

Analysis of liver development, regeneration, and carcinogenesis by genetic marking studies

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Analysis of liver regeneration by genetic marking studies provides an exciting method not only to identify newly replicated cells, but also to determine their migration patterns within the hepatic acinus. This review addresses timely issues such as hepatocyte "streaming" and the origin in hepatocellular carcinoma. We now realize that a single hepatocyte can potentially replace 90% of the liver. This unique ability supports the use of hepatocyte transplantation as a modality to replace damaged liver and as a vehicle to deliver genes for replacement therapy. With the advent of methods to genetically mark cells of different lineages, it has become possible to follow their growth and migration characteristics. The results suggest, in fact, that the adult parenchymal cell is responsible for postnatal replication of hepatocytes and that streaming from periportal to pericentral regions does not occur. Does this imply, however, that the fully differentiated hepatocyte is the cellular origin of hepatocellular carcinoma? This review finds growing evidence to implicate both hepatocytes and oval cells as precursors of primary hepatic cancers. However, if oval cells are truly bipotential in their ability to replicate into hepatocytes or bile ductular cells, one might expect to see some incidence of cholangiocarcinoma in the models of multistep hepatic carcinogenesis. In fact, such is the case! The use of retroviral vectors and marker genes such as β -galactosidase are of unquestionable benefit in answering basic questions regarding the regenerative capacity of the liver, cell differentiation, and the cellular origin of hepatocellular cancers. But the definitive studies of the existence and growth characteristics of multipotent stem cells *in vivo* are, in fact, lacking. Future studies to address these issues will undoubtedly use genetic marking, transgenic models, and the regenerating liver.

—Clifford J. Steer, Coordinating Editor

ABSTRACT The mechanism of generating new hepatocytes and bile ductule cells in the liver has been controversial. Oval cells are found in the periportal region under some circumstances and may represent multipotent stem cells. The role of stem cells in generating new liver cells in normal and pathological conditions is unclear, however. Genetic

marking can be used to determine the ability of a particular cell to replicate and to migrate. Cells of known lineage are marked at an initial time point, and their developmental potential determined by the cluster size, position, and phenotype of marked cells at a later time point. Recently, genetic marking studies have demonstrated that the hepatocyte itself is the source of new hepatocytes in the normal postnatal liver and that daughter cells do not migrate. These studies have also demonstrated that the hepatocyte can replicate extensively when stimulated. Finally, genetic marking studies suggest that either hepatocytes or oval cells can develop into a hepatocellular carcinoma or cholangiocarcinoma if a sufficient number of genetic mutations accumulate. The implications of these results for hepatic gene therapy, treatment of liver insufficiency states, and liver cancer are discussed. Future genetic marking studies may help to address some remaining questions in liver biology.—Ponder, K. P. Analysis of liver development, regeneration, and carcinogenesis by genetic marking studies. *FASEB J.* 10, 673–684 (1996)

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THE LIVER IS AN EPITHELIAL ORGAN that contains two major differentiated cell types: the hepatocyte and the bile ductule cell (1). Hepatocytes produce blood proteins, perform metabolic functions, and contribute to bile production. Bile ductules collect the bile, which flows into the common bile duct. Hepatocytes are present throughout the liver. Bile ductule cells are limited to the periportal region, which is where the blood enters the liver via the portal vein and hepatic artery. Although the phenotype of differentiated hepatocytes and bile ductule cells is different, they are thought to derive from a common stem cell known as the oval cell, as detailed below. It is unclear, however, what role stem cells play in normal postnatal liver growth, in the chronically regenerating liver, or in liver carcinogenesis. The subject of this re-

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view is the recent use of genetic marking studies to address some of these questions.

GENETIC MARKING TECHNIQUES

Genetic marking involves the transfer of a gene into the chromosome of a cell. The developmental fate of that cell can then be determined by identifying what cell types express the marker gene at a later time point. Three types of technology have been used for genetic marking. The first method involves the developmental analysis of expression in organs that exhibit mosaic expression of a gene. This can be achieved by mixing embryonic cells from histochemically distinguishable strains to create chimeric animals (2). Alternatively, transgenic mice can be generated by injecting a gene into oocytes. The gene will sometimes integrate into the chromosome and be expressed in organs that activate the promoter upstream of the gene. Such transgenic mice can exhibit mosaic expression of the gene, even in later generation animals that contain the gene in all cells. Mosaic expression of a transgene has been used to study lineage relationships in several organs, where clusters of positive cells have been demonstrated to be clonally related (3, 4). A second method for genetic marking involves the transplantation of genetically marked cells into unmarked recipients (5). The source of cells can be either transgenic animals or a histochemically distinguishable strain. The third method for genetic marking is to deliver a retroviral vector to specific cell types in animals in vivo (6, 7). A retroviral vector is generated in which the retroviral *gag*, *pol*, and *env* genes are replaced with a marker gene, but sequences necessary for retroviral packaging and integration into the chromosome remain. After introduction of this vector into a packaging cell line that expresses retroviral proteins, retroviral particles are generated that can undergo one, and only one, round of infection. One advantage of genetic marking is the fact that cells will still be labeled regardless of the time after gene transfer and the number of replication events that occur. Second, the use of genes that encode histochemically visible marker proteins enables one to evaluate a large number of sections with a minimal amount of effort.

