Quantitative Analysis of Cell Allocation During Liver Development, Using the *spf*^{ash}-Heterozygous Female Mouse

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Mosaicism of ornithine transcarbamylase (OTC) expression in hepatocytes was quantitatively analyzed during liver development of the *spf*^{ash}-heterozygous female mouse. Because the mosaic patterns depend on cell migration and cell mixing, such analysis could give insights on the growth pattern or allocation pattern of hepatocytes during liver development. Complex mosaic patterns of OTC-positive and -negative hepatocytes were observed in sections of fetal and postnatal livers. Sizes of patches, which were aggregates of OTC-positive or -negative hepatocytes, increased during development. Patches were slender and comparatively simple in 15.5- and 17.5-day fetal and neonatal livers. Quantitative analysis of patch shapes demonstrated that undulation of patches was maximal at 7 postnatal days. Patches with nodular shapes also started to increase in number at this stage. Isolated patches in sections of fetal livers and postnatal livers three-dimensionally connected with one another. However, especially in fetal livers, in which OTC-positive patches were minor, due to the presence of abundant hemopoietic cells, isolated three-dimensional patches consisting of approximately 5 to 70 cells were often found. They were shaped like slender branching or zigzag-shaped cords, but no definite orientation such as portal-central was observed in them at any stage. These results suggest that hepatocytes contiguously allocate their daughter cells as zigzag-shaped or branching cords at younger stages. Some hepatocytes grow with nodular formation after 7 postnatal days. Migration and mixing of hepatocytes appear to be more extensive at fetal stages than in the adult liver. Immunohistochemical analysis of intercellular junction proteins (E-cadherin, connexins 26 and 32, occludin, and ZO-1) also revealed that their expression and distribution changed in hepatocytes during development, which may be correlated with

the OTC mosaic patterns. (*Am J Pathol* 2000, 156:65-75)

Chimeric animals, in which cells of two genotypes or two species are mixed, provide a powerful system to study cell lineages, cell migration, and cell-cell interactions in organogenesis during development and in pathogenesis.¹⁻⁷ Studies with such animals have demonstrated the monoclonal origin of the cryptic stem cells in the small intestine^{8,9} and the migration pathways and the developmental fates of neural crest cells.^{10,11} Analysis of adult rodent chimeric livers has demonstrated that patches in the liver, which are cell aggregates of either genotype, are very complicated and possess no definite orientation, such as portal-central or surrounding blood vessels.¹²⁻¹⁴ We have also shown that heterozygotes of the *spf*^{ash} mutation, which is located on the X chromosome and causes a deficiency of ornithine transcarbamylase (OTC),¹⁵⁻¹⁷ is a useful animal model for the mosaic analysis of liver development,¹⁸ because the random inactivation of either X chromosome in heterozygous females results in mosaic expression of hepatic OTC.¹⁸⁻²⁰ OTC also starts to be expressed in the liver at mid-gestational stages.²¹ Our three-dimensional analysis with postnatal heterozygous livers revealed that hepatocytes allocate their daughter cells randomly and contiguously during liver development.¹⁸ However, quantitative analysis of the patch sizes and shapes, which can suggest the extent of cell migration and cell mixing,²² has not been carried out during liver development. Mosaicism in the fetal liver, in which hemopoiesis culminates²³⁻²⁵ and extensive cell migration and cell mixing might occur, has not been examined either. Such studies may reveal the allocation pattern of hepatocytes during development more accurately. Three-dimensional analysis is also important because isolated patches in sections may be three-dimensionally connected with one another.

Cell mixing and cell migration in histogenesis may depend on cell-cell and cell-matrix interactions,^{2,22,26}

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which are controlled by adhesion molecules and intercellular junctions, including adherens junctions, gap junctions, and tight junctions. Some molecules that are localized at the intercellular junctions have been well characterized; for example, E-cadherin in adherens junctions, connexins 26 and 32 in gap junctions, and occludin and ZO-1 in tight junctions of hepatocytes.^{27–29} Therefore, it would be intriguing to compare expression patterns of the intercellular junction proteins with developmental changes of the OTC mosaicism.

