Cell Lineages in Hepatic Development and the Identification of Progenitor Cells in Normal and Injured Liver (43659)

NELSON FAUSTO,[1] JOAN M. LEMIRE,[2] AND NOBUYOSHI SHIOJIRI[3]
Department of Pathology and Laboratory Medicine, Brown University, Providence, Rhode Island 02912

The search for liver stem or progenitor cells has a long history, but there is now sufficient evidence to indicate that such cells do exist in adult animals and probably also in humans (see Refs. 1–6 for reviews). They constitute a reserve compartment that is activated in situations of severe liver injury in which hepatocytes cannot mount an appropriate proliferative response. Proliferation of cells of this compartment is seen at the early stages of hepatocarcinogenesis induced by many chemicals as well as in noncarcinogenic toxic injury, such as that produced by galactosamine administration. The nonparenchymal epithelial cells that can be detected in these conditions received the name "oval cells" because of their shape. Oval cells are not, however, a homogeneous population, but rather form a cellular compartment that contains cells at various stages of differentiation, either in the hepatocyte or bile duct lineages. A very small proportion of these cells seem capable of serving as progenitors for both lineages and could be considered to be stem cells (1–6).

The search for liver stem cells, which started with the analysis of cell lineages in hepatocarcinogenesis, has now been extended to encompass work in fetal and adult normal livers as well as acutely injured liver. The central concept that emerged from these studies is that the adult liver contains facultative stem cells located in the small ductular segments (Hering canals) that connect hepatocytes with the ducts of the biliary tree. These cells appear capable, at least under certain conditions, of functioning as bipotential progenitors and to give rise to normal or transformed hepatocytes. It is a matter of debate whether the term stem cell is entirely appropriate because such cells may be part of a well-defined ductular segment. Regardless of the terminology used to describe these cells (stem cells, progenitor cells, etc.) there is good experimental evidence to indicate that: (i) they originate during fetal development from hepatoblasts which form primitive intrahepatic bile ducts; and (ii) they give origin to the oval cell compartment, which is prominent in carcinogenesis and some forms of noncarcinogenic injury in which hepatocyte replication is inhibited or slow.

In the normal liver, hepatocytes located near portal spaces have a higher proliferative capacity than perivenous cells and slow cell streaming occurs (7, 8). These observations do not, however, imply that the normal liver is a typical stem cell system with slow kinetics because: (i) almost all hepatocytes in the lobule are capable of proliferating (9); (ii) even hepatocytes with the highest proliferative capacity are highly differentiated cells (10, 11); (iii) regeneration of the liver after partial hepatectomy does not seem to depend upon the proliferation of a localized stem cell compartment (12); (iv) generation of hepatocytes from progenitor cells in carcinogenesis and galactosamine injury are abnormal or atypical proliferative processes that disrupt the hepatic architecture (13, 14).

There have been remarkable advances in basic concepts and experimental data related to liver stem cells and an exciting component of these studies has been the realization that the bile duct and hepatocyte lineages that appear to be completely separate in their differentiated forms are in reality interconnected lineages with a common origin (15, 16). Furthermore, some ductular cells in the adult liver and pancreas retain a high degree of plasticity, as indicated by the generation of intestinal glands and pancreatic acini in the liver and the formation of hepatocytes from pancreatic ductules in conditions of severe injury to each of these organs (17). Nevertheless, enormous gaps in our knowledge remain. Some of the more pressing problems include:

[1] To whom requests for reprints should be addressed at Department of Pathology and Laboratory Medicine, Brown University, Providence, RI 02912.

[2] Present address: Department of Pathology, University of Washington, Seattle, WA 98195.

[3] Present address: Biological Institute, Shizuoka University, Shizuoka 422, Japan.

P.S.E.B.M. 1993, Vol 204: 237–241

0037-9727/93/2043-0237$3.00/0

(i) understanding the mechanisms that activate the facultative stem cell compartment (these may include growth factors, cell-cell and cell-matrix interactions, transcriptional activators, etc.); (ii) development of *in vitro* systems to study the steps involved in hepatocyte differentiation from precursor cells and the mechanisms that regulate each step; (iii) development of efficient systems for introducing genetic markers in putative stem cells in the normal liver and study their developmental fate; and (iv) better definition of the transactivating factors required for cell lineage (hepatocyte and bile duct) and tissue (liver, pancreas, intestine) specification.

Where Do Progenitor Cells in Adult Liver Come From?

