# Cell Lineage Analysis during Liver Development Using the *spf*^ash-Heterozygous Mouse

Nobuyoshi Shiojiri, Sachiko Inujima, Kiyoshi Ishikawa, Kazutoyo Terada, and Masataka Mori

*Department of Biology (NS, SI, KI), Faculty of Science, Shizuoka University, Shizuoka, and Department of Molecular Genetics (KT, MM), Kumamoto University School of Medicine, Kumamoto, Japan*

**SUMMARY:** Biliary epithelial cells differentiate from periportal hepatoblasts during fetal mouse liver development. It remains to be determined whether each hepatoblast is equivalent for differentiation into hepatocytes and biliary epithelial cells in normal liver development. To resolve this question, the mosaic pattern of ornithine transcarbamylase (OTC) expression was analyzed in the hepatoblast population of *spf*^ash (sparse-fur with abnormal skin and hair)-heterozygous fetal mouse livers, in which random inactivation of either the X chromosome carrying the *spf*^ash gene (causing OTC deficiency) or its wild-type gene occurs. Aggregates (patches) of OTC-positive hepatoblasts showed very complex patterns, and their shapes and size distributions were similar in sections from periportal regions and nonperiportal regions of the fetal liver in which bile duct differentiation by periportal hepatoblasts occurred. Average sizes of periportal patches were larger than those of nonperiportal patches because of the presence of more hemopoietic cells in the latter region. The OTC mosaicism in periportal bile duct progenitors and hepatoblast islands of other liver parenchyma was also similar. These results suggest that the growth patterns of hepatoblasts are similar in both periportal and nonperiportal regions. Isolated three-dimensional patches comprising hepatoblasts giving rise to only biliary epithelial cells or hepatoblasts giving rise to both hepatocytes and biliary epithelial cells were observed in periportal regions. In nonperiportal regions, patches consisting of hepatoblasts differentiating into hepatocytes were also seen. Thus, it is likely that there are three lineages for the developmental fates of hepatoblasts: hepatoblasts giving rise to only biliary epithelial cells, hepatoblasts giving rise to only hepatocytes, and hepatoblasts giving rise to both of them. (*Lab Invest 2001, 81:17–25*).

*H*epatocytes and biliary epithelial cells constitute the liver as endodermal components (Shiojiri, 1981). Biliary epithelial cells differentiate from a histochemically homogeneous cell population of hepatoblasts under the influence of periportal connective tissue at the fetal stage, and other hepatoblasts give rise to mature hepatocytes during mammalian liver development (Bloom, 1926; Enzan et al, 1974; Haruna et al, 1996; Horstmann, 1939; Shiojiri, 1994; Shiojiri et al, 1991; Terada et al, 1998; Terada and Nakanuma, 1994; Van Eyken et al, 1988a, 1988b). It has also been experimentally demonstrated that hepatoblasts can give rise to both hepatocytes and biliary epithelial cells during in vivo transplantation experiments using immature fetal mouse liver fragments (Shiojiri, 1984; Shiojiri et al, 1995). Cell culture studies also indicate that hepatoblasts can differentiate into cells expressing bile duct markers or mature hepatocyte markers, depending on culture conditions (Blouin et al, 1995; Brill et al, 1995; Germain et al, 1988). However, it still remains to be resolved whether each hepatoblast gives rise to both hepatocytes and biliary epithelial cells during fetal liver development or whether special
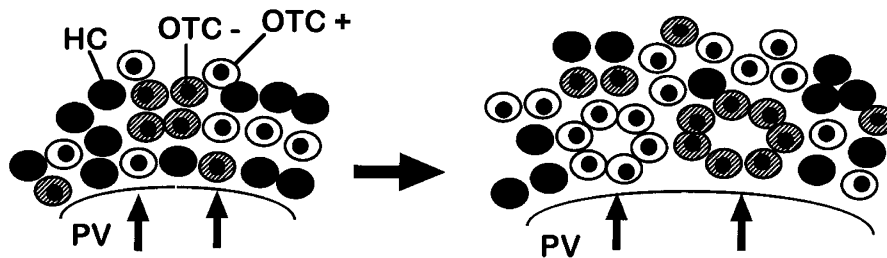
hepatoblasts for biliary epithelial cells are present around the portal area.

Mosaic analysis in chimeric animals, in which cells of two genotypes or two species are mixed, can reveal cell lineages and cell migration in development. Such analysis in rodent livers suggests that hepatocytes randomly allocate their progeny in liver development and regeneration (Iannaccone et al, 1987; Khokha et al, 1994; Ng and Iannaccone, 1992). We have immunohistochemically demonstrated that, in *spf*^ash (sparse-fur with abnormal skin and hair)-heterozygous mouse liver (DeMars et al, 1976; Hodges and Rosenberg, 1989; Ohtake et al, 1987), ornithine transcarbamylase (OTC) expression is mosaic in hepatocytes because of the random inactivation of either the X chromosome carrying the *spf*^ash gene or the wild-type gene (Lyon, 1961, 1972; Wareham and Williams, 1986), and that the mosaic pattern can be analyzed in both two and three dimensions (Shiojiri et al, 1997). Analysis of the OTC mosaic pattern in the fetal mouse liver, where biliary cell differentiation occurs, may be helpful in resolving the issues mentioned above. OTC has already been shown to be expressed in fetal hepatoblasts and hepatocytes (Dingemanse et al, 1996; Ryall et al, 1986). If special hepatoblasts reside in periportal regions and give rise only to biliary epithelial cells during liver development, mosaic patterns of OTC expression in the precursor cell populations of biliary epithelial cells would be different from those of cells giving rise to mature hepatocytes (Fig. 1). Mosaicism of OTC expression in precursor structures of bile ducts may also be