There are three potential pitfalls to the use of genetic marking for developmental analyses, however. First, because identification of marked cells usually requires expression of the marker protein, the promoter must be expressed. If differentiation leads to inactivation of the promoter, an investigator might falsely conclude that a cell did not derive from its genetically marked progenitor. Second, there are no techniques in mammals for studying a single cell at more than one time point. This makes it important to mark a homogeneous population of cells of known phenotype at the initial time point. Failure to do so raises uncertainties as to which cell begat a cell that is present at later times. Third, if wild-type retrovirus is present, a cluster of marked cells could result from additional rounds of infection instead of replication of the cell

that was marked at the initial time point. Thus, it is imperative to demonstrate that the retroviral vectors are replication incompetent.

EVIDENCE FOR A BIPOTENTIAL STEM CELL IN THE LIVER KNOWN AS THE OVAL CELL

Hepatocytes and bile ductule cells are thought to derive from a common stem cell for the following reasons (reviewed in refs 1, 8–14). 1) Cells with histochemical characteristics of both hepatocytes [α -fetoprotein and albumin (8)] and bile ductule cells [γ -glutamyl-transpeptidase and cytokeratin 19 (8)] are present in embryonic livers (15, 16). 2) Some carcinogenic protocols result in the appearance of cells of unknown origin with oval-shaped nuclei in the periportal region. These so-called oval cells have characteristics of both hepatocytes and bile ductule cells (8, 16–20). 3) Oval cells can differentiate into either hepatocytes or bile ductule cells in vitro depending on the culture medium used (21). Furthermore, a mixed preparation of oval cells differentiated into bile ductule cells (but not hepatocytes) after implantation into the fat pads of syngeneic rats (22), whereas pulse:chase DNA labeling studies have demonstrated that oval cells appear to differentiate into hepatocytes in vivo (23, 24). 4) Liver cancers with characteristics of both bile ductule cells and hepatocytes can develop in human patients (25) and in rats treated with a carcinogenic protocol (26).

Although there is evidence in support of a bipotential stem cell that can generate both hepatocytes and bile ductule cells, its existence has not been conclusively proved. First, some so-called lineage-specific markers are expressed in cells derived from other organs. For example, although cytokeratin 19 (CK19)² is specific for oval cells and bile ductule cells in the liver, it is expressed in other organs, including the skin and bladder (12). This raises the possibility that the coexpression of CK19 and hepatic markers such as AFP and albumin does not necessarily identify a bipotential stem cell. Second, the apparent movement of label from oval cells into hepatocytes might be observed if label reutilization had occurred (27) and hepatocytes themselves replicated at a slightly later time point. Finally, oval cell preparations are quite heterogeneous, and a single cell has never been demonstrated to give rise to cells of both the hepatic and the bile ductule lineage. Thus, the progenitor cells that gave rise to hepatocytes in vitro (21) might be different from the progenitor cells that gave rise to bile ductule cells in vitro (21) or in vivo (22). Thus, a variety of data support but do not definitively prove the hypothesis that there is a bipotential stem cell that can generate both hepatocytes and bile ductule cells. Furthermore, even if such a stem

²Abbreviations; CK19, cytokeratin 19; LTR, long-terminal repeat; hAAT, human α_1 -antitrypsin; Alb-uPA, urokinase plasminogen activator cDNA from the albumin promoter; HCC, hepatocellular carcinoma; CC, cholangiocarcinoma; DEN, diethylnitrosamine.

cell exists, it is unclear what role it plays in normal postnatal liver growth, in a chronic regenerating liver, or in liver carcinogenesis. It has recently become possible to mark liver-derived cells genetically and to observe their developmental potential in animals. Such studies have led to important insights regarding the mechanism of liver growth under normal and pathological conditions.

HEPATOCYTES ARE THE SOURCE FOR NEW CELLS IN THE NORMAL POSTNATAL LIVER AND DO NOT MIGRATE

The source of new cells in the normal liver has been controversial. Some investigators have favored the hypothesis that periportal stem cells give rise to daughter cells that "stream" toward the pericentral region and undergo apoptosis after approximately one year (11, 28, 29). This model is shown in Fig. 1 A–C. It resembles what occurs in other epithelial organs such as the intestines, which undergo rapid regeneration in response to a continuous loss of cells (3). An alternative viewpoint of liver development is that mature hepatocytes replicate during nor-

mal liver growth and that newly formed cells do not migrate (14), as shown schematically in Fig. 1D–F.

Although the streaming liver theory has its disciples, there are only two pieces of data that support it. First, tritiated thymidine labeling in either normal livers (28–30) or in livers that were replicating in response to partial hepatectomy (31) demonstrated that labeled cells appeared to migrate from the periportal to the pericentral region over time. However, other investigators reported no differences in the frequency of labeling between various regions of the hepatic lobule in normal livers, and failed to see migration over time (32–34). It is likely that the low frequency of labeling of hepatocytes due to their slow replication rate (35) and problems of label reutilization (27) accounts for this discrepancy. A second fact that is cited by supporters of the streaming liver theory is that several liver-specific genes exhibit a gradient in expression from the periportal to the pericentral region (11). Some genes that are normally expressed in the periportal region cannot be activated in pericentral hepatocytes by reversing the blood flow (and thus the concentration of metabolites) in the liver. This fact has led some investigators to conclude that pericentral hepatocytes represent a more differentiated cell that cannot reacquire expression of genes expressed in "younger" cells (11).

