Conditional gene modification in mouse liver using hydrodynamic delivery of plasmid DNA encoding Cre recombinase

Huan Zhang Zhu*, Wei Wang, Deng Min Feng, Yin Sai, Jing Lun Xue*

State Key Laboratory of Genetic Engineering, Institute of Genetics, School of Life Sciences, Fudan University, Shanghai 200433, China

Abstract The success of Cre-mediated conditional gene targeting in liver of mice has until now depended on the generation of Cre recombinase transgenic mice or on viral-mediated transduction. Here, we sought to establish the feasibility of using hydrodynamic gene delivery of Cre recombinase into liver, using a *ROSA26 EGFP* mouse. The expression of EGFP and β-galactosidase was exclusively detected in the liver of mice treated with hydrodynamic gene delivery of Cre recombinase, as assessed with fluorescence microscopy and X-Gal staining, respectively; Southern blotting also showed that Cre mediated recombination occurred specifically in the liver and not in other organs. The Cre mediated recombination reached about 61% of hepatocytes of mouse after repeated injection, as analyzed by flow cytometry. These results demonstrate that Cre recombinase can be transferred to the liver of mice through a simple hydrodynamic gene-delivery approach and can mediate efficient recombination in hepatocytes. Thus, hydrodynamic gene delivery of the Cre recombinase provides a valuable approach for Cre-loxP-mediated conditional gene modification in the liver of mice.
© 2006 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

1. Introduction

The Cre/*lox*P system from bacteriophage P1 has become an essential tool for conditional gene activation and/or inactivation in mouse [1–3]. It has been successfully used in dissecting gene function during development and adulthood, or in a particular tissue/cell type [4–6]. The liver has crucial functions in the regulation of energy homeostasis, detoxification, and the production of serum proteins. The ability to create defined genetic modifications in vivo provides a powerful strategy for understanding the relevant functions of numerous hepatic genes in health and disease. A common approach for achieving conditional gene modification in mouse liver (besides generating mice with *lox*P-flanked genes of interest) is to generate transgenic mice specifically expressing Cre recombinase in liver. Several groups have already described the generation and characterization of transgenic mice carrying the Cre recombinase under the control of constitutive liver-specific promoters [7,8]. Recently, mice have been engineered to allow spatially and temporally regulation of Cre recombinase using a ligand-inducible system under the control of liver-specific promoters [9,10]. Mice expressing Cre recombinase are mated to mice engineered with *lox*P sites to allow inducible gene ablation [11,12].

Although the transgenic approach is promising, it has certain limitations [13–15]. First, a transgenic construct integrated at different genomic locations often varies because of position effects, necessitating generation and analysis of multiple transgenic founder lines. Second, the Cre recombinase-expressing mouse line must be crossed with mice engineered with *lox*P sites, a resource-intensive process that can increase variation in the genetic background of the offspring, potentially confounding behavioral and other analyses. Third, inducible systems may be leaky, resulting in low-level Cre recombinase expression in the absence of ligand and allowing gene knockout to occur without induction. Finally, Cre transgenic mice may reveal subtle phenotypes related to Cre toxicity, such that the phenotypic consequences of Cre overexpression may confound phenotypic analysis of the conditional mutation.

Another approach is to use recombinant viruses to deliver Cre recombinase to the liver of the adult animal. Previous studies from other laboratories have shown the utility of adenovirus [16–18]. However, when using E1-deleted adenoviral vectors complications may arise from the expression of viral functions on the vector genomes, resulting in host immune responses and loss of transduced cells. Moreover, incorporation of tissue-specific promoters in first generation adenoviral vectors often results in loss of specificity because of viral elements influencing the promoter activity. Thus, traditional delivery of Cre by either transgenics or viral vectors represents a limiting step of conditional mutagenesis employing Cre/*lox*P technology.

To overcome these problems, we adapted previously described hydrodynamic delivery technique to transfer DNA efficiently to the livers of living mice [19–24]. We demonstrate that Cre recombinase can be transferred to mouse liver with a simple hydrodynamic gene delivery approach and can mediate efficient recombination in liver. This approach will prove useful for selective ablation of genes in the adult mouse liver without necessitating generation of transgenic mouse lines expressing Cre recombinase.

