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Cell Injury, Repair, Aging and Apoptosis

A Mouse Model of Inducible Liver Injury Caused by Tet-On Regulated Urokinase for Studies of Hepatocyte Transplantation

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Mouse models of liver injury provide useful tools for studying hepatocyte engraftment and proliferation. A representative model of liver injury is the albumin-urokinase (Alb-uPA) transgenic model, but neonatal lethality hampers its widespread application. To overcome this problem, we generated a transgenic mouse in which transcription of the reverse tetracycline transactivator was (rtTA) driven by the mouse albumin promoter, and backcrossed the rtTA mice onto severe combined immunodeficient (SCID)/bg mice to generate immunodeficient rtTA/SCID mice. We then produced recombinant adenoviruses Ad.TRE-uPA, in which the urokinase was located downstream of the tetracycline response element (TRE). The rtTA/SCID mouse hepatocytes were then infected with Ad.TRE-uPA to establish an inducible liver injury mouse model. In the presence of doxycycline, uPA was exclusively expressed in endogenous hepatocytes and caused extensive liver injury. Enhanced green fluorescent protein-labeled mouse hepatocytes selectively repopulated the rtTA/SCID mouse liver and replaced over 80% of the recipient liver mass after repeated administration of Ad.TRE-uPA. Compared with the original uPA mice, rtTA/SCID mice did not exhibit problems regarding breeding efficiency, and the time window for transplantation was flexible. In addition, we could control the extent of liver injury to facilitate transplantation surgery by regulating the dose of Ad.TRE-uPA. Our inducible mouse model will be convenient for studies of hepatocyte transplantation and hepatic regeneration, and this system will facilitate screening for potential genetic factors critical for engraftment and proliferation of hepatocytes *in vivo*. *(Am J Pathol 2009, 175:1975–1983; DOI: 10.2353/ajpath.2009.090349)*

Hepatocyte transplantation has been proposed as an alternative therapy to orthotropic liver transplantation for patients with acute liver failure and metabolic disorders. Compared with orthotropic liver transplantation, hepatocyte transplantation is less expensive and less invasive.[1,2] Furthermore, hepatocytes from one donor can benefit multiple patients, partly compensating for the shortage of livers available for transplantation.[3] Some clinical trials of hepatocyte transplantation have demonstrated partial improvement of liver function, but transplanted hepatocytes cannot rescue patients because of inadequate levels of engraftment.[1,4,5] In addition, there has been little evidence of proliferation of transplanted hepatocytes in patient livers.[1] Therefore, more studies are required to investigate methods of improving the repopulation level of transplanted hepatocytes.

Mouse models of liver injury provide useful tools for studying hepatocyte engraftment and proliferation.[6–10] A representative model is the albumin–urokinase-type plasminogen activator (Alb-uPA) mouse, which has been widely used for hepatocyte transplantation.[6,11,12] Alb-uPA mice were initially developed to study hemorrhagic

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and thrombotic disorders.[13] Unexpectedly, uPA is hepatotoxic and causes severe liver injury via intracellular activation of the uPA substrate plasminogen.[14,15] Neonatal hemorrhaging and liver injury leads to the death of half of the hemizygous transgenic mice and to the death of almost all of the homozygous mice shortly after birth.[13,14] The mice breed so poorly that it is difficult to obtain adequate numbers for experiments. Additionally, the transplantation time window is immobile. In Alb-uPA mice, some hepatocytes delete the transgene through recombination and proliferate rapidly, restoring the entire liver by 8 to 12 weeks after birth.[14] To avoid competing with recipient hepatocytes, donor hepatocytes must be transplanted as soon as possible. Therefore, transplantation is usually performed within the first two weeks after birth.[6,11]

Despite the disadvantages of the original Alb-uPA mouse model, the uPA gene may be the most suitable toxic gene to produce an ideal liver injury model. uPA is involved in multiple physiological and pathological processes. In liver regeneration, uPA activates plasminogen, which removes necrotic cell debris and degrades the extracellular matrix to promote reorganization of the hepatic architecture.[15–17] In urokinase-deficient mice, liver regeneration is transiently impaired, suggesting that uPA participates in initiating hepatocyte proliferation.[18] In addition, hepatocyte growth factor can be activated by uPA, which is a prominent hepatic mitogen.[19,20] These studies suggest that uPA facilitates the engraftment and proliferation of transplanted hepatocytes.

To overcome the disadvantages of the original Alb-uPA model, an inducible uPA model would be a better strategy. The tetracycline-regulated gene expression system is broadly used to conditionally express genes *in vivo* and *in vitro*,[21,22] including both the "tet-on" and "tet-off" systems. The tet-on system is composed of two elements: a reverse tetracycline-controlled transactivator (rtTA), and a tetracycline-response element (TRE). In the presence of doxycycline (Dox), rtTA binds to TRE and activates the transcription of target genes. An adenoviral vector, which predominantly and effectively infects liver infused by tail vein,[23,24] has been used to deliver the tet system.[25,26] In addition, adenoviral vectors do not integrate into the genome of target cells, and more importantly, adenoviral vectors are able to repeatedly infect immunosuppressed mice.[27,28]

Here, we generated transgenic mice that specifically expressed rtTA in the liver via the mouse albumin promoter, and backcrossed the rtTA mice on severe combined immunodeficient (SCID)/bg mice to generate immunodeficient rtTA/SCID mice. We next delivered TRE-uPA to the transgenic mice via an adenoviral vector to establish an inducible uPA mouse model, in which uPA was specifically expressed in endogenous hepatocytes and caused extensive liver injury. We subsequently transplanted enhanced green fluorescent protein (EGFP) transgenic mouse hepatocytes into the model to determine whether the model could be reconstituted by transplanted hepatocytes.

