Liver disease and compensatory growth: unexpected lessons from genetically altered mice

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ABSTRACT   Over the last decade, several animal models have been established that permit exploration of liver biology and disease. Although these models have been developed using diverse strategies, including transgene targeting, homozygous gene disruption and administration of hepatotoxic chemicals, each approach creates an animal with hepatocyte damage, resulting in an hepatic microenvironment that supports proliferation of healthy hepatocytes. These models have been used to demonstrate: (1) the remarkable ability of adult hepatocytes to clonally proliferate in response to liver growth signals, (2) the effectiveness of transplanted donor hepatocytes in repopulating damaged liver parenchyma, and (3) the feasibility of reconstituting liver with xenogeneic hepatocytes. This paper reviews the development and use of these models, and outlines their potential future application to the study of hepatic stem cells, therapy of liver disease and hepatic toxicology.

KEY WORDS: *hepatic stem cells, hepatocyte transplantation, liver disease, liver regeneration, transgenic mice*

Introduction

Often enough as scientists we discover things by accident. This review describes our entry into the field of liver growth regulation and hepatocyte transplantation almost 10 years ago. By pursuing an unexpected finding in transgenic mice, the result of a "failed" experiment, we identified a remarkable experimental system that vividly highlighted the striking regenerative capability of liver cells. Subsequent studies employing this model system, and more recently developed models with conceptually similar characteristics, have contributed substantially to our understanding of the biology of liver growth, and in the future they may be critical tools for identifying hepatic stem cells, the therapeutic potential of hepatocyte transplantation, the nature of interactions between different cell populations in liver, and, ultimately, the biological and toxicological features of mouse chimeric livers that are composed of human hepatocytes. The history of our work with this system, initiated in the laboratory of Ralph Brinster, together with a summary of more recent studies employing related systems, is reviewed below.

Urokinase and metastasis

The most lethal characteristic of many neoplasms is their ability to spread. Often, elevated expression of proteases, and in particular urokinase, accompanies the metastatic phenotype. Urokinase is a protease, one of two physiological plasminogen activators, that cleaves plasminogen to plasmin. Activated plasmin is also a proteolytic enzyme with broad substrate specificity that is best known for its role in degrading fibrin clots, but it has been hypothesized that plasmin production within neoplasms can serve a pathological function of enhancing tumor cell invasiveness and metastasis.

To examine this hypothesis, transgenic mice were generated carrying a transgene linking the albumin enhancer/promoter with the mouse urokinase coding sequence (AL-uPA; Fig. 1). The human growth hormone (hGH) 3' UTR with polyadenylation sequence was added to enhance message stability. AL-uPA should target uPA expression to hepatocytes, in which the endogenous

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uPA gene is not typically active. These mice, established in Ralph Brinster's laboratory in collaboration with Drs. Jay Degen and Richard Palmiter, were to be mated with mice expressing oncogenic transgenes in hepatocytes. Experimental analysis would compare metastasis between two groups of resulting offspring: those carrying the oncogenic transgene alone and those carrying both the oncogenic transgene and AL-uPA. An association between uPA expression and metastasis should have been manifest as a higher incidence of metastasis in bitransgenic mice. We recognized that this approach would be problematic if liver-targeted uPA expression caused a phenotype, but we believed it to be a necessary first step in assessing whether uPA could modulate cancer metastasis. Although the original objectives were never attained, the actual outcome turned out to be much more interesting than expected.

Neonatal bleeding in AL-uPA transgenic mice

One potential outcome of uPA overproduction in hepatocytes could be altered hemostasis. As noted above, uPA proteolytically activates plasminogen to plasmin. Plasmin, in turn, may also mediate direct fibrinogenolysis. In fact, founder AL-uPA transgenic mice did exhibit aberrant hemostasis. Many founder animals displayed spontaneous intestinal and intra-abdominal bleeding, and died within 4 days of birth. However, two lineages were established from surviving founder mice and these animals exhibited a unique phenotype. Mice in each lineage transmitted an incompletely penetrant bleeding phenotype through multiple generations: while approximately one-half of the transgenic offspring from these lines bled and died within 4 days of birth, the surviving transgenic siblings showed a gradual abatement of circulating uPA level and a restoration of clotting function between 3 and 6 weeks of age (Heckel *et al.*, 1990). This reduction in uPA level was surprising, since albumin was expected to continuously promote expression of the transgene as long as hepatocytes remained functional.