*Corresponding authors. Fax: +86 21 6564 9899.
E-mail addresses: HZ_Zhu@hotmail.com (H.Z. Zhu), jlxue@fudan.ac.cn (J.L. Xue).

2. Materials and methods

2.1. Animals

The homozygous *ROSA26 EGFP* reporter strain was kindly provided by Prof. S.H. Orkin of Harvard Medical School (Boston, MA) [25]. Animals were treated according to Guidelines for Animal Care and the guidelines of laboratory animals of Fudan University. Mice were used at 6–8 weeks of age.

2.2. Plasmid DNA

To generate an alb-Cre expression vector containing the Cre recombinase gene under the control of the mouse albumin enhancer and promoter, a 0.6-kb *Sma*I–*Sac*II (blunted) fragment containing the cytomegalovirus (CMV) promoter from plasmid pCMV Cre-ERt [26] (kindly provided by Prof. P. Chambon) was replaced with a 2.3-kb *Sac*II (blunted)-*Eco*RV fragment containing the albumin enhancer and promoter (alb e/p) from p2335A (kindly provided by Prof. R.D. Palmiter). The ERt fragment was then deleted to form the alb-Cre plasmid DNA. The identity of the construct was confirmed by restriction enzyme mapping and DNA sequence analysis.

2.3. Hydrodynamics based transfection in mice

The hydrodynamic gene-transfer procedure was carried out as described previously by Liu et al. [19]. In brief, animals were kept at a high temperature to dilate the tail veins prior to treatment. Naked DNA was administered to the animals by injection into the tail vein a volume of sterile 147 mM NaCl, 4 mM KCl, 1.13 mM CaCl$_2$ (Ringer's solution) corresponding to 8% of the bodyweight. Mice were randomly assigned to five groups: groups were injected with 2.2–2.6 ml of Ringer's solution containing either 10 μg of alb-Cre plasmid DNA (experiment group 1, $n = 10$), 50 μg of alb-Cre plasmid DNA (experiment group 2, $n = 10$), or without DNA (control injection group, $n = 8$); the fourth group was not injected (normal mice, $n = 5$). Injection via the tail vein enter into the circulation within 5–7 s. The injection with 50 μg of alb-Cre plasmid DNA was repeated 8 and 24 h later for experiment group 5 ($n = 6$).

2.4. Histological procedures

At 1, 3, and 5 days after gene delivery, mice from each group were euthanized, and the liver, brain, heart, lung, kidney, and spleen were collected and assessed for the presence of green fluorescence. The remaining tissue was frozen in liquid nitrogen and stored for frozen section and DNA assay. In addition, 5 days after gene delivery, liver from each group of mice ($n = 3$) was perfused in situ through the portal vein with either 0.05% collagenase dissolved in D-Hank's solution (for hepatocyte isolation) or 4.0% paraformaldehyde (for X-Gal staining and immunofluorescence). Isolation of hepatocytes was performed as described by Lecocq et al. [21]. Briefly, the perfused liver was gently homogenized and further incubated in 0.025% collagenase for 30 min at 37 °C. This suspension was filtered through stainless steel gauze and centrifuged for 10 min at $50 \times g$ to remove parenchymal cells, after which the cells were cultured for the indicated time and prepared flow cytometry.

2.5. Visualization of EGFP

For collected organs, green fluorescence was observed on a Leica Wild M3C stereo microscope with an MAA-02 Universal light source from BLS-Ltd. Tissue sections were viewed using a Leitz DMRB microscope equipped with epifluorescence lighting, using the FITC filter set. All microscope samples were photographed using a Nikon E2 digital camera.

2.6. X-Gal staining

Frozen sections from organs including liver, lung, brain, heart, spleen and kidney were stained by X-Gal using the method described previously [27]. Briefly, sections were fixed in phosphate-buffered saline (PBS) containing 1% formaldehyde and 0.2% glutaraldehyde (Sigma). *lacZ* expression was detected by in situ X-Gal staining. The sections were incubated overnight in PBS with 5 mM potassium ferricyanide (Sigma), 5 mM potassium ferrocyanide (Sigma), 2 mM MgCl$_2$, and 1 g/L X-Gal (Sangon, Shanghai, China), washed twice with PBS, and then dehydrated and mounted.