Other investigators favor the alternative theory that all hepatocytes can replicate regardless of their position. This theory has been based primarily on the fact that a partial hepatectomy induces one (31, 34) or more (36) rounds of replication of most hepatocytes within 48 h, implying that all have proliferative potential. Such labeling studies cannot address the role that the hepatocyte plays in the nonregenerating liver, however, due to the above-mentioned difficulties in labeling a significant number of cells. In addition, DNA labeling studies cannot determine the number of replication events an individual hepatocyte can perform due to dilution of label and difficulties in achieving a continuous stimulus for replication.

Determination of the source of new cells in the normal liver required an improved method for marking individual cells and following them over time. Recent genetic marking studies have provided such a tool, and they present unequivocal support for the theory that the parenchymal hepatocyte is responsible for postnatal replication of hepatocytes and streaming does not occur. Bralet et al. (6) delivered a retroviral vector containing the β -galactosidase (β -gal) gene downstream of the retroviral long-terminal repeat (LTR) promoter to hepatocytes *in vivo*. X-gal staining results in a blue color in β -gal-expressing cells. It was performed on liver sections obtained at various times after retroviral delivery to determine the cluster size and position of the retroviral-transduced hepatocytes. The frequency of cells in various zones of the liver did not change for 15 months after transduction, suggesting that hepatocytes cannot stream from the periportal to the pericentral region. Similar results were obtained using a retroviral vector containing the liver-specific transthyretin promoter upstream of β -gal. In this study, retroviral

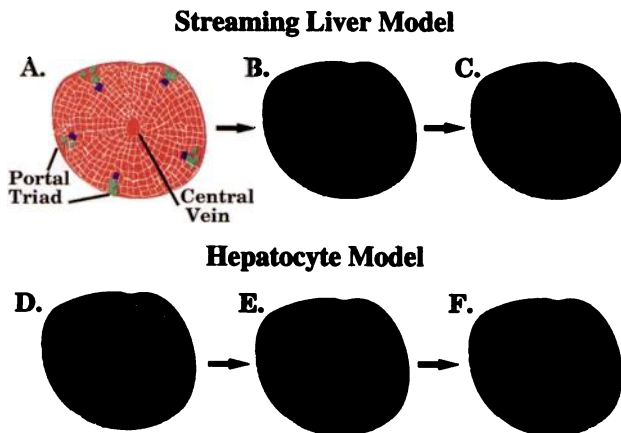


Figure 1. Models for normal liver growth. A–C) Streaming liver model. In this model, new hepatocytes are generated in the periportal region and migrate to the central vein over the period of 1 year. The periportal region surrounds the portal triad, which is shown in green in panel A. This contains the portal vein, hepatic artery, and bile ductule. The pericentral region surrounds the central vein, which is where the blood collects after it percolates through the liver. Panel A depicts new hepatocytes as blue. These were recently generated in the periportal region from stem cells. Other hepatocytes are shown in red. Panel B demonstrates the position of the marked hepatocytes several months later. They have migrated slowly toward the central vein, as indicated by the blue cells. Panel C demonstrates the position of the marked cells 1 year after they were generated. The blue cells are now close to the central vein, and will undergo apoptosis in the near future. D–F) Hepatocyte model. In this model, hepatocytes give rise to additional hepatocytes, and do not migrate. The position of three individual hepatocytes at the initial time point is indicated by their blue color in panel D. To accommodate normal liver growth, these hepatocytes replicate into two cells, as shown in panel E. To accommodate further liver growth, these hepatocytes replicate again to generate four blue cells, as shown in panel F. The time course of replication will depend on the age of the animal and its need for liver replication.

transduced cells remained as single cells or small clusters that did not migrate for up to 10 months after transduction (7), as shown in **Fig. 2**. In both studies, most clusters contained less than seven blue cells at 10 months or later after transduction. This makes it very unlikely that retroviral-marked stem cells were responsible for producing additional blue periportal cells, as such a stem cell should result in large clusters or linear streaks of blue cells.

A second method for genetic marking provides further evidence against the streaming liver hypothesis. In these studies, hepatocyte cluster size was analyzed in animals expressing a transgene in a subset of liver cells or in mosaic animals containing cells from histochemically distinguishable strains. Transgenic mice containing the β -gal gene downstream of the liver-specific human α_1 -antitrypsin promoter were mosaic for expression in the liver (37). Mosaic expression of a transgene has been used to study