Materials and Methods

Plasmid Construction and Generation of Alb-rtTA2S-M2 Transgenic Mice

The coding sequence rtTA2S-M2 and sv40 poly(A) was excised from the pUHrt62-1 plasmid (a kind gift from Prof. Hillen and Prof. Bujard Germany) with *EcoR* I and *Hind* III and then cloned into plasmid pbluscript sk(+) via *EcoR* I and *Hind* III to generate plasmid pSK-rtTA2S-M2. A 2.3-kb mouse albumin promoter and enhancer fragment was excised from pAlb-EGFP (a kind gift from Dr. Ochiya Japan) by *Sac* I and *BamH* I (blunted with Klenow) and inserted upstream of rtTA2S-M2 via *Sac* I and *EcoR* I (blunted with Klenow) to generate pSK-Alb-rtTA2S-M2.

The 3.5-kb Alb-rtTA2S-M2-poly(A) fragment was isolated by restriction digest with *Sac* I and *Kpn* I and microinjected into the fertilized eggs (CD1 × CD1 Vital River China) to produce transgenic mice. Founder animals and their progenies were identified by PCR analysis of genomic DNA obtained from tail biopsies. PCR analysis was performed in 25-μl reaction mixtures. The primers for Alb-rtTA2S-M2 (forward: 5′-GCTCCAGATGGCAAACATACGC-3′, reverse: 5′-TTCCTCCAATACGCAGCCCAGT-3′) were designed to amplify a 645 bp region. Primers for mouse GAPDH (forward: 5′-GGGTGGAGCCAAAAGGGTCATC-3′, reverse: 5′-AGAGGGGCCATCCACAGTCTTC-3′) were used to amplify a 372bp region as internal control. Amplification was performed on a thermocycler for 5 minutes at 94°C, 30 cycles of 30 seconds at 94°C, 30 seconds at 57°C, 30 seconds at 72°C, and a final 10-minute extension at 72°C.

Production of Recombinant Adenovirus Ad.TRE-uPA

Mouse uPA (NM_008873) was amplified from mouse kidney by reverse transcription (RT)-PCR using primers (forward: 5′-GGCGCTAGCCACC ATGAAAGTCTGGCTGGCGAG-3′ reverse: 5′-TCTGGTACCGAGAGGACGGTCAGCATGGG-3′). PCR conditions were 5 minutes at 94°C, 30 cycles of 30 seconds at 94°C, 30 seconds at 57°C, 30 seconds at 72°C, and a final 10-minute extension at 72°C. A 1.4-kb uPA fragment was cloned into T vector, and sequenced. The *Nhe* I-*Kpn* I of uPA was inserted into pTRE-Shuttle via *Nhe* I and *Kpn* I. The TRE-uPA cassette was excised from pTRE-uPA by restriction digest with PI-*Sce* I and I-Ceu I and ligated into the E1/E3-deleted adenovirus type 5 vector (Vector Laboratories, Burlingame, CA) to generate Ad.TRE-uPA, which was lined with *Pac* I and transfected into HEK 293 cells that contain E1 of the adenovirus to package recombinant adenovirus. Recombinant adenoviruses were harvested, purified by CsCl ultracentrifugation and titered by plaque assay. The viral titer of Ad.TRE-uPA was 5×10^{10} plaque-forming units/ml. Primers for Ad.TRE-uPA (forward: 5′-GTCGAGTAGGCGTGTACGGT-3′ reverse: 5′-CCATTTCCATGATAGCAGGT-3′) were used to amplify a 407-bp region of TRE-uPA. PCR conditions included denaturation at 94°C for 4 minutes, followed by 30 cycles at 94°C for 30 sec-

onds, 57°C for 30 seconds, 72°C for 30 seconds, and a final extension of 72°C for 10 minutes.

RT-PCR

Total RNA was isolated from mouse tissues using the Trizol reagent (Invitrogen, Carlsbad, CA). Residual genomic DNA was removed with DNase (Promega, Madison, WI), and the RNA was then reverse transcribed to cDNA using oligo d(T) primers with a reverse transcription system (Promega), according to the protocol provided by the manufacturer. Primers for rtTA2S-M2 (forward: 5′-CAAGTCATTCCGCTGTGCTCTC-3′ reverse: 5′-TCCAAACTCATCAATGTATCTTATC-3′) were used to amplify a 544-bp region of rtTA2S-M2. Primers for uPA (forward: 5′-GCTGTCAGAACGGAGGTGTA-3′, reverse: 5′-TTGGGAGTTGAATGAAGCAG-3′) were used to amplify a 600-bp region of uPA. Primers for β-actin (forward: 5′-CTGACCCTGAAGTACCCCATTGAAC-3′, reverse: 5′-TGTGTTGGCATAGAGGTCTTTACGG-3′) were used to amplify a 699-bp region as an internal control. PCR conditions included denaturation at 94°C for 4 minutes, followed by 30 cycles at 94°C for 30 seconds, 57°C for 30 seconds, 72°C for 30 seconds, and a final extension of 72°C for 10 minutes.

Western Blot Analysis of rtTA2S-M2

Total protein was extracted from livers using RIPA buffer (Beyotime, Shanghai, China), separated in 12% SDS-polyacrylamide gel, and transferred to a nitrocellulose filter. Membranes were immunoblotted with an anti-TetR mouse monoclonal antibody (MoBiTec, Göttingen, Germany) at a dilution of 1:1000, revealed with a peroxidase-conjugated anti-mouse IgG (Zymed Laboratories, San Francisco, CA). All immunoblots were detected by chemiluminescence (Amersham, Buckinghamshire, England) and normalized with an anti-β-actin antibody (Santa Cruz, CA).