2.7. Immunofluorescence

Sections were incubated for 1 h in 1% H$_2$O$_2$ in PBS to inactivate endogenous peroxidases and then rinsed twice for 20 min with PBS. Sections were coincubated overnight at 4 °C with a rabbit polyclonal anti-Cre antibody at (1:1000) (Novagen, San Diego, CA) and a mouse monoclonal anti-EGFP antibody at 1:400 (Diasorin, Stillwater, MN), both in PBS/0.3% Triton X-100. After two washes with PBS for 20 min at room temperature, sections were incubated in PBS/0.3% Triton X-100 containing goat anti-mouse IgG coupled to fluorescein at 1:400 (Boehringer Mannheim, Indianapolis, IN), and goat anti-rabbit IgG coupled to rhodamine at 1:400 (Boehringer Mannheim). Finally, sections were washed twice in PBS for 20 min at room temperature, mounted on microscope slides by floating them on PBS, and coverslipped under FluorSave reagent.

2.8. Southern blotting

Southern blotting was performed as described previously [28]. Total DNA was isolated from liver, heart, spleen, kidney, lung, and brain tissue using a standard phenol–chloroform extraction method. DNA was digested with the restriction enzyme *Xba*I, electrophoresed through a 0.7% (w/v) agarose gel, and blotted onto a Magnacharge nylon membrane (Frisenette, Ebeltoft, Denmark). The membrane was hybridized with a ^{32}P random-labeled neomycin probe (Amersham Pharmacia Biotech Inc). The hybridized bands were visualized by autoradiography.

2.9. Flow cytometry

Isolated hepatocytes were analyzed on a Becton Dickinson FACScan Flow Cytometer, using a 480-nm laser for excitation, 530-nm emission for GFP, and FACS data were analyzed with FLOWJO software (Tree Star, CA).

2.10. Liver toxicity assays

To monitor liver damage in mice that received plasmids or Ringer's solution, blood was collected at 1, 3, and 5 days after gene delivery. Samples were analyzed for the levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) by enzyme assay kits (ALT/AST assay kit; KeHua, Shanghai, China). Histological examination was performed on formalin-fixed mouse liver sections harvested at various times following injection and prepared by routine histological staining (hematoxylin/eosin) procedures.

2.11. Statistical analysis

The data are presented as the mean and standard error of the mean for each group. Statistical analysis of the data were performed by Student's *t*-test using SigmaStat software (Jandel Scientific, San Rafael, CA). A P value of less than 0.05 was considered significant.

3. Results

3.1. Cre-mediated gene expression in ROSA26 EGFP mice

We first examined the resected specimens, including the brain, heart, lung, kidney, liver, and spleen, by fluorescence stereoscopic microscopy. At 1 day after hydrodynamic delivery, expression of enhancer green fluorescent protein (EGFP) was not seen in any resected specimens from the various groups. At 3 days after hydrodynamic delivery, low but definite EGFP expression was first seen only in the livers of mice treated with alb-Cre plasmid DNA (50 μg and 10 μg), indicating recombination had occurred. On day 5 after hydrodynamic delivery, expression of EGFP was markedly seen only in the livers of mice treated with alb-Cre plasmid DNA (Fig. 1). There are more cells expressing EGFP at 5 days than at 3 days (Fig. 2A and B). This increase over time indicates that there is a time-lag from Cre entry to gene expression and recombination. No EGFP expression was observed in the rest of the organs of the experimental group, nor in any organs from control animals that received the Ringer's solution.
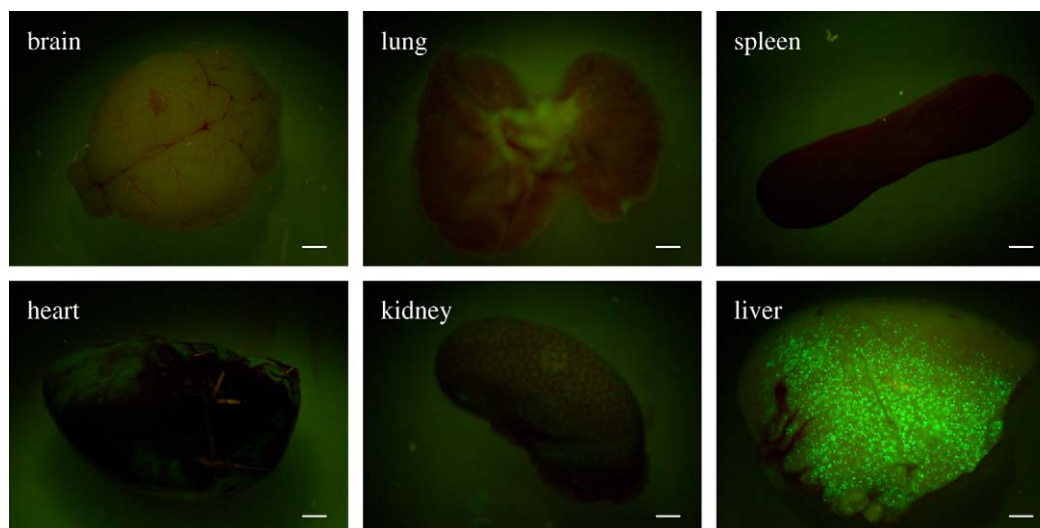
Fig. 1. Cre-mediated gene expression in *ROSA26 EGFP* reporter mice. EGFP expression was examined by fluorescence stereoscopic microscopy in organs from reporter mice 5 day after tail vein rapid injection of alb-Cre plasmid. Scale bar = 1 mm.