**Figure 1.**
Mosaic analysis for bile duct differentiation. If periportal progenitors for biliary epithelial cells grow more extensively than hepatoblasts in other regions in the mosaic liver, in which hepatoblasts of two genotypes for ornithine transcarbamylase (OTC) are mixed, the OTC mosaicism in bile duct cells would be biased toward either OTC-positive or -negative patches (*arrows*). Periportal mosaicism would also become different from that in other hepatic regions. Periportal connective tissue is not drawn. *HC*, hemopoietic cells; *OTC+*, OTC-positive hepatoblasts; *OTC−*, OTC-negative hepatoblasts; *PV*, portal vein.

biased toward either OTC-positive or -negative cells if special cell proliferation occurs more extensively in progenitor cells of biliary epithelial cells than in cells of nonperiportal liver parenchyma.

In the present study, we analyzed the mosaic pattern of OTC expression in fetal livers of *spf* [ash]-heterozygotes, and we report here that there was no special zone for proliferation of hepatoblasts in the fetal liver parenchyma and that hepatoblasts may be classified into three cell lineages: hepatoblasts giving rise to only biliary epithelial cells, hepatoblasts differentiating into only hepatocytes, and hepatoblasts differentiating into both hepatocytes and biliary epithelial cells.

## Results

### OTC Expression in Fetal Mouse Livers of the Wild Type

In 15.5-day fetal livers of wild-type mice, bile duct differentiation by hepatoblasts occurred around portal veins, and their progenitor cells expressed OTC and bile duct markers (laminin and strong expression of cytokeratins) (Figs. 2 and 3). Hepatoblasts differentiating into hepatocytes in nonperiportal regions were positive for OTC immunostaining but were negative for laminin and were weakly or moderately positive for cytokeratin immunostaining. Hemopoietic cells, which were abundant in fetal livers, were negative for OTC, laminin, and cytokeratin immunostainings. At 17.5 days, squamous biliary epithelial cells differentiated and became negative or weakly positive for OTC immunostaining (Fig. 2). Neonatal biliary epithelial cells of the wild-type did not express OTC. Although our anticytokeratin antibodies reacted weakly or moderately with hepatocytes, laminin expression was specific for biliary epithelial cells and their progenitors around portal veins among hepatic endodermal cells throughout development (Fig. 3).

### OTC Mosaicism in Fetal spf [ash]-Heterozygous Livers

Mosaic expression of OTC was already seen not only in hepatoblasts of nonperiportal regions, but also in periportal bile duct progenitors in 15.5-day *spf* [ash]-heterozygous livers (Fig. 4). The percentage of OTC-positive regions in the liver parenchyma at this stage was very low (10%–25%) because of the presence of many OTC-negative hemopoietic cells, which accounted for

approximately 50% of the liver volume. Patches were very small compared with those in postnatal livers. OTC-positive cells and OTC-negative cells were complicatedly mixed in periportal bile duct progenitors and hepatoblast islands in the liver parenchyma (Fig. 4). There was no special orientation of patch shapes in sections in either the periportal or nonperiportal region.

Double immunofluorescence analysis of OTC and cytokeratins or laminin demonstrated that OTC-positive or -negative biliary cell progenitors sometimes connected with OTC-positive or -negative hepatoblasts differentiating into hepatocytes, respectively (Fig. 5). OTC-positive patches remaining only within biliary cell progenitors were also found.

Mosaicism in OTC expression in squamous epithelial cells of bile ducts was seen in 17.5-day fetal livers (Fig. 4). However, because in some biliary epithelial cells OTC expression was suppressed as a result of bile duct differentiation at this stage, quantitative analysis was done only for 15.5-day livers.

### Comparison of Patch Sizes between Periportal Regions and Nonperiportal Regions of Fetal Livers

Patch sizes in the liver parenchyma of 15.5-day *spf* [ash]-heterozygous mice were measured quantitatively using the computer-assisted image analyzer, and those in periportal and nonperiportal regions were compared (Fig. 6). In both regions, many small patches were observed, though average patch sizes of periportal regions were larger than those of nonperiportal regions (1.4 times). Histologically, more hemopoietic cells were observed in nonperiportal regions, which may have decreased the patch sizes. There were no clear differences in the shapes of the patch size distributions between periportal and nonperiportal regions (Fig. 6). The shapes of the patches themselves were also similar.

