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Differentiation of Biliary Epithelial Cells from the Mouse Hepatic Endodermal Cells Cultured in Vitro

NOBUYOSHI SHIOJIRI and TOHRU KOIKE*

Department of Biology, Faculty of Science, Shizuoka University, Shizuoka 422

SHIOJIRI, N. and KOIKE, T. *Differentiation of Biliary Epithelial Cells from the Mouse Hepatic Endodermal Cells Cultured in Vitro.* Tohoku J. Exp. Med., 1997, **181** (1), 1-8 —— Differentiation of biliary epithelial cells from hepatic endodermal cells of the mouse embryo was examined with a special attention to the role of the connective tissue. When the whole liver primordium of the 9.5-day mouse embryo was cultured in vitro for 5 days, the endodermal cells differentiated into mature hepatocytes expressing carbamoylphosphate synthetase I (CPSI) and accumulating glycogen. Intrahepatic bile duct cells and connective tissue were poorly developed in this culture. However, when the hepatic endoderm was recombined with the 4-day embryonic chick lung mesenchyme and cultured in vitro, the endodermal cells differentiated into many ductal epithelial cells as well as mature hepatocytes with abundant connective tissue development. These results suggest that the ducts might be bile ducts, and that connective tissue is very important for bile duct development. In addition, this in vitro culture system might be useful for the study of mechanisms of bile duct differentiation and congenital biliary atresia. —— liver primordium; biliary epithelial cells; hepatocytes; induction; mesenchyme

During mammalian liver development, intrahepatic bile ducts originate from periportal hepatocytes (Bloom 1926; Du Bois 1963; Wilson et al. 1963; Wood 1965; Enzan et al. 1974; Shiojiri 1984a; Van Eyken et al. 1988; Shiojiri et al. 1991), and it has been proposed that the periportal connective tissue induces adjacent hepatocytes to differentiate into intrahepatic biliary epithelial cells (Du Bois 1963; Wilson et al. 1963; Wood 1965; Enzan et al. 1974; Shiojiri 1984a). However, it remains to be demonstrated experimentally whether connective tissue induces ductal formation from fetal hepatocytes, though Doljanski and Roulet (1934) showed that adult hepatocytes differentiate into biliary epithelial cells

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*Present address: Department of Biology, Faculty of Science, Tokyo Metropolitan University, Minamiohsawa, Hachiohji, Tokyo 192-03, Japan.

Address for reprints: Nobuyoshi Shiojiri, Department of Biology, Faculty of Science, Shizuoka University, 836 Oya, Shizuoka 422, Japan.

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under the influence of connective tissue cells in vitro. Some substances such as sodium butyrate and dexamethasone, and basal laminar components can promote expressions of bile-duct-specific markers in fetal hepatocytes cultured in vitro (Germain et al. 1988; Shiojiri and Mizuno 1993; Blouin et al. 1995).

To investigate the mechanisms of bile duct differentiation and the cause of congenital biliary atresia, which is unknown, it would be useful to develop an in vitro culture system in which the intrahepatic bile ducts differentiate from fetal hepatocytes. Using a culture system of this type it would be possible to screen the effects of various substances on bile duct formation and bile duct obstruction.

Because embryonic chick lung mesenchyme not only induces bronchus formation in lung endoderm but also allows mouse hepatic endoderm to differentiate into hepatocytes (Houssaint 1980), it was postulated that it may induce bile duct differentiation in differentiated hepatocytes. In the present study, we cultured the whole liver primordium and the recombinants of the hepatic endoderm with embryonic chick lung mesenchyme in vitro, and report that the heterologous mesenchyme induces abundant ductal development from hepatic endoderm.

Materials and Methods

Animals. C3H/HeSlc strain mice (SLC, Shizuoka) were used. Mice were mated during the night and copulation was confirmed by the presence of a vaginal plug the next morning. Noon of the day the vaginal plug was found was considered 0.5 days of gestation. Embryos at 9.5 days of gestation were staged by their somite number (15–23 somites). Chick embryos were obtained from a local breeder (Oohata Shever Co., Ltd., Yaizu).