To elucidate the population and location of cells expressing reporter gene in the resected specimen, β-galactosidase gene was identified by X-Gal staining of frozen sections. X-Gal stained only the livers of the alb-Cre plasmid DNA injected mice (Fig. 2C and D), but did not stain the livers of mice that received only the Ringer's solution (data not shown). This finding agrees with the findings of Cre-mediated EGFP expression. The number of positive cells was greater with 50 μg than with 10 μg alb-Cre plasmid DNA (Fig. 2C and D). Repeated injection of 50 μg produced a further increase (Fig. 2E). It is evident that under our experimental conditions, β-galactosidase gene expression in the liver seems restricted to certain areas, as X-Gal-positive cells clustered around the hepatic vein. The distribution of the cells expressing the reporter gene was heterogeneous, as some areas of the liver showed more positive cells than others. The reasons for the uneven distribution are not understood. Uneven recombination or uneven expression of the recombined reported gene may be involved. No X-Gal-positive cells were observed in the heart, lungs, kidney, brain, or spleen of the alb-Cre plasmid DNA injected mice (data not shown).

To further assess the Cre-mediated recombination, Southern blotting was performed on genomic DNA from reporter mice treated with alb-Cre plasmid DNA. Cre activity was reflected by the conversion of the 10.3-kb floxed allele fragment into a 5.3-kb excised allele fragment. Fig. 2F shows that Cre recombination occurred specifically in the liver of Cre-treated animals only. No recombination was observed in brain, heart, lung, kidney, or spleen, in mice treated with alb-Cre plasmid DNA. These results are consistent with the data described above.

To determine whether Cre and EGFP were expressed within the same cell, we performed double immunofluorescence on the frozen sections of the livers obtained from reporter mice after alb-Cre plasmid DNA injection. Colocalization was identified by double immunofluorescence using a mouse monoclonal anti-EGFP antibody and a rabbit polyclonal anti-Cre antiserum, followed by a fluorescein-coupled anti-mouse IgG antibody and a rhodamine-coupled anti-rabbit IgG antibody. As expected, all cells that showed Cre expression were also EGFP positive (data not shown). In addition, the GFP and Cre signals were predominantly localized to the nucleus, indicating that Cre and EGFP were colocalized. This suggests that Cre recombinase can be expressed in hepatocytes and mediates recombination between the *lox*P sites to splice out the stuffer sequence, thereby allowing EGFP reporter gene expression in hepatocytes.