The cause of variability in the bleeding phenotype in these mice remains unclear. The incomplete penetrance of the bleeding phenotype in neonates did not appear to be explained by quantitative differences in transgene expression or plasma plasminogen activator activity. Northern blots and zymography assays comparing the levels of transgene-derived uPA mRNA and protein activity between bleeding and non-bleeding neonates showed no significant differences. Furthermore, despite the fact that clotting function was lost in these transgenic mice until around 21 days of age, the animals only appeared to be susceptible to hemorrhage during the first 4 days of life. Together, these data suggest that, while overexpression of uPA may cause the bleeding phenotype, the increased uPA levels are not sufficient to induce hemorrhage. It appears that an epigenetic effect may be involved. One hypothesis is that microvascular trauma suffered during parturition may initiate the bleeding in some pups. Since all of the transgenic neonates are severely hypofibrinogenemic, those with significant internal hemorrhage initiated during birth and not stopped by platelet plugs subsequently die.

uPA-induced hepatic lesions

Surviving transgenic AL-uPA mice displayed additional lesions that were in some ways more striking than altered hemostasis. The
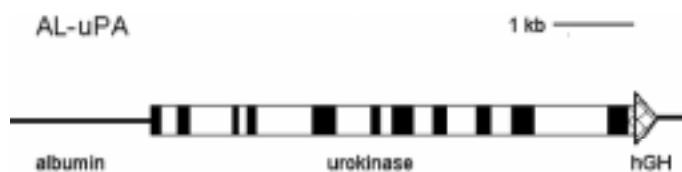

Fig. 1. AL-uPA transgene construct. *See text for details.*

age-dependent decrease in uPA level was accompanied by unexpected morphological changes in the transgenic mouse liver. Young transgenic mouse liver was pale tan to white compared to non-transgenic littermate liver, and electron microscopy revealed the presence of vacuolations within hepatocyte rough endoplasmic reticulum (RER) (Sandgren *et al.*, 1991). The targeting of uPA expression to hepatocytes creates a situation in which both plasminogen and its activator are present within the same cellular compartment; thus, plasminogen activation within the RER may produce proteolytic damage within this organelle and ultimately cause hepatotoxicity. At around 2 weeks of age, red foci began to appear within the white parenchyma of AL-uPA transgenic mice (Fig. 2); these foci gradually expanded until the white areas were replaced by confluent red nodules at 6 to 10 weeks of age (Sandgren *et al.*, 1991). In contrast to white parenchyma, the red parenchyma lacked transgene expression. The overgrowth of liver by transgene-deficient red parenchyma explained the gradual decrease of uPA levels and recovery of clotting function as these mice aged. Similar gross and molecular phenotypes were initially seen in two lineages with this transgene, and more recently in four additional lineages, so the phenomenon is not an artifact of transgene integration site. Microscopically, there appear to be two cellular populations present in the liver of transgenic mice that have expanding red foci. In the areas of red parenchyma, the cells appear mildly pleomorphic, but otherwise resemble normal hepatocytes. These cells also display occasional mitotic figures. In contrast, the grossly white areas contain small hepatocytes with prominent cytoplasmic vacuolation. At interfaces between red and white parenchyma, it appears that red tissue is compressing the white tissue, suggesting expansion of the red foci.

We next determined that red parenchyma lacked transgene expression due to a physical loss of the integrated transgene DNA within individual hepatocytes; this was calculated to be a stochastic event occurring with a frequency of 1×10^{-4} or less per cell division (Sandgren *et al.*, 1991). Southern blot analysis was used to identify transgene status in red nodules versus white liver. Transgene deletion was shown to involve (a) intrachromosomal recombination (Fig. 2) within the transgene array, which deleted the internal, intact transgene copies and resulted in a shortened tandem transgene array, or (b) in some cases loss of the entire transgene array. Furthermore, individual red nodules displayed only one type of deletion, suggesting that each nodule represented a clone of cells derived from a single transgene-deficient progenitor.

Thus, following transgene loss, transgene-deficient hepatocytes, liberated from the toxic effects of uPA expression, proliferated at the expense of surrounding transgene-expressing tissue, eventually leading to repopulation of the entire liver by non-expressing hepatocytes. [³H]-Thymidine labeling in 1-month-old transgenic mice showed that hepatocytes in red parenchyma

had a selective growth advantage, as they were labeled twice as frequently as cells in white parenchyma. Typically, the liver of AL-uPA mice was replaced by tens to hundreds of foci that expanded until they became confluent. Occasionally, one regenerative nodule replaced up to 95% of the liver, indicating that liver cells have tremendous replicative potential. The stimuli that control the growth of the red nodules are unknown. Presumably, replication is initiated in response to a functional hepatic deficit caused by uPA hepatotoxicity. The replication process continues even when 90 to 95% of the liver has been repopulated by transgene-deficient parenchyma (Sandgren *et al.*, 1992), but then stops when the liver is completely repopulated and at the appropriate weight, indicating that the cells are exquisitely sensitive to negative growth-regulatory signals. Several observations suggest that clonal regeneration in this model is limited to the hepatocyte lineage, and not due to transgene loss in a multipotent stem cell precursor. First, even cells in the smallest microscopically visible red foci (~30 cells) appeared morphologically identical to non-transgenic hepatocytes when examined using both light and electron microscopy. Second, the red nodules lacked bile ducts until after hepatic repopulation was complete, consistent with the conclusion that the nodules represented expanding hepatocytic structures. Finally, transgene loss in a non-hepatocytic cell should not influence selective cellular amplification, since albumin (and transgene) expression is limited to hepatocytes in neonatal and adult mice. Once repopulation is complete, the mouse liver is a genetic chimera, composed of two distinct cell populations: (a) hepatocytes, which have deleted the transgene DNA, and (b) non-hepatocytic cells, which should possess an intact transgene array.