Organ culture. Mouse embryos at 9.5 days of gestation were dissected in HEPES (Nakarai Chemical Co., Ltd., Tokyo)-buffered Dulbecco's modified MEM (DMEM) (Gibco, Grand Island, NY, USA) under a dissecting microscope (model SZH131, Olympus, Tokyo), and liver rudiments were isolated. The liver rudiments were placed on membrane filters (RA-type; Nihon Millipore Kogyo K.K., Yonezawa) on stainless grids, and were cultured organotypically in the interface between the culture medium and a gas phase of 5% CO_2 in air (Trowell 1954, 1959). DMEM supplemented with 10% fetal calf serum (Gibco), epidermal growth factor (10 ng/ml, Upstate Biotechnology Inc., Lake Placid, NY, USA), insulin (5 μg/ml, Sigma, St. Louis, MO, USA), dexamethasone (10^{-7} M, Sigma), penicillin G (100 U/ml, Meiji Seika Co., Ltd., Tokyo) and streptomycin (100 μg/ml, Meiji Seika Co., Ltd.) was used as the culture medium. The medium was changed every 3 days.

The endoderm of the liver rudiment was separated from hepatic mesenchyme using 0.01% collagenase (CLSI; Worthington Biochem. Corp., Freehold, NJ, USA). Lung mesenchyme was also prepared from 4-day chick embryos with the aid of collagenase. The hepatic endoderm was recombined with hepatic mesenchyme or lung mesenchyme on the membrane filter, and then covered with

Matrigel (Collaborative Research, Bedford, MA, USA). Hepatic endoderm was also cultured in Matrigel in the absence of mesenchyme.

Histochemistry. Tissues for histochemical analyses were fixed in a chilled mixture of ethanol and glacial acetic acid (99 : 1 v/v) overnight and embedded in paraffin at 53°C. Expressions of α-fetoprotein (AFP), albumin, carbamoylphosphate synthetase I (CPSI), bile-duct-specific cytokeratin and laminin were examined immunohistochemically (Shiojiri 1981, 1984b, 1994; Shiojiri and Katayama 1987). Rabbit anti-mouse AFP antiserum, rabbit anti-mouse albumin antiserum (Miles Lab., Inc., Elkhart, IN, USA) (1/50 dilution), rabbit anti-rat CPSI antiserum (a generous gift from Dr. W.H. Lamers; 1/1000 dilution) (Gaasbeek Janzen et al. 1984, 1985), rabbit anti-calf keratin antiserum (Dako, Carpinteria, CA, USA) (1/300 dilution) and rabbit anti-mouse laminin antiserum (E-Y Lab., San Mateo, CA, USA) (1/50 dilution) were used as primary antibodies. Fluorescein isothiocyanate-labeled goat anti-rabbit IgG antibodies (Organon Teknika Corp., West Chester, PA, USA) (1/50 dilution) were used as secondary antibodies.

Lectin-binding sites were also histochemically examined with fluorescein-conjugated peanut agglutinin (PNA), soybean agglutinin (SBA) and *Dolichos biflorus* agglutinin (DBA) (Vector Lab., Burlingame, CA, USA) (Shiojiri and Nagai 1992). These lectins bound specifically to biliary epithelial cells (Shiojiri and Nagai 1992). The control sections were incubated with the lectin solutions containing each haptenic sugar.

Demonstration of glycogen with periodic acid-Schiff (PAS) staining was carried out according to the method of McManus (1948).

Results

The endodermal cells of the liver primordia (with 15–23 somites) used in the present study started to express AFP, but did not express albumin and CPSI, or store glycogen. When the liver primordia were cultured for 5 days in vitro, however, the endodermal cells differentiated into large hepatocytes expressing AFP, albumin and CPSI, and storing glycogen (Fig. 1). Histologically, hepatocytes formed aggregates (ca. 8 hepatocytes in a section) with large lumina in their center, and these aggregates were surrounded by connective tissue or endothelial cells. However, the development of the connective tissue was poor. Intrahepatic bile ducts did not differentiate in the liver parenchyma (no explants showing intrahepatic bile duct differentiation among 6 explants examined) (Fig. 1), though simple columnar epithelial cells, which originated from the cells of contaminated stomach and intestinal primordia, existed in the explants. Anti-calf cytokeratin antibodies, which reacted specifically with intrahepatic biliary epithelial cells in adult liver, and anti-laminin antibodies did not stain the liver parenchyma differentiated in vitro. In prolonged cultivation of the liver primordium for 7 days or 10 days, intrahepatic bile duct differentiation did not occur.

