Dixon, in (*37*)] with the following modifications: Fusion proteins were bound to glutathione agarose, and the recombinant portion was eluted for 30 min in 10 ml of 50 mM tris (pH 8.0), 150 mM NaCl, 2.5 mM $CaCl_2$, and 0.1% β-mercaptoethanol with thrombin (4 μg ml^{-1}). Phenylmethylsulfonyl fluoride (0.6 mM) was added to the protein elution, and the sample concentrated to 0.5 ml. The protein was further purified by gel filtration on a Superose 12 sizing column (Pharmacia, Piscataway, NJ) in HBS-T20 buffer. Control preparations were prepared identically from lysates expressing only the GST protein.

39. SPR detection experiments were performed with the BIAcore apparatus (Pharmacia Biosensor). All protein immobilizations were performed in 25 mM acetate buffer (pH 4.0), 15 mM NaCl, 0.05% Tween-20. The carboxylated dextran matrix of the flow cell was first activated with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and *N*-hydroxysuccinimide, which allowed the subsequent cross-linking of injected protein through primary amine groups [B. Johnsson *et al.*, *Anal. Biochem.* **198**, 268 (1991)]. After the protein was cross-linked to the flow cell, the reactive groups were blocked by the injection of an excess of primary amines, 1 M ethanolamine (pH 8.5). Data for BIAcore SPR detection experiments were collected at 5 Hz. All binding was done in HBS-T20 buffer at room temperature.
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Replacement of Diseased Mouse Liver by Hepatic Cell Transplantation

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Adult liver has the unusual ability to fully regenerate after injury. Although regeneration is accomplished by the division of mature hepatocytes, the replicative potential of these cells is unknown. Here, the replicative capacity of adult liver cells and their medical usefulness as donor cells for transplantation were investigated by transfer of adult mouse liver cells into transgenic mice that display an endogenous defect in hepatic growth potential and function. The transplanted liver cell populations replaced up to 80 percent of the diseased recipient liver. These findings demonstrate the enormous growth potential of adult hepatocytes, indicating the feasibility of liver cell transplantation as a method to replace lost or diseased hepatic parenchyma.

Despite its highly specialized function, the liver is unique in its capacity to regenerate, even within the adult organism. The primary model of this process, two-thirds partial hepatectomy in the rat, has allowed investigation of molecular events that underlie the regenerative process (*1*). While much has been learned about liver regeneration, many questions regarding the proliferative capacity of adult liver cells remain. After partial hepatectomy, much of the lost liver mass is restored by the division of fully differentiated hepatocytes (*1, 2*). However, because only one to two rounds of hepatocellular division are required to restore the liver mass after partial hepatectomy, the extent of the regenerative capacity of hepatocytes is unknown. Certain pathological processes may reflect a finite capacity for hepatocyte replication. In gene therapy protocols, in which therapeutic genes are delivered to the liver via genetically modified hepatocytes, expression of the introduced gene declines over time (*3, 4*). This diminution of gene expression may be due to gradual loss of the modified cells because of a limited capacity to divide, either as a result of an inherent restriction in their replicative potential or as a result of changes acquired during their manipulation in culture, and is an especially serious problem given the very low colonization of host liver (less than 1%) that has been achieved in experimental models using hepatocellular transplantation (*3–5*). Finally, in conditions of severe liver injury, restoration of liver mass may be primarily accomplished not by hepatocytes but by the expansion of hepatocyte precursors (stem cells) that subsequently differentiate into hepatocytes. The existence of liver stem cells, their replicative potential, and their role in liver regeneration remain controversial (*6, 7*).

We recently described a model of liver regeneration in albumin-urokinase (Alb-uPA) transgenic mice [TgN(Alb1Plau)-144Bri, TgN(Alb1Plau)145Bri], in which hepatocyte-targeted expression of a hepatotoxic transgene creates a functional liver deficit (*8*) resulting in a chronic stimulus for liver growth. Because of the stimulus for liver growth present in these mice, a small number of hepatic cells in young mice that stochastically delete the deleterious transgene are selectively expanded. Each cell gives rise to a clone of hepatocytes that forms a nodule (*8*). By 8 to 12 weeks of age, transgenic mouse livers have been completely replaced by the nodular, clonal growth of these transgene-deficient cells (referred to as "red nodules"). The observation that the regenerating nodules in these livers were composed of normal appearing hepatocytes suggested that mature hepatocytes may indeed possess considerable replicative capacity. However, we could not rule out the possibility that the progenitor cells of these nodules, instead of being hepatocytes, were actually undifferentiated cells with a much greater replicative capacity than the hepatocytes to which they gave rise. Furthermore, even if nodule progenitor cells were hepatocytes, they were of fetal or early postnatal origin; cells at this stage display a much higher mitotic index than the adult hepatocytes, which are of greatest interest biologically and medically.

To determine the replicative capacity of adult liver cells, we transplanted cells isolated from adult mouse livers into Alb-uPA transgenic mice, then studied their growth. Marking of donor cells at the genetic level can be accomplished with retroviral transduction of hepatocytes (*9*), but for our studies we wished to avoid methods that might alter the replicative potential of the cells or mark only subpopulations of liver cells. We therefore transplanted cells freshly isolated from two groups of transgenic mice. The first, EL-*myc* [TgN(Ela1Myc)159Bri], carried the rat elastase enhancer-promoter fused to the mouse c-*myc* structural gene (*10*). This transgene is not expressed in liver; however, there are approximately nine copies per cell, facilitating detection of donor hepatocyte DNA within recipient livers. This lineage is congenic in C57BL/6 (B6). Second, we generated B6 donor transgenic mouse lines that carried the mouse metallothionein-I gene promoter plus flanking sequences fused to a modified β-galactosidase (β-gal) structural gene (MT-*lacZ*) (*11*). The protein product of this transgene is targeted to the cell nucleus as a result of the presence of the SV40 large T-antigen nuclear localization signal peptide. The MT promoter is active in liver of transgenic mice, and its expression can be induced up to 10-fold by administration of heavy metal

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ions (*12*). β-Galactosidase activity can be visibly detected with the synthetic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal).

Two MT-*lacZ* lineages (containing the transgene at different genomic locations) were established and designated 4560-2 [TgN(MTnlacZ)203Bri] and 4580-2 [TgN-(MTnlacZ)204Bri]. After induction with cadmium and incubation with X-gal (*13*), transgenic livers were stained homogeneously blue. Microscopic examination revealed β-gal activity localized to hepatocyte nuclei (Fig. 1A).

For all transplantation experiments, liver cell suspensions were freshly prepared by two-step EDTA-collagenase perfusion (*14*), then centrifuged twice at 50*g* to enrich for hepatocytes. Cellular viability ranged from 50 to 85% as measured by trypan blue exclusion. Each suspension was kept on ice and transferred within 2 hours. Approximately 80 to 85% of the cells in the preparations were hepatocytes morphologically; however, small nonhepatocytic cells were also present (Fig. 1B).

In the hemizygous Alb-uPA transgenic mice used in our experiments, the regenerative stimulus is present as long as transgene-expressing liver remains, for ~6 to 8 weeks after birth. Thus, to optimize assessment of cellular replicative ability, cells must be transferred into transgenic recipients as soon after birth as possible. Donor cells were introduced into the spleen of mice within the first 2 weeks of life (*15*). Recipient mice were generally maintained for an additional 4 to 6 weeks and killed; livers were then analyzed for the presence of donor cells and their progeny.

In two initial experiments, ~10^4 liver cells isolated from adult El-*myc* transgenic mice were injected into spleens of 5- to 11-day-old Alb-uPA recipients. Four to 6 weeks later, the recipients were killed and their livers removed and analyzed by polymerase chain reaction and Southern (DNA) blot hybridization. All six recipient mice transplanted with adult El-*myc* cells displayed evidence of donor cells in one or more portions of liver. Phosphoimage analysis of Southern blots indicated that, in some liver samples, more than 50% of the cells were donor-derived (*16*). In contrast, no signal was observed in livers of similarly treated control mice lacking the Alb-uPA transgene. Given that individual samples examined were often greater than 2.0 mm^3 in size and contained many more cells than the total number transplanted, these results strongly suggested that extensive donor cell expansion occurred within recipient liver.

To determine both the extent and pattern of colonization of donor cells and their progeny in the recipient livers, we injected donor cells harvested from liver of adult

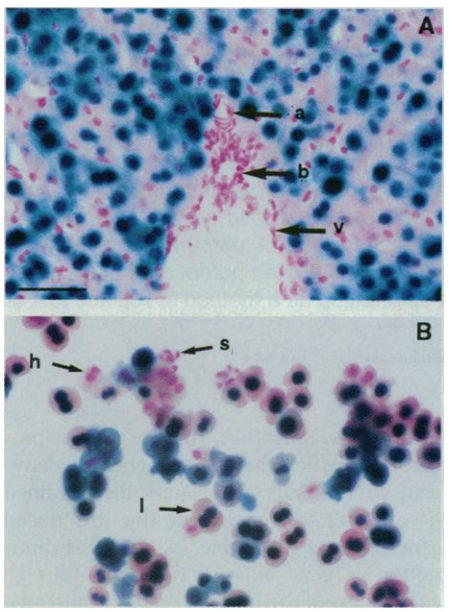

Fig. 1. X-gal staining of MT-*lacZ* donor liver after cadmium induction. A frozen section (**A**) or a liver cell suspension (**B**) from MT-*lacZ* transgenic liver was stained with X-gal (*13*) and counterstained with nuclear fast red. In the frozen section (A), a portal triad is shown containing an arteriole (a), a bile duct (b), and a vein (v). Hepatocyte nuclei show strong X-gal staining. Neither biliary cell nor endothelial cell nuclei had any detectable β-gal activity; they are stained red. Scale bar, 40 μm. In the cell preparation (B), over 80% of the cells are hepatocytes (l); they are large, round to polygonally shaped cells with abundant cytoplasm and one or two centrally placed nuclei. In most hepatocytes the β-gal activity is confined to the nucleus; in others, the activity is also present within the cytoplasm. A few hepatocytic cells showed no evidence of β-gal activity (h); their nuclei stained red. In addition to hepatocytes, other cells with smaller nuclei, less cytoplasm, and no evidence of β-gal activity were also present (s). We found that the location and intensity of X-gal staining in hepatocytes varied with different fixation protocols for cell suspensions. Staining was much more uniform and predictable in frozen sections where variation in intensity appeared related primarily to the plane of section relative to the hepatocyte nucleus.

Fig. 2. X-gal staining of control and transgenic livers transplanted with MT-*lacZ* cells. Livers from a nontransgenic control (top, left), an MT-*lacZ* positive control (top, center), a control mouse lacking the Alb-uPA transgene transplanted with MT-*lacZ* cells (top, right), and three livers from Alb-uPA transgenic mice receiving MT-*lacZ* cells (bottom) were stained with X-gal after cadmium induction. Unstained liver represents red areas of endogenous cells (r) that had lost transgene expression as well as residual transgene-expressing white parenchyma (w). No gross blue staining was detected in the nontransgenic liver transplanted with MT-*lacZ* cells (top, right).

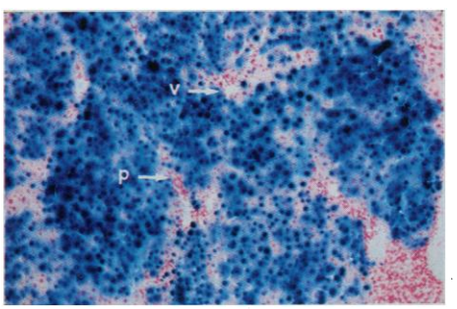

Fig. 3. Histology of Alb-uPA transgenic liver receiving MT-*lacZ* cells. A frozen section of Alb-uPA transgenic liver transplanted with MT-*lacZ* cells 4.5 weeks previously was incubated with X-gal and counterstained with nuclear fast red. The liver parenchyma was almost completely replaced by MT-*lacZ* hepatocytes, nearly all of which displayed intense nuclear blue staining with little variation among cells. The MT-*lacZ* cells have become associated with portal triads, central veins, and sinusoidal cells. Biliary nuclei from a portal area (p), endothelial nuclei from a central vein (v), and sinusoidal cell nuclei were stained red. A small residual area of transgene-expressing liver is seen in the lower right corner of the figure.

MT-*lacZ* mice into spleens of 10-day-old Alb-uPA mice. Transgenic recipients were administered cadmium 4 to 5 weeks after transplant, then killed, and their livers stained with X-gal. No gross blue staining was present in livers of control recipients lacking the Alb-uPA transgene (Fig. 2, top right). Microscopic examination of these livers did show single isolated blue cells (*16*). In contrast, large areas of blue staining, comprising up to 80% of the liver, were readily apparent on gross examination of transgenic recipient livers (Fig. 2, bottom), indicating that the donated MT-*lacZ* cells had expanded substantially. Microscopically, the blue stained areas were composed of hepatocytes with blue nuclear staining (Fig. 3). Examination of the spleens of recipient animals showed no evidence of donated liver cells (*16*).

Close inspection of chimeric livers revealed that the growth of the transplanted cells was nodular (Fig. 2). The apparently solid blue areas were themselves composed of several thousand confluent blue nodules. The nodules were of markedly uniform size (Table 1). This uniformity of donor nodule size contrasts with the much greater size variation of endogenous (transgene-deficient) red nodules identified in two of the mice (Table 1), caused by either the variable onset of growth of red nodules in these mice or by a difference in the growth potential of red nodule progenitor cells.

Table 1. Donor and endogenous nodule size. Nodule diameter was measured from X-gal–stained livers of three Alb-uPA mice transplanted with MT-*lacZ* cells in each of two separate experiments by means of a dissecting microscope equipped with a calibrated measuring reticle. Fifty donor cell–derived (blue) nodules were measured from each liver. Twenty spontaneously arising, endogenous nodules (unstained) were measured in two livers. Note the large standard deviation in endogenous nodule size compared to that of donor nodules. The number of donor cell doublings needed to generate a nodule of average size was calculated for each animal by determining the number of MT-*lacZ* cells within the nodule and assuming that each nodule was clonally derived (*17*).

Experiment number	Percentage of liver replaced	Type of nodules measured	Average nodule diameter ± SD (mm)	Average doublings per donor cell
1	5	Donor	0.70 ± 0.24	14
	40	Donor	0.50 ± 0.15	12
	80	Donor	0.64 ± 0.16	13
		Endogenous	1.25 ± 1.08	16
3	30	Donor	0.56 ± 0.15	13
		Endogenous	1.26 ± 1.02	16
	60	Donor	0.57 ± 0.18	13
	70	Donor	0.52 ± 0.11	12

Table 2. Fraction of transplanted liver cells giving rise to recipient liver nodules. MT-*lacZ* liver cells were injected intrasplenically into day 10 Alb-uPA transgenic pups. After 32 days the livers were stained with X-gal and the number of blue nodules calculated by dividing the volume of the replaced portion of liver by the volume of the average blue nodule (*19*). Each nodule was assumed to be clonally derived (*8*). In experiments 2 and 3 and in a separate experiment, nontransgenic pups were injected with MT-*lacZ* cells. Their livers were removed after 3 days, stained with X-gal, and the number of MT-*lacZ* cells determined and used to calculate the seeding efficiency (number of cells detected in nontransgenic recipient livers divided by the number of viable cells injected into the spleen) of the liver. The efficiencies were as follows: experiment 2, 0.1 to 1.2% ($n = 2$); experiment 3, 3.5 to 17.0% ($n = 3$); and additional experiment, 2.0 to 6.4% ($n = 2$). Because only a small fraction of the injected cells reach the liver, the calculated percentage of injected cells giving rise to nodules (shown in the last column) is likely to significantly underestimate the fraction of injected cells capable of giving rise to nodules.

Experiment number	Viable cells injected	Recipient animal	Percentage of liver replaced	Number of nodules per recipient liver	Percentage of injected cells giving rise to nodules
1	120,000	1	5	400	0.3
		2	40	3300	2.8
		3	80	6500	5.4
2	88,000	1	5	400	0.5
		2	70	5700	6.5
		3	70	5700	6.5
3	75,000	1	30	2400	3.2
		2	60	4900	6.5
		3	70	5700	7.6
4	170,000	1	50	4100	2.4
		2	60	4900	2.9
		3	70	5700	3.4

The nodular appearance of the donor-derived cells suggested that they, like their transgene-deficient endogenous counterparts, grew by clonal expansion at the expense of the surrounding diseased, transgene-expressing liver. By comparing the volume of a blue nodule to the volume of a blue cell, the number of cells comprising the nodule can be calculated. Using this result, one can determine the number of cell doublings required to form the nodule (*17*), assuming that blue nodules are derived from the expansion of one progenitor cell. On the basis of these calculations, the progenitor cells appear mitotically equivalent (Table 1, last column); that is, each gave rise to approximately the same number of descendants during the 4.5-week in vivo incubation period. Thus, donated liver cells underwent extensive growth within the transgenic liver, dividing at least 12 times. Reconstitution of an entire liver from one hepatocyte would require 28 cell doublings.

The growth of transgenic livers transplanted with MT-*lacZ* cells was appropriately regulated. By 6 weeks after transplant, the liver weight to body weight ratio of transgenic recipients was no different than that of nontransgenic controls (*16*). In addition, the livers of older animals were not grossly enlarged. No tumors have been observed in animals up to 6 months after transplantation.

To assess the functional competency of Alb-uPA/MT-*lacZ* chimeric livers, we performed two experiments. First, the blood concentrations of albumin, total protein, and bilirubin, molecules whose concentration is dependent on hepatic sufficiency, were compared in recipient and control mice; the values were similar (*16*). Second, a sensitive test of liver cell function and adequacy is the mitotic index of hepatocytes after injury or loss of liver mass (*1*). Therefore, Alb-uPA/MT-*lacZ* chimeric livers of 49-day-old mice were subjected to two-thirds partial hepatectomy, and the mitotic index was determined 44 hours later (the time of peak mitotic activity in mouse hepatocytes). We also determined the responses of donor and endogenous liver present in the same mouse, thus providing an internal control to eliminate variation between animals. The overall mitotic response of the Alb-uPA/MT-*lacZ* chimeric livers (including both donor and endogenous areas) was elevated compared to non-

transgenic control livers (1.5 ± 0.4%, n = 3, and 0.7 ± 0.1%, n = 2, respectively), possibly reflecting the regenerative state of the transgenic livers. Hepatocytes of donor (1.6 ± 0.3%, n = 3) and endogenous (1.4 ± 0.6%, n = 3) origin within transgenic host liver responded nearly identically to this test of regenerative function.

By comparing the number of blue nodules in the recipient liver to the number of injected cells, it is possible to estimate the fraction of cells with extensive growth potential within the donor cell population. Of 12 animals from four separate experiments, the percentage of injected cells giving rise to blue nodules ranged from 0.3 to 7.6% (Table 2). These percentages strongly support the conclusion that hepatocytes were the progenitors of at least some of the blue nodules, since 0.3%, and certainly 7.6%, is an unreasonably high frequency for a distinct class of cells with unique replicative potential (that is, stem cells). Although there were nonparenchymal contaminant cells within our donor cell preparations (Fig. 1B), it is very unlikely that this population of cells would be selectively taken up by the recipient liver and account for all of the blue nodules. Indeed, the percentage of adult hepatocytes that can function as nodule progenitor cells is most likely appreciably higher than 7.6% considering that the percentage of injected cells reaching the livers of seven control mice evaluated over three experiments never exceeded 17.0% (Table 2, legend).

We have shown that progeny of nonautologous transplanted adult liver cells can replace almost the entire mouse hepatic parenchyma because of the substantial replicative capacity of the mature hepatocyte. On the basis of our results, we propose that transplanted adult hepatocytes can undergo at least 12 cell doublings and still retain the ability to respond to partial hepatectomy with one or two additional divisions. Although we cannot rule out the possibility that the progenitor cells of the nodules represent a subpopulation of hepatocytes, we note that, should such subpopulations exist, our model is ideal for determining their replicative potential.

Our findings suggest that preparation of a donor cell population will not be the principal difficulty facing medical use of hepatic cell transplantation; rather, success is likely to depend on provision of a suitable internal environment in which transplanted cells can survive and grow. Transgene-expressing Alb-uPA hepatocytes are at a growth disadvantage compared to regenerating transgene-deficient liver (*8*) and are apparently also functionally compromised, since a strong hepatic growth environment is present in Alb-uPA liver despite a normal hepatic mass (*8*). However, hepatic architecture and the complement of liver cells (including endothelium, biliary epithelium, Kupffer cells, and Ito cells) remain relatively normal. Thus, in young Alb-uPA recipients, the physical microenvironment is permissive for survival and function of transplanted cells, while the functional deficit of endogenous hepatocytes stimulates the subsequent expansion of this donor cell population. These characteristics of the model suggest it will be extremely valuable to test the replicative potential of various cell donor populations, including hepatocytes manipulated for gene therapy.

Our findings have several medical implications. First, immediate transplantation of currently available human liver cell populations may save patients with fulminant hepatic failure where liver architecture is preserved (*18*). Second, the approach of transplanting genetically engineered hepatocytes to correct a metabolic defect appears likely to succeed as long as a suitable microenvironment for growth of these cells can be provided. Finally, Alb-uPA mice backcrossed onto an immunodeficient genetic background should accept xenogeneic cell transplants and could in principle be reconstituted with human liver cells. Thus, this approach of cell transplantation into Alb-uPA mice can also be used to characterize the regenerative potential of human liver cell populations and to study the biology of human hepatocytes within an intact liver in a controlled experimental setting.

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15. Liver cell suspensions were introduced into 10-day-old mice by intrasplenic injection. Mice were anesthetized by inhalation of methoxyflurane (Metofane). The spleen was exteriorized through a small left flank incision. A 30-gauge needle stabilized by a micromanipulator was introduced into the spleen, and the chilled cell suspension was slowly injected in a volume of 5 to 10 μl. The spleen was then gently returned to the abdominal cavity and the abdominal musculature and skin sutured. The mice were allowed to recover overnight before being returned to their mother. Mice were housed and maintained in accordance with the institutional animal care and use committee guidelines of the University of Pennsylvania.
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17. Providing that each nodule is clonally derived, the number of cell doublings can be calculated on the basis of the number of MT-*lacZ* cells within a nodule. For the average blue nodule with a diameter of 0.58 mm (representing the average of all the nodule diameters shown in Table 1), the volume is 0.102 mm^3. For an average blue hepatocyte with a diameter of 0.03 mm, the volume is 0.000014 mm^3. Therefore, the number of blue cells in the average nodule equals the volume of a whole nodule divided by the volume of a blue hepatocyte = 7300 cells per average nodule. The number of cell doublings needed to give 7300 cells is $2^n = 7300$, or $n = 13$.
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