### OTC Mosaicism in Bile Duct Progenitors and Hepatoblast Islands of the Liver Parenchyma

OTC mosaicism in bile duct progenitors and hepatoblast islands in liver parenchyma was examined in 15.5-day fetal livers. If each patch were developmentally derived from one cell, it would be composed only of cells of either genotype. If cell proliferation patterns differed in hepatoblast populations between periportal
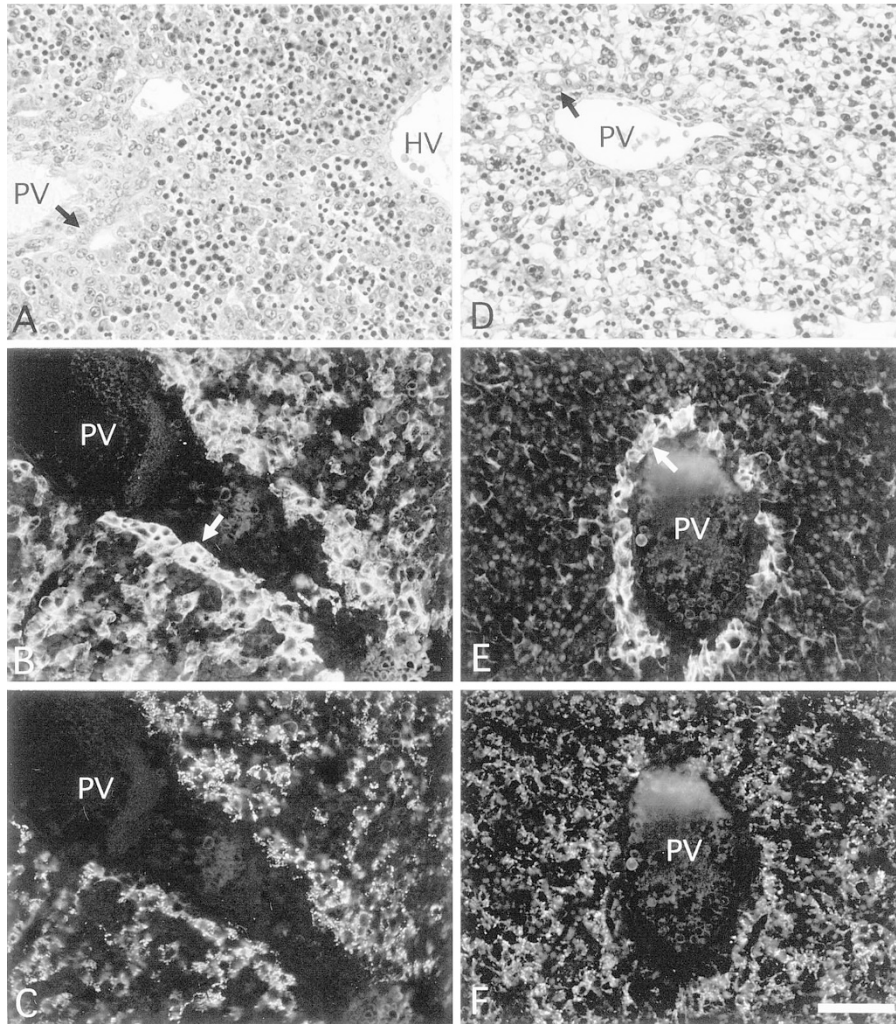
**Figure 2.**
Development of bile ducts in wild-type mouse livers. A, Bile duct progenitors (*arrow*) at 15.5 days of gestation. Many hemopoietic cells are also seen. Hematoxylin-eosin staining. B, Cytokeratin immunostaining in 15.5-day liver. Precursor cells for biliary epithelial cells (*arrow*) around the portal vein express cytokeratins strongly. Hepatoblasts in other parts are weakly or moderately stained for the cytokeratins. C, OTC immunostaining in B. Precursor cells of biliary epithelial cells also express OTC. D, Squamous biliary epithelial cells (*arrow*) in 17.5-day fetal liver. Hematoxylin-eosin staining. E, Cytokeratin immunostaining in 17.5-day fetal liver. Cytokeratins are expressed strongly in biliary epithelial cells (*arrow*). F, OTC immunostaining in E. Biliary epithelial cells either express OTC or do not. *PV*, portal vein. *Bar* indicates 50 $\mu$m.

and nonperiportal regions, different mosaicisms would be obtained in them (Fig. 1).

The percentages of OTC-positive cells in periportal bile duct progenitors and hepatoblast islands of nonperiportal areas, which were strongly cytokeratin-positive and laminin-positive or moderately cytokeratin-positive and laminin-negative, respectively, were analyzed using double immunofluorescent pictures (Fig. 7). A biased pattern of OTC mosaicism was not obtained in hepatoblast populations for either region. Distribution patterns of OTC mosaicism were similar in the two cell populations. The average percentage was approximately 42% for both regions (42% $\pm$ 19% in bile duct progenitors; 42% $\pm$ 23% in hepatoblast islands in 18 sections of 3 heterozygous livers).

### Three-Dimensional Analysis of Periportal Patches

Three-dimensional analysis also showed no definite shapes of three-dimensional patches in either region in

15.5-day fetal livers (Fig. 8). Isolated three-dimensional patches were often seen (Table 1). Their population sizes were approximately 5 through 70 cells in the smallest patches. Periportal patches consisted only of laminin-positive cells or of both laminin-positive biliary cell progenitors and laminin-negative hepatoblasts differentiating into hepatocytes. In nonperiportal regions, three-dimensional patches of laminin-negative hepatoblasts differentiating into hepatocytes were observed.