Studies of stem cells in adult liver seek to define their developmental potential, identify the cells by marker expression and functional assays, and understand the factors that control their differentiation. None of these objectives can be achieved without a proper understanding of liver development. How endodermal cells become determined to form the liver and how these committed cells differentiate into the liver cell lineages are two of the fundamental issues that relate directly to the formulation of stem cell concepts applicable to the adult liver. Little is known about the mechanisms of endodermal cell commitment, mostly because it is difficult to establish appropriate mammalian cell systems to study this question. Induction by cardiac mesoderm is required for liver formation, but it is conceivable that the commitment process of endodermal cells starts with an even earlier event that gives the cell the capacity to react to the inducive stimuli (18, 19). Much more is known about the generation of hepatoblasts from committed endodermal cells and the establishment of specific lineages from hepatoblasts, but the control mechanisms for both of these developmental steps are still poorly understood. Several investigators have examined in rats and mice the expression of markers in the endodermal cells of the liver primordium before cord formation, the formation of intra- and extrahepatic biliary ducts and the steps involved in hepatocyte maturation (15, 20–23).

The liver develops from a diverticulum of the ventral floor of the foregut. The founder cells invade the mesenchyma of the septum transversum containing the vitelline veins. Endodermal cells eventually generate hepatocytes and nonparenchymal epithelial cells whereas the mesenchyma (which also contains elements of the celomic mesothelium) gives rise to sinusoidal lining cells (18, 19). The hemopoietic cells of the fetal liver have an extrahepatic origin derived from stem cells that migrate from the yolk sac (24). Hepatic development from the foregut diverticulum depends on interactions between endodermal cells and the surrounding mesoderm. In avian embryos, commitment of these cells to becoming hepatic progenitors requires the inductive effect of cardiac mesoderm at the five-somite stage. At a later state of development (20 somites), differentiation of already committed hepatic endodermal cells into hepatoblasts depends on interactions with the hepatic mesoderm (18, 19, 24).

At 10.5 days of development in rats (10- to 14-somite stage), the liver primordium can be recognized as a thickening of the ventral foregut containing about 100 cells (15). These cells, but not those of the adjacent endoderm, contain α-fetoprotein (AFP) mRNA as shown by *in situ* hybridization. Expression of the protein is detectable 1 day later at 11.5 days of development. Albumin mRNA expression is first detected at 11.5 days, followed 1 day later by the protein. Thus, AFP mRNA transcription occurs in committed cells before liver morphogenesis, while translation of the protein coincides with the formation of hepatic cords. Albumin mRNA and protein appear 1 day later than the respective AFP molecules (15).

Shiojiri and collaborators (22) have done extensive studies on AFP and albumin expression in the liver primordium of mouse embryos and analyzed the formation of intra- and extrahepatic bile ducts. Using immunofluorescence methods, they detected AFP at 9–9.5 days of development and both AFP and albumin at 10.5–11.5 days. Based on this work, Shiojiri *et al.* (22) concluded that the earliest liver cell in mice is AFP^+, $albumin^-$ and that these cells become AFP^+, $albumin^+$ at the time of cord formation. Cascio and Zaret (20) studied the expression of albumin mRNA in mice by *in situ* hybridization and found that mRNA expression was first detected in the liver primordium at 9.5 days of development. They suggested that the initial expression of albumin mRNA in endodermal cells requires close contact with the mesenchyme and showed that expression of albumin mRNA was highly enhanced by the cell interactions that occur during organogenesis.

Parenchymal cells forming hepatic cords from Days 10 to 17 in rat liver development are generally referred to as hepatoblasts. 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 (1–3). 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 (15, 23). These structures contain cells that express AFP

and albumin as well as cytokeratins. In the rat, the pattern of cytokeratin expression is a particularly good marker for these cells, as surrounding hepatoblasts contain only cytokeratins 8 and 18 and expression of cytokeratins 7 and 19 is present exclusively in the newly formed ducts (15, 23). In humans, a significant proportion of hepatoblasts express cytokeratin 19 in early gestation (16, 25). The expression of this marker increases in cells near vascular spaces forming ducts, but progressively decreases and is no longer detectable after 14 weeks of gestation in the hepatoblasts that mature into hepatocytes in the rest of the parenchyma. In adult livers of rodents and humans, hepatocytes express only cytokeratins 8 and 18, while bile ducts express cytokeratins 7, 8, 18, and 19 (16).

The cells of primitive intrahepatic bile ducts can be considered as transitional because they express markers of both hepatocyte (AFP, albumin, and the cell surface antigen HES6) and bile duct (cytokeratin 7 and 19, γ-glutamyltransferase, and the cell surface antigen BDS7) lineages, a pattern of expression also found in the oval cell compartment of hepatocarcinogenesis (26–29). On the basis of these data, we have suggested that the primitive intrahepatic bile ducts formed from AFP^+, $albumin^+$ hepatocytes at 15.5–18 days of development in the rat are the embryological counterparts of progenitor or stem-like cells in the oval cell compartment and constitute the source of such cells present in normal liver (see below). The developmental interconnection between the hepatocyte and bile duct lineages explains why lineage transitions in either direction may commonly occur in normal and pathologic states.