Generation of Alb-rtTA2S-M2/SCID/bg Mice

All animal procedures followed the guidelines of the Institutional Animal Care and Use Committees of Peking University. The Tg2-10 line was crossed with SCID/bg mice (Vital River Inc., Beijing, China), and Alb-rtTA2S-M2 positive mice were selected from the offspring by genomic PCR and backcrossed with SCID/bg mice 5 to 7 times to generate Alb-rtTA/SCID/bg mice. Genotyping of the SCID/bg mice was performed as previously described.[29] Briefly, the amplified DNA samples were treated with the restriction enzyme AluI. Samples without the mutation exhibited 64-bp-long bands, and samples with the mutation displayed 38- and 26-bp-long bands.

Immunohistochemical Analysis of uPA Expression

Liver tissues obtained from the mice were embedded in OCT compound (Sakura Finetek, Tokyo, Japan) and frozen in liquid nitrogen. The frozen specimens were cut into 5-μm sections with the microtome portion of the cryostat (Leica CM 1850, Germany). The slides were incubated with 10% goat blocking serum (Zymed Laboratories) in PBS for 30 minutes to suppress nonspecific binding of IgG and were then incubated with rabbit polyclonal anti-uPA antibody (Bethyl, Montgomery, TX) diluted 1:200 in blocking solution for 1 hour at 37°C in a humidified chamber. The slides were then washed three times with PBS and incubated with tetramethylrhodamine B isothiocyanate-labeled goat anti-rabbit immunoglobulins (Zymed Laboratories) at a dilution of 1:200 in PBS for 1 hour at room temperature in the dark. The slides were washed three times with PBS, incubated with 4,6-diamidino-2-phenylindole (Roche, San Diego, CA) for 5 minutes at room temperature and washed three times with PBS. Confocal images were acquired using a Nikon confocal laser scanning microscope equipped with an ORCA CCD camera (Hamamatsu Photonics, Shizuoka, Japan).

Histological Assessments

Tissues were fixed with 4% formaldehyde for 1 day and washed with PBS. They were embedded in paraffin, and 10-μm serial sections were cut and stained with H&E.

Measurement of uPA and Transaminase Activity in the Blood

Blood was collected by retro-orbital puncture, using a one-tenth volume of 0.1 M/L sodium citrate as an anticoagulant, at different time points after Ad.TRE-uPA treatment. Samples were centrifuged at 2000 $\times$ *g* for 10 minutes to separate the plasma and centrifuged at 8000 $\times$ *g* for 10 minutes to separate serum. Serum alanine aminotransferase (ALT) activity was measured using a transaminase assay kit (Baixiang, Shanghai, China) and uPA was measured using a mouse uPA total antigen assay (Innovative, Novi, MI) according to the manufacturer's protocol. Data are expressed as means $\pm$ SD.

Hepatocyte Isolation and Cell Transplantation Design

Six- to eight-week-old rtTA/SCID mice and nontransgenic mice were treated with 2.5×10^9 plaque forming units (pfu) Ad.TRE-uPA via tail vein injection and with 1 mg/ml Dox in their drinking water. About 24 hours later, EGFP hepatocytes were isolated from 6- to 8-week-old EGFP transgenic CD1 mice using a two-step EDTA/collagenase perfusion.[30] Cell viability was over 80% as determined by trypan blue exclusion. Recipients were anesthetized with tribromoethanol, and through a small left-flank incision, 5×10^5 viable hepatocytes suspended in 100 μl Dulbecco's Modified Eagle Medium (DMEM) were injected into the inferior splenic pole.

The injection site was tied with a line for hemostasis, and the incision was closed. After transplantation, 5×10^9 pfu Ad.TRE-uPA were administered via tail vein every 7 days to promote proliferation of the transplanted hepatocytes.

Monitoring the Repopulation of EGFP Hepatocytes in rtTA/SCID Mice

Two weeks after each Ad.TRE-uPA injection, that is, 2 weeks, 3 weeks, 4 weeks, 5 weeks, and 6 weeks after transplantation, livers (n = 10 to 14 per time point) were harvested and green fluorescence microscopy was performed on cryosections (5 μm) to detect EGFP-positive cells. Three random sections from each liver lobe of each mouse were analyzed. Sections were fixed with 4% paraformaldehyde for 20 minutes, and then washed three times with PBS, incubated with 4,6-diamidino-2-phenylindole for 5 minutes, and washed three more times with PBS. The number of EGFP cells was quantified in 10 view fields/section ($\times$200 magnification) and the extent of liver repopulation by transplanted EGFP cells was estimated with the assistance of a computer-assisted image analyzer.

Results

Generation of Alb-rtTA2S-M2/SCID/bg (rtTA/SCID) Mice and the Ad.TRE-uPA Recombinant Adenovirus

The experimental design is outlined in Figure 1A. In the present study, an improved rtTA mutant, rtTA2S-M2, driven by the mouse albumin promoter,[31,32] was used to generate the transgenic mice. The rtTA2S-M2 mutant was more stable and more sensitive to Dox than the original rtTA, and it demonstrated low basal activity.[22] After the DNA construct was microinjected into fertilized eggs, four transgenic lines (Tg1-5, Tg2-9, Tg2-10, and Tg4-6) were identified using PCR analysis (Figure 1B). RT-PCR results were obtained for diverse tissues (heart, lung, liver, spleen, pancreas, and kidney), and inspection of these tissues revealed that rtTA2S-M2 was exclusively expressed in the livers of all four transgenic lines (Figure 1C). Western blot analysis confirmed the expression of rtTA2S-M2 in Tg2-10 (Figure 1D). Semiquantitative PCR showed that the expression level of rtTA2S-M2 was highest in Tg2-10 (data not shown). Therefore, Tg2-10 was chosen for further studies. To accept exogenous hepatocytes, Tg2-10 was crossed with SCID/bg mice to obtain immunodeficient rtTA/SCID mice via five to seven selective backcrosses.