In 17.5-day livers, isolated three-dimensional patches were also detectable, but a strict analysis of the three-dimensional connections of patches in sections could not be done for periportal areas because of the partial suppression of OTC expression in them.

### Discussion

It is well established that bile duct differentiation by hepatoblasts takes place in periportal areas in the fetal
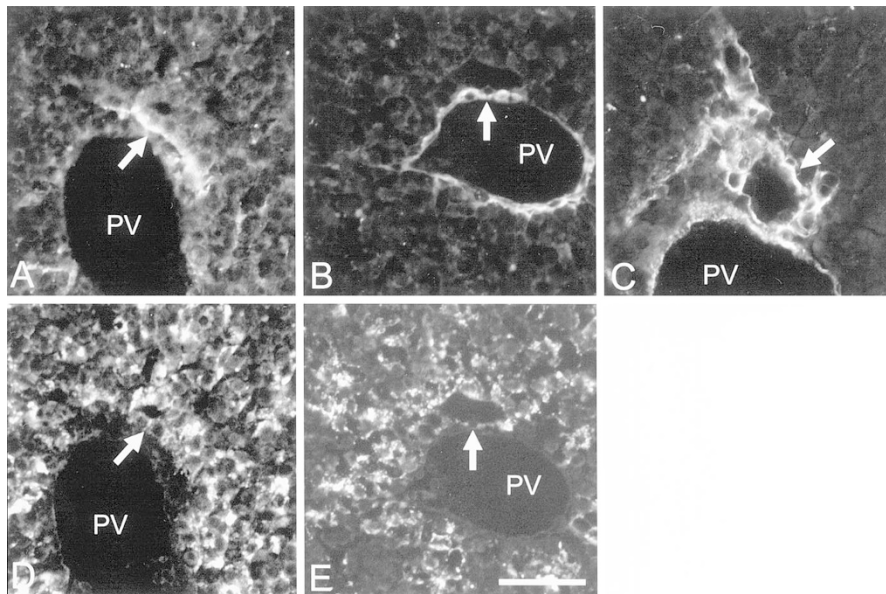
**Figure 3.**
Biliary epithelial cells and their progenitors express laminin in development of wild-type mouse livers (A, 15.5-day; B, 17.5-day; C, 2-week-old). *Arrows* indicate biliary epithelial cells and their progenitors. D and E, OTC immunostaining in A and B, respectively. Biliary cell progenitors at 15.5 days coexpress OTC and laminin. *PV*, portal vein. *Bar* indicates 50 μm.
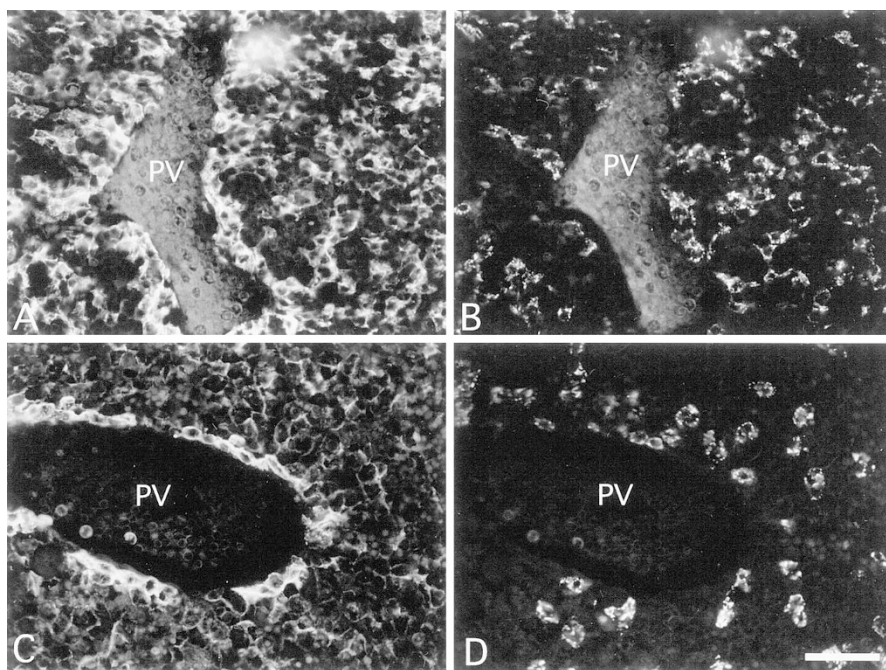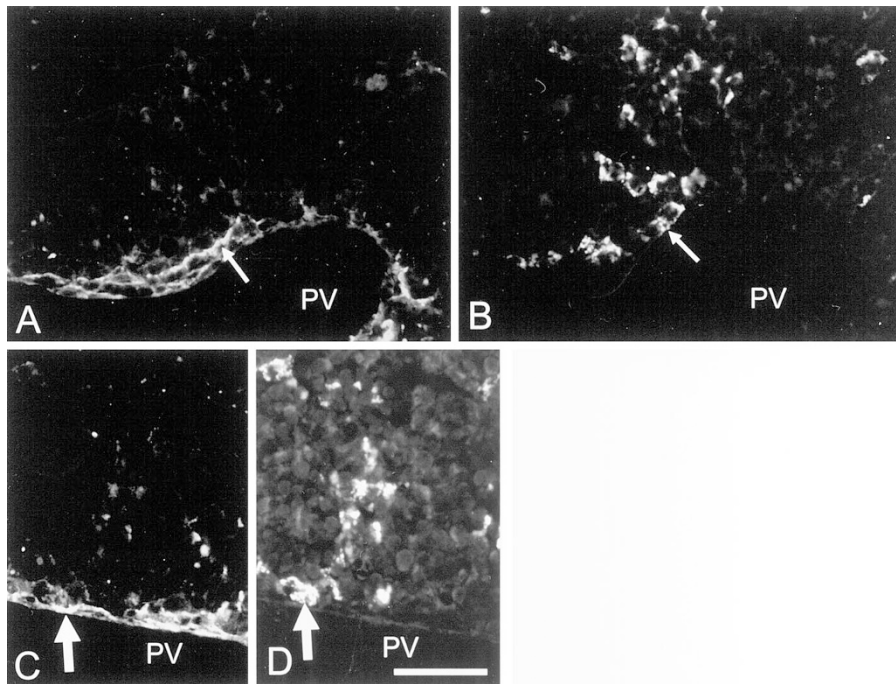