In considering additional models in which transgene deletion (or gain of function in mutant mice) can change organ morphology and function, it is useful to think about the special requirements that this model fulfills. First, loss of transgene expression imparted a selective growth advantage to those cells that had deleted the transgene. Second, the tissue permitted amplification of these cells. The liver is unusual in this context, in that fully differentiated hepatocytes are able to re-enter the cell cycle in response to certain mitotic stimuli.

Hepatocyte transplantation in AL-uPA mice

The ability of endogenous transgene-deficient hepatocytes to clonally expand and repopulate the AL-uPA mouse liver indicated that it was likely that exogenous liver cells introduced into this environment should have the same proliferative advantage. We confirmed this hypothesis (Rhim *et al.*, 1994) by demonstrating that the AL-uPA liver can be up to 80% reconstituted with donor hepatocytes from a different mouse (Fig. 3). Donor cells were derived from a transgenic mouse carrying a marker transgene, typically the MT-lacZ transgene. When incubated with the synthetic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal), hepatocytes from these mice turned blue. Donor mice underwent a two-step EDTA-collagenase perfusion to isolate liver cells. The population was then enriched for hepatocytes by two slow speed centrifugations. Hepatocytes were injected into the spleens of transgenic AL-uPA mice within the first 2 weeks of life (to minimize competition with endogenous regenerative nodules). These mice were examined 4 to 6 weeks later for evidence of donor
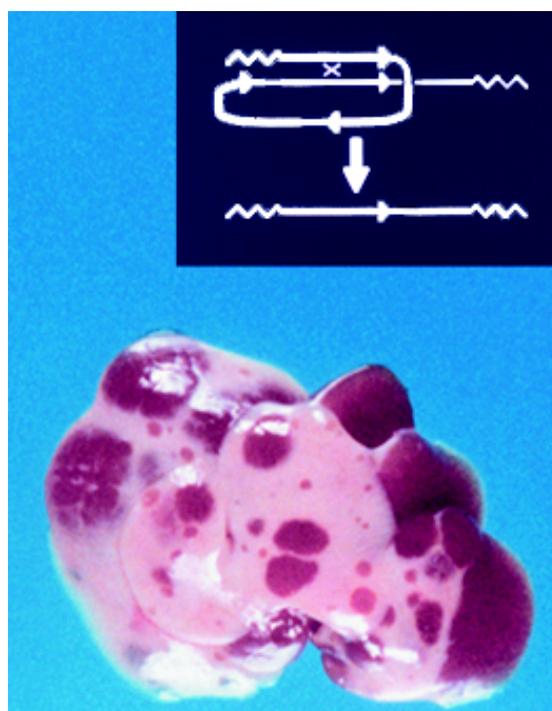

Fig. 2. Gross appearance of AL-uPA transgenic mouse liver; representative transgene rearrangement. *The gross appearance of young transgenic AL-uPA mouse liver reflects the genetic chimerism of the hepatic parenchyma, with (a) pale tan tissue consisting of hepatocytes that contain the transgene array, and (b) red nodules clonally derived from hepatocytes that have undergone transgene rearrangement and therefore no longer express the hepatotoxic uPA gene product. The schema in the upper right illustrates one type of intrachromosomal transgene re-arrangement that can abrogate transgene expression within a hepatocyte. Transgenes typically integrate into chromosomal DNA as head-to-tail arrays. In this illustration, wavy lines represent mouse chromosomal DNA, and the transgene DNA consists of an albumin promoter (thin line), uPA coding region (thick line) and 3' hGH (arrow-head). Following homologous recombination between transgenes within this array, the integrated transgene DNA that remains is incomplete and can no longer be expressed; as drawn, the transgene fragment on the left lacks a promoter region, while the transgene fragment on the right lacks the 3' hGH sequence.*

hepatocytes in liver. Transgenic recipients had between 5 and 80% of their livers replaced by donor hepatocytes. Growth of the transplanted hepatocytes was nodular, and nodules reached a very uniform size (approximately 1 mm in diameter), although sometimes nodules would become confluent. The nodular appearance of the donor-derived cells suggested that they, like the endogenous transgene-deficient nodules, grew by clonal expansion; it was calculated that donor liver cells could undergo at least 12 cell doublings in the recipient liver.