In this paper, we quantitatively analyzed mosaic OTC expression patterns during development of the *spf^{ash}*-heterozygous mouse liver and report here that patch sizes increased and patch shape changes occurred during development. Patches in sections were often isolated through development, but they were three-dimensionally well connected with one another to form cell aggregates with no definite orientations, such as portal-central. In fetal livers small isolated three-dimensional patches were observed, and they may correspond to clones. Cell migration and mixing may be extensive in fetal stages. The present study also demonstrated that intercellular junction proteins poorly developed at early stages of liver development, when the patch size was very small.

Materials and Methods

Materials

(B6×C3H/He)F₁-*spf^{ash}* mice were used. Livers (left lobes) of *spf^{ash}*-heterozygotes (females) of 15.5-day and 17.5-day fetuses, neonates (1 day old), and 1-, 2-, 3-, and 8-week-old (adult) animals were examined for mosaicism of OTC expression. Noon of the day a vaginal plug was found was considered 0.5 days of gestation. At least five animals were examined at each stage. Homozygous animals could survive, although their growth was highly retarded due to hyperammonemia, and wild-type and heterozygous animals showed similar growth and development to each other. In addition, histological and immunohistochemical analyses of heterozygous livers (30–70% mosaicism in hepatocytes) during development showed that these livers develop normally in terms of growth, histogenesis and expression of hepatocyte markers such as other urea cycle enzymes and serum proteins and glycogen accumulation (data not shown). Thus patch sizes probably were not influenced by potential differences in the survival rate and proliferation rate of cells that varied in the OTC status in our heterozygous livers.

OTC expression was seen at from 14.5 to 15.5 days of gestation during mouse liver development, and major developmental changes occurred at the following stages in our liver samples. Hepatocyte maturation occurred at 17.5 days of gestation to neonatal stages,^{30–34} liver lobules developed at 1 to 2 postnatal weeks,^{35,36} and hepatic hemopoiesis abruptly declined after 17.5 days of gestation and persisted poorly in postnatal (2-week-old) livers.^{23–25}

Immunohistochemistry

Tissues for OTC immunofluorescence were fixed in Gendre's fixative (a mixture of saturated 90% ethanol of picric acid, formalin, and acetic acid at 80:15:5, v/v/v) overnight, dehydrated, and embedded in paraffin (melting point, 51–54°C).¹⁸ Serial paraffin sections 6 μ m thick were cut. Dewaxed sections were incubated with rabbit anti-human recombinant OTC antiserum (1:1000 dilution in 0.01 mol/L phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA)) for 1 hour at room temperature. After thorough washing with PBS, sections were incubated with fluorescein isothiocyanate-labeled goat anti-rabbit immunoglobulin G (IgG) antibodies (Organon Teknika Corp., West Chester, PA; 1:100 dilution with PBS containing 1% BSA) for 1 hour at room temperature. Sections were again washed with PBS and then mounted in buffered glycerol containing *p*-phenylenediamine.³⁷ The specific immunofluorescence in the section was observed with a fluorescence microscope (model BHS-RF; Olympus, Tokyo, Japan). Under this immunostaining protocol, hepatocytes in which the *spf^{ash}* gene was active were negative for OTC, whereas hepatocytes in which the wild-type OTC gene was active were strongly positive. Control slides were incubated with PBS containing 1% BSA or nonimmune rabbit serum (1:1000 dilution in PBS) in place of the primary antibodies. Anti-human OTC antiserum gave a single band in immunoblots of mouse liver extract (data not shown).

Expression of E-cadherin, occludin, ZO-1, and connexins 26 and 32 was examined in frozen sections of developing livers by indirect immunofluorescence. Tissues were frozen in *n*-hexane chilled with dry ice-ethanol. Frozen sections 8 μ m thick were cut and then fixed in acetone at –20°C for 10 minutes. Rat monoclonal antibodies (mAbs) against mouse E-cadherin (Takara Biomedicals, Otsu, Japan; 1:200 dilution in 0.02 mol/L Tris-buffered saline (TBS) containing 10 mmol/L CaCl₂ and 1% BSA), rabbit anti-human occludin antibodies (Zymed Laboratories, Inc., San Francisco, CA; 1:100 dilution), rat mAbs against mouse ZO-1 (Chemicon International Inc., Temecula, CA; 1:100 dilution), rabbit anti-rat connexin 26 antibodies (Zymed; 1:100 dilution), and rabbit anti-rat connexin 32 antibodies (Zymed; 1:100 dilution) were used as primary antibodies, and fluorescein isothiocyanate-labeled goat anti-rat IgG antibodies (Organon Teknika; 1:100 dilution) or anti-rabbit IgG antibodies (1:100 dilution) were used as secondary antibodies. Incubation with primary and secondary antibodies was carried out for 1 hour at room temperature. Control slides were incubated with TBS containing 10 mmol/L CaCl₂ and 1% BSA or PBS containing 1% BSA in place of the primary antibodies.