**Figure 4.**
OTC mosaicism in livers of *spf* [ash]-heterozygous fetuses. A, Cytokeratin immunostaining in 15.5-day fetal liver. B, OTC expression in A. Note the small patches and the lack of special orientation of patch shapes to portal veins. C, Cytokeratin immunostaining in 17.5-day fetal liver. D, OTC expression in C. Patches are very small. *PV*, portal vein. *Bar* indicates 50 μm.

stages of humans and rodents (Bloom, 1926; Enzan et al, 1974; Haruna et al, 1996; Horstmann, 1939; Shiojiri, 1981, 1994; Shiojiri et al, 1991; Terada et al, 1998; Terada and Nakanuma, 1994; Van Eyken et al, 1988a, 1988b). Immunohistochemical studies have shown that hepatoblasts are a homogeneous population in terms of expression of alpha-fetoprotein, albumin, and cytokeratins before the appearance of periportal bile duct pro-genitors (Shiojiri, 1981; Shiojiri et al, 1991; Van Eyken et al, 1988a, 1988b). However, strict analysis of the cell lineages during fetal bile duct development has not been carried out. The present study on OTC expression with a double immunofluorescent technique in mosaic mouse reported on similar growth patterns of hepatoblasts located in periportal areas and nonperiportal areas and on the cell lineages of hepatoblasts.

**Figure 5.**

Two patterns of OTC mosaicism in the periportal region of 15.5-day *spf* ^ash^-heterozygous fetal livers. A, Laminin immunostaining. B, OTC immunostaining in A. Note the OTC-positive patch within a bile duct progenitor (*thin arrow*). C, Laminin immunostaining. D, OTC immunostaining in C. OTC-positive patches composed of laminin-positive cells and -negative cells are seen (*thick arrows*). PV, portal vein. *Bar* indicates 50 $\mu$m.
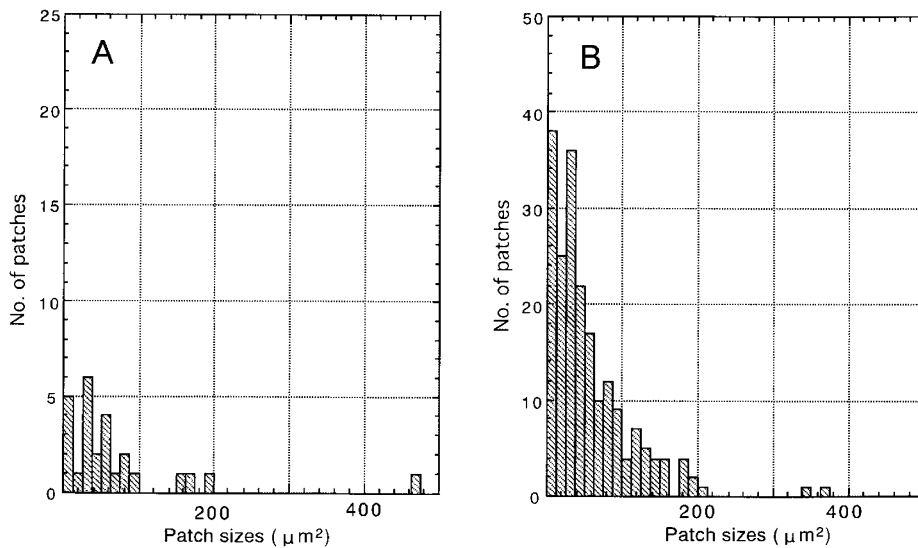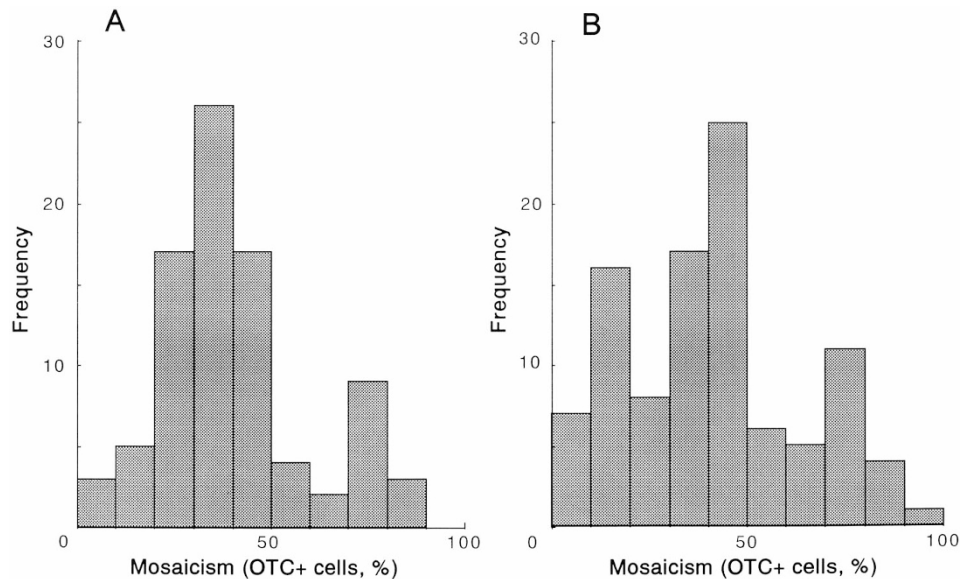