We totally obtained about 350 transgenic positive rtTA/SCID mice. All of these mice survived after birth and had no problem of viability. In the 350 transgenic offspring, about 150 were used for experiments and 200 for further breeding. All of the offspring used for breeding had reproductive capacity. On the contrary, the original albumin-uPA mice have poor breeding efficiency, of which approximately one half or two thirds die shortly after birth due to neonatal hemorrhaging[13,14] and infertility exists in homozygous uPA/SCID mice.[33]

The target gene uPA was cloned into pShuttle-TRE to generate the responsive element TRE-uPA, which was then inserted into an adenovirus type 5 vector (Ad5ΔE1ΔE3) to generate adenovirus Ad.TRE-uPA. The

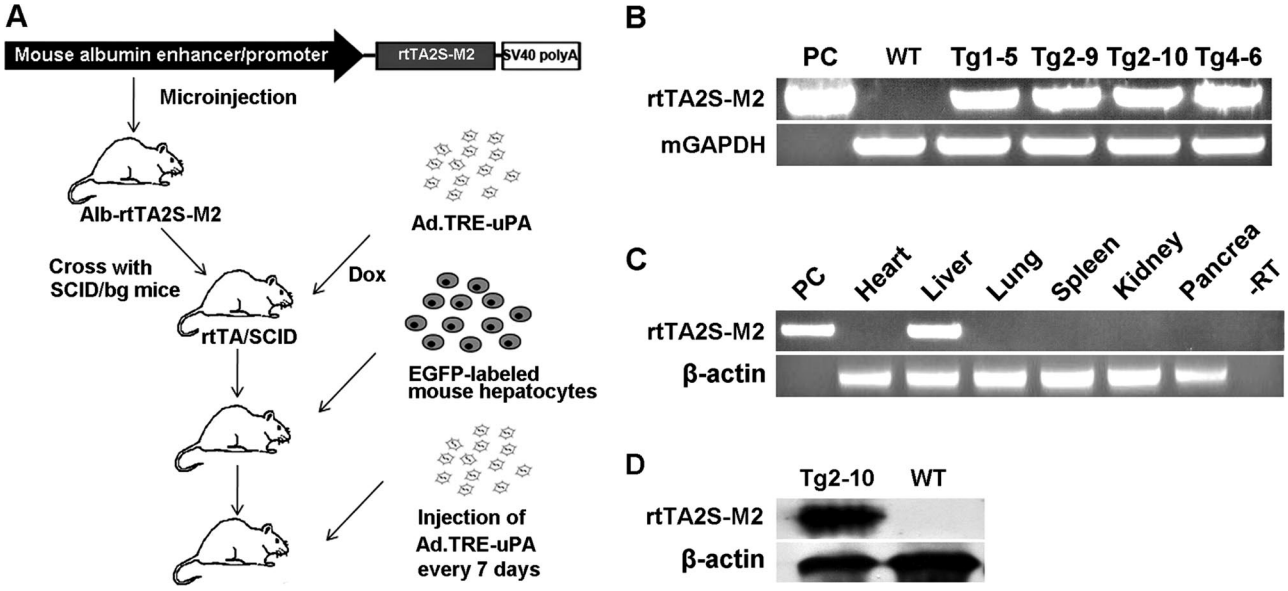

Figure 1. Experimental design and analysis of Alb-rtTA2S-M2 transgenic mice **A**: Experimental design used to characterize Ad.TRE-uPA recombined adenovirus-mediated liver injury in Alb-rtTA2S-M2/SCID/bg mice, which were used for hepatocyte transplantation. **B:** PCR analysis of the Alb-rtTA2S-M2 transgenic mouse line. Four lines were identified after microinjection. PC: plasmid pAlb-rtTA2S-M2, WT: wild-type mice. **C:** RT-PCR analysis of rtTA2S-M2 mRNA expression in different organs of the Tg2-10 transgenic line. Note that rtTA2S-M2 mRNA was detected exclusively in the liver. β-actin served as an internal control. PC: plasmid pAlb-rtTA2S-M2. –RT: RNA from the liver of Tg2-10 without reverse transcription. **D:** Western blot analysis of liver samples showing the expression of rtTA2S-M2 in Tg2-10, but not in wide type mice. β-actin used as an internal control of the loaded amounts of liver proteins. WT: wild-type mice.