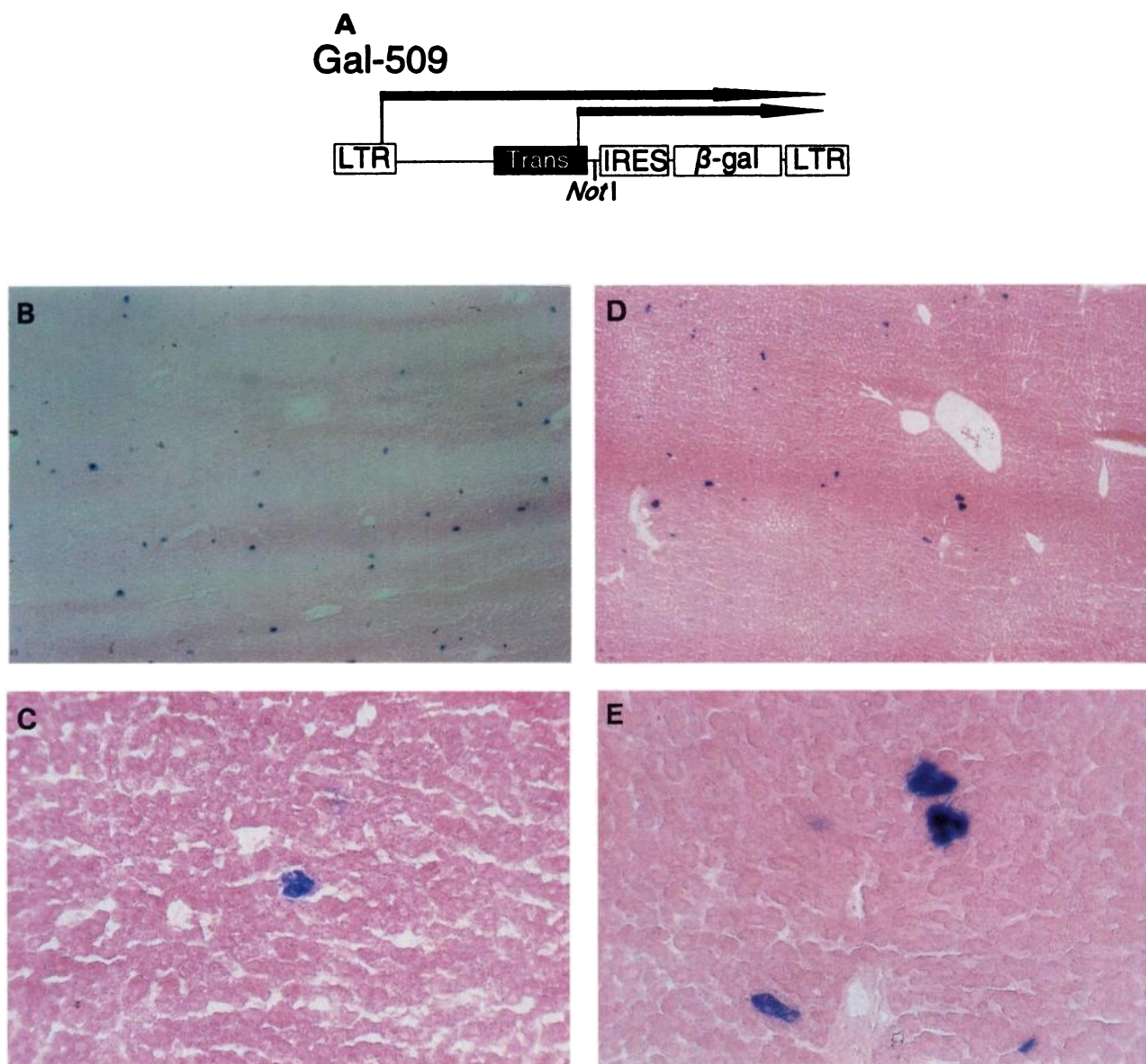


Figure 2. Evidence against the streaming liver model. Retroviral-transduced hepatocytes do not migrate. *A*) Retroviral vector Gal-509. The LTRs are necessary for producing a retroviral vector. The transthyretin promoter (Trans) is a liver-specific promoter that is expressed long-term from a retroviral vector in the rat liver. The encephalomyocarditis internal ribosome entry site sequence (IRES) is an element that allows the downstream gene of a dicistronic mRNA to be translated into two proteins. The β -gal gene encodes an enzyme that allows expressing cells to be visualized by X-gal staining. The arrows indicate that transcription can initiate from either the LTR or the internal transthyretin promoter; either transcript can be translated into the protein. *B–E*) X-gal staining of Gal-509-transduced rat liver sections. Rat hepatocytes were transduced *in vivo* with Gal-509. A 70% partial hepatectomy was performed on adult rats to induce hepatocyte replication, and a replication-incompetent retroviral vector was injected into the portal vein 24 h later. X-gal staining performed on a frozen section of a liver obtained 1 month after transduction identifies retroviral-transduced cells. They are present throughout the liver, as shown in panel *B* (40 \times). These blue cells are present as singlets and doublets, as shown at higher power in panel *C* (200 \times). The frequency and position of blue cells was similar at 10 months after transduction, as shown in panel *D* (40 \times). Although the average cluster size increased slightly, as shown in panel *E* (200 \times), the clusters were still less than seven cells.

lineage relationships in several other organs, where clusters of positive cells have been demonstrated to be clonally related (3, 4). A developmental study was therefore performed to determine the cluster size and position of blue cells in the livers of human α_1 -antitrypsin (hAAT) - β -gal transgenic mice at various times after birth. In neonatal animals, blue cells were present in singlets or doublets randomly scattered throughout the liver, as shown in Fig. 3A, B. In older animals, blue cells were present in larger clusters, as shown in Fig. 3C, D. This result is consistent with the theory that hepatocytes from all regions replicated to form a clonally derived cluster. No linear streaks between the periportal and the pericentral region were observed, making it unlikely that streaming of daughter cells had occurred. Similar results were obtained in chimeric livers that were created by morula aggregation between histochemically distinguishable strains of rats, in which adult livers had large clusters of donor cells whose location was unrelated to the liver architecture (2). Thus, analysis of livers with mosaic expression of a marker gene provides further evidence that

the hepatocyte is responsible for postnatal replication and streaming does not occur.