**Figure 6.**

Representative of patch size distribution in periportal (A) and nonperiportal regions (B) of a 15.5-day heterozygous liver. Patches at this stage are small, and their size distributions in both regions are similar. Periportal patches are larger than nonperiportal patches on average.

The present study demonstrated that OTC mosaicism was similar in both periportal bile duct precursors and nonperiportal hepatoblast islands at 15.5 days of gestation, suggesting that hepatoblasts in both areas grow similarly. Furthermore, the shapes of patch size distributions were similar in periportal and nonperiportal hepatoblasts, and patch shapes in both areas had no special orientation to the landmarks of the liver at this stage. These results also support the similar growth of both periportal and nonperiportal hepatoblasts. Although the average patch sizes were smaller in nonperiportal regions than in periportal regions, this difference probably stemmed from the presence of more hemopoietic cells in nonperiportal regions; OTC-negative hemopoietic regions became larger in nonperiportal regions, which resulted in smaller patch sizes. Therefore, there may be no special zones for growth of hepatoblasts in the liver parenchyma before 15.5 days of gestation. This conclusion agrees well with findings of the random allocation of progeny by hepatocytes during postnatal liver develop-

**Figure 7.**
Representative of OTC mosaicism in periportal progenitors of biliary epithelial cells (A) and hepatoblast islands (B) of the nonperiportal area. The average ratio of OTC mosaicism (42% for each region) is similar.
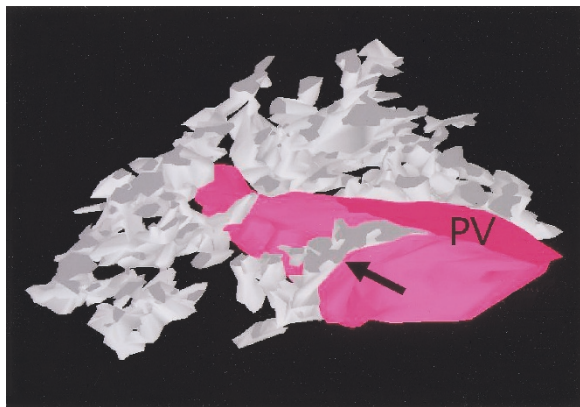


**Figure 8.**
Three-dimensional reconstruction of OTC patches from six serial sections of 15.5-day *spf* ash-heterozygous mouse liver. Note irregular shapes of three-dimensional patches. *Arrow* indicates a three-dimensional patch comprising a ductal plate and cells giving rise to hepatocytes. *PV*, portal vein.

**Table 1. Isolated Three-Dimensional Patches of Periportal Regions in 15.5-Day Fetal Livers of spf ash-Heterozygotes**

| Patch sizes (No. of cells) | Progenitors of | |
| --- | --- | --- |
| | Biliary epithelial cells | Hepatocytes |
| 5 | 2 | 3 |
| 5 | 5 | 0 |
| 8 | 8 | 0 |
| 8 | 3 | 5 |
| 9 | 8 | 1 |
| 10 | 10 | 0 |
| 10 | 5 | 5 |
| 10 | 3 | 7 |
| 10 | 6 | 4 |
| 12 | 7 | 5 |
| 14 | 9 | 5 |
| 21 | 3 | 18 |
| 22 | 2 | 20 |
| 40 | 25 | 15 |
| 53 | 19 | 34 |
| 74 | 37 | 37 |

In nonperiportal regions, 22 isolated patches were observed, and they were all composed of laminin-negative hepatocyte progenitors.
Progenitors of biliary epithelial cells were identified by laminin immunostaining.

ment (Iannaccone et al, 1987; Khokha et al, 1994; Ng and Iannaccone, 1992; Shiojiri et al, 2000).