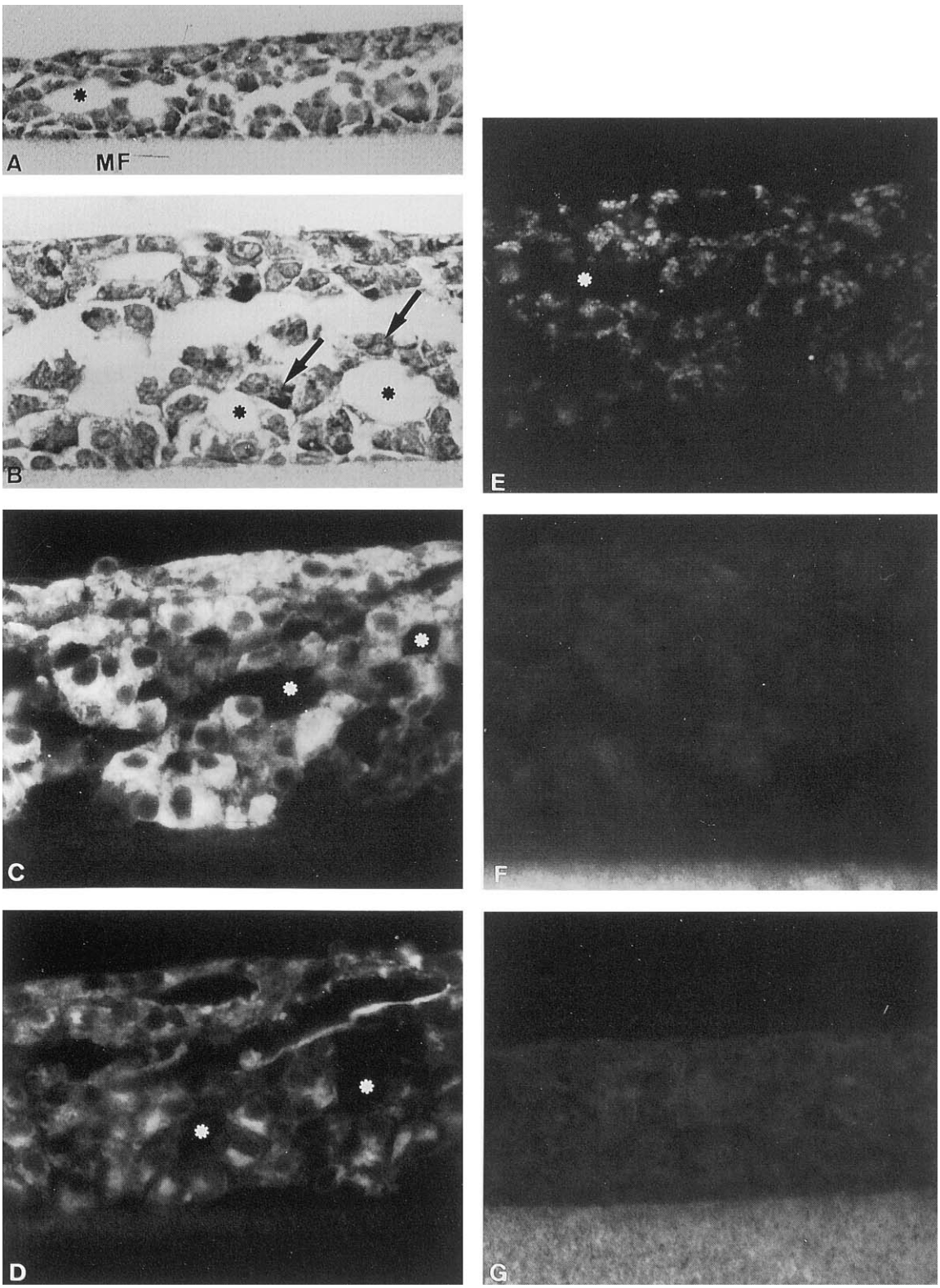

Fig. 1. Differentiation of the whole liver primordium cultured in vitro for 5 days. A, B: PAS staining. C: AFP immunostaining. D: Albumin immunostaining. E: CPSI immunostaining. F: Bile-duct-specific cytokeratin immunostaining. G: Laminin immunostaining. Hepatocytes form large lumina (A-E, ∗) and are positive for AFP, albumin, CPSI and glycogen (arrows). Note that there are no ductal cells in the liver parenchyma with poor development of connective tissue. Hepatocytes are negative for bile-duct-specific cytokeratin and laminin. MF: Millipore filter. A: ×215. B-G: ×430.