These studies have been expanded to demonstrate that livers of immunodeficient AL-uPA athymic nude mice can be reconstituted with xenogeneic hepatocytes. Rat liver cells were isolated by two step EDTA-collagenase perfusion, enriched for hepatocytes, and injected into the spleens of 10- to 15-day-old nude AL-uPA transgenic mice. Animals were sacrificed between 6 and 14
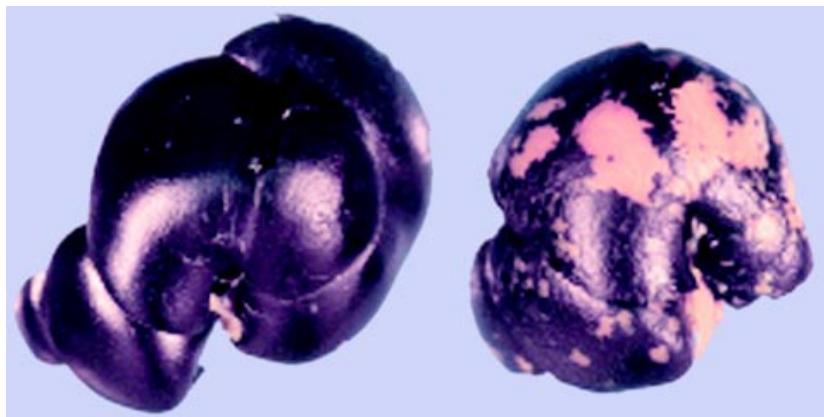
Fig. 3. Gross appearance of AL-uPA liver repopulated with exogenous mouse hepatocytes. (Left) *MT-lacZ donor transgenic mouse (positive stain control; hepatocytes that express the lacZ transgene stain blue when exposed to the X-gal substrate).* **(Right)** *AL-uPA transgenic mouse transplanted with MT-lacZ hepatocytes. The unstained areas in this liver consist of endogenous regenerative nodules derived from cells that have deleted the uPA transgene.*

weeks of age and shown to have up to 100% reconstitution by rat hepatocytes in AL-uPA homozygous recipients (Rhim *et al.*, 1995). Immunohistochemistry was used to morphologically demonstrate rat cell repopulation, while gene expression studies showed that up to 100% of hepatocellular gene expression was of rat origin. The animals were clinically healthy and had a normal ratio of liver to body weight, indicating that the rat cells were responsive to mouse growth control signals. While the hepatocytes were rat-derived, the biliary epithelial cells and other accessory cells appeared to be of mouse origin, yielding a bi-species chimeric liver.

AL-uPA mice and hepatocyte liver biology

The AL-uPA transgenic mouse model provided several important insights into growth and its regulation in liver. First, the clonal growth potential of individual hepatocytes is remarkable: during clonal nodular replacement of diseased liver in this model, each transgene-deficient progenitor cell can undergo at least twelve cell doublings, equivalent to the expected average cell doubling following eight sequential two-thirds partial hepatectomies (assuming one and one-half cell doublings after each surgery to restore full liver mass). In these mice, this is accomplished as a continuous process extending over a period of only several weeks. Second, the selective expansion of healthy hepatocytes continues until there is total replacement of diseased parenchyma. Once subsequent remodeling (i.e., extension of bile ducts and associated structures into new parenchyma) is complete, the liver appears almost entirely normal. This finding underscores the liver's exquisite responsiveness to growth stimulatory and growth inhibitory influences, and its ability to recreate appropriate microscopic architecture following recovery from injury. Third, the cell transfer studies illustrate the practicality and ease of hepatocyte transplantation as a method to replace diseased hepatic parenchyma. Prior to this result, there appeared to be two hurdles to therapeutic use of hepatocyte transplantation: acquisition of a medically effective donor cell population, and presence of an appropriate stromal network within which donor cells could survive and function. Following this result, it appears that the former requirement will not be problematic, since routinely isolated hepatocytes can reconstitute host liver when stroma is present. These observations in AL-uPA transgenic mice foreshadowed the characteristics of additional animal models of liver disease that are described below.

Recent models

AL-uPA transgenic mice remain a technically challenging model with which to work. In particular, the loss of one-half of all transgenic progeny due to neonatal hemorrhage makes it difficult to generate a large number of experimental subjects. Also, transplants must be done into very young mice to minimize competition between donor cells and endogenous regenerative nodules. Furthermore, although homozygous AL-uPA mice are outstanding recipients for achieving high levels of repopulation in transplant studies (since each endogenous cell must delete two copies of the transgene to escape the toxic effects of uPA, so the number of endogenous regenerating nodules is minimized), these mice are difficult to generate and tend to develop severe, widespread edema between 4 and 6 weeks of age. More recently, additional animal models have been described in which liver function is compromised and the damaged hepatic microenvironment is able to support clonal outgrowth of healthy hepatocytes. These models, together with the uPA model described above, are being used extensively to study liver biology.

Fah$^{-/-}$ mice

Hereditary tyrosinemia type I (HT1) is a human disease caused by a recessive inborn error of metabolism that blocks the tyrosine catabolism pathway, leading to a build-up of toxic by-products that result in liver and kidney damage. Specifically, this disease results from a deficiency of fumarylacetoacetate hydrolase (FAH), which is the final enzyme in the five-step pathway that catabolizes tyrosine. Patients with this metabolic disorder suffer from progressive hepatic failure during infancy; they also are susceptible to development of hepatocellular carcinoma (HCC) during early childhood.