That hepatocytes obtained from 11- to 13-day fetal rat and mouse liver are bipotential lineage progenitors has been demonstrated in two ways. Transplantation of fragments of 11- to 13-day liver containing only hepatocytes (intrahepatic bile ducts have not yet appeared at this stage) into ectopic sites leads to the appearance of both mature hepatocytes and ductular structures (15). Furthermore, Germain *et al.* (21) 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, cytokeratin 8, and an antigen recognized by monoclonal antibody 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 dimethyl sulfoxide, transforming growth factor-β, and insulin-like growth factor-II, while sodium butyrate induced ductular differentiation. The bipotential progenitor capacity of these cells decreased progressively during development until differentiation became restricted only to the hepatocyte lineage. The proportion of parenchymal cells capable of differentiating into both lineages dropped from 70% at Day 12 to 20% and 5%, respectively, at Day 18 and 5 days after birth (21).

In summary, studies of liver development have shown that: (i) committed endodermal cells that will form the liver contain AFP mRNA and that albumin mRNA expression appears at about the same time as AFP or a day later; (ii) hepatocytes formed from these endodermal cells have dual lineage progenitor capacity and give rise to mature hepatocytes, intrahepatic bile ducts, and portions of the extrahepatic ducts; (iii) formation of primitive intrahepatic ducts, which starts at 15.5 days in rat liver and at 9 weeks of gestation in humans, involves transitional cells that may correspond to progenitors in adult tissue which are capable of expressing hepatocyte and bile duct markers and are the source of the oval cell compartment of carcinogenesis and liver injury.

Stem Cells in Normal Liver

The analysis of liver development and the studies of cell populations in hepatocarcinogenesis provide a framework for predicting the localization of putative liver stem cells in adult normal liver and the types of markers they may express. Given the pattern of proliferation of the oval cell compartment in carcinogenesis and the histogenesis of primitive intrahepatic bile ducts in development, it is logical to expect that putative stem cells should be localized in the smallest units of the biliary tree closest to the parenchyma. These units are the canals of Hering, structures that are lined by both hepatocytes and duct cells and extend through the limiting plate to form a connection between parenchymal hepatocytes and interlobular ducts. They are also referred to as cholangioles, terminal ducts, or ductules, although some authors make a distinction between canals of Hering and ductules (30).

Ductular stem cells in normal liver should express AFP as well as typical ductal cell markers, such as cytokeratins 7, 8, 18, and 19. However, it is possible that the degree of differentiation and the expression of markers are heterogeneous among ductules, reflecting the time of their embryologic origin. Intrahepatic bile duct formation during liver development in rats starts at 15.5 days and continues up to about the second postnatal week. Although the process of duct formation is the same throughout this period, the cells from which the ductular structures originate progressively change (15). At first, primitive intrahepatic bile ducts are generated from AFP^+ hepatoblasts. At later stages, ductules originate from immature hepatocytes and postnatally they are formed from more mature hepatocytes that no longer express AFP. As indicated by the work of Germain *et al.* (21) on the lineage-generating properties of hepatoblasts, intrahepatic bile ducts formed during the neonatal period probably do not contain bipotential progenitor cells, in contrast to ductules formed earlier

in development. Studies using monoclonal antibodies generated against oval cell and fetal liver antigens have shown that there is considerable heterogeneity in the reactivity to these antibodies by bile ducts and ductules of the adult liver (6). It remains to be established whether any of these monoclonal antibodies specifically recognize bipotential progenitor cells.