Measurement of Patch Sizes and Shapes in Sections

OTC immunofluorescent pictures of three sections of each liver with relatively low magnification (10× or 20× objective lenses), were taken using Tri-X pan film (East-

man Kodak Co., Rochester, NY). Then contours of the positive cells and blood vessels on the photographic prints were manually traced onto transparent sheets. Such traces were input into a computer-assisted image analysis system (Luzex F; Nireco, Hachioji, Japan), using a 3CCD RGB camera (XC-009/P; Sony, Atsugi, Japan). The areas, the perimeters, and the maximum lengths (MLs) of OTC-positive patches and the ratios of OTC-positive and -negative regions in sections were measured by using programs running on the Luzex F. Because fetal mouse liver is a hemopoietic organ, fetal livers and neonatal livers contained many hemopoietic cells. Areas of hemopoietic cells in wild-type fetal and neonatal mouse livers, which were negative in OTC immunostaining, were also similarly measured. Patch sizes in heterozygous livers were also expressed as cell numbers by dividing the area of each patch by the average cell size.

The corrected maximum lengths (CMLs) of patches in sections were calculated by the following equation, to show the increase of the patch size during liver development:

$$CML = ML / [1 / (1 - p)],$$

where p is the ratio of the OTC-positive hepatocyte region in all tissue areas of a section. The ML of OTC-positive patches (see Figure 3) depended on the ratio of OTC mosaicism. It increased with the increase of OTC-positive hepatocytes in sections of heterozygous livers. However, the CML value was not affected by the ratio of mosaicism (see *Results* below).

We also used the shape factor (α), which is defined as follows, to demonstrate changes of patch shapes during liver development:

$$\alpha = PM^2 / (4\pi \cdot A),$$

where PM is the perimeter of a patch, and A is the area of a patch. If the patch is round, the value is close to 1. In patches with complicated, undulated shapes, the value would increase. This analysis was carried out for patches consisting of more than 4 or 10 cells in sections, because small patches of a few cells generally have a simple shape. Although the fractal dimension has also been shown to be useful as a measure of patch complexity,¹³ it could not be applied owing to the very weak signal of OTC immunofluorescence in our samples under low magnification (1.6 $\times$ or 4 $\times$ objective lens). Accurate fractal-dimension analysis needs such data.

The data on patch sizes, CML values, and shape factor values during liver development were analyzed statistically by the Kruskal-Wallis nonparametric analysis of variance test and Dunn's multiple-comparisons test, using Instat (Graphpad Software, San Diego, CA). Differences were considered significant when $P < 0.05$.

Computer-Aided Three-Dimensional Reconstruction

Contours of OTC-positive cells and blood vessels on the photographic prints were manually traced onto transpar-

ent sheets. The traces were input into a computer-assisted image analysis system (TRI for Windows, Ratoc System Engineering Co., Ltd., Tokyo, Japan) by using the 3CCD RGB camera. Cross sections of blood vessels were used as landmarks and reference points. Connections of each patch were also analyzed by manual piling of traces of it onto transparent sheets.

Results

Developmental Changes of Patch Sizes

Figure 1 shows developmental changes of mosaic expression of OTC in hepatocytes of *spf^{ash}*-heterozygous mouse livers and the areas and cell numbers of patches, which increased with development. Although patches were generally complex at all stages, they were very small and mostly consisted of a few OTC-positive hepatocytes in fetal stages. The patches were also comparatively simple and appeared as slender branching or meandering cords in fetal livers. Because the fetal liver is a hemopoietic organ, the OTC-negative regions, including OTC-negative hepatocyte patches and abundant hemopoietic cells, were in the majority, and the OTC-positive patches were small. After 7 postnatal days, some nodular patches were observed. No patch shapes that had a definite relationship to any landmark in the liver including portal veins and hepatic veins were seen in sections throughout liver development. Control slides for OTC immunostaining were invariably negative.