One facet of the streaming liver theory is that the life span of a hepatocyte is limited, with apoptosis reported to occur at approximately 1 year after a hepatocyte is generated in the periportal region (28, 33). Thus, genetic marking data demonstrating that hepatocytes can survive for up to 2 years in rodents provide further evidence against the streaming liver theory. Transgenic hepatocytes expressing the serum protein hAAT and β -gal genes were transplanted into congenic mice by intrasplenic injection (5). These hepatocytes localized to the recipient liver, as demonstrated by X-gal staining. Serum hAAT levels in recipient mice were stable for as long as 2 years after transplantation, demonstrating that the mouse hepatocytes were long-lived (K. Ponder and S. L. C. Woo, unpublished data). Similarly, stable expression of hAAT was observed for up to 2 years from a retroviral vector delivered to rat hepatocytes *in vivo* (38, 39). Thus, these experiments demonstrate that the hepatocyte does not undergo programmed cell death after 1 year, as was hypothesized by the streaming liver theory.

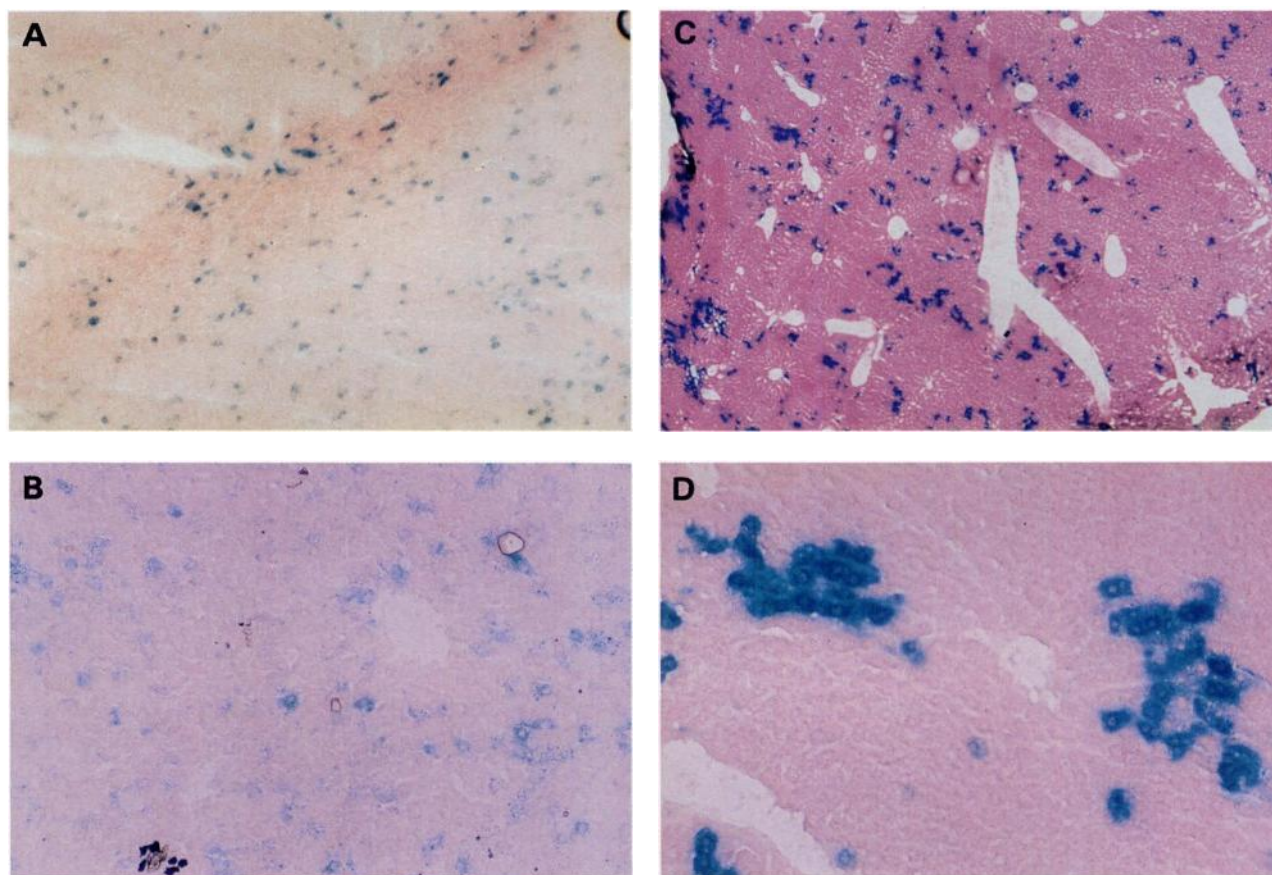


Figure 3. Evidence against the streaming liver model. Analysis of transgenic mice demonstrates that individual hepatocytes replicate to form a clonally derived cluster during postnatal growth. Transgenic mice were generated in which the liver-specific α_1 -antitrypsin promoter was upstream of the β -gal gene. The livers were mosaic for expression of the transgene, even in later generation mice that contained the transgene in all cells. X-gal staining performed 1 day after birth demonstrated single blue cells and doublets that were scattered randomly throughout the liver, as shown in panels A (40 \times) and B (200 \times). The blue cells were present in much larger clusters 9 months after birth, as shown in panels C (40 \times) and D (200 \times). This suggests that individual hepatocytes replicated to form a clonally derived cluster.