In contrast, when the hepatic endodermal cells were recombined with 4-day embryonic chick lung mesenchyme, and cultured for 5 days, they differentiated into many ductal cells in addition to large hepatocytes (6 explants showing ductal differentiation among 6 explants examined) (Fig. 2). The ductal cells were lined with well-developed connective tissue, and also connected with hepatocytes expressing CPSI and accumulating glycogen. Histochemical analyses revealed that the ductal cells were negative for CPSI and albumin, but positive for AFP (Fig. 2). Bile-duct-specific cytokeratin was expressed in some of the ductal cells, and anti-laminin antibodies also reacted with the basal side of some ductal cells. However, basal laminar components such as PNA- and SBA-binding sites, which also characterized differentiated biliary epithelial cells, were absent in these ductal cells. The differentiation state of hepatocytes in this culture was comparable to that of the culture of the whole liver primordium.

The hepatic endodermal cells recombined and cultured with the hepatic mesenchyme showed histology similar to that of the culture of whole liver primordium. The hepatic endoderm did not survive in the culture without mesenchyme.

Discussion

In the present study, we demonstrated that heterologous mesenchyme induces the hepatic endodermal cells to differentiate into many ductal cells as well as large hepatocytes. Although the ductal cells did not express the basal laminar components such as PNA- and SBA-binding sites, they were negative for hepatocyte markers (CPSI and albumin), and some of them were positive for bile-duct-specific cytokeratin and laminin. In addition, they connected with hepatocytes. Thus, they might be bile ducts, though they appeared to be immature. These results support the idea that periportal connective tissue plays an important role in bile duct differentiation during normal liver development (Du Bois 1963; Wilson et al. 1963; Wood 1965; Enzan et al. 1974; Shiojiri 1984a).

Bile duct differentiation did not occur in the cultures of whole liver primordium and the recombinants of the hepatic endoderm and the hepatic mesechyme. The reason for the lack of success of bile duct differentiation is unknown at present. Because the connective tissue did not develop abundantly from the hepatic mesenchyme in these cultures, this may lead to a poor differentiation of bile ducts. Our culture conditions may not be suitable for periportal connective tissue development. We have also shown that when fetal mouse liver fragments, in which intrahepatic bile ducts have not developed, are cultured in vitro, abundant connective tissue and fully differentiated bile ducts do not differentiate (Shiojiri and Mizuno 1993).

Although mechanisms of abundant ductal cell induction by the embryonic chick lung mesenchyme are unknown and are questions to be resolved in the future, we can screen the effects of various substances on the formation of bile