Patch size depended on the ratio of mosaicism even at the same developmental stage; sections with a higher ratio of the OTC-positive regions had larger patches (Figure 2). Thus, strictly speaking, patch size analysis in development should be done in livers with the same ratio of OTC mosaicism. We calculated patch sizes at the ratio of 50% mosaicism for postnatal livers from the data of patch sizes with various ratios of mosaicism and showed that patch size increased during postnatal development (Table 1). However, accurate similar calculations for fetal livers were impossible because the OTC mosaicism at 15.5 and 17.5 days of gestation was highly unbalanced (the ratio of OTC-positive regions in all tissue areas was 15–40%) owing to the presence of abundant hemopoietic cells.

The CML value of patches was constant regardless of the ratio of mosaicism (Figure 3). The CML value increased with increases in patch size and liver development (Figure 4). The CML value and the calculated patch sizes at 50% mosaicism were smaller in adult livers than those of 3-week-old livers, because of the very large sizes of patches in adult liver and exclusion of large patches exceeding the lattice of the measured area.

Developmental Changes of Patch Shapes

Quantitative analysis of patch shape changes during development was carried out for patches consisting of more than 4 or 10 OTC-positive hepatocytes, by measuring the

Figure 1. Developmental changes of OTC mosaic patterns in *spf^{ash}*-heterozygous mouse livers. **A:** 15.5-day fetal liver. **B:** 17.5-day fetal liver. **C:** Neonatal liver (1 day old). **D:** 1-week-old liver. **E:** 2-week-old liver. **F:** Adult liver (8 weeks old). Patch sizes increase with development. Small and slender patches are the major ones in 15.5-day and 17.5-day livers (**A**, **B**). Nodular patches start to be seen in 1-week-old liver (**E**). Scale bar, 50 μ m.

shape factor value of each patch (Figure 5). At early stages of liver development (15.5 and 17.5 days), the values of the shape factor for patches were small, and the patches were slender. In the 1-week-old liver, the value was maximal, and patches were highly undulated. In 2-week-old, 3-week-old, and adult livers, the value decreased, indicating that their patches were rounder than those seen in the 1-week-old-liver.

Three-Dimensional Analysis of Patches

Isolated patches were often observed in sections of both fetal and postnatal livers, but each isolated patch also connected well with others in three dimensions. The orientation of the three-dimensional patches was also irregular and had no clear relationships to the anatomy in livers at any stages (Figure 6). However, when patches in

Figure 2. Developmental changes of patch sizes presented by area. **A:** 15.5-day livers. **B:** Neonatal livers (1 day old). **C:** One-week-old livers. **D:** Adult livers (8 weeks old). Patch sizes increase during development, but they are also dependent on the ratio of the OTC mosaicism. A higher ratio of OTC-positive hepatocytes results in larger patch sizes at each stage.

a section was located near veins or in the mid-zone of the liver parenchyma, their connectants had a tendency to take similar positions in adjoining sections (Figure 6).

In 15.5-day and 17.5-day livers, in which OTC mosaicism was highly unbalanced, isolated three-dimensional patches were often observed. They consisted of approximately 5 to 70 OTC-positive cells and also had irregular shapes such as slender meandering or branching cords (Figures 6 and 7). Fetal three-dimensional patches

around veins tended to be allocated along their long axes.

Intercellular Junction Protein Expression during Liver Development

Developmental changes of intercellular junction protein expression in hepatocytes are summarized in Table 2.

Table 1. Developmental Changes of Patch Sizes of OTC-Positive Hepatocytes in *spf^{ash}*-Heterozygous Mouse Liver

Developmental stage	Calculated patch sizes (μm^2)
1 day old	1970
1 week old	12,980
2 weeks old	11,220
3 weeks old	18,550
8 weeks old (adult)	18,910

Patch sizes at 50% mosaicism ($P = 0.5$) were calculated from the data for various ratios of mosaicism.

E-cadherin

E-cadherin was expressed in hepatocytes throughout mouse liver development. At fetal stages, E-cadherin was localized in the cell membrane of hepatocytes, but its immunostaining pattern was heterogeneous (linear or finely granular in the cell membrane; Figure 8A). The shape of fetal hepatocytes was various and flattened, compared with that of adult hepatocytes. In neonatal livers, hepatocytes became more cuboidal and larger, and E-cadherin immunostaining was more homogeneous

Figure 3. The CML values and OTC mosaicism in 17.5-day livers (A) and adult livers (B). The CML values are constant in 17.5-day and adult livers regardless of the OTC mosaicism, whereas the ML values increase with the ratio of OTC-positive hepatocytes.

and finely linear in the cell membrane in sections (Figure 8B). The linear staining of E-cadherin was also longer than that in the cell membrane of fetal hepatocytes. In 2-week-old livers and adult livers, half of the hepatocytes

Figure 4. Developmental changes of patch size by the CML values. The CML values of patches increase with development. The value in adult livers is smaller than that in 3-week-old livers, owing to the presence of very large patches in adult liver exceeding the lattice of the measured area. The number in each bar is the number of livers examined. Vertical lines above the bars indicate standard deviations from the means. g.d., gestational days.

Figure 5. Developmental changes of the shape factor (α) values of OTC-positive patches. The data are for patches comprising more than 10 cells. The value was maximal at 1 postnatal week. Means marked by the same letter are significantly different at $P < 0.05$ (a), $P < 0.01$ (b), or $P < 0.001$ (c). The number in each bar is the number of livers examined. Vertical lines above the bars indicate standard deviations from the means. g.d., gestational days.

that were upstream of liver lobules (portal) expressed E-cadherin (Figure 8, C and D), but other hepatocytes were negative for E-cadherin expression. At these stages, E-cadherin immunostaining appeared to be localized also in the sinusoidal cell membrane (Figure 8D).

Connexins 26 and 32

Both connexins 26 and 32 showed similar developmental expression patterns. At 15.5 days of gestation, their expression was absent in hepatocytes (Figure 9A). At 17.5 days, connexin 26- or 32-positive granules or spots started to be observed in cell membranes of hepatocytes, although the number of connexin 26-positive hepatocytes was fewer than that of connexin 32-positive hepatocytes. In neonatal livers, expression of connexins 26 and 32 in hepatocytes increased, but connexin-positive granules were heterogeneous in size (Figure 9, B and C). More homogeneous sizes of connexin 32-positive granules started to be observed in hepatocytes of 2-week-old livers (Figure 9D). Each adult hepatocyte had a uniform distribution of connexin 26- or 32-positive granules in their lateral cell membrane (Figure 9E).

Occludin and ZO-1

Occludin was expressed in 15.5-day fetal livers, but its expression was granular or linear in the cell membrane of hepatocytes (Figure 10A). With development, occludin-positive linear staining corresponding to the bile canaliculus region increased (Figure 10B). In 2-week-old and adult livers, sinusoidal and other lateral cell membranes of hepatocytes were also occludin-positive in addition to the bile canaliculus region (Figure 10, C and D).

Figure 6. Three-dimensional reconstruction of OTC-positive patches from 18 serial sections of a 15.5-day *spf^{ash}*-heterozygous mouse liver. **A:** Complex shapes of three-dimensional patches are seen. **Arrow** indicates an isolated patch (**purple**) in the liver parenchyma. **B:** Some patches have a tendency to be allocated along a blood vessel. The patches are slender and exhibit meandering or branching patterns. **Arrow** indicates an isolated patch (**brown**) around the portal vein. HV, hepatic vein; PV, portal vein.

ZO-1 showed a more localized distribution in the mouse liver throughout development, which corresponded to the bile canaliculus region, as compared with the occludin distribution. At 15.5 days of gestation, linear or granular immunostaining of ZO-1 was seen (Figure 10E). With development, ZO-1 immunostaining corresponding to the bile canaliculus region became well connected (Figure 10, F, G, and H).

Figure 7. Size distribution of isolated three-dimensional patches of OTC-positive hepatocytes in 15.5-day fetal livers. Isolated three-dimensional patches mainly comprised from 5 to 20 cells. The largest one among patches of which sizes were known had 70 cells.

Discussion

During mammalian liver development, hepatocytes derived from the hepatic endoderm grow extensively and mature under the influence of their surrounding microenvironment, which eventually leads to liver lobule formation.^{30-34,38-40} The growth pattern of hepatocytes during development has been estimated by analyzing mosaicism in chimeric adult rodent livers; it occurs randomly and coherently and is fractal.^{12,13,18} However, there are no studies on mosaic analysis of fetal liver development. The current quantitative immunohistochemical studies could trace the mosaic development to 15.5 days of gestation.