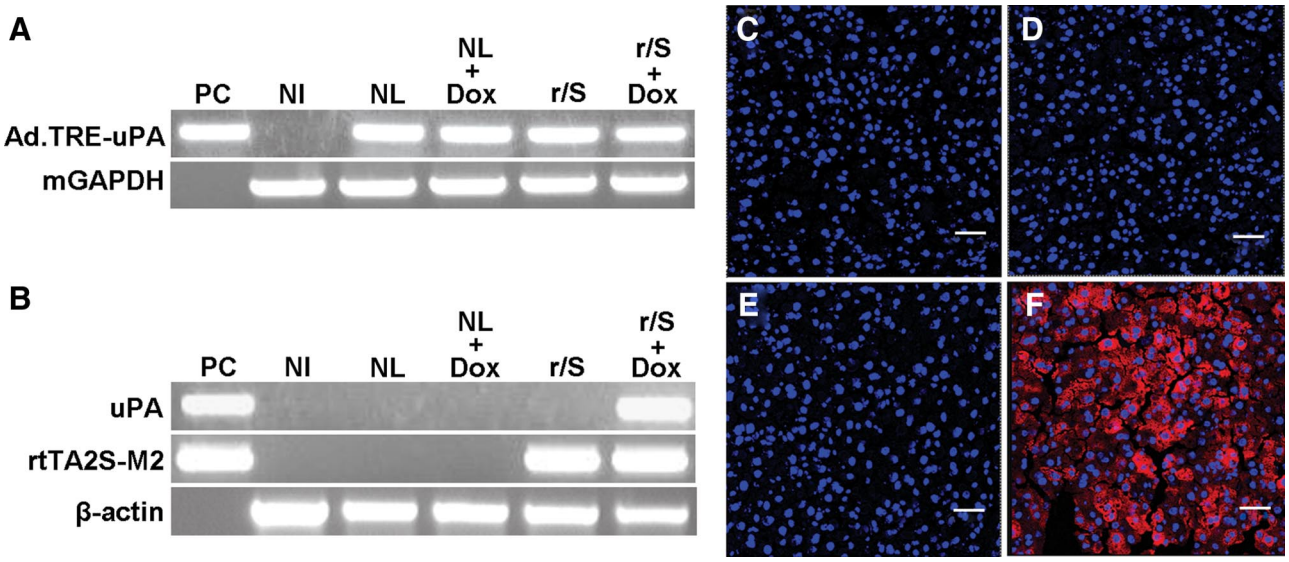

Figure 2. Analysis of uPA expression. Four groups of mice, (**C**) NL, nontransgenic littermates; (**D**) NL+Dox, nontransgenic littermates treated with doxcycline; (**E**) r/S, Alb-rtTA2S-M2/SCID/bg mice; (**F**) r/S+Dox, Alb-rtTA2S-M2/SCID/bg mice treated with doxycycline, were administered Ad.TRE-uPA (5×109 pfu/mouse). Two days later, the livers were removed for analysis. **A:** PCR amplification of the Ad.TRE-uPA in DNA samples from mouse livers showed that all four groups of mice were infected with the Ad.TRE-uPA. Mouse GAPDH served as an internal control. PC, plasmid pAd.TRE-uPA. NI, DNA samples from nontransgenic littermates not infected with Ad.TRE-uPA. **B:** RT-PCR analysis to examine the expression of uPA mRNA revealed that only rtTA/SCID mice treated with Dox expressed uPA. β-actin served as an internal control. PC, plasmid pAd.TRE- uPA and plasmid pAlb-rtTA2S-M2. NI, cDNA samples from nontransgenic littermates not infected with Ad.TRE-uPA. **C–F:** Immunofluorescent staining for uPA. The nuclei were counterstained with 4,6-diamidino-2-phenylindole (blue). Note that only rtTA/SCID mice treated with Dox (**F**) showed more than 90% of hepatocytes expressing uPA (red), while the other three groups (**C–E**) exhibited no basal uPA staining. Scale bars = 40 μm.

recombinant adenovirus was administered via tail vein injection into rtTA/SCID mice to generate a Tet-on–regulated uPA mouse model.

Liver-Specific Expression of uPA in rtTA/SCID Mice Regulated by Dox

To ensure that the recipient hepatocytes were destroyed by inducible uPA and that the transplanted hepateocytes selectively proliferated, we assessed whether the expression of uPA was restricted to Alb-rtTA2S-M2 transgenic hepatocytes. Mice that were 6 to 8 weeks old were divided into four groups: nontransgenic littermates, nontransgenic littermates with Dox, rtTA/SCID mice, and rtTA/SCID mice with Dox. All mice were injected via tail vein with 5×10^9 pfu Ad.TRE-uPA, which is enough to transduce 90% of hepatocytes.[34] After two days, livers were removed from the treated mice for analysis. PCR results showed that all mice were infected with Ad.TRE-uPA (Figure 2A). RT-PCR demonstrated that uPA mRNA was specifically expressed in rtTA/SCID mice treated with Dox, but no uPA mRNA was detected in the other groups (Figure 2B). Liver sections were immunohistochemically stained for uPA. The results showed that over 90% of the hepatocytes expressed uPA in the liver of rtTA/SCID mice treated with Dox (Figure 2F), whereas no uPA staining was detected in the other three groups (Figure 2, C–E), which was consistent with the RT-PCR results. Nontransgenic littermates treated with Dox and injected with Ad.TRE-uPA demonstrated no basal expression of uPA, suggesting that Ad.TRE-uPA administration was unable to induce uPA expression in hepatocytes lacking rtTA2S-M2.

Specific Ablation of Hepatocytes in rtTA/SCID Mice by Inducible uPA

To examine the damage caused by the inducible uPA, rtTA/SCID mice and nontransgenic littermates were injected with 5×10^9 pfu Ad.TRE-uPA via tail vein and administered Dox (1 mg/ml) in their drinking water. Both groups of mice were sacrificed 4 days later for analysis.

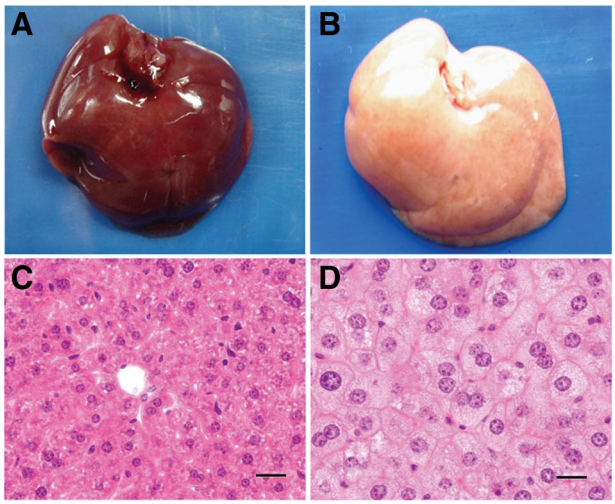

Figure 3. Histological analysis of liver injury. Four days after Ad.TRE-uPA and Dox treatment, liver sections from nontransgenic littermates and rtTA/SCID mice were stained with H&E. **A:** The liver from nontransgenic littermate. **B:** The liver from rtTA/SCID mouse. **C:** Liver sections from nontransgenic littermates displayed normal histological appearance. **D:** Liver sections from rtTA/SCID mice showed less eosinophilic and cytoplasmic vacuolization. Scale bars = 20 μm.

On gross morphology, the livers of rtTA/SCID mice were pale nearly to white (Figure 3A), a typical characteristics of the livers of the original Alb-uPA transgenic mice,[13,14] whereas livers of nontransgenic littermates appeared normal and dark red (Figure 3B). Liver sections from both types of mice were stained with H&E. Microscopically, less eosinophilic and cytoplasmic vacuolization were seen in the sections from rtTA/SCID mice (Figure 3C), suggesting that the uPA-expressing hepatocytes had the characteristic histological hepatocellular injury and degenerative changes observed in previous studies.[35] Conversely, liver sections from nontransgenic littermates revealed hepatocytes with a normal histological appearance (Figure 3D). These results demonstrated that specific expression of uPA in rtTA/SCID mouse hepatocytes induced severe hepatocellular injury.

Kinetic Study of uPA Expression and Live Injury

Mice were injected with Ad.TRE-uPA repeatedly to promote proliferation of exogenous hepatocytes after transplantation. To determine the frequency of injections, we investigated the length of time required to restore the liver mass after one injection. rtTA/SCID mice and nontransgenic littermates were administered Dox (1 mg/ml) and treated with 5×10^9 pfu Ad.TRE-uPA. At different time points, blood samples were collected to determine the plasma urokinase concentration and ALT activity. In rtTA/SCID mice, after injection, uPA began to increase at day 2, reached a peak value of 500 ng/ml (100 to 140 times greater than endogenous levels) at day 3, significantly decreased at day 7, and returned to basal levels at day 13 (Figure 4A). We also measured serum ALT activity to monitor the process of liver injury. The results showed that variations in serum uPA were associated with changes in ALT activity, which began to increase at day 2 and reached a peak value of 300 U/L at day 4 (1 day after the peak of uPA) before beginning to decrease at day 7 (Figure 4B). No uPA was detected in nontransgenic littermates, and ALT activity remained at basal levels. Therefore, injection of Ad.TRE-uPA every 7 days was determined to be the regimen necessary to sustain the liver in a damaged state, providing a continuous stimulus for the proliferation of transplanted hepatocytes in the model.

Repopulation of rtTA/SCID Mice with EGFP Labeled Mouse Hepatocytes

To determine whether the injured livers could be efficiently reconstituted by donor hepatocytes, EGFP transgenic mouse hepatocytes were transplanted into rtTA/SCID mice. In our experiments, when 5×10^9 pfu Ad.TRE-uPA was administered before transplantation, some rtTA/SCID mice could not survive after surgery. We then decreased the dose and found that administration of 2.5×10^9 pfu Ad.TRE-uPA before transplantation was nonlethal and appropriate for induction of liver damage (data not shown). After transplantation, 5×10^9 pfu

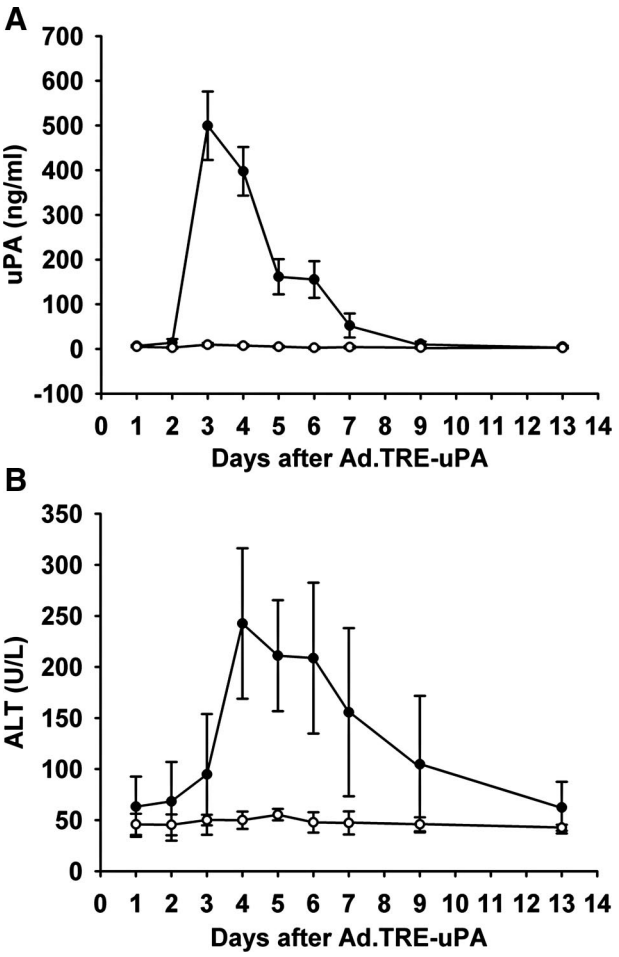

Figure 4. Biochemical analysis after Ad.TRE-uPA adiministration. rtTA/SCID mice (filled circle) and Nontransgenic littermates (open circle) were injected with 5×10^9 pfu Ad.TRE-uPA and administered Dox (1 mg/ml) in their drinking water. At different time points, blood samples were collected for measurement of the plasma uPA concentration (**A**) and analysis of serum ALT activity (**B**). Vertical lines represent that SD Four independent samples were analyzed for each point.

Ad.TRE- uPA was injected weekly to promote the proliferation of transplanted hepatocytes.

To monitor the repopulation of GFP hepatocytes in recipient livers, the mice were sacrificed about 2 weeks after each adenovirus administration, and liver sections were analyzed by examining green fluorescence. Transplantation was performed in a total of 79 mice. Two weeks after the first injection of Ad.TRE-uPA and after transplantation (without additional adenovirus promotion), single or small clusters containing 2 to 6 EGFP hepatocytes were observed scattered throughout the recipient liver in 8 of 10 mice (Figure 5A). Promoted by one additional injection of Ad.TRE-uPA, the clusters became larger and typically consisted of 10–20 EGFP hepatocytes in 9 of 13 mice (Figure 5B). The clusters grew into nodules and replaced about 20% of the host liver in 7 of 14 mice after the third injection of Ad.TRE-uPA (Figure 5C). After the fourth injection of Ad.TRE-uPA, the adjacent clusters became confluent and replaced about 50% of the parenchymal mass in 5 of 14 mice (Figure 5D). After the fifth injection of Ad.TRE-uPA, approximately 6 weeks after transplanta-

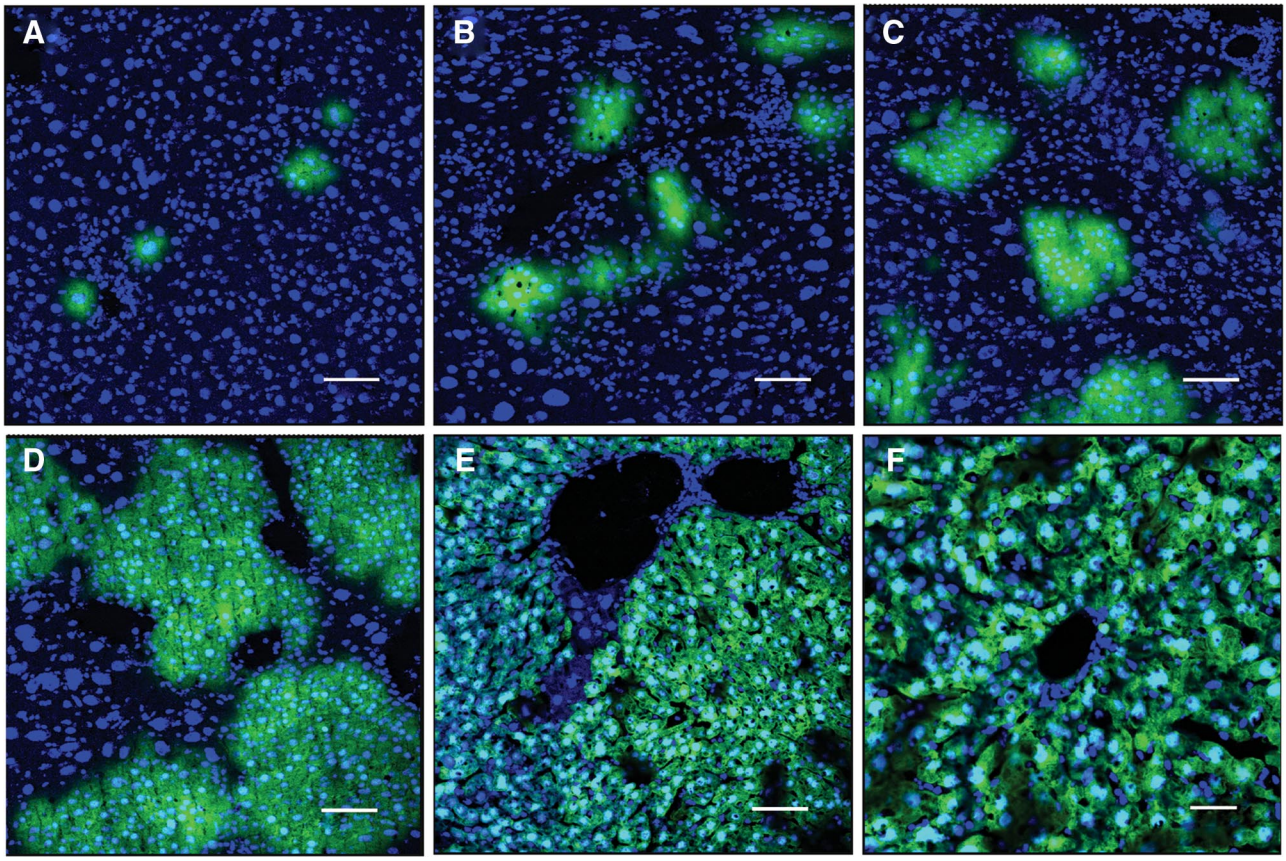

Figure 5. Detection of EGFP transgenic mouse hepatocytes in rtTA/SCID mouse livers. Representative liver sections from the recipient mice transplanted with EGFP hepatocytes, 2 weeks after being injected with Ad.TRE-uPA for 1 time (**A**), 2 times (**B**), 3 times (**C**), 4 times (**D**), 5 times (**E–F**). Scale bars = 80 μm (**A–E**); 40 μm (**F**).

tion, the EGFP hepatocytes had replaced more than 80% of the host livers in 4 of 13 mice (Figure 5E). In control groups consisting of mice transplanted with the same quantity of EGFP mouse hepatocytes, no engraftment was detected in nontransgenic littermates that underwent repeated administration of Ad.TRE-uPA (n = 10) or in untreated rtTA/SCID (n = 5) mice (data not shown). The repopulation level rose progressively with the increasing times of Ad.TRE-uPA injection (Figure 6). The reconstituted liver by transplanted EGFP hepatocytes showed normal hepatic architecture (Figure 5F), and the serum concentration of albumin, bilirubin and total protein were similar to control mice (data not shown).

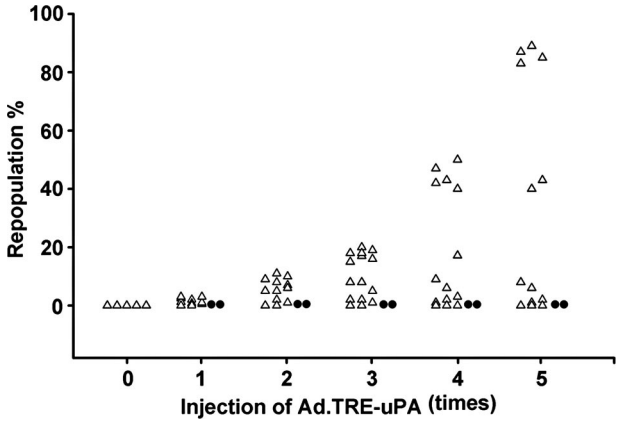

Figure 6. The percentage of liver repopulation by EGFP transgenic hepatocytes related with the times of Ad.TRE-uPA treatment. Open triangles, rtTA/SCID mice; filled dots, nontransgenic littermates.

Discussion

Here we described a mouse model of inducible liver injury created by administering Ad.TRE-uPA to immunodeficient rtTA/SCID mice. In this model, recipient hepatocytes were destroyed by the regulated uPA, whereas the transplanted hepatocytes had a selective advantage to repopulate the recipient liver. After transplantation, the mice were injected with Ad.TRE-uPA every 7 days to maintain the liver in a damaged state. Consequently, the transplanted hepatocytes repopulated recipient liver, replacing over 80% of the host parenchyma, similar to the repopulation rates obtained in the original Alb-uPA mice.[6]

Our model has distinct advantages over the original uPA model. First, we can conveniently obtain adequate numbers of rtTA/SCID mice for experiments. In contrast to the poorly breeding efficiency of the original uPA mice, rtTA/SCID mice bred similarly to normal mice, because rtTA is nontoxic to mice. Second, compared with the inflexible window for transplantation in the original uPA mice, transplantation time point can be more flexibly

chosen by administering Ad.TRE-uPA to rtTA/SCID mice to initiate uPA expression. Furthermore, controlling the extent of liver injury could facilitate transplantation surgery. In our experiments, some rtTA/SCID mice pretreated with 5×10^9 pfu Ad.TRE-uPA died after transplantation, because transplanted hepatocytes resulted in additional injury to the mice by eliciting portal hypertension and transient ischemia-reperfusion.[5] Therefore, we decreased the dose of Ad.TRE-uPA to 2.5×10^9 pfu before transplantation so that no mice died after surgery. After the mice recovered from surgery, a high dose of Ad.TRE-uPA (5×10^9 pfu) was injected to promote repopulation. This dose did not result in the death of mice, but more efficiently promoted the proliferation of transplanted hepatocytes.

Other conditional liver injury models have been reported, but they have certain limitations. Weglarz et al[36] published the MUP-uPA mice, which used MUP promoter to initiate the uPA expression at 2 to 4 weeks of age. Therefore' the MUP-uPA mice overcame the problem of breeding efficiency and can be transplanted and repopulated after weaning. However, the MUP-uPA mouse livers were restored by 8 weeks old, to achieve high level repopulation, transplantation had to be performed between 2 and 5 weeks of age, thus the time window for transplantation was not flexible as for our rtTA/SCID mice.[36] In the Fas/CD95 model, Bcl-2 transgenic hepatocytes selectively repopulate mice treated with anti-Fas antibody, but only one third of the host hepatocytes were destroyed by nonlethal doses of the antibody. Even after 12 injections, the repopulation level was no more than 30%.[10] In our model, over 90% of the rtTA/SCID hepatocytes were eliminated by the inducible uPA. Consequently, after five injections, over 80% of the host liver was replaced by transplanted hepatocytes.

Although our model overcomes the main disadvantages of the original uPA mice, further improvement can be envisioned. In the study, we repeatedly treated rtTA/SCID mice with Ad.TRE-uPA to promote proliferation of the transplanted hepatocytes. However, not all recipient mice achieved high levels of repopulation (Figure 6). We found that these mice produced neutralizing antibodies that inhibited the re-administration of Ad.TRE-uPA (data not shown). Another potential cause of the heterogeneous levels of repopulation is that the transgenic mice we used for transplantation were heterozygous rtTA/SCID mice, deletion or silencing of the transgene could happen in some host hepatocytes. *Rag2*$^{-/-}$*/Il2rg*$^{-/-}$ mice, which completely lack T, B, and NK cells, are reported to be excellent recipients of xenografts.[37] Therefore, breeding homozygous rtTA mice and crossing the mice onto the *Rag2*$^{-/-}$*/Il2rg*$^{-/-}$ background would facilitate to acquire high levels of repopulation. We also detected that the transplanted EGFP hepatocytes were infected with Ad.TRE-uPA (Supplemental Figure S1 A and B, see *http://ajp.amjpathol.org*). After 5 injections of Ad.TRE-uPA, there were about 10 copies per transplanted EGFP hepatocyte.[38] While the copy number was about 25 per hepatocyte in SCID/bg mice treated with 5×10^9 pfu Ad.TRE-uPA (Supplemental Figure S1 C, see *http://ajp.amjpathol.org*), which is in agreement with published data.[39,40] The reduction of copy number of Ad.TRE-uPA could be due to the proliferation of the EGFP hepatocytes. To reduce the potential side-effect of Ad.TRE-uPA, it is better to employ the third generation adenoviral vector, which completely deleted all viral coding sequence,[41] to replace the adenoviral vector used in this study.

Hepatocyte transplantation is a complicated process, comprising multiple steps. Transplanted hepatocytes first accumulate in periportal vessels and hepatic sinusoids after infusion, and then cross the sinusoidal barrier and integrate into the liver parenchyma, where they proliferate under proper conditions.[42] Many factors (integrin, epidermal growth factor, hepatocyte growth factor, transforming growth factor-α, etc) are involved in this process.[43–45] Germline transgenesis is expensive and time-consuming, which constrain its application for screening. Using our strategy, we were able to substitute the uPA of the Ad.TRE-uPA vector with potential candidates to conveniently determine whether they could improve the engraftment level of the transplanted hepatocytes or promote their proliferation, eliminating the need to spend time producing transgenic mice.

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