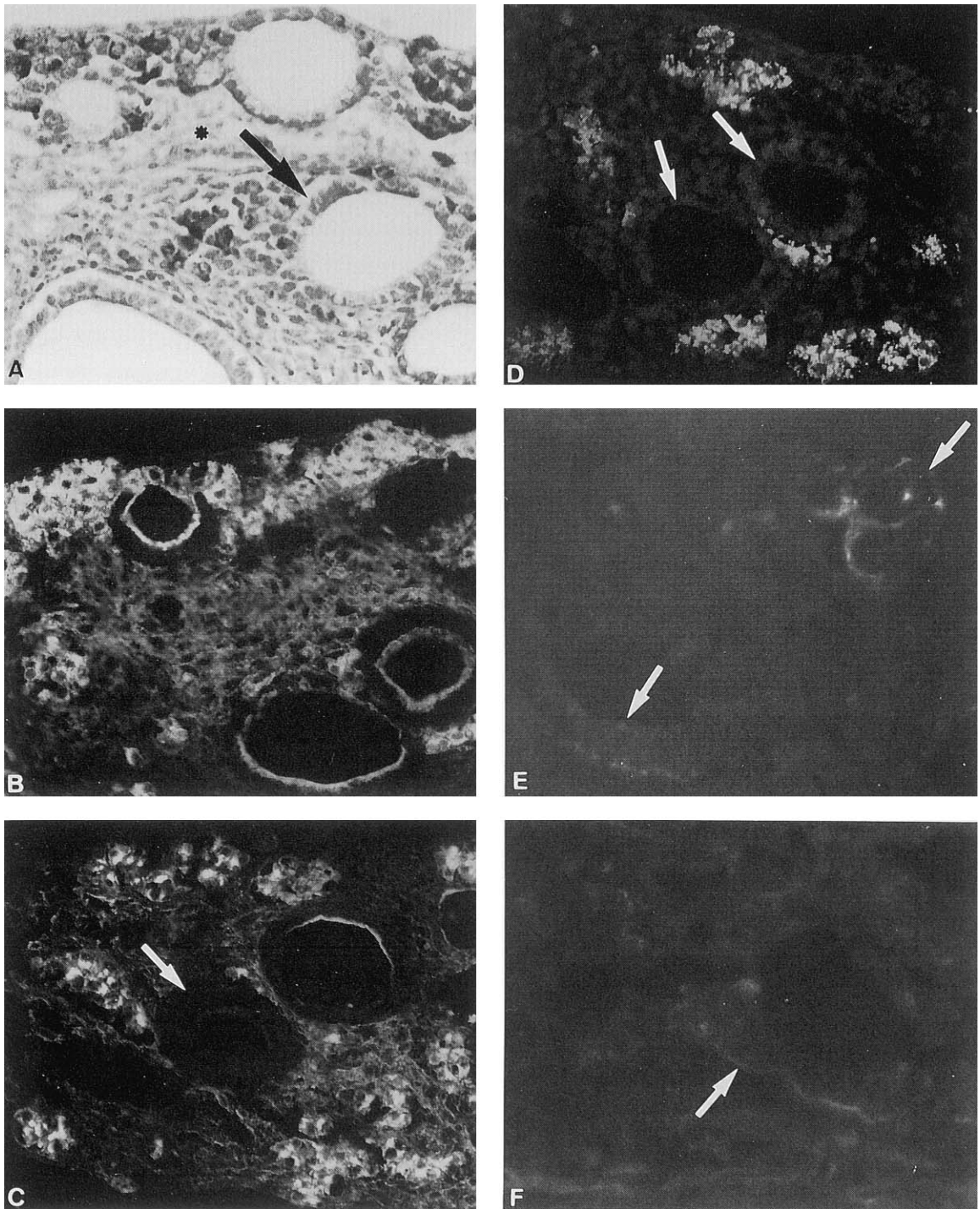

Fig. 2. Culture of a recombinant of the hepatic endoderm with the embryonic chick lung mesenchyme for 5 days. A: PAS staining. B: AFP immunostaining. C: Albumin immunostaining. D: CPSI immunostaining. E: Bile-duct-specific cytokeratin immunostaining. F: Laminin immunostaining. Abundant ductal cells differentiate (A, arrows). They are negative for albumin and CPSI (C, D arrows), and some of them are reactive to bile-duct-specific cytokeratin antibodies (E, arrows) and anti-laminin antibodies (F, arrow). Connective tissue (A, ∗) is well developed. A-D: ×215. E, F: × 430.

ducts and bile duct obstruction using our culture system.

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References

1) Bloom, W. (1926) The embryogenesis of human bile capillaries and ducts. *Am. J. Anat.*, **36**, 451-465.
2) Blouin, M.J., Lamy, I., Loranger, A., Noel, M., Corlu, A., Guguen-Guillouzo, C. & Marceau, N. (1995) Specialization switch in differentiating embryonic rat liver progenitor cells in response to sodium butyrate. *Exp. Cell Res.*, **217**, 22-30.
3) Doljanski, L. & Roulet, Fr. (1934) Über die gestaltende Wechselwirkung zwischen dem Epithel und dem Mesenchym, zugleich ein Beitrag zur Histogenese der sogenannten "Gallengangswucherungen". *Virchows Arch. Path. Anat. Physiol.*, **292**, 256-267.
4) Du Bois, A.M. (1963) The embryonic liver. In: *The Liver, Morphology, Biochemistry, Physiology*, vol. 1, edited by Ch. Rouiller, Academic Press, New York & London, pp. 1-39.
5) Enzan, H., Ohkita, T., Fujita, H. & Iijima, S. (1974) Light and electron microscopic studies on the development of periportal bile ducts of the human embryo. *Acta Path. Jpn.*, **24**, 427-447.
6) Gaasbeek Janzen, J.W., Lamers, W.H., Moorman, A.F.M., de Graaf, A., Los, J.A. & Charles, R. (1984) Immunohistochemical localization of carbamoylphosphate synthetase (ammonia) in adult rat liver: Evidence for a heterogeneous distribution. *J. Histochem. Cytochem.*, **32**, 557-564.
7) Gaasbeek Janzen, J.W., Moorman, A.F.M., Lamers, W.H. & Charles, R. (1985) The development of the heterogeneous distribution of carbamoylphosphate synthetase (ammonia) in rat-liver parenchyma during postnatal development. *J. Histochem. Cytochem.*, **33**, 1205-1211.
8) Germain, L., Blouin, M.-J. & Marceau, N. (1988) Biliary epithelial and hepatocytic lineage relationships in embryonic rat liver as determined by the differential expression of cytokeratins, α-fetoprotein, albumin and cell surface-exposed components. *Cancer Res.*, **48**, 4909-4918.
9) Houssaint, E. (1980) Differentiation of the mouse hepatic primordium. I. An analysis of tissue interactions in hepatocyte differentiation. *Cell Differ.*, **9**, 269-279.
10) McManus, J.F.A. (1948) Histological and histochemical uses of periodic acid. *Stain Technol.*, **23**, 99-108.
11) Shiojiri, N. (1981) Enzymo- and immunocytochemical analyses of the differentiation of liver cells in the prenatal mosue. *J. Embryol. Exp. Morphol.*, **62**, 139-152.
12) Shiojiri, N. (1984a) The origin of intrahepatic bile duct cells in the mouse. *J. Embryol. Exp. Morphol.*, **79**, 25-39.
13) Shiojiri, N. (1984b) Analysis of differentiation of hepatocytes and bile duct cells in developing mouse liver by albumin immunofluorescence. *Develop. Growth Differ.*, **26**, 555-561.
14) Shiojiri, N. (1994) Transient expression of bile-duct-specific cytokeration in fetal mouse hepatocytes. *Cell Tissue Res.*, **278**, 117-123.
15) Shiojiri, N. & Katayama, H. (1987) Secondary joining of the bile ducts during the hepatogenesis. *Anat. Embryol.*, **177**, 153-163.
16) Shiojiri, N. & Nagai, Y. (1992) Preferential differentiation of the bile ducts along the portal vein in the development of mouse liver. *Anat. Embryol.*, **185**, 17-24.

17) Shiojiri, N. & Mizuno, T. (1993) Differentiation of functional hepatocytes and biliary epithelial cells from immature hepatocytes of the fetal mouse in vitro. *Anat. Embryol.*, **187**, 221-229.
18) Shiojiri, N., Lemire, J. M. & Fausto, N. (1991) Cell lineages and oval cell progenitors in rat liver development. *Cancer Res.*, **51**, 2611-2620.
19) Trowell, O.A. (1954) A modified technique for organ culture in vitro. *Exp. Cell Res.*, **6**, 246-248.
20) Trowell, O.A. (1959) The culture of mature organs in a synthetic medium. *Exp. Cell Res.*, **16**, 118-147.
21) Van Eyken, P., Sciot, R. & Desmet, V. (1988) Intrahepatic bile duct development in the rat: A cytokeratin-immunohistochemical study. *Lab. Invest.*, **59**, 52-59.
22) Wilson, J.W., Groat, C.S. & Leduc, E.H. (1963) Histogenesis of the liver. *Ann. N.Y. Acad. Sci.*, **111**, 8-24.
23) Wood, R.L. (1965) An electron microscope study of developing bile canaliculi in the rat. *Anat. Rec.*, **151**, 507-530.