3.2. Cre-mediated recombination efficiency

The use of EGFP as the Cre reporter also allows the use of flow cytometry analysis for quantification of cells that have undergone Cre excision. To analyze the recombination efficiency in liver, hepatocytes were isolated from reporter mice treated with alb-Cre plasmid or from untreated control reporter mice. In mice injected with 50 μg DNA, 33.27% ± S.D. 3.55% ($n = 3$) of hepatocytes were EGFP positive; in mice injected with 10 μg DNA, 7.38% ± S.D. 0.54% ($n = 3$) of hepatocytes were EGFP positive; whereas, in mice repeatedly injected with 50 μg DNA 61.1% ± S.D. 3.5% ($n = 3$) of hepatocytes were EGFP positive (Fig. 3).

3.3. Tests for liver damage

The potential toxic effects of our procedure on animals were assessed by a comprehensive clinical biochemical analysis. The tests included the concentration of the liver specific enzymes – AST and ALT. After the injection of Ringer's solution with or without plasmid DNA, the serum ALT of mice sharply increased on day 1 after the injection and returned to normal levels by day 5 (Table 1). A similar pattern was seen for serum AST (Table 1). Histological sections of livers from each group of mice showed no apparent pathological changes in the liver sections of mice sacrificed on day 3 and day 5 after injection, compared with the uninjected mice (data not shown).

4. Discussion

Recent evidence demonstrates that hydrodynamic-based gene delivery is a powerful tool for manipulating gene expression in liver. Accordingly, we sought to establish the feasibility
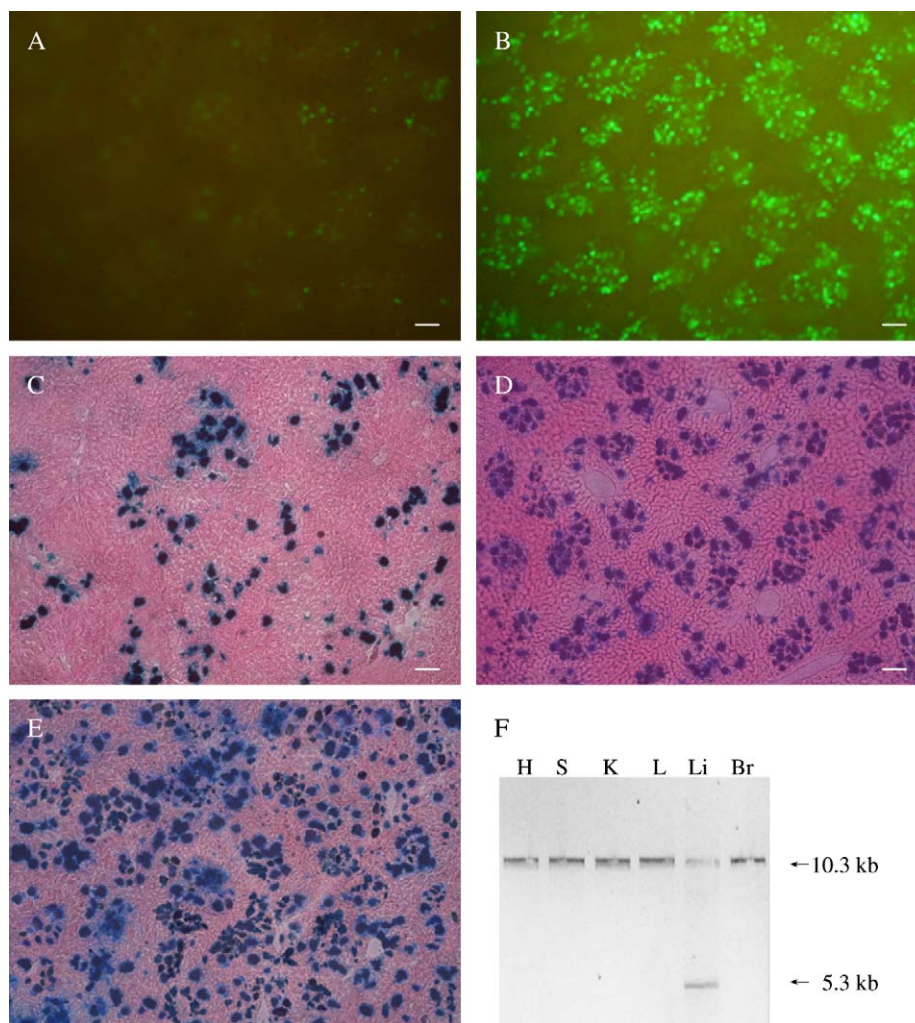
Fig. 2. Cre-mediated EGFP/β-galactosidase gene expression in liver of *ROSA26 EGFP* reporter mice. EGFP expression was examined by fluorescence stereoscopic microscopy in liver at 3 day (A), 5 day (B) after tail vein rapid injection of 50 μg alb-Cre plasmid. β-galactosidase gene expression was examined by X-Gal stained on the frozen sections of the livers from reporter mice treated with 10 μg (C), 50 μg (D), and repeated 50 μg DNA (E). Southern blot analysis of Cre mediated excision in genomic DNA from heart (H), spleen (S), kidney (K), lung (L), liver (Li) and brain (Br), in reporter mice treated with DNA; The sizes of floxed allele and excised allele are 10.3 kb and 5.3 kb (F), respectively. Scale bar: 250 μm (A and B), 200 μm (C–E).

of using hydrodynamic Cre gene delivery into liver for conditional gene targeting. Cre-mediated conditional gene targeting requires precise knowledge of sites of recombination in order to interpret mutant phenotypes. For this reason, we used the *ROSA26 EGFP* strain as Cre recombinase reporter mice, which enabled detection of recombination by fluorescence microscopy and X-gal staining to determine sites of Cre gene expression in mouse. We observed that liver was the only organ that expressed the EGFP/β-galactosidase gene at detectable levels after hydrodynamic injection of alb-Cre plasmid DNA. Cells positive for EGFP and X-Gal were mainly localized in areas surrounding the central vein. Our results are similar to other reports [22,23] in which the *lacZ* gene and human alpha1-antitrypsin gene expression after hydrodynamic injection were observed exclusively in the liver. Liver-specific gene transfer probably results from a reflux of DNA solution into the liver via the hepatic vein as a result of a transient cardiac congestion. It may also be related to using liver-specific promoters, such as the albumin promoter.

To precisely analyze Cre-mediated recombination efficiency in liver, we quantified cells from isolated hepatocytes with EGFP by using flow cytometry. Our data showed that Cre-mediated recombination occurred in about 33% of hepatocytes of the mice treated with 50 μg DNA using hydrodynamic gene delivery, whereas only about 7% of hepatocytes of the mice treated with 10 μg DNA were EGFP positive, indicating a dose–response relationship between EGFP-positive cells and the amount of injected DNA. Similarly, in a previous study in which mice were subjected to hydrodynamic gene transfer, approximately 40% of the cells in the liver expressed the lacZ gene, and there is a clear correlation between the number of X-gal-positive cells in each section and the amount of plasmid DNA each animal received [19]. Recently, Song et al. [24] verified delivery of synthetic siRNA duplexes into mouse hepatocytes in vivo by repeated hydrodynamic tail vein injection of Cy5-labeled Fas siRNA. Twenty-four hours after the last of three injections, 88 ± 6% of hepatocytes had taken up the siRNA and were Cy5-positive, as determined by flow cytometry.
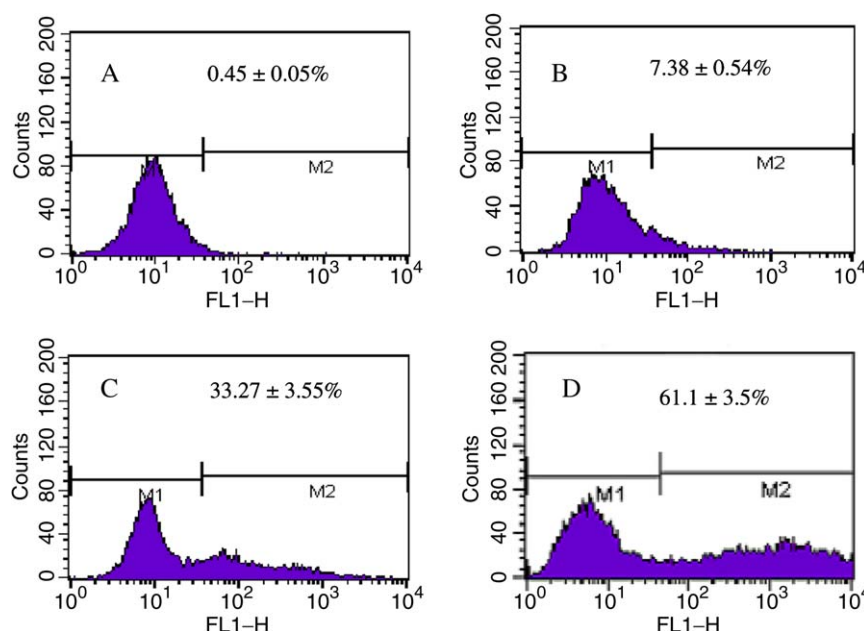
Fig. 3. Measurement of Cre-mediated EGFP expression in isolated hepatocytes by flow cytometry. Hepatocytes from report mice treated without DNA (A), or with 10 µg (B), 50 µg DNA (C) and repeated 50 µg DNA (D). The percentage indicated is the number of cells positive for EGFP fluorescence. Data represent means ± S.D. (n = 3).

Table 1
Effect of the tail vein injection technique on serum concentration of ALT and AST

Time after injection (day)	ALT (µ/l)			AST (µ/l)		
	1	3	5	1	3	5
Experiment group 1	887 ± 54***	64 ± 15**	26 ± 3	164 ± 32***	88 ± 16**	68 ± 6
Experiment group 2	906 ± 44***	68 ± 16**	25 ± 4	174 ± 36***	89 ± 17**	67 ± 5
Control injection group	877 ± 48***	62 ± 15**	23 ± 4	166 ± 33***	85 ± 13**	70 ± 4
Normal mice	24 ± 2	23 ± 2	24 ± 2	66 ± 5	67 ± 3	66 ± 4

Note. The toxicity assay was assessed at various time points after tail vein quickly injection of experiment group1 (10 µg alb-Cre plasmid, n = 4), experiment group 2 (50 µg alb-Cre plasmid, n = 4), control injection group (without DNA, n = 4), and normal mice (n = 4). Data represent means ± S.D. **$P < 0.05$, ***$P < 0.001$, for comparisons with normal mice.

Transduction efficiency was higher than the 40% efficiency observed with a single injection of reporter plasmid DNA [19,20]. To further increase the efficiency of Cre-mediated recombination in our experiment, reporter mice were treated with repeated hydrodynamic tail injections. Our result showed that Cre-mediated recombination reached about 61% of hepatocytes in the reporter mouse, indicating the feasibility of repeated hydrodynamic tail injections.

In transgenic mice carrying the Cre recombinase under the control of a liver-specific promoter, the efficiency of recombination is only about 40% immediately after birth and is complete by 6 weeks of age. To perform temporally controlled hepatocyte-selective recombination of floxed genes, Tannour-Louet et al. established a transgenic line expressing a tamoxifen-inducible Cre recombinase under the control of the transthyretin promoter. Recombination of floxed DNA occurred in approximately 50% of hepatocytes after treatment with 4-hydroxytamoxifen (4-OHT). Only after a series of 4-OHT injections can the recombination efficiency reach 80–100% in the liver of these transgenic mice [9]. An alternative approach for the temporal regulation of the Cre activity in the liver is to use adenoviruses carrying the Cre transgene. Akagi et al. used an adeno Cre virus to monitor recombina-

tion in vivo in *LacZ* reporter mice. Inoculation of 10^9 p.f.u. vector into the tail vein resulted in massive staining of the liver. About 65% of hepatocytes were stained by X-gal 5 days after injection. However, the fraction of X-gal-positive hepatocytes was reduced to 40, 4 and finally <1% of hepatocytes on days 15, 30 and 60 post-injection, respectively, probably as the result of the expression of viral functions on the vector genomes resulting in host immune responses and loss of transduced cells. Reduction of the virus dose to 10^6 p.f.u. led to a much lower frequency of recombination [17]. To circumvent some of these problems, Badorf et al. used an adenoviral vector in which all viral coding sequences were absent. With this vector, injection of 5×10^9 infectious units resulted in recombination in approximately 10–20 % of hepatocytes [18]. In addition, although adenoviral vectors expressing Cre can transduce liver, other tissues are simultaneously infected by this vector [17,18]. Therefