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Robust expansion of human hepatocytes in *Fah*$^{-/-}$/*Rag2*$^{-/-}$/*Il2rg*$^{-/-}$ mice

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Mice that could be highly repopulated with human hepatocytes would have many potential uses in drug development and research applications. The best available model of liver humanization, the uroplasminogen-activator transgenic model, has major practical limitations. To provide a broadly useful hepatic xenorepopulation system, we generated severely immunodeficient, fumarylacetoacetate hydrolase (Fah)-deficient mice. After pretreatment with a urokinase-expressing adenovirus, these animals could be highly engrafted (up to 90%) with human hepatocytes from multiple sources, including liver biopsies. Furthermore, human cells could be serially transplanted from primary donors and repopulate the liver for at least four sequential rounds. The expanded cells displayed typical human drug metabolism. This system provides a robust platform to produce high-quality human hepatocytes for tissue culture. It may also be useful for testing the toxicity of drug metabolites and for evaluating pathogens dependent on human liver cells for replication.

The liver is the principal site for the metabolism of xenobiotic compounds, including medical drugs. Because many hepatic enzymes are species specific, it is necessary to evaluate the metabolism of candidate pharmaceuticals using cultured primary human hepatocytes[1,2]. Today, hepatocytes are isolated primarily from cadaveric organs and then shipped to the location where testing will be performed. The condition (viability and state of differentiation) of hepatocytes from cadaveric sources is highly variable, and many cell preparations are of marginal quality. The availability of high-quality human hepatocytes is further hampered by the fact that they cannot be substantially expanded in tissue culture[3,4]. Hepatocytes from readily available mammalian species, such as the mouse, are not suitable for drug testing because they have a different complement of metabolic enzymes and respond differently in induction studies. Immortal human liver cells (hepatomas) or fetal hepatoblasts are also not an adequate substitute for fully differentiated adult cells.

To overcome these difficulties, several groups have attempted to engraft and expand primary human hepatocytes in rodents. In all of these reports, the animals used were immunodeficient transgenics that express uroplasminogen activator (uPA) under the transcriptional control of an albumin promoter, rendering it hepatotoxic[5–9]. Although engraftment levels of up to 70% have been reported in these models, the system has several major disadvantages that have prevented its widespread use. These include poor breeding efficiency, a narrow time window for transplantation and renal disease in repopulated mice[7]. Thus, a more robust model of humanizing mouse liver is desirable.

We decided to create an immunodeficient strain of mice deficient in the tyrosine catabolic enzyme fumarylacetoacetate hydrolase (Fah). *Fah* mutant mice develop liver disease only when the protective drug 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC) is withdrawn; they have proven to be a reliable model of liver repopulation with transplanted cells[10–12]. Here we report that mice triply mutant for *Fah*, *Rag2* and the common γ-chain of the interleukin receptor can be efficiently repopulated with human hepatocytes. In contrast to uPA transgenic mice, mutant breeders are fully viable, repopulation can be done at any age, cell expansion by serial transplantation is possible and no renal disease was present.

RESULTS

Generation of *Fah*$^{-/-}$/*Rag2*$^{-/-}$/*Il2rg*$^{-/-}$ (FRG) mice

We initially generated several strains of immunodeficient *Fah* knockout mice by crossing with the nude, *Nod/Scid*[13] or *Rag1*$^{-/-}$[14] backgrounds. However, none were successfully repopulated with human hepatocytes despite multiple attempts (data not shown). No human-cell engraftment was seen in the *Fah*/nude and *Fah*/*Rag1* mutant mice, probably owing to immune rejection. In contrast, occasional human cells did engraft in the *Fah/Nod/Scid* mice. However, most of these animals developed rapid hepatic failure and died after withdrawal of the liver-protective drug NTBC, probably due to the DNA repair defect present in *Scid* mice[15]. We therefore sought to generate an immunodeficient *Fah*$^{-/-}$ mouse strain completely lacking T cells, B cells and natural killer cells, but without a DNA repair defect.

Rag2$^{-/-}$/*Il2rg*$^{-/-}$ mice have been reported to be excellent recipients of human hematopoietic xenografts[16,17]. Therefore the *Fah* mutation was crossed into this background, resulting in *Fah*/*Rag2*/*Il2rg* (*FRG*) triple mutants. *FRG* mice grew well and were fully fertile if they were

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Table 1

Donor	Origin	Age (years)	Number of mice transplanted	Human albumin positive (%)
A	Cadaveric	1.8	6	N/A[a]
B	Resection	55	9	3 (33)
C	Resection	50	5	1 (20)
D	Cadaveric	1.2	2	1 (50)
E	Cadaveric (cryopreserved)	55	5	2 (40)
G	Commercial (cryopreserved)	N/A	8	1 (13)
H	Cadaveric	64	6	2 (33)
I	Cadaveric	59	5	3 (60)
J	Cadaveric	1.3	6	4 (67)

[a]Albumin levels were not measured in these mice, but repopulation was shown by immunohistochemistry in 2/6 (33%).

continuously given NTBC in their drinking water[12]. As in conventional *Fah*$^{-/-}$ mice, NTBC withdrawal in *FRG* mice resulted in gradual hepatocellular injury and eventual death after 4–8 weeks[11].

Repopulation of *FRG* mouse liver with human hepatocytes

Overexpression of urokinase has been shown to enhance hepatocyte engraftment in several systems by inducing cell-autonomous hepatotoxicity[18]. We therefore performed pilot experiments to determine whether administration of a urokinase-expressing adenovirus[19] in *FRG* mice before transplantation of human hepatocytes would be beneficial. In three separate transplantations, we were able to observe primary engraftment of human hepatocytes only in recipients that had first received the uPA adenovirus, whereas murine hepatocytes repopulated regardless of uPA expression (data not shown). The uPA-pretreatment regimen was therefore used in subsequent experiments.

We successfully transplanted human hepatocytes from nine different donors. Of these, seven samples were isolated from the livers of brain-dead organ donors and two were from surgical liver resections. Donor ages varied from 1.2 years to 64 years (**Table 1**). Donor cells were isolated at the University of Pittsburgh and shipped overnight to Oregon for transplantation. However, in two experiments, cryopreserved hepatocytes were used (**Table 1**). The viability and quality of donor hepatocytes upon arrival was highly variable, with plating efficiencies ranging from 10% to 60%. For transplantation, the following general protocol was used. Adult (6–15 week old) *FRG* mice were given an intravenous injection (retroorbital) of uPA virus (5×10^9 plaque-forming units (p.f.u.)/mouse) as soon as a liver cell shipment was announced. One million human hepatocytes were injected intrasplenically 24–48 h after the adenovirus administration, and NTBC was then gradually withdrawn over the next 5 d. Two weeks after stopping NTBC, animals were put back on the drug for 5 d and then taken off again permanently.

In all experiments, at least one recipient became clearly engrafted (>1% human cells) with human hepatocytes, regardless of the cell batch used. Engraftment was demonstrated by different methods, including histology, DNA analysis, enzyme assay and human serum albumin level (**Figs. 1** and **2**). In the transplantations monitored by albumin levels, 17 of 43 primary recipients (39.5%; range 12–67%) became repopulated, that is, they had more than 1% human hepatocytes and rising albumin levels (**Table 1** and **Fig. 3**). Of these, seven were highly repopulated (30–90%) and achieved albumin levels >1 mg/ml. Importantly, hepatocytes not only from cadaveric livers but also from hepatic resections engrafted. Furthermore, two batches of cryopreserved cells also worked.

In highly engrafted mice (>30% repopulation), the weight of transplanted *FRG* mice stabilized during the second NTBC withdrawal, whereas less immunodeficient litter mates heterozygous for *Il2rg* (*Fah*$^{-/-}$/*Rag2*$^{-/-}$/*Il2rg*$^{+/-}$) given the same cells continued to lose weight (**Fig. 1a**). This weight stabilization (**Fig. 1a**) in triple mutant mice suggested that the transplanted human hepatocytes were replacing the

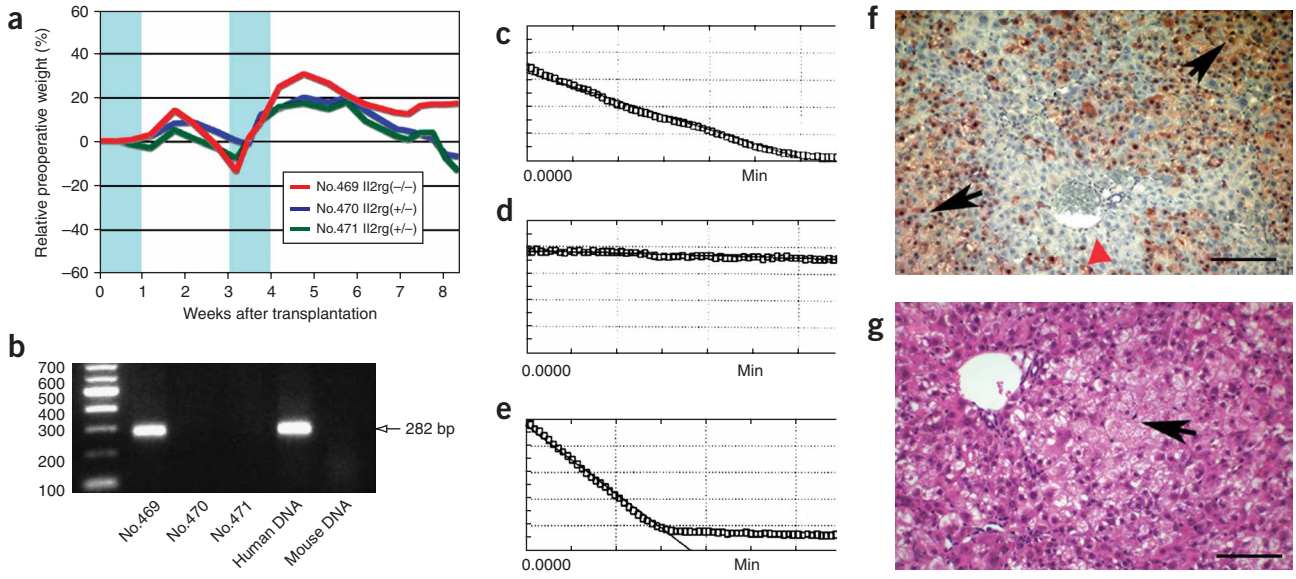

Figure 1 Engraftment and repopulation of *Fah*$^{-/-}$/*Rag2*$^{-/-}$/*Il2rg*$^{-/-}$ mice with human hepatocytes. (**a**) A triple-mutant mouse (no. 469, red line) maintained its weight 6 weeks after transplantation whereas *Il2rg* gene heterozygote littermates (no. 470, no. 471) lost weight continuously after NTNC withdrawal. NTBC was given in weeks 1 and 4 (blue area), and was withdrawn at other times (white area). (**b**) Human Alu sequence PCR on genomic DNA from recipient livers. Only triple mutant mice were positive. (**c**–**e**) FAH enzyme assay with equal protein concentrations. Substrate concentration was measured at 330 nm, declined in wild-type mouse liver (**c**) but did not change with Fah$^{-/-}$ mouse liver (**d**); a humanized mouse liver showed high enzyme activity (**e**). (**f**) FAH immunostaining (brown; black arrow) in a repopulated liver showed more than 80% of hepatocytes positive for FAH. Red arrow demarks FAH-negative cells. (**g**) H&E staining of the same liver section shows the less eosinophilic human hepatocytes (black arrow). Scale bars, 100 μm (**f**,**g**).

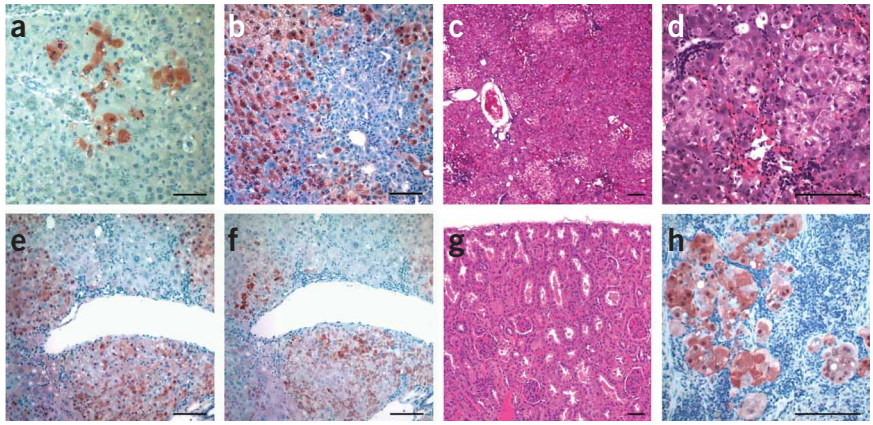

Figure 2 Histology and immunohistochemistry of chimeric mice. (**a**) FAH-positive human hepatocytes were integrated in mouse liver tissue and did not disturb recipient liver microstructure. (**b**) Highly repopulated chimeric livers also retained normal structure. (**c**,**d**) H&E staining readily distinguishes human hepatocyte clusters, which are less eosinophilic and hence appear paler than surrounding mouse cells. (**e**,**f**) Serial sections were stained for FAH (**e**) and HepPar (**f**). (**g**) Congruent staining is seen. Kidney section of highly repopulated mouse shows no tubular or glomerular destruction even 4 months after transplantation. (**h**) FAH-positive human hepatocytes in the spleen. Scale bars, 100 μm.

functions of the diseased *Fah*$^{-/-}$ recipient hepatocytes. Upon complete weight stabilization (2–3 months after initial transplantation), the recipient livers were harvested. Macroscopically, *FRG* livers were normal in shape and weight and lacked macroscopic nodules. Genomic PCR for human-specific Alu DNA sequences was positive in FRG recipient livers, whereas Il2rg heterozygotes were all negative (**Fig. 1b**).

To directly confirm hepatocytic function of the repopulating cells, FAH enzyme activity was assayed[20]. Recipient mouse livers had considerable FAH enzyme activity, equaling or exceeding normal mouse liver (**Fig. 1c–e**). As FAH is expressed in fully differentiated hepatocytes, this suggested that the transplanted human hepatocytes were not dedifferentiated or abnormal when engrafted in mouse liver. FAH immunostaining confirmed that >70% of liver parenchyma was repopulated with FAH-positive human hepatocytes (**Fig. 1f**,**g**).

FAH-positive human hepatocytes appeared completely integrated into the structure of the recipient liver (**Fig. 2a–d**). In several recipients, the engrafted hepatocytes occupied >80% of the parenchyma without disturbing the recipient liver organization (**Fig. 2b**,**e**,**f**). Clonally expanding human hepatocytes could be clearly distinguished from mouse hepatocytes morphologically, that is, by size, and by their pale cytoplasm (**Fig. 2c**,**d**) as previously described[21]. The FAH-positive hepatocytes were also positive for HepPar antibody, which specifically labels human but not mouse hepatocytes (**Fig. 2e**,**f**).

To examine whether repopulating human hepatocytes expressed mature hepatocyte-specific genes, we performed RT-PCR on messenger RNA extracted from recipient livers. The human albumin (*ALB*), *FAH*, transferrin (*TF*), transthyretin (*TTR*), tyrosine aminotransferase (*TAT*), and bilirubin UDP-glucuronosyltransferase *(UGT1A1)* genes were abundantly expressed in recipient livers (**Fig. 3a**). Hepatocyte functionality was also assessed by measuring blood concentration of human albumin. Human albumin was first detected between weeks 4 and 10 after transplantation in primary recipients and then it

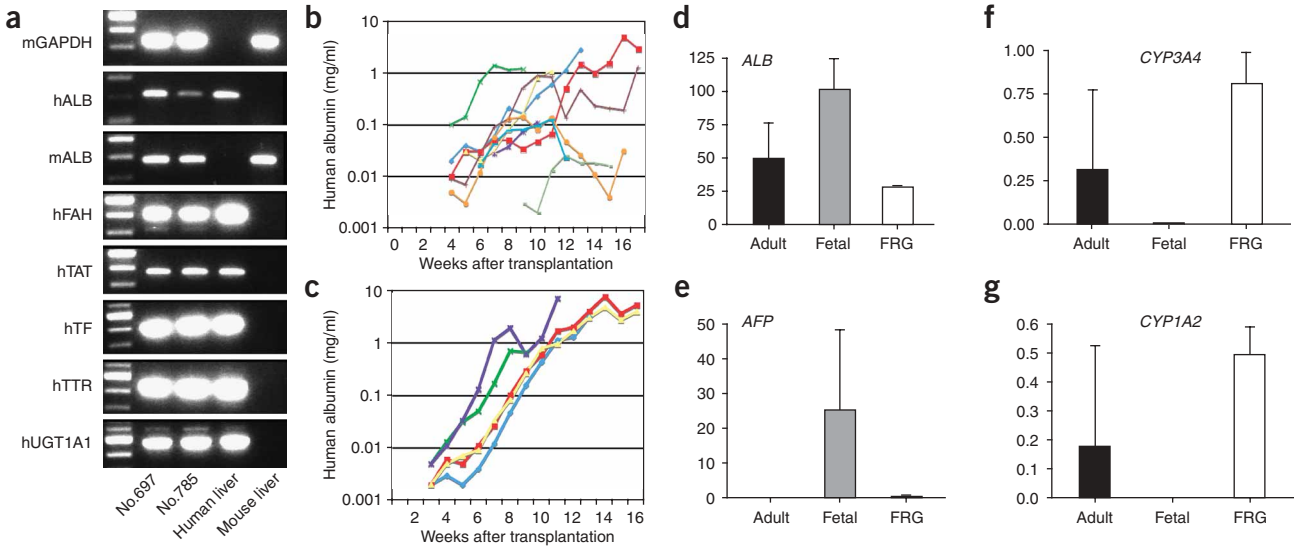

Figure 3 Human gene and protein expression in chimeric mice. (**a**) RT-PCR of chimeric liver. The human *ALB*, *FAH*, *TAT*, *TF*, *TTR* and *UGT1A1* genes were expressed in chimeric mice livers (no. 697 and no. 785). Human hepatocytes and mouse hepatocytes were used as positive and negative control, respectively. (**b**,**c**) Blood human albumin concentration of human cell recipients was assayed by enzyme-linked immunosorbent assay (ELISA). The threshold concentration of our system is around 0.005 μg/ml. Primary recipients (**b**); secondary recipients (**c**). The degree of difference among secondary recipients was much smaller than primary recipients. Logarithmic plotting shows the doubling time of albumin concentration was around 1 week. (**d**–**g**) Human liver–specific gene expression levels (displayed on the *y*-axis) determined by quantitative RT-PCR and normalized to human cyclophilin (= 1). Adult, average of nine adult human cell donors; Fetal, five human fetal livers; FRG, average of three highly humanized (>10%) FRG mouse livers. (**d**,**e**) All samples show high levels of albumin (**d**), but alpha-fetoprotein (AFP) is expressed highly only in fetal liver (**e**). (**f**) CytochromeP450 *CYP3A4* (**f**) is highly expressed in adult liver and FRG mice, but not in fetal liver. (**g**) Similarly, *CYP1A2* levels in FRG mice mimic those of adult liver.

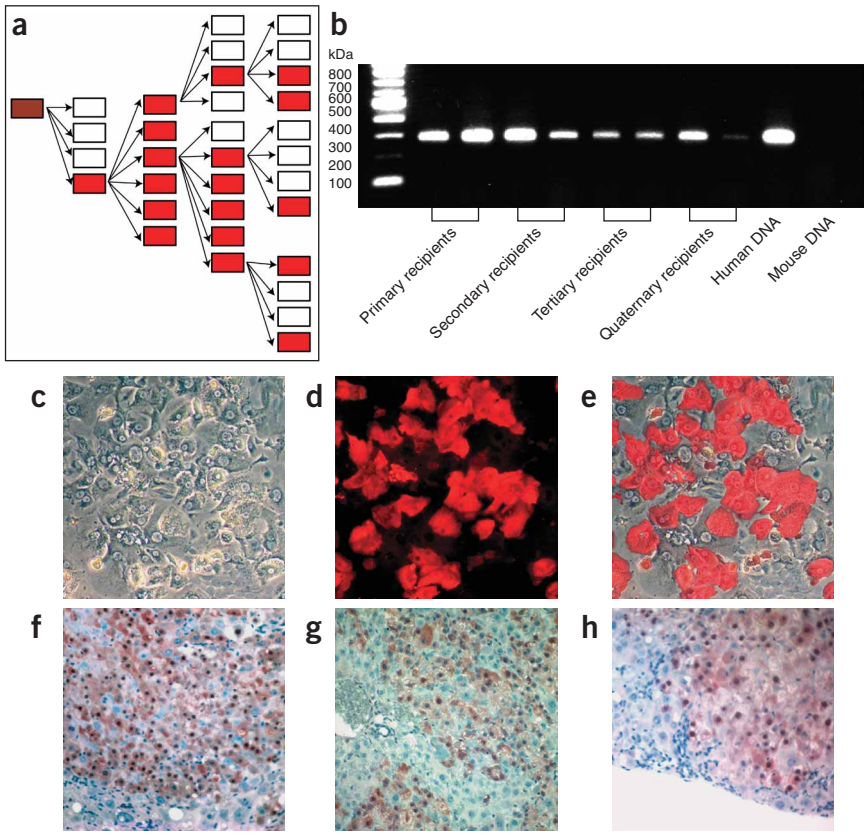

Figure 4 Serial transplantation of human hepatocytes. (**a**) Serial transplantation scheme starting with the primary cells (brown). Red boxes indicate repopulated serial recipients, white boxes are nonengrafted mice. Only 1/4 of the primary recipients was repopulated but all 6 secondary recipients were engrafted. (**b**) Alu sequence PCR of serially transplanted recipient livers. (**c**–**e**). More than 70% of cultured hepatocytes from a tertiary mouse were positive for FAH. Phase contrast (**c**); Fah immuno stain (red) (**d**); merge (**e**). (**f**–**h**) FAH immunohistochemistry (brown stain) of serially transplanted mice liver. Primary (**f**), secondary (**g**) and tertiary (**h**) recipient livers were repopulated by human hepatocytes.

increased steadily for several more weeks (**Fig. 3b**,**c**). To further assess the maturation state of repopulating hepatocytes, we performed quantitative RT-PCR to compare gene expression levels in adult human hepatocytes, fetal human hepatocytes and chimeric FRG livers (**Fig. 3d**–**g**). For this analysis RNA was isolated from the same nine donor samples used for transplantation, from five fetal cell preparations (gestational age 16–18 weeks) and three FRG mice (5%, 30% and 70% repopulation). In all cases, genes highly expressed in adult liver were also highly expressed in FRG samples. Conversely, fetal-specific genes (e.g., alpha-fetoprotein) were suppressed in the FRG samples.

One previous report indicates that a pharmacological proteinase inhibitor is necessary to keep highly humanized mice alive. It was suggested that human complement produced by the donor hepatocytes injured recipient kidneys[7]. We therefore observed several ($n = 3$) highly repopulated mice for 4 months off NTBC. Weight and general health remained normal and their kidneys were macroscopically and histologically intact at harvest (**Fig. 2g**).

Serial transplantation of human hepatocytes

One of the limitations of the current liver xenorepopulation models is the inability to further expand engrafted human hepatocytes. To test the feasibility of serial transplantation in the FRG mouse system, we perfused the liver of a single highly repopulated primary recipient with collagenase and transplanted one million viable hepatocytes into secondary *FRG* recipients without separating the FAH-positive human and Fah-negative mouse hepatocytes (**Fig. 4**). In contrast to the cells used for primary engraftment, the viability of human hepatocytes harvested in this fashion was >80%, and they readily attached to collagen-coated culture plates (**Fig. 4c**–**e**). After engraftment of the secondary recipient, serial transplantation was continued in similar fashion into tertiary and quaternary recipients. In each generation, some, but not all, recipient mice became highly positive for blood human albumin (**Fig. 4a**). The percentage of highly repopulated mice was higher in serial transplant recipients (17/28 versus 7/43), and the rate of albumin increase was more consistent (**Fig. 3b**,**c**). This may indicate that serial passaging enriches for the most transplantable human hepatocytes or it may simply reflect the higher quality and viability of cells harvested freshly from a donor mouse. Genomic PCR of the liver samples from albumin-positive mice showed the presence of human DNA in each generation (**Fig. 4b**). Liver repopulation by human hepatocytes was also confirmed by fluorescent immunostaining against FAH (**Fig. 4c**–**e**). Histological examination showed that engrafted human hepatocytes were morphologically similar in each generation and were distinctly FAH positive (**Fig. 4f**–**h**).

Absence of cell fusion

A recent report of liver repopulation with primate cells in urokinase transgenic mice demonstrated that cell fusion could potentially account for apparent "hepatocyte repopulation"[22]. To confirm that the repopulated hepatocytes were truly human in origin, we performed double immunostaining against human- or mouse-specific albumin and FAH. Most (>95%) mouse albumin–positive hepatocytes were indeed negative for FAH, and most FAH-positive hepatocytes were negative for mouse albumin (**Fig. 5a**–**c**). On the other hand, almost all (>90%) human albumin–positive hepatocytes were also FAH-positive, whereas the rest of the hepatocytes were double-negative (**Fig. 5d**–**f**). To further confirm the lack of cell fusion, we used flow cytometry to detect human and mouse anti-major histocompatibility complex antigens. Each antibody was confirmed to be species specific (**Fig. 5g**–**j**). No hepatocytes positive for the surface markers of both species were found in highly repopulated livers (**Fig. 5k**,**l**). Finally, we also performed fluorescent *in situ* hybridization with human and mouse whole genome probes on hepatocytes from highly repopulated serial transplant recipients. In these samples, the percentages of cells positive for human or mouse DNA added up to 100%, indicating the absence of a significant double-positive population (**Supplementary Fig. 1**). Furthermore, human hepatocytes were present in the spleens of all highly repopulated mice (**Fig. 2h**) despite the fact that the spleen is devoid of murine hepatocytes that could serve as fusion partners for human cells. Taken together, these results indicate that fusion events, if they occurred, were rare and that the

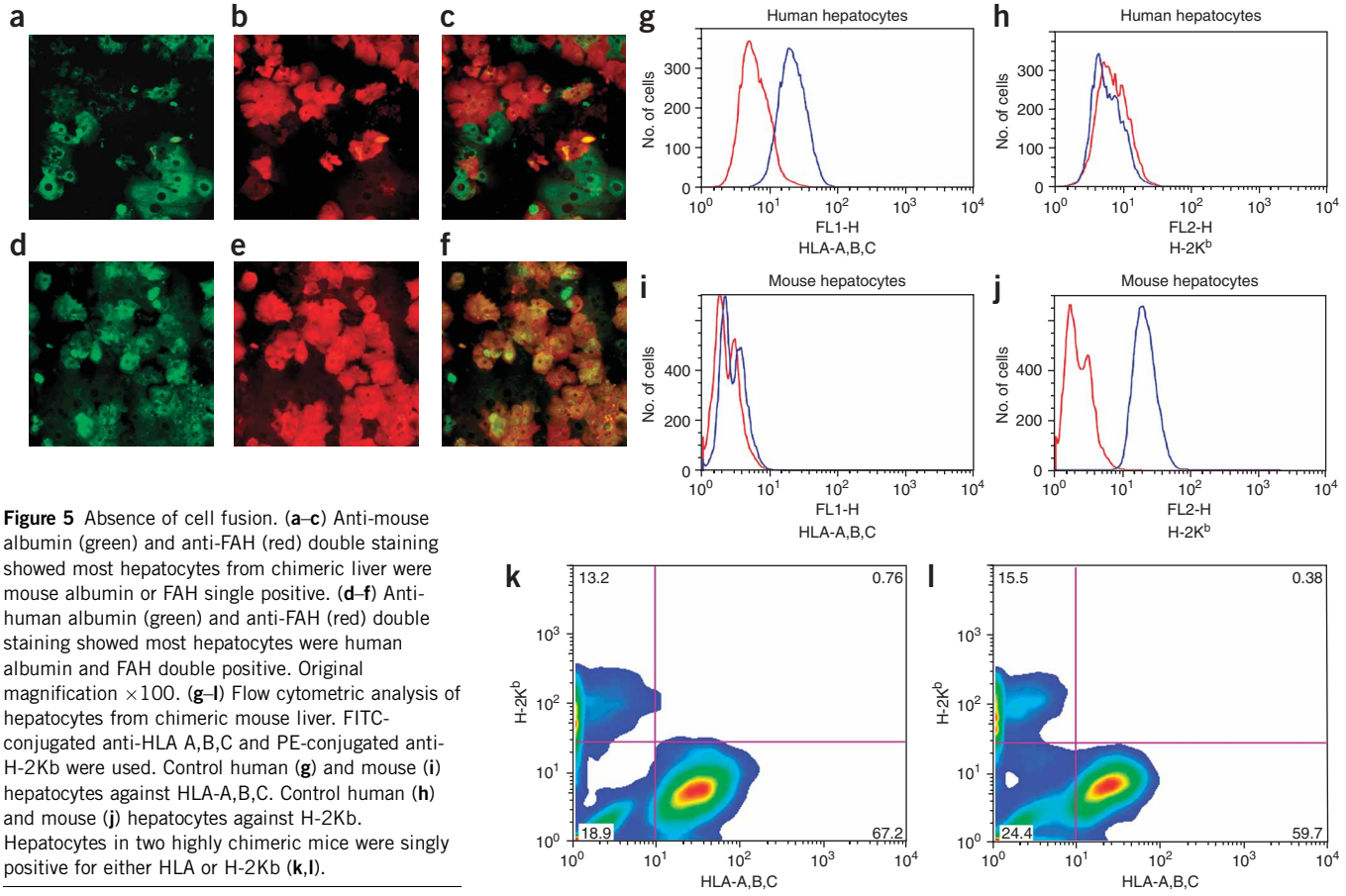

Figure 5 Absence of cell fusion. (**a**–**c**) Anti-mouse albumin (green) and anti-FAH (red) double staining showed most hepatocytes from chimeric liver were mouse albumin or FAH single positive. (**d**–**f**) Anti-human albumin (green) and anti-FAH (red) double staining showed most hepatocytes were human albumin and FAH double positive. Original magnification ×100. (**g**–**l**) Flow cytometric analysis of hepatocytes from chimeric mouse liver. FITC-conjugated anti-HLA A,B,C and PE-conjugated anti-H-2Kb were used. Control human (**g**) and mouse (**i**) hepatocytes against HLA-A,B,C. Control human (**h**) and mouse (**j**) hepatocytes against H-2Kb. Hepatocytes in two highly chimeric mice were singly positive for either HLA or H-2Kb (**k**,**l**).

majority of repopulating cells were of purely human origin even when serial transplantation was performed.

Characterization of drug metabolism in humanized mice

We examined the basal expression and induction of human liver–specific genes in chimeric mice. Notably, the levels of genes critical for drug metabolism (*CYP1A2, CYP3A4)* in repopulated mice were typical for adult human liver and different from fetal liver (**Fig. 3d**–**g**). To further test the cells' utility in drug testing, we established cultures of isolated hepatocytes and exposed them to prototypical inducers of the cytochrome P450 genes. The basal gene expression levels of cytochrome (*CYP1A1, CYP1A2, CYP2B6, CYP3A4, CYP3A7*), transporter (*ABCB11* (formerly known as *BSEP*), *ABCC2* (formerly known as *MRP2*) and drug-conjugating enzymes (*UGT1A1*) were equivalent to those found in cultured normal adult human hepatocytes (**Fig. 6c** and **Supplementary Fig. 2** online). Furthermore, the pattern of genes induced by compounds such as beta-naphthoflavone (BNF), phenobarbital and rifampicin was as expected from normal human cells (**Supplementary Fig. 2**). In addition to the mRNA expression levels of human drug metabolism genes, we measured the enzymatic activity of the human CYP1A and 3A family members. Ethoxyresorufin-*O*-deethylase activity (EROD) is known to be mediated by CYP1A1 and CYP1A2 in human liver. EROD activity was specifically and robustly induced by prior exposure to BNF in humanized mouse liver cells (**Fig. 6a**). Conversely, prior exposure to phenobarbital or rifampicin specifically induced the conversion of testosterone to 6-beta-hydroxyltestosterone, a specific measurement of CYP3A4-mediated metabolism (**Fig. 6b**). Thus, hepatocytes from repopulated FRG livers were indistinguishable from normal human adult hepatocytes in these standard drug metabolism assays.

DISCUSSION

Many recent publications have unambiguously demonstrated the value of humanized mouse livers for the study of drug metabolism and hepatitis research[7–9,21,23–29]. However, the albumin-uPA transgenic mouse used for these studies has several major practical limitations. The FRG liver humanization model described here is superior to the uPA model in several ways.

First, the extent of liver disease and selective pressure can be controlled by administering and withdrawing NTBC[12]. This simplifies animal husbandry and surgery. Second, the Fah-deficiency mutation is a deletion[10] and cannot revert back to wild-type by transgene inactivation. Therefore, competition from endogenous revertant mouse cells does not exist in *Fah* knockout livers. This means that *FRG* mice can be engrafted with human hepatocytes at any age and that serial transplantation is feasible[30]. The latter is a major advantage of our new system, because serial transplantation permits human cells of the same genotype to be expanded through several generations of recipient mice. It also means that a high-quality source of human hepatocytes for further transplantation is always in hand. Here we demonstrated that four rounds of transplantation were feasible. Based on an estimate of 10% engraftment efficiency (100,000 cells) and the final harvest of about 15 million human hepatocytes after completed repopulation, an *in vivo* expansion of at least 150-fold was achieved in each round. The total expansion was therefore at least $150^4 = 500$ million-fold. Third, FRG mice can be highly repopulated

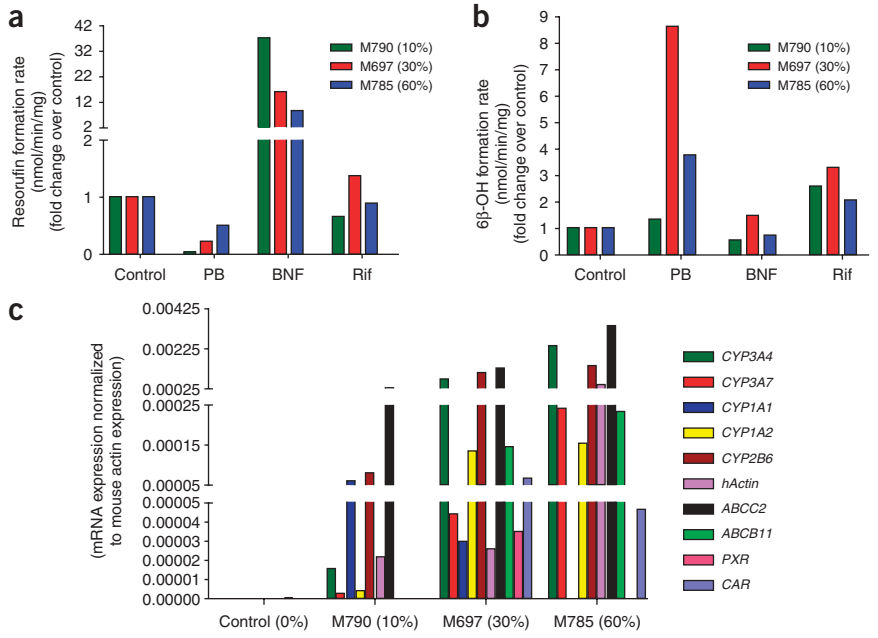

Figure 6 Drug metabolism. Cultured hepatocytes from three mice were analyzed. M790 had 10%; M697, 30%; and M785, 60% human repopulation. (**a**,**b**) Induction of drug metabolism enzymes in response to beta-naphthoflavone (BNF), phenobarbital (PB) and rifampicin (Rif). Control, noninduced cultures. EROD (CYP1A1 dependent) (**a**). Conversion of testosterone to 6-beta-hydroxyltestosterone (CYP3A4 mediated) (**b**). (**c**) mRNA levels of human-specific genes relevant to drug metabolism, transport and conjugation were determined by quantitative RT-PCR. The ratios of human drug metabolism genes are typical of adult human hepatocytes.

without administration of a complement inhibitor[7]. This is not only of practical importance in animal husbandry but also removes a potential source of pharmacological interference. This feature could be particularly important in virology applications. Finally, we are able to achieve engraftment and liver repopulation with human hepatocyte preparations of very variable quality and from donors of all ages. All of the cells used here were isolated at least 24 h before transplantation and were shipped in the mail. Despite this delay, we achieved primary engraftment from all batches. Repopulation was not limited to young cadaveric donor sources but was also obtainable from liver resection specimens and cryopreserved samples. Our oldest donor was 64 years of age. These findings indicate the robustness of the FRG xenorepopulation model and suggest that it will be useful in a wide variety of settings, including academic and commercial laboratories with no access to freshly isolated human hepatocytes.

The issue of cell fusion has not been addressed in previous reports of mouse liver repopulation with human cells. Recently it was shown that liver repopulation by embryoid body–derived monkey hepatocytes was due almost entirely to cell fusion between the murine hepatocytes and the donor cells[22]. Since uPA transgenic mice were used in that study, these findings raised the possibility that cell fusion was also the mechanism in other reports of mouse liver humanization[31]. Here we implemented three different approaches to explore the possibility of fusion between donor human hepatocytes and the FRG recipients. All three approaches consistently indicated that cell fusion was rare or nonexistent. We therefore conclude that human hepatocytes expanded in FRG mice have only human genetic and biochemical properties.

Chimeric mice generated in the uPA transgenic system have been reported by others to express basal and inducible human cytochrome P450 genes, including *CYPs 1A1, 1A2, 2D6* and 3A4 (refs. 7,25). Data presented here confirm these findings and extend the observation of human gene expression to several important new genes, including the nuclear hormone receptors, *PXR* and *CAR*, which regulate the expression of many liver-enriched genes. In addition, we found expression of genes involved in drug conjugation and detoxification, including several of the hepatocyte transporter proteins. Recent studies have shown the critical role played by these conjugation pathways[32] and hepatocyte transporter proteins[33] in predicting drug toxicity. As the human hepatocytes in our chimeric mice show a normal human response to CYP induction by drugs such as rifampicin, phenobarbital and BNF, and express hepatic nuclear hormone receptor transcription factors, conjugation pathways and major transport proteins, they open the possibility of assessing the role of these gene products in human drug metabolism and toxicity *in vivo*.

Despite its advantages, further improvements of the FRG liver humanization model can be envisioned. Primary engraftment did not occur in 100% of recipients, even with urokinase adenovirus pre-administration. The reasons for this stochastic behavior are not clear. However, we speculate that hepatic macrophages present in normal numbers in FRG mice may limit human cell engraftment through an innate immune response. Macrophage depletion using chemicals[34] or antibodies[35] may further enhance repopulation by human cells. It is also possible that the background strain of the mice (C57/BL6) is suboptimal for xenorepopulation[17]. Although further improvements of the FRG model are likely, we have demonstrated here that human hepatocytes expanded in this system are useful for common drug metabolism studies. We expect that humanized FRG mice will be useful for the same broad spectrum of pharmacological and virological studies as the existing models.

METHODS

Generation of *Fah*$^{-/-}$/*Rag2*$^{-/-}$/*Il2rg*$^{-/-}$ mice. Male *Fah*$^{-/-}$129S4 mice[10] were crossed with female *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ mice (Taconic). All animals were maintained with 2-(2-nitro-4-trifluoro-methyl-benzoyl)-1,3 cyclohexanedione (NTBC)-containing drinking water at a concentration of 16 mg/l[12]. PCR-based genotyping was carried out on 200 ng genomic DNA isolated from toe tissue as previously described[10,16].

Isolation of human hepatocytes. Human hepatocytes were isolated from donor livers that were not used for liver transplantation. The details of the isolation method have been described previously[36,37] with modifications as outlined in ref. 38. Pelleted hepatocytes were transferred into cold University of Wisconsin solution (Viaspan). Cell isolation procedures were carried out at the University of Pittsburgh and the cells were shipped by overnight express mail to Oregon Health and Science University. Cryopreservation of human hepatocytes was done as previously described[7].

Transplantation of human hepatocytes. All procedures performed on the animals were approved by the Institutional Animal Care and Utilization Committee at Oregon Health & Science University. The adenoviral vector expressing the secreted form of human urokinase (uPA) was previously

described[18]. Transplant recipients were injected intraperitoneally with 5×10^9 p.f.u. as soon as a cell shipment was announced. Thus shipped hepatocytes were transplanted immediately upon arrival, that is, 24–48 h after isolation. Cell number and viability were determined by Trypan blue exclusion in a hemocytometer. One million viable hepatocytes in 100 µl of Dulbecco's modified essential medium were injected intrasplenically via a 27-gauge needle. The concentration of NTBC was gradually decreased (1.6 mg/l, day 0–2; 0.8 mg/l, day 3–4; 0.4 mg/l, day 5–6) and completely withdrawn one week after transplantation.

Serial transplantation. Cells from repopulated primary recipients were harvested with a standard collagenase perfusion protocol. Briefly, the liver was perfused with calcium- and magnesium-free Earle's balanced salt solution (EBSS) supplemented with 0.5 mM EGTA and 10 mM HEPES for 5 min. The solution was changed to EBSS supplemented with 0.1 mg/ml collagenase XI (Sigma) and 0.05 mg/ml DNase I (Sigma) for 10 min. The liver was gently minced in the second solution and filtered through 70 µm and 40 µm nylon mesh sequentially. After 150*g* centrifugation for 5 min, the pellet was washed twice at 50*g* for 2 min. The number and viability of cells were assessed by the Trypan blue exclusion test. One million viable cells suspended in 100 µl DMEM were injected into recipient spleen via 27-gauge needle.

Histology and immunocytochemistry. FAH immunohistochemistry was done as previously described[39]. The HepPar antibody (DAKO) was used according to the manufacturer's specifications. For immunocytochemistry hepatocytes from humanized mouse livers were plated on collagen type1–coated 6-well plates. Attached cells were fixed with 4% paraformaldehyde for 15 min and blocked with 5% skim milk. Rabbit anti-FAH (Grompe lab), goat anti-human albumin (Bethyl), goat anti-mouse albumin (Bethyl) were used as primary antibodies at dilution of 1/200. Alexa Fluoro 488 anti-goat IgG (Invitrogen) or Alexa Fluoro 555 anti-rabbit IgG (Invitrogen) were used as secondary antibodies. The images were captured with an Axiovert 200 microscope and a Nikon digital camera.

FAH enzyme assay. Fumarylacetoacetate was incubated with cytosolic liver fractions from recipient liver, and disappearance speed was measured spectroscopically at 330 nm. Wild-type and *Fah*$^{-/-}$ livers were used as positive and negative control, respectively. Fumarylacetoacetate is not commercially available and was prepared enzymatically from homogentisic acid[20].

Genomic PCR for Alu sequences. Genomic DNA was isolated from the liver using the DNeasy tissue kit (Qiagen). Human Alu sequences were amplified by PCR with the following primers; 5′-GGCGCGGTGGCTCACG-3′ and 5′-TTTTTTGAGACGGAGTCTCGCTC-3′.

Human albumin measurement. Small amounts of blood were collected once a week from the left saphenous vein with a heparinized blood capillary. After 1,000 or 10,000× dilution with Tris-buffered saline, human albumin concentration was measured with the Human Albumin ELISA Quantitation Kit (Bethyl) according to the manufacturer's protocol.

FACS analysis. After dissociation of the recipient livers, parenchymal cells were incubated at 4 °C for 30 min with FITC-conjugated anti-human HLA-A,B,C (BD Pharmingen) and PE-conjugated anti-mouse H2-K(b) (BD Pharmingen) antibodies. They were then rinsed with PBS twice and analyzed with a FACS Calibur (Becton Dickinson). FITC-conjugated and PE-conjugated IgG were used as negative controls.

Drug metabolizing enzymes in cultured hepatocytes. EROD is mediated by CYP1A1 and 1A2 in human liver. Conversely prior exposure to phenobarbital or rifampicin specifically induces the conversion testosterone to 6-beta-hydroxytestosterone, a specific measurement of CYP3A4 mediated metabolism. Hepatocyte isolation and culture was conducted as described in detail by Strom *et al.*[37]. Testosterone metabolism and EROD activity on cultured hepatocytes were conducted as described by Kostrubsky, *et al.*[40] and Wen *et al.*[41], respectively. Briefly, hepatocytes were isolated from control, non-transplanted mice or those showing repopulation with human hepatocytes using a standard collagenase perfusion protocol[42]. Cells were then plated in HMM media (hepatocyte maintenance media; Lonza, USA) at a density of 1.5 million cells per well in collagen I -coated 6 well plates. Cells were cultured for 48 h without inducers and then for 48 additional hours in the presence of 1 mM Phenobarbital, 10 µM rifampicin or 25 µ M beta-naphthoflavone as previously described[40,41]. Following the induction period, cells were washed 3 times with PBS to remove inducers, and media was replaced with fresh media (without inducers). For the measurement of CYP1A activity, cells were exposed to media containing 20 µM 7-ethoxyresorufin and 1.5 mM salicylamide for 30 min. The conversion of 7-ethoxy to 7-hydroxyresorufin in the media was quantified by the fluorescence of the 7-hydroxy metabolite measured at 535 nm (Ex) and 581 nm (Em). Salicylamide was used to prevent the conjugation of resorufin formed by de-ethylation. The analysis of CYP3A activity was measured by exposing cultures of cells to 350 µM testosterone for 30 min. The conversion of testosterone to 6β-hydroxytestosterone was measured by a high-performance liquid chromatographic method (HPLC) as previously described by Kostrubsky *et al.*[40].

CYP3A (testosterone) and CYP1A (EROD) activities were measured in cells from both nontransplanted (control FAH mice) and from the mice with humanized liver. Control, FAH mice displayed low levels of metabolic activity and these activities were subtracted from the values presented in **Figure 6**. The results show that EROD activity was specifically induced by prior exposure to BNF, whereas phenobarbital and rifampicin specifically induced CYP3A activity.

RT-PCR for hepatocyte specific gene expression. Total RNA was isolated from the liver using the RNeasy mini kit (Qiagen). Complementary DNA was synthesized by reverse transcriptase with a oligo dT primer. The following primers were used for 30 cycles of human or mouse specific cDNA amplification; 5′-ATGGATGATTTCGCAGCTTT-3′ (human *ALB* forward), 5′-TGGCTTTACACCAACGAAAA-5′ (human *ALB* reverse), 5′-TACAGCGGAGCAACTGAAGA-3′ (mouse *Alb* forward), 5′-TTGCAGCACAGAGACAAGAA-3′ (mouse *Alb* reverse), 5′-CCGGGAGAGTTTTACCACAA-3′ (human *TAT* forward), 5′-CCTTCCCTAGATGGGACACA-3′ (human *TAT* reverse), 5′-CTGACCTCACCTGGGACAAT-3′ (human *TF* forward), 5′-CCTCCACAGGTTTCCTGGTA-3′ (human *TF* reverse), 5′-TTTGGGACCACTGTCTCTCC-3′ (human *FAH* forward), 5′-CTGACCATTCCCCAGGTCTA-3′ (human *FAH* reverse), 5′-ATGGCTTCTCATCGTCTGCT-3′ (human *TTR* forward), 5′-GCTCCTCATTCCTTGGGATT-3′ (human *TTR* reverse), 5′-GTGCCTTTATCACCCATGCT-3′ (human *UGT1A1* forward), 5′-TCTTGGATTTGTGGGCTTTC-3′ (human *UGT1A1* reverse).

For quantitative gene expression analysis in cultured hepatocytes and repopulated liver, the RNA isolation, cDNA synthesis and real-time PCR were also conducted as described in reference 38, with primers obtained from Applied Biosystems. Hepatocytes were cultured under the conditions described for drug-induction studies above. Primers used in these studies were specific for human *CYP1A1* (Hs00153120_m1), *CYP1A2* (Hs00167927_m1), *CYP3A4* (Hs00430021_m1), *CYP3A7* (Hs00426361_a1), CYP2B6 (Hs00167937_g1), CYP2D6 (Hs00164385_a1), Multidrug resistance–associated protein *ABCC2* (Hs00166123_m1), Bile Salt export Pump *ABCB11*, (Hs00184829_m1), *CAR* (Hs00231959_m1) *ALB* (Hs00609411_m1), *HNF4α* (Hs00230853_m1), P_{pid} cyclophilin (Hs99999904_m1), mouse[38] (Ma00607939_s1).

Note: Supplementary information is available on the Nature Biotechnology website.

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AUTHOR CONTRIBUTIONS

H.A., primary experimenter, performed all transplantations and analyzed repopulated mice; N.P., FISH in Supplementary Figure 2; A.R., E.E., drug metabolism and drug metabolism gene expression; C.D., FACS analysis; M.A.-D., mouse breeding and transplantation assistance; S.S., provided human hepatocytes and supervised drug metabolism work; M.A.K., provided urokinase adenovirus; M.F., all tissue histology; M.G., overall project planning and coordination.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturebiotechnology/.

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