We demonstrated that, during development, patches varied in cell number and areas and also that CML values increased, which is consistent with contiguous growth of hepatocytes during development as has been estimated.^{12,13,18} The mosaic pattern had no definite relationships to anatomical landmarks such as portal veins and hepatic veins throughout development. Although it has been suggested that extensive migration and mixing of cells can occur in tissues that have small patches in mosaic or chimeric animals,²² this might not be applicable to for the mosaic fetal liver in the current study. Abundant hemopoietic cells occupied large areas of fetal mouse livers (~50% at 15.5 days of gestation), and the spaces for hepatocytes were very limited, which is prob-

Table 2. Expression of Intercellular Junction Proteins in Hepatocytes during Mouse Liver Development

Intercellular junction protein	Development stage					
	15.5 g.d.	17.5 g.d.	1 day old	1 week old	2 weeks old	8 weeks old
E-cadherin	+	+	+	+	±	±
Connexin 26	—	±	+	ND	ND	+
Connexin 32	—	±	+	+	+	+
Occludin	±	±	+	+	+	+
ZO-1	±	±	+	+	+	+

—, no immunofluorescence; +, positive immunofluorescence (all cells); ±, positive cells and negative cells; g.d., gestation days; ND, not determined.

Figure 8. E-cadherin expression during mouse liver development. **A:** 15.5-day fetal liver. **B:** Neonatal liver (1 day old). **C:** 2-week-old liver. **D:** Adult liver (8 weeks old). Whereas E-cadherin immunostaining varies (linear or finely granular) in cell membranes of adjoining hepatocytes in 15.5-day liver (**A**), that in cell membranes of neonatal, 2-week-old, and adult hepatocytes is linear and more homogeneous in sections (**B–D**). The sinusoidal cell membrane (**D**, arrow) of adult hepatocytes also appears to be positive for E-cadherin. PV, portal vein. Scale bar, 50 μ m.

ably one of the causes of the small patch sizes in fetal livers. In addition, isolated patches in sections were often connected even in fetal stages to form cell aggregates of branching cords or zigzag-shaped cords, suggesting that hepatocytes allocate their daughter cells contiguously also in fetal livers and that the allocation of hepatocytes occurs in the direction of the longitudinal axis of hepatic cords. When a patch in a section was located near veins, its connectants also took a similar position,

implying that fetal hepatocytes grow focally in the liver parenchyma and that their migration and cell mixing are not highly extensive. This also suggests that fetal hepatocytes around veins tend to allocate their daughter cells in the direction of the veins.

However, we often observed isolated three-dimensional patches in fetal livers, and their frequency was higher than that in older livers. This may derive from the large imbalance of OTC mosaicism in the fetal liver,

Figure 9. Connexin 26 (**B**) and 32 (**A**, **C–E**) expression during mouse liver development. **A:** 15.5-day fetal liver. **B** and **C:** Neonatal liver (1 day old). **D:** 2-week-old liver. **E:** Adult liver (8 weeks old). Connexin 32 was not expressed in 15.5-day liver (**A**). Connexin 26- or -32-positive granules in neonatal hepatocytes are heterogeneous in size (**B** and **C**). In 2-week-old livers (**D**), distribution and sizes of connexin 32-positive granules become more homogeneous compared with those in neonatal liver (**C**). PV, portal vein. Scale bar, 50 μ m.

Figure 10. Expression of tight junction proteins during mouse liver development. **A** and **E**: 15.5-day fetal liver. **B** and **F**: Neonatal liver (1 day old). **C** and **G**: 2-week-old liver. **D** and **H**: Adult liver (8 weeks old). Occludin immunostaining was used in panels **A** to **D**. ZO-1 immunostaining was used in panels **E** to **H**. At 15.5 days of gestation, positive immunoreaction for occludin and ZO-1 is linear or spotty in cell membranes of hepatocytes (**A** and **E**). Whereas ZO-1 immunostaining continues to be localized in the bile canaliculus region (**F** to **H**), that of occludin was localized in the bile canaliculus region at neonatal stage (**B**) but became positive also in other lateral and sinusoidal cell membranes of 2-week-old and adult hepatocytes (**C** and **D**). Scale bar, 50 μ m.