In an attempt to produce an animal model of HT1, Grompe and colleagues created mice that were homozygous for *Fah* gene disruption. The *Fah* null mice exhibited a neonatal lethal phenotype caused by hepatic failure (Grompe *et al.*, 1993). However, treatment of the affected mice with 2-(2-nitro-4-trifluoro-methylbenzyol)-1,3-cyclohexanedione (NTBC) rescued mice from the neonatal lethality by disabling the tyrosine catabolism pathway at its second step (Grompe *et al.*, 1995), thereby blocking the accumulation of hepatotoxic intermediates in the mutant mice. Treatment of *Fah$^{-/-}$* mice with NTBC normalized liver function and partially corrected aberrant hepatic RNA expression patterns. When adult *Fah$^{-/-}$* mice

were removed from NTBC treatment, the animals suffered rapidly advancing hepatic failure ultimately leading to death; this phenotype resembled human HT1. Also as for HT1, $Fah^{-/-}$ animals developed early-onset hepatocellular carcinoma, even when continuously treated with NTBC.

In addition to providing a murine model of HT1, the $Fah^{-/-}$ mice proved beneficial for studying gene therapy and liver cell regeneration. The first evidence that this model might be useful for these studies came from observations made in human HT1 patients. HT1 patients frequently contained discrete nodules of normal appearing hepatic parenchyma in which Fah activity was present. These nodules arose following a somatic reversion event in which one allele of the Fah gene was restored to wild-type in an individual cell (Kvittingen *et al.*, 1993,1994). The revertant Fah^+ cell, freed from the build-up of hepatotoxic metabolites, clonally proliferated at the expense of the surrounding $Fah^{-/-}$ parenchyma to form discrete nodules, reminiscent of the response of transgene-deficient cells in AL-uPA mice.

These results suggested that Fah^+ cells would also be positively selected to proliferate in the $Fah^{-/-}$ mouse liver. To test this concept, Grompe and colleagues demonstrated that transplantation of as few as 1000 congenic wild-type donor hepatocytes was able to repopulate the mutant liver following removal from NTBC (Overturf *et al.*, 1996). Not only did the donor hepatocytes proliferate in this environment, but they also were able to become appropriately organized and to rescue hepatic function. While removal of $Fah^{-/-}$ mice from NTBC uniformly led to death within 2 months in non transplanted mice, mice that received 10^4 or more donor cells always survived, as did 5 out of 6 mice that received 10^3 donor cells.

To test whether a gene therapy approach could rescue these mice, $Fah^{-/-}$ mice were infused via the portal vein with retroviruses expressing Fah. Mutant animals that had been removed from NTBC and treated with multiple injections of retroviruses contained greater than 90% Fah^+ cells two months post-treatment (Overturf *et al.*, 1996). These animals had normal liver function and survived following removal from NTBC. These results are particularly exciting for the field of hepatic gene therapy because they demonstrate that "*in vivo* selection" can be used to expand a small population of permanently corrected hepatocytes into a therapeutically significant fraction of hepatic parenchyma, as occurs spontaneously in the AL-uPA model (Wilson, 1996). This overcomes the traditional problem of the limiting number of hepatocytes that can be harvested and infected with a retroviral construct. While this *in vivo* gene therapy selection strategy is exciting, its use is limited to situations in which: (a) the population of corrected cells is capable of replicating and has a growth advantage over diseased cells, and (b) the effects of the disease are cell-autonomous so that the corrected cells are not inhibited by adjacent, diseased cells.

As a means to study hepatocyte biology, the Fah model has several advantages over the uPA model. Due to the nature of the null mutation, spontaneous revertants have not been observed in the $Fah^{-/-}$ mice; therefore, donor hepatocytes do not have to compete to repopulate the liver with endogenous nodules of hepatocytes that have reverted to normal. In addition, treatment with NTBC allows both the timing and the severity of the hepatic failure to be adjusted in these mice. This is in contrast to the uPA mice, in which hepatic disease occurs within the first month of life, making surgical manipulation of the animals technically challenging.
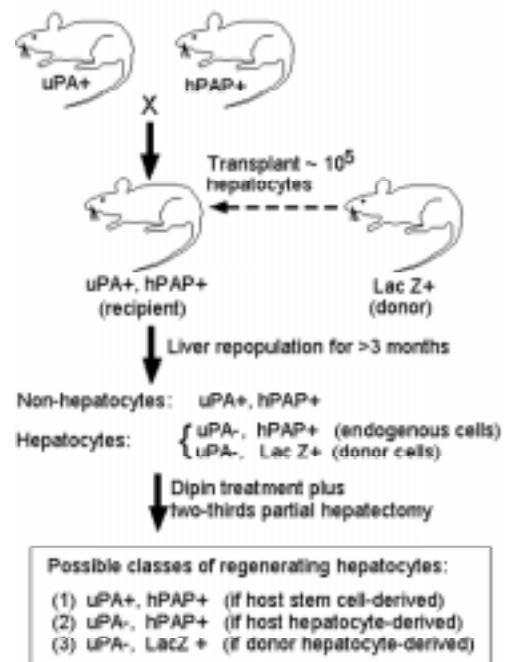