We also observed isolated three-dimensional patches in 15.5-day fetal livers of *spf* ash-heterozygotes whose population sizes were approximately 5 to 70 cells in the smallest patches. As discussed elsewhere (Shiojiri et al, 2000), they might correspond to hepatoblast clones. These could be observed because of the high imbalance of OTC-positive hepatoblasts in fetal livers in which hemopoiesis was climaxing. In the present study, we analyzed the differentiation states of cells in these isolated patches using laminin and cytokeratin immunohisto-chemistry and observed three types of patches: patches consisting of only biliary cell progenitors, patches of only hepatocyte progenitors, and patches of both progenitors. Therefore, there may be three cell

lineages for hepatoblasts in the fetal liver: hepatoblasts that give rise to only biliary epithelial cells, hepatoblasts that give rise to only hepatocytes, and hepatoblasts that give rise to both biliary epithelial cells and hepatocytes.

The three possible cell lineages for hepatoblasts in fetal mouse livers may indicate the importance of the microenvironment surrounding each hepatoblast in its

differentiation (Doljanski and Roulet, 1934; Wilson et al, 1963; Wood, 1965), which could be equivalent in its potential to differentiate into hepatocytes and biliary epithelial cells. It has been stated that periportal connective tissue is crucial for bile duct differentiation (Doljanski and Roulet, 1934; Wilson et al, 1963), but its mechanism is still uncertain. Molecular genetic studies of Allagile syndrome, which is an autosomal-dominant disorder characterized by intrahepatic cholestasis and abnormalities of the heart, eye, and vertebrae, recently revealed that mutations in the human Jagged1 gene are responsible for this disease, thus offering insight into the mechanisms of hepatobiliary development (Li et al, 1997; Oda et al, 1997).

On the other side, our observation of patches consisting of only biliary cell progenitors may also suggest the presence of cells that are already committed to differentiation into biliary epithelial cells long before 15.5 days of gestation. Previous immunohistochemical analyses demonstrated that the hepatoblast population has a homogeneous morphology and expression of several molecular markers before 13.5 days of gestation and that biliary cell progenitors transiently express mature hepatocyte markers (Shiojiri, 1981, 1994), including OTC, as shown in the present study. These data suggest that, at early stages of development, hepatoblasts could be equivalent in terms of their potential to differentiate into biliary epithelial cells and hepatocytes. The similar OTC mosaicism in cell populations of both periportal and nonperiportal regions at 15.5 days shown in the present study also seems to support the idea of the equivalency of young hepatoblasts. Because mature hepatocyte markers (bile canalicular alkaline phosphatase and 5′-nucletidase activities) and biliary cell markers (laminin, PNA-binding sites, and cytokeratins) start to be expressed at around 13.5 days (Shiojiri, 1981, 1994; Shiojiri and Katayama, 1987; Shiojiri and Nagai, 1992), their commitment or determination may commence at around 13.5 days of gestation. Thus, at around 15.5 days of gestation, biliary cell progenitors and hepatocyte progenitors may already be determined in mouse liver development. Because intrahepatic bile duct development continues during the remainder of fetal life along the developing branches of the portal vein towards the periphery of the liver organ (Wilson et al, 1963; Wood, 1965), commitment or determination of biliary cells may proceed successively. In any event, experimental studies, such as clonal cultures of hepatoblasts for strict demonstration of the differentiation potency of each hepatoblast, are required in the future.

In conclusion, we demonstrated that the growth pattern of hepatoblasts was not different between periportal and nonperiportal regions and that there may be three lineages of hepatoblasts in fetal liver: hepatoblasts differentiating into only biliary epithelial cells, hepatoblasts differentiating into only hepatocytes, or hepatoblasts differentiating into both cells. These data will be helpful for the study of the mechanisms of bile duct differentiation.

## Materials and Methods

### Materials

(B6xC3H/He)F$_1$-*spf*$^{ash}$ mice were used. Livers (left lobes) of heterozygous females of 15.5-day and 17.5-day fetuses, neonates (1-day-old), and youngs (2-week-old) were examined for mosaicism of OTC expression. Wild-type mouse livers were also examined. Noon of the day a vaginal plug was found was considered 0.5 days of gestation. At least five animals were examined at each developmental stage.

### Immunohistochemistry

Tissues were fixed in Gendre's fixative (a mixture of saturated 90% ethanol of picric acid, formalin, and acetic acid [80:15:5 v/v/v]) overnight, dehydrated, and embedded in paraffin (melting point [mp], 51–54° C). Serial paraffin sections 6 $\mu$m thick were used for immunohistochemistry. Immunohistochemistry for OTC was carried out according to Shiojiri et al (1997). Briefly, dewaxed sections were incubated with rabbit antihuman recombinant OTC antiserum (1/1000 dilution with PBS containing 1% BSA) for 1 hour at room temperature, washed thoroughly with PBS, incubated with fluorescein isothiocyanate (FITC)-labeled goat antirabbit immunoglobulin G (IgG) antibodies (Organon Teknika Corporation, West Chester, Pennsylvania) (1/100 dilution) for 1 hour at room temperature, washed again with PBS, and then mounted in buffered glycerol containing *p*-phenylenediamine (Johnson and Nogueira Araujo, 1981). The specific immunofluorescence in the section was observed with a fluorescence microscope (Model BHS-RF; Olympus, Tokyo, Japan). Control slides were incubated with PBS containing 1% BSA or nonimmune rabbit serum (1/1000 dilution in PBS) in place of the primary antibodies.