In summary, three pieces of data demonstrate that the parenchymal hepatocyte is responsible for postnatal replication in the normal liver and that hepatocytes do not migrate: 1) retroviral marking of hepatocytes *in vivo* demonstrates that labeled cells do not move, and remain primarily as small clusters of cells for up to 15 months after transduction; 2) developmental analysis of expression patterns in mosaic livers demonstrates that individual hepatocytes replicate to form a large cluster of hepatocytes during postnatal liver growth; and 3) the life span of a differentiated hepatocyte is at least 2 years in rodents.

THE HEPATOCYTE CAN PROLIFERATE EXTENSIVELY

These experiments demonstrate that the hepatocyte is responsible for most, if not all, postnatal replication in normal animals. The number of replication events that occur after birth in the liver is limited, however. Because differentiated cells have a limited proliferative potential in many other organs (12), some investigators believe that a stem cell must become involved when extensive replication occurs (reviewed in refs 9–14), as shown in Fig. 4A–C. Indeed, cultured hepatocytes cannot replicate more than a few times *in vitro* (11). Furthermore, chronic liver regeneration results in the appearance of oval cells, which are the putative multipotent stem cells of the liver (8, 16–19). An alternative model is that the hepatocyte itself is capable of many rounds of replication if a sufficient stimulus is applied. This model is shown in Fig. 4D–F. These models are not mutually exclusive, making it possible that both stem cells and differentiated hepatocytes play a role in extensive hepatocyte regeneration.

Determination of the replicative potential of postnatal hepatocytes requires a method for marking cells as well as a technique for applying a continuous stimulus for replication. As noted, transgenic hepatocytes that express β -gal can be transplanted into a congenic recipient liver to create genetically marked cells present on a background of normal cells (5). A system for applying a continuous stimulus for hepatocyte replication was fortuitously discovered in a transgenic mouse line expressing the urokinase plasminogen activator cDNA from the albumin promoter (Alb-uPA). The expression of uPA was toxic for hepatocytes, which resulted in continuous regeneration of the liver after birth (40). Rhim et al. (40) transplanted otherwise normal transgenic mouse hepatocytes that expressed β -gal into congenic Alb-uPA recipient mouse livers. The transplanted hepatocytes replicated extensively because of their selective advantage over the uPA-expressing hepatocytes. By quantitating the size of the blue nodules that developed in the recipient mice, they calculated that the β -gal expressing hepatocytes underwent up to 12 doublings (40). The major caveat to this study is the fact that less than 7% of the injected cells gave rise to nodules upon transplantation. This raises the possibility that rare stem cells present in their preparation of

hepatocytes, and not parenchymal cells, were what proliferated extensively. Alternatively, the low seeding efficiency may have been due to the fact that not all cells injected into the spleen reached the liver and survived. Similar results were obtained after transplantation of rat hepatocytes into immunosuppressed Alb-uPA mice. This resulted in the replacement of most of the liver with rat hepatocytes, although the bile ductule cells remained of mouse origin (41). These results provide strong but not conclusive evidence that the hepatocyte itself can proliferate extensively. They do not exclude the possibility that oval cells also play a role in some models of extensive liver regeneration.

THE CELLULAR ORIGIN OF HEPATOCELLULAR CARCINOMA

The cellular origin of hepatocellular carcinoma (HCC) has been controversial (9, 12). Some investigators believe that oval cells undergo developmental arrest and develop into HCC (11, 42), as diagrammed in Fig. 5A–D. Evidence for this hypothesis includes the fact that some carcinogenic protocols induce oval cell proliferation without apparent proliferation of hepatocytes. In addition, oval cell proliferation is observed in the intermediate stages of development of cancers in some transgenic mice that express the SV40 T antigen (43, 44). An alternative theory states that the hepatocyte can develop into HCC if a sufficient number of mutations accumulate (45–47), as dia-

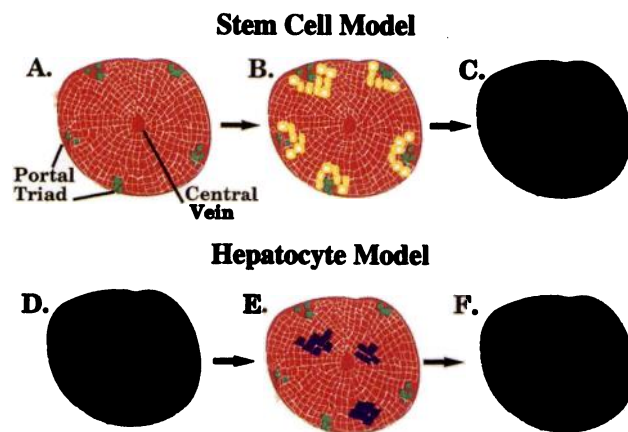


Figure 4. Models for extensive liver regeneration. A–C) Stem cell model. In this model, extensive liver regeneration requires stem cells. A normal liver is shown in panel A. A stimulus for extensive liver regeneration results in the proliferation of oval cells in the periportal region, as shown by the yellow oval-shaped cells in panel B. These cells differentiate into new hepatocytes, which are indicated as blue cells in panel C. D–F) Hepatocyte model. In this model, the hepatocyte itself can replicate multiple times during extensive liver regeneration. The position of an individual hepatocyte is designated by its blue color in panel D at the initial time point, before a need for extensive liver regeneration. The appropriate stimulus results in multiple rounds of replication, resulting in a large cluster that derived from that single cell, as indicated by the blue cells in panel E. Further stimulus for replication results in more rounds of replication, resulting in a larger blue cluster, shown in panel F.