Fig. 4. Investigating the existence and growth potential of stem cells in adult mouse liver. *See text for details of experimental design.*

Grompe and colleagues have taken advantage of these characteristics of $Fah^{-/-}$ mice to provide a remarkable demonstration of the replicative potential of hepatocytes. Hepatocytes not only have the ability to re-enter the cell cycle in response to mitotic stimuli, but they also have the ability to divide numerous times in response to repeated partial hepatectomies within a single animal (Stocker *et al.*, 1973). Studies in AL-uPA (Rhim *et al.*, 1994) and $Fah^{-/-}$ (Overturf *et al.*, 1996) mice have demonstrated that adult hepatocytes are able to undergo 12-18 cell doublings when transplanted into a diseased liver microenvironment that supports clonal expansion of donor hepatocytes. More recently, Overturf *et al.* (1997) used a serial transplantation approach to explore the replicative potential of adult mouse hepatocytes. In their experiments, 10,000 lacZ-marked hepatocytes were transplanted into spleen of $Fah^{-/-}$ mice. The mutant mice were removed from NTBC 2 days post-transplantation. Following 8 weeks of repopulation by donor hepatocytes, the livers of the recipient mice were collagenase perfused, hepatocytes were isolated, and 10,000 donor cells were transplanted into new mutant recipient mice. After six rounds of serial transplantation, there was still no sign of donor hepatocyte senescence, indicating that the upper limit of the ability of hepatocytes to divide had not yet been reached. The authors calculated that hepatocytes had undergone a minimum of 69 cell doublings after the sixth serial transplant, or assuming that only 15% of transplanted hepatocytes actually seed the liver following splenic transplantation, at least 85 cell doublings. Despite the large number of cell doublings observed in these studies, the hepatocytes were fully functional and the morphology of the lobules was normal. The repopulating cells showed no evidence of altered differentiation or malignant transformation. However, it is not clear whether all hepatocytes have the same clonogenic potential, or whether

sub-populations of hepatocytes exist, only some of which have extensive replicative potential. The results of this experiment not only demonstrate that hepatocytes have a tremendous replicative potential, but also suggest that transplantation of small numbers of hepatocytes, followed by *in vivo* expansion, may someday be an appropriate therapeutic treatment for humans with hepatocytic metabolic disorders (Fausto, 1997).

Retrorsine treated rats

The second recent model of hepatic toxicity/regeneration involves chemically treated rats. Pyrrolizidine alkaloids, such as lasiocarpine and retrorsine, are processed by the liver to form their active metabolites within hours to days; these metabolites can alkylate DNA. Administration of these toxins can induce long-term blocks on the hepatocyte cell cycle, and also lead to chronic hepatic lesions including megalocytosis, fibrosis, and neoplasms. Laconi *et al.* (1995) investigated whether syngeneic donor hepatocytes, transplanted into lasiocarpine-treated rats, could diminish the chronic effects of toxin treatment in these rats. The authors believed that donor cells transplanted after lasiocarpine metabolism was complete would not be subject to the inhibitory influences of chemical treatment; this hypothesis assumes, reasonably, that the inhibitory effects of lasiocarpine treatment are cell autonomous, so that the donor cells would not be affected by the adjacent, toxin-inhibited hepatocytes. Fischer 344 rats were treated once with lasiocarpine (80 μmol/kg) then subjected to 2/3 partial hepatectomy 4 weeks later. At the time of partial hepatectomy, one group of rats received 1×10^6 hepatocytes infused into the portal vein while the other group of rats received media. All animals were sacrificed 14 weeks post-hepatectomy. Relative to media-treated control animals, rats that received hepatocyte transplants showed milder hepatic lesions, including increased total liver size, lack of nodular proliferation, and significant reductions in hepatocyte megalocytosis and bile ductular proliferation. One explanation proposed by the authors was that the donor hepatocytes responded to mitotic stimuli by undergoing replication to replace some of the chemically damaged, mito-inhibited parenchyma in the recipient mice. Unfortunately, donor cells were not uniquely marked so this hypothesis could not be proven.

