$, C/EBP$\alpha$, and C/EBP$\beta$ may be necessary for hepatocytes to acquire highly differentiated functions (Mizuguchi *et al*. 1998).

In our culture system (L-15 + EGF + 2% DMSO), unlike under 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 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 cell-cell adhesion structures 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 the 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 advances in culture conditions and the discovery of proliferating hepatocytes are great steps forward toward the dream of freely regulating the proliferation and differentiation of true hepatocytes.  In the near future, the cells will be used for artificial liver, in gene therapy, as biosensors, etc, and finally, for reconstruction of a 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 a 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 a 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 $\mu$M 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.