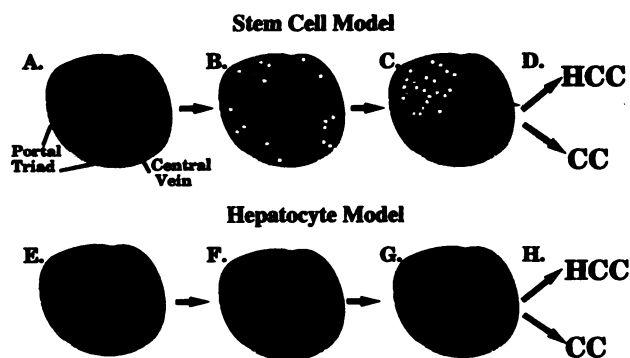


Figure 5. Models for liver carcinogenesis. *A–D*) Stem cell model. In this model, oval cells are the cell of origin for HCC and CC. *A*) Normal liver; *B*) a liver early after the administration of a carcinogen. Oval cells have replicated in the periportal region of the liver, as shown in yellow. Panel *C* demonstrates a later time point when most yellow cells are no longer visible because they underwent apoptosis or differentiated into hepatocytes. Rare oval cells acquire mutations that arrest their development and prevent them from differentiating into hepatocytes. These altered oval cells continue to replicate to form a larger cluster, shown in yellow. Panel *D* demonstrates that these mutated oval cells can result in cancers with some characteristics of the lineages that normally develop from that cell. Thus, either HCC or CC can occur. *E–H*) Hepatocyte model. In this model, the hepatocyte is the cell of origin for HCC and CC. Several hepatocytes are indicated in blue in a normal liver in panel *E*. Administration of a carcinogen results in the replication of these cells, as shown in panel *F*. These foci are usually identified because of alterations in the expression of one or more enzymatic functions. Panel *G* demonstrates that most of these enzyme-altered hepatic foci revert to normal at later times. Some enzyme-altered foci continue to replicate, which results in a larger cluster of blue cells. Panel *H* demonstrates that these cells can result in a cancer with characteristics of hepatocytes (HCC). Alternatively, the modified cell can retrodifferentiate into a more immature cell and acquire characteristics of cells of the bile ductule lineage (CC).

grammed in Fig. 5E–H. This theory is supported by the fact that the administration of some carcinogenic agents results in the appearance of foci of replicating hepatocytes with altered gene expression before the development of cancer. It has been difficult to prove either of these hypotheses because the cells that participate in cancer formation are extremely rare, and carcinogenic protocols probably induce replication of both oval cells and hepatocytes, although to a varying extent. This makes it difficult to know if the cancer originated from the most prevalent cell type for a particular model of carcinogenesis. Indeed, the majority of the proliferating oval cells will differentiate into hepatocytes or undergo apoptosis. Similarly, the majority of the enzyme-altered hepatic foci revert to phenotypically normal hepatocytes or die by apoptosis.

Differentiation between these theories of liver carcinogenesis requires a method to mark cells of known lineage and determine whether or not they can develop into HCC under the appropriate conditions. Cells of different lineages can be transduced with an oncogene in vitro and analyzed in animals for their ability to develop into cancers of a particular phenotype. Studies of this sort have demonstrated that cultured cells with characteristics of oval cells can develop into cancers in animals if they are

modified to express an oncogene (48–53). Although the phenotype of the cancers has varied dramatically depending on the particular cell line and the site of implantation, several of these tumors were typical HCC, suggesting that the oval cell can be the cell of origin for HCC. Cholangiocarcinomas (CC) were observed in some studies, which suggests that the oval cell can also develop along the bile ductule lineage (51, 52). Similar in vitro marking/transplantation studies have demonstrated that hepatocytes can develop into HCC. Implantation of SV40-immortalized hepatocytes containing no (54) or one additional (55–57) oncogene into recipient rodents resulted in the appearance of cancers. Some tumors clearly resembled HCC, suggesting that the hepatocyte itself can be the cell of origin for HCC (54, 56). Other cancers were morphologically undifferentiated (55, 57). In these studies, no CC were observed.

The above studies suggested that either hepatocytes or oval cells could develop into HCC, whereas oval cells could develop into CC. However, the common expression of some genes in both cell types (8) and the fact that culture conditions can alter the expression of liver-specific genes (11) could complicate the definitive identification of a particular cell type. In addition, the marked heterogeneity in the phenotype of the cancers that developed from different clones suggests that not all "oval cells" or "hepatocytes" were identical. A method for transferring an oncogene into a known cell type in vivo eliminates the problems associated with in vitro culture.

We recently developed a retroviral vector that expressed an activated *K-Ras* oncogene in addition to the β -gal gene (*Ras-gal*), as shown in Fig. 6A. This was delivered to rat hepatocytes in vivo using a retroviral transduction protocol that appears to be specific for hepatocytes (7). Some rats were also treated with a low dose of the carcinogen diethylnitrosamine (DEN) to induce the additional mutations that are needed for multistep carcinogenesis. In this study, rare cells that replicated as often as every 3–5 days were observed in all zones of the liver at 25 days after transduction with *Ras-gal* and DEN treatment, as shown in Fig. 6B, C. These large clusters represented cells that replicated rapidly due to the expression of activated *Ras* and additional mutations induced by DEN or insertional mutagenesis from the retroviral vector. These cells were hepatocytes because of their morphological appearance, their location within the liver acinus, and their expression of several liver enzymes such as lactate dehydrogenase and glucokinase (R. V. Pearline, Y.-Z. Lin, K. J. Shen, E. M. Brunt, W. Bowling, et al., unpublished results). Some rats treated with *Ras-gal* and DEN developed cancers at 3–5 months after transduction. The phenotype of these cancers was primarily HCC, although one mixed cancer and one CC was seen (7). It is likely that a retroviral-transduced hepatocyte was the cell of origin for the HCC and CC. This study cannot rule out the possibility that the cell that developed into a cancer was a rare stem cell that was transduced with the retroviral vector in vivo, however. No

large clusters of blue cells or cancers were observed in animals treated with *Ras-gal* alone or in DEN-treated animals transduced with a control retroviral vector containing β -gal without an oncogene.