Retrorsine treatment also has been shown to persistently inhibit hepatocyte DNA replication. Rats injected twice with retrorsine (30 mg/kg, 2 weeks apart) were subjected to two-thirds partial hepatectomy 4 weeks later to induce cell proliferation. Total DNA levels in the retrorsine treated animals were significantly lower than levels observed in vehicle treated controls at 3 days and 2 weeks post partial hepatectomy, indicating that hepatocyte proliferation was inhibited in retrorsine treated animals following partial hepatectomy (Pani *et al.*, 1997). To investigate the fate of transplanted hepatocytes in this model, 2×10^6 hepatocytes isolated from Fischer 344 rats containing the enzyme dipeptidyl peptidase IV (DPPIV) were transplanted into liver of retrorsine treated, partially hepatectomized syngeneic DPPIV⁻ recipient rats. Rats were sacrificed at various times post-transplantation, and a histochemical assay was used to detect DPPIV⁺ donor hepatocytes. The results demonstrated that donor hepatocytes were able to replace up to 99% of the retrorsine-compromised livers by 9 months post-transplant (Laconi *et al.*, 1998). Furthermore, the identification of canaliculi between DPPIV⁺ and DPPIV⁻ cells indicated that donor hepatocytes were able to form normal hepatic architecture. Thus, as demon-

strated in AL-uPA transgenic and Fah null mice, healthy hepatocytes isolated from adult animals and transplanted into animals with liver disease can proliferate and ultimately replace a significant portion of the host diseased liver. This model will likely become of increasing importance in view of the long-standing use of the rat in studies of liver growth and regeneration.

Future directions

The AL-uPA and related systems can be used to address questions of liver cell growth regulation as well as cell-cell interactions and cell lineage potential within liver in response to both mitotic stimuli and toxins. This section will discuss several future applications of these model systems that rely on their use to create chimeric livers with distinct and uniquely marked hepatocytic and non-hepatocytic somatic genotypes.

Existence and replicative potential of liver stem cells

While hepatocytes in the normal adult liver are mitotically quiescent, these fully differentiated cells have a remarkable ability to re-enter the cell cycle in response to mitotic stimuli. Following two-thirds partial hepatectomy (via removal of the left and median hepatic lobes), nearly all hepatocytes in the adult liver undergo cell division; this is followed by a second round of replication in which about half of the hepatocytes divide again to fully restore the liver to its original mass within seven to ten days. This response to surgical injury is termed "regeneration", although the term is not precisely accurate since the response involves a compensatory hyperplasia within remaining lobules, not a recreation of the original lobular morphology. Replication of differentiated hepatocytes and biliary epithelial cells accounts for this regeneration. In contrast, several treatments have been developed in the rat and mouse that couple hepatic injury with hepatocyte mitoinhibition. Regeneration under these conditions may rely on undifferentiated stem cells (reviewed in Fausto, 1994; Thorgeirsson, 1995; Sell and Ilic, 1997). Subsequent to these treatments a population of small ovoid cells with pale-staining nuclei (oval cells) is observed bordering biliary ductules in liver. Oval cells multiply and migrate into the hepatic parenchyma during regeneration. Once regeneration is complete the population of oval cells disappears. For example, treatment with 1,4-Bis[N,N'-di(ethylene)-phosphamide]-piperazine (Dipin) followed by two-thirds partial hepatectomy has been shown to induce an oval cell response in mice (Engelhardt *et al.*, 1990; Factor and Radaeva, 1993). Dipin is an alkylating agent that causes chromosomal breakage in cells undergoing DNA synthesis. Hepatectomy-stimulated hepatocytes are unable to divide and are irreversibly damaged. Periportal oval cells appear 1 to 3 weeks following surgery and this response becomes maximal at between 8 and 10 weeks. At this time, basophilic hepatocytes can be seen adjacent to the oval cells. Approximately 5 to 6 months post-surgery, the liver is morphologically normal.

It has been proposed that a facultative stem cell lineage exists that can generate hepatocytes in cases of serious hepatic injury, such as the Dipin protocol described above. Several observations suggest that oval cells may be transitional cells in this lineage. Oval cells are often found adjacent to cells that exhibit morphology "intermediate" between hepatocytes and biliary epithelial cells. Furthermore, when [³H]-thymidine was used to pulse-label oval cells, the label was found later in small hepatocytes, suggesting

that they are oval cell progeny. Finally, many oval cells express hepatocyte related genes, in particular alphafetoprotein (see reviews above). However, despite this evidence supporting the existence of a facultative stem cell lineage, this hypothesis remains controversial. The principal difficulty is the fact that the hypothesis has been based upon circumstantial evidence: putative transitions of individual cells from one form to another cannot be directly observed in live animals. The existence of facultative stem cells in liver will be difficult to prove until a model is developed in which hepatocytic and non-hepatocytic cells can be differentially marked *in vivo*.