which was not ordinarily seen in older livers (30–70% mosaicism). Smaller clone sizes at fetal stages may also be one of the causes. The cell number of the isolated three-dimensional patches was approximately 5 to 70, which may correspond to the clone size of hepatocytes at this stage. Although data on the generation time of the cell cycle of hepatocytes during fetal development are not available, their clone size would be less than 100 cells at this stage if we postulate that a fetal hepatocyte continues to divide every day from 9.5 days of gestation, when the liver formation starts.^{39,40} The estimated cell number is larger than the observed values of population sizes of isolated three-dimensional patches. This suggests that fragmentation of hepatocyte clones (cell mixing) may take place in the fetal liver. It is unknown precisely how extensive cell migration and mixing occur in the fetal liver. The migrating in and extensive growth of hemopoietic cells in fetal liver^{23–25} could be involved in such fragmentation.

Although patches in sections were small and slender at fetal stages, they became more complex in early postnatal development, and nodular patches also started to be observed after 1 week. The postnatal nodular cell proliferation reported in the present study is also consistent with a report on transgenic mouse liver.⁴¹ This

change of patch patterns may be related to the migrating out of hemopoietic cells and/or the development of liver lobules, which start around the perinatal stage.^{23–25,35,36} Migrating out of most hemopoietic cells at perinatal stages might cause hepatocytes to adhere tightly.

The current study demonstrated that expression and distribution of intercellular junction proteins in hepatocytes changed with development. At 15.5 days of gestation, gap junction proteins were not expressed, and expression and localization of E-cadherin and tight junction proteins were immature in hepatocytes. These results suggest a weak adhesion between fetal hepatocytes, which can generate small patches in the OTC mosaic livers at this stage. We also have indicated that hepatocyte maturation for intercellular junction proteins, including developmental changes of immunostainings of E-cadherin, connexins, occludin, and ZO-1 in the cell membrane, occurred during perinatal and postnatal periods, which is consistent with previous biochemical studies of adhesion molecules in developing hepatocytes.^{27,29,42,43} Such maturation for the intercellular junction proteins may be involved in the transient, remarkable undulation of patches and in the nodular patch formation during postnatal development. Cell-matrix interactions may also be important for the developmental

changes of OTC mosaic patterns. These possibilities require future study.

The current study showed that, although fetal and neonatal hepatocytes had an occludin immunoreaction only in the bile canaliculus region, the lateral and sinusoidal membranes of 2-week-old and adult hepatocytes were positive for occludin. It has been demonstrated that occludin is localized to the bile canaliculus region in newly hatched chicken hepatocytes.²⁸ The reason for this difference is unknown at present.

The work by Iannaccone et al¹² and Khokha et al¹³ showed that patch patterns in chimeric rat livers are fractal, suggesting that repeated random and contiguous allocation of daughter cells may occur during liver development. If this is the case, the fetal OTC mosaic pattern would be consistent with that of the adult liver observed under very low magnification. However, our data on fetal and neonatal livers demonstrated that patches at these stages were more slender than those at older stages, including the adult stage (8 weeks old). The values of the shape factor changed developmentally and were maximal in the 1-week-old liver, as shown in the current study. Thus, the OTC mosaic pattern in the fetal liver may be different from that of the adult liver, which rather resembles the mosaic pattern in 2- or 3-week-old livers.

Several animal models have been developed for engraftment of cellular transplants for treatment of hepatic disease, in which transplanted hepatocytes can proliferate extensively and replace those of the host.^{44–46} Their growth pattern is nodular, and our mosaic analysis may be useful in explanation of the data of such transplantation.

In conclusion, our developmental studies of OTC mosaicism revealed growth patterns of hepatocytes throughout liver development. At fetal stages, they allocated their daughter cells along the long axis of the hepatic cords, and, in postnatal development, they sometimes formed nodular structures. Although no definite orientation of patch shapes to the landmarks of the liver was found throughout development, portal-central migration by hepatocytes seldom occurred; rather they settled around veins or in the mid-zonal parenchyma. The allocation of daughter cells of hepatocytes is coherent throughout development, although cell migration and cell mixing might be extensive in fetal livers compared with adult livers. At mid-gestational stages, the clone sizes of hepatocytes may be small and from 5 to 70 cells. These developmental changes of OTC mosaicism can be partially explained by the strength of cell-cell adhesion.

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