The OTC mosaic pattern and expression of cytokeratins 8 and 18, which was seen in all endoderm-derived cells, including hepatoblasts, hepatocytes, and biliary cell progenitors in fetal livers, were also analyzed in the same sections of the developing livers. Progenitor cells of biliary epithelial cells are strongly positive for cytokeratins 8/18 (Shiojiri, 1994; Van Eyken et al, 1988a, 1988b) and also express hepatocyte markers such as carbamoylphosphate synthetase I, OTC, and albumin (Shiojiri, 1981; Shiojiri et al, 1991). Hepatoblasts and hepatocytes in nonperiportal liver parenchyma are weakly positive for cytokeratins 8/18. Laminin expression, which was a marker of biliary epithelial cells (Shiojiri and Katayama, 1987), was also analyzed in OTC patches by using double immunofluorescent method.

For double immunofluorescence of OTC and cytokeratins 8/18, dewaxed sections were incubated for 20 minutes with 0.0125% trypsin (Sigma, St. Louis, Missouri) in 10 mM Tris-HCl (pH 7.4)-buffered saline containing 10 mM CaCl$_2$ at room temperature and washed with PBS. Sections were then incubated as follows: (a) anti-OTC antiserum (1/1000 dilution with PBS containing 1% BSA) for 1 hour, (b) PBS washes, (c) lissamine rhodamine-labeled donkey antirabbit IgG

antibodies (Jackson ImmunoResearch Laboratory, Inc., West Grove, Pennsylvania) (1/500 dilution) for 1 hour, (d) PBS washes, (e) guinea pig anticytokeratins 8/18 (Progen Biotechnik Gmbh, Heidelberg, Germany) (1/200 dilution) for 1 hour, (f) PBS washes, (g) FITC-labeled donkey antiguinea pig IgG antibodies (Jackson ImmunoResearch) (1/500 dilution) for 1 hour, (h) PBS washes.

Double immunofluorescence analysis of laminin and OTC was done according to Carl et al (1993). Briefly, dewaxed sections were incubated as follows: (a) anti-OTC antiserum (1/2000 dilution) for 1 hour, (b) PBS washes, (c) Cy3-labeled donkey antirabbit IgG antibodies (Jackson ImmunoResearch) (1/500 dilution) for 1 hour, (d) PBS washes, (e) normal rabbit serum (Rockland, Gilbertsville, Pennsylvania) (1/20 dilution) for 1 hour, (f) PBS washes, (g) nonlabeled Fab' fragment of goat antirabbit IgG antibodies (Protos Immunoresearch, San Francisco, California) (1/20 dilution) for 2 hours, (h) PBS washes, (i) rabbit antimouse laminin antiserum (E-Y Laboratories, Inc., San Mateo, California) (1/200 dilution) for 1 hour, (j) PBS washes, (k) FITC-labeled goat antirabbit IgG antibodies (1/400 dilution) for 1 hour, (l) PBS washes.

### Quantitative Analysis of Patch Sizes and OTC Mosaicism

Immunofluorescent pictures of OTC and cytokeratins 8/18 or laminin in five or six sections of each liver, with relatively low magnification ($\times$10 or $\times$20 objective lenses), were taken on Tri-X pan film (Eastman Kodak Company, Rochester, New York). Then contours of the positive cells around portal veins and in nonperiportal regions on the photographic prints were manually traced onto transparent sheets. Such traces were input into a computer-assisted image analysis system (Luzex F; Nireco Corporation, Hachioji, Japan) using a 3CCD RGB camera (XC-009/P; Sony, Atsugi, Japan). The areas of each OTC-positive patch, strongly cytokeratin-positive or laminin-positive biliary cell progenitors in periportal regions, and weakly cytokeratin-positive or laminin-negative hepatocyte islands in nonperiportal regions were measured using programs running on the Luzex F. OTC mosaicism was also calculated in each bile duct progenitor and hepatoblast island in the sections.

### Computer-Aided Three-Dimensional Reconstruction

Computer-aided three-dimensional reconstruction was done according to Shiojiri et al (1997). OTC immunofluorescent pictures of serial sections, with relatively low magnification, were taken using Tri-X pan film, and then contours of the positive cells, portal veins, and central veins on the photographic prints were manually traced onto transparent sheets. Such traces were input into a computer-assisted image analysis system (TRI for Windows; Ratoc System Engineering Company, Ltd., Tokyo, Japan) using a 3CCD RGB camera. Connections of each patch were also analyzed by manually piling traces of it onto transparent sheets. Laminin and strong cytokeratin

expression was examined in such three-dimensional patches.

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