In summary, in vitro transfer of an oncogene into cultured oval cells resulted in HCC and CC after implantation into animals. Although similar in vitro transfer of an oncogene into cultured hepatocytes only resulted in HCC in animals, transfer of an oncogene into hepatocytes in vivo resulted in both HCC and CC. The finding that both oval cells and hepatocytes can be the cell of origin for HCC is not surprising, as they both express hepatic genes such as albumin (8). The finding that hepatocytes can be the cell of origin of CC is consistent with the model of retrodifferentiation proposed by Uriel (47). In this model, a cell can revert to a more primitive cell in response to an oncogene (or oncogenes). This more primitive cell can then differentiate along a parallel pathway, resulting in a cancer that resembles that lineage.

IMPLICATIONS OF THESE RESULTS FOR LIVER DISEASE

These results have important implications for the treatment of genetic disorders and liver insufficiency states. Hepatic gene therapy is currently being used to treat

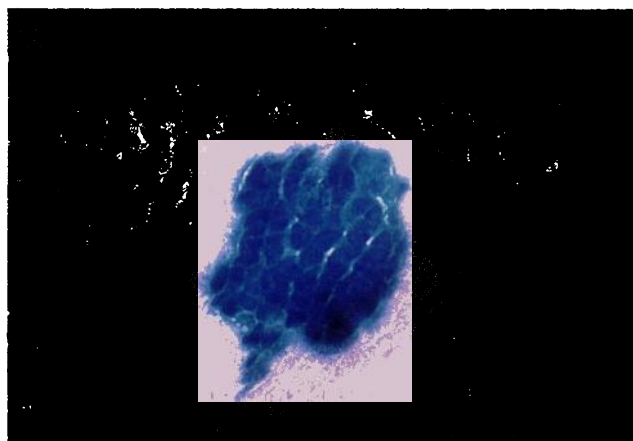


Figure 6. Evidence that the hepatocyte can be the cell of origin for HCC and CC. **A)** Retroviral vector *Ras-gal*. This construct is identical to Gal-509 (Fig. 2A), except that a *K-Ras* gene with a point mutation at codon 12 that results in a constitutively active protein has been inserted downstream of the transthyretin promoter and upstream of the IRES- β -gal cassette. **B, C)** X-gal staining of a *Ras-gal* transduced DEN-treated rat liver. *Ras-gal* was used to transduce rat liver cells in vivo, as described in the legend to Fig. 2. Animals were also treated with a low dose of DEN (30 mg/kg) to induce the additional mutations necessary for multistep carcinogenesis. X-gal staining of a section of liver obtained 25 days after transduction identifies retroviral-transduced cells, as shown in panel **B** (40 \times). Because the *K-Ras* gene is coexpressed with β -gal, blue cells also express the activated *Ras* gene. The average blue cluster size was larger than what was observed in cells that were transduced with a retroviral vector without the *Ras* gene (see Fig. 2), demonstrating that most *Ras-gal*-transduced cells had replicated a few times. Occasional blue clusters were quite large, as shown by the arrow in panel **B** and at higher magnification in panel **C** (200 \times). These large clusters are retroviral-transduced hepatocytes with markedly abnormal growth characteristics due to *K-Ras* and additional mutations.

monogenic deficiencies in both animals and humans (58). The long-term success of gene therapy requires that genetically modified cells be present several years after the gene transfer procedure is performed. As reviewed here, genetic marking studies demonstrate that hepatocytes are the source of new hepatocytes in the normal postnatal liver in rodents. If this is the case in larger animals, gene transfer into hepatocytes of infants should result in permanent correction of a genetic defect. The studies reviewed here have also demonstrated that the hepatocyte can replicate extensively if a sufficient stimulus for replication is applied. This implies that hepatocytes might be used to restore liver function in liver insufficiency states such as cirrhosis if their replication could be appropriately manipulated.

REMAINING QUESTIONS IN LIVER BIOLOGY THAT MIGHT BE ADDRESSED BY GENETIC MARKING

Although genetic marking studies have resolved some controversial issues in liver biology, others remain unanswered. For example, there is no definitive proof that a multipotent stem cell exists that can produce both hepatocytes and bile ductule cells, although some data support its existence. Demonstration that adjacent bile



ductule cells and hepatocytes can express a genetic marker that was delivered to only a small fraction of cells should constitute proof that a bipotential stem cell exists. Unfortunately, such a study has not yet been performed successfully. Although a β -gal-expressing oval cell line acquired characteristics of a hepatocyte upon transplantation into a normal rat liver (59), these cells did not develop into bile ductule cells in vivo. Future studies in which genetically marked oval cells are introduced into livers under conditions that favor their differentiation into bile ductule cells might help to resolve this issue. [F]

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