The AL-uPA and related model systems permit this condition to be fulfilled. One potential experiment takes advantage of the chimeric genetic status of repopulated uPA mouse liver (see Fig. 4). Liver in adult uPA mice will be composed of two genetically distinct somatic cell populations including: (a) hepatocytes that have lost the transgene DNA and (b) non-hepatocytic cells that possess intact, albeit unexpressed, transgene DNA. Using hepatocyte transplantation, donor cell-derived parenchyma can be further marked by a lacZ transgene, while the recipient is marked by a different transgene, such as human placental alkaline phosphatase (hPAP). Thus, for this experiment, bitransgenic mice will be generated carrying the uPA and hPAP transgenes (Fig. 4). The uPA/hPAP mice will receive hepatocyte transplants (via intrasplenic injection of ~10^5 cells) from a lacZ donor mouse. After 3 months, the liver will be completely repopulated by two populations of uPA transgene-deficient hepatocytes: (a) hepatocytes derived from the recipient mouse that have physically deleted the uPA transgene (genotype: uPA$^-$, hPAP$^+$) and (b) hepatocytes derived from the donor mouse that did not carry the uPA transgene (genotype: uPA$^-$, lacZ$^+$). In contrast, non-hepatocytic cells in the recipient mouse, including those in the liver, should still contain an unexpressed uPA transgene array plus the hPAP transgene (genotype: uPA$^+$, hPAP$^+$). At this time, the uPA mice will be treated with Dipin (60 mg/kg) and hepatectomized 2 hours later. Over the course of the next 6 months, liver will be collected from treated mice and examined by histochemical staining to identify the source of the newly developing hepatocytes. Possible outcomes include: (a) repopulation by hepatocytes that have not been irreversibly damaged by Dipin, in which case the hepatic parenchyma should display both lacZ- and hPAP-expressing cells in proportion to their relative presence prior to Dipin/hepatectomy; or (b) repopulation by hepatocytes derived from proliferation and differentiation of a stem cell compartment, in which case all hepatocytes should express hPAP. This conceptually straightforward experimental design, though in practice more complex given the potential for reactivation of the uPA transgene in stem cell-derived hepatocytes, provides one means to resolve the long-standing controversy regarding the presence and growth potential of stem cells in adult liver. This approach also illustrates the power of differential somatic cell marking methodology in the mouse, a tool of historic importance in analysis of development and disease in other species.

Therapeutic hepatocyte transplantation

Current protocols to treat severely damaged liver in humans include whole liver transplants; however, this treatment faces many problems, including a shortage of healthy donor livers. Therefore, alternative treatment options need to be explored. In particular, the ability of transplanted hepatocytes to produce a therapeutic effect in animals with pre-existing liver lesions can be investigated using animals with genetically engineered liver disease. To rescue animals with lethal hepatic disease, donor hepatocytes would need not only to clonally repopulate liver, but also to become integrated into the hepatic architecture and take over liver function from the damaged host hepatocytes. As noted earlier, findings in the AL-uPA model regarding the proliferative potential of routinely isolated adult hepatocytes suggest that medically appropriate donor cell populations can be readily obtained. Future studies in these and similar mice will be directed at optimizing collection, storage, and delivery of donor cells to maximize therapeutic effect.

Toxicology and xenobiology studies

In 1995, Rhim and colleagues demonstrated that livers of immunocompromised AL-uPA mice could be repopulated by rat hepatocytes. An extension of these studies includes testing xenogeneic hepatocytes from multiple species for their ability to reconstitute damaged rodent liver. By comparing the ability of hepatocytes from different species to repopulate these models, we may be able to learn more about the signals involved in the regenerative response and whether these signals are conserved across species boundaries. The bi-species chimeric livers also should be useful in studies of toxin metabolism and induction of lesions. For example, will a rat-reconstituted mouse liver respond to toxins in a manner resembling that of the donor rat species or the recipient mouse species? The answers to questions like this will begin to differentiate responses to toxins mediated by the target cell (cell-autonomous responses) from those responses influenced by the overall host environment. A final critical objective of xenobiotic transplant studies is to determine whether human hepatocytes can be used to create a liver in which the hepatocyte population is human-derived and non-hepatocytic cells are rodent-derived. Rodent livers reconstituted with human hepatocytes would be invaluable for testing the *in vivo* effects of toxins on human hepatocytes, as well as addressing questions of human hepatocyte biology.

Summary

The creation of AL-uPA transgenic mice nearly a decade ago, combined with the subsequent development of Fah null mice and retrosine-treated rats, provide us with powerful animal models of liver disease that can be used to address long-standing questions in hepatic biology. Already, we have learned that the adult hepatocyte has a stunning replicative potential, rivaling that of certain stem cells in other tissues. Studies of hepatocyte transplantation have shown that diseased mouse liver can be replaced by both exogenous mouse hepatocytes and xenogeneic rat hepatocytes. Not only can healthy donor hepatocytes clonally proliferate in diseased livers, but the recipient liver integrates donor hepatocytes into a normal hepatic architecture; in fact, the reorganized lobular structures are sufficiently functional so that hepatocyte transplants can rescue FAH$^{-/-}$ mice that would otherwise die from hepatic failure. Future investigations using these models will likely include: (a) studies of hepatic growth control signals, (b) characterization of liver cell-cell interactions, (c) assessment of the existence and growth potential of an adult hepatic stem cell population, (d) investigations to improve the utility of therapeutic hepatocyte

transplantation and (e) toxicity testing in chimeric livers that may one day include human hepatocytes. Recall that the lines of investigation outlined above, plus additional applications of rodent models with engineered liver disease, received their initial impetus from an experiment that failed to achieve its stated goals. This observation highlights the fact that, at times, we may be wise to put aside our own questions in favor of those raised serendipitously by the experimental systems with which we work.

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