Because AFP is expressed by the earliest liver cells at the time of formation of the hepatic primordium, this protein should be a good marker for the identification of putative stem cells in normal adult livers. However, there are some complications with the use of AFP as a marker because the rat liver contains multiple forms of AFP mRNA and some truncated species of the protein are present in adult normal liver (31). In addition to the 2.1-kb AFP mRNA encoding the 68- to 70-kDa protein, which is abundantly expressed in fetal liver and the oval cell compartment, other less abundant AFP RNA forms were identified in fetal and adult rat liver. These AFP mRNA variants are produced by differential exon usage of the AFP gene and code for smaller proteins that can be precipitated by antibodies generated against the full-length molecule. A 1.7-kb mRNA is found only in fetal liver, whereas the adult normal liver contains variants of 1.4 and 1.0 kb corresponding to 3′-exons of the gene, which encode truncated forms of the protein of approximately 44–58 kDa. In normal rat hepatocytes, transcription of the 2.1-kb AFP mRNA is shut off at the early neonatal stages, but transcription of the smaller variants, possibly initiated by internal promoters in the gene, is active in hepatocytes of the adult liver (31). Given these findings, our search for stem cells in normal liver using AFP as a marker needed to be formulated more precisely and was guided by the following questions: (i) Are there cells in adult liver that express 2.1-kb AFP mRNA, indicating that transcription of the fetal form of AFP was not shut off in such cells? (ii) Would these cells be localized in the canals of Hering? And (iii) do these cells have bipotential progenitor properties?

Studies by Lemire and Fausto (31) demonstrated, by serial sectioning and *in situ* hybridization with probes that are specific for 2.1-kb AFP mRNA, that a very small number of cells localized in bile ductules (Hering canals) inside portal spaces in adult rat liver express this mRNA. The fetal form of AFP mRNA was also found in some rat hepatocytes in the parenchyma (approximately 1 in 20,000 cells), with no preferential zonal distribution in the lobule. Labeling studies with bromodeoxyuridine revealed that these hepatocytes were not undergoing replication. Although the significance of these hepatocytes is at this time entirely unknown, the ductular cells that express the fetal form of AFP mRNA in normal adult rat liver could conceivably be liver stem cells. Caution is necessary, however, in accepting this interpretation. These cells have not been isolated and conclusions about their developmental potential are assumptions that need to be verified experimentally. Marceau *et al.* (5) have suggested that ductular cells that express fetal AFP mRNA in adult rat liver are unlikely to be bipotential progenitors because they lack certain markers, and in contrast to hepatoblasts, they do not react with monoclonal antibody BPC5. Another point for further study is that Sell and Salman (32) found that the first cells to divide when the proliferation of the oval compartment starts in early carcinogenesis are localized in periductular spaces rather than in ductular structures. All of these uncertainties can be resolved by tracing the developmental fate of specific ductular cells after introduction of genetic markers both *in vivo* and *in vitro* and when methods to isolate these cells in reasonably pure form become available. These goals, although difficult to achieve in view of the small proportion of putative stem cells which are present in adult rat liver, are being actively pursued in this and other laboratories.

1. Fausto N. Liver stem cells. In: Arias I, Boyer J, Fausto N, Jakoby W, Schacter D, Shafritz D, Eds. The Liver: Biology and Pathobiology, 3rd ed. New York: Raven Press, in press.
2. Fausto N. Hepatocyte differentiation and liver progenitor cells. Curr Opinion Cell Biol **2**:1036–1042, 1990.
3. Fausto N, Lemire JM, Shiojiri N. Oval cells in liver carcinogenesis: Cell lineages in hepatic development and the identification of stem cells in normal. In: Sirica AE, Ed. The Role of Cell Types in Hepatocarcinogenesis. Boca Raton, FL: CRC Press, pp89–108, 1992.
4. Thorgeirsson SS, Evarts RP. Growth and differentiation of stem cells in adult rat liver. In: Sirica AE, Ed. The Role of Cell Types in Hepatocarcinogenesis. Boca Raton, FL: CRC Press, pp109–120, 1992.
5. Marceau N, Blouin M-J, Noël M, Török N, Loranger A. The role of bipotential progenitor cells in liver ontogenesis and neoplasia. In: Sirica AE, Ed. The Role of Cell Types in Hepatocarcinogenesis. Boca Raton, FL: CRC Press, pp121–149, 1992.
6. Hixson DC, Faris RA, Yang L, Novikoff P. Antigenic clues to liver development, renewal, and carcinogenesis: An integrated model. In: Sirica AE, Ed. The Role of Cell Types in Hepatocarcinogenesis. Boca Raton, FL: CRC Press, pp151–182, 1992.
7. Zajicek G, Oren R, Weinreb MJ. The streaming liver. Liver **5**:293–300, 1985.
8. Reid L, Fiorino A, Sigal S, Brill S, Holst P. Extracellular matrix gradients in the space of Disse: Relevance to liver biology. Hepatology **15**:1198–1203, 1992.
9. Rabes H. Kinetics of hepatocellular proliferation after partial resection of the liver. Prog Liver Dis **5**:83–89, 1976.
10. Gumucio J. Hepatocyte heterogeneity: The coming of age from the description of a biological curiosity to a partial understanding of its physiological meaning and regulation. Hepatology **9**:154–160, 1989.
11. Gebhardt R. Metabolic zonation of the liver: Regulation and implications for liver function. Pharmacol Ther **53**:275–354, 1992.
12. Klinman NR, Erslev AJ. Cellular response to partial hepatectomy. Proc Soc Exp Biol Med **112**:338–340, 1963.
13. Lemire J, Shiojiri N, Fausto N. Oval cell proliferation and the

origin of small hepatocytes in liver injury induced by D-galactosamine. Am J Pathol **139**:535–552, 1991.
14. Sell S, Dunsford H. Evidence for the stem cell origin of hepatocellular carcinoma and cholangiocarcinoma. Am J Pathol **134**:1347–1363, 1989.
15. Shiojiri N, Lemire JM, Fausto N. Cell lineages and oval cell progenitors in rat liver development. Cancer Res **51**:2611–2620, 1991.
16. van Eyken P, Desmet VJ. Development of intrahepatic bile ducts, ductular metaplasia of hepatocytes, and cytokeratin patterns in various types of human hepatic neoplasms. In: Sirica AE, Ed. The Role of Cell Types in Hepatocarcinogenesis. Boca Raton, FL: CRC Press, pp227–263, 1992.
17. Rao M, Dwivedi R, Yeldani A, Subbarao V, Tan X, Usman M, Thangada S, Nemali M, Kumar S, Scarpelli D, Reddy J. Role of periductal and ductular epithelial cells of the adult rat pancreas in pancreatic hepatocyte lineage: A change in differentiation commitment. Am J Pathol **134**:1069–1086, 1989.
18. Le Douarin NM. An experimental analysis of liver development. Med Biol **53**:427–455, 1975.
19. Houssaint E. Differentiation of the mouse hepatic primordium. I. An analysis of tissue interactions in hepatocyte differentiation. Cell Differ **9**:269–279, 1980.
20. Cascio S, Zaret KS. Hepatocyte differentiation initiates during endodermal-mesenchymal interactions prior to liver formation. Development **113**:217–225, 1991.
21. Germain L, Blouin M-J, Marceau N. Biliary epithelial and hepatocytic cell 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, 1988.
22. Shiojiri N., Enzymo, immunocytochemical analyses of the differentiation of liver cells in prenatal mouse. J Embryol Exp Morphol **62**:139–152, 1981.
23. van Eyken P, Sciot R, Desmet VJ. Intrahepatic bile duct development in the rat: A cytokeratin-immunohistochemical study. Lab Invest **59**:52–59, 1988.
24. Houssaint E. Differentiation of the mouse hepatic primordium. II. An analysis of tissue interactions in hepatocyte differentiation. Cell Differ **9**:269–279, 1980.
25. Gerber MA, Thung SN. Cell lineages in human liver development, regeneration, and transformation. In: Sirica AE, Ed. The Role of Cell Types in Hepatocarcinogenesis. Boca Raton, FL: CRC Press, pp209–226, 1992.
26. Fausto N, Thompson NL, Braun L. Purification and culture of oval cells from rat liver. In: Pretlow TG, Pretlow TPP, Eds. Cell Separation: Methods and Selected Applications. New York: Academic Press, pp45–77, 1987.
27. Goyette M, Faris R, Braun L, Hixson D, Fausto N. Expression of hepatocyte and oval cell antigens in hepatocellular carcinomas produced by oncogene-transfected liver epithelial cells. Cancer Res **50**:4809–4817, 1990.
28. Evarts RP, Nagy P, Marsden E, Thorgeirsson SS. In situ hybridization studies on expression of albumin and α-fetoprotein during the early stage of neoplastic transformation in rat liver. Cancer Res **47**:5469–5475, 1987.
29. Hixson DC, Faris Ra, Thompson NL. An antigenic portrait of the liver during carcinogenesis. Pathobiology **58**:65–77, 1990.
30. Sirica AE, Elmore LW, Williams TW, Cole SL. Differentiation potential of hyperplastic bile ductular epithelial cells in rat models of hepatic injury and cholangiocarcinogenesis. In: Sirica AE, Ed. The Role of Cell Types in Hepatocarcinogenesis. Boca Raton, FL: CRC Press, pp183–208, 1992.
31. Lemire JM, Fausto N. Multiple α-fetoprotein RNAs in adult rat liver: Cell type-specific expression and differential regulation. Cancer Res **51**:4656–4664, 1991.
32. Sell S, Salman J. Light- and electron-microscopic autoradiographic analysis of proliferating cells during the early stages of chemical hepatocarcinogenesis in the rat induced by feeding N-2-fluorenylacetamide in a choline-deficient diet. Am J Pathol **114**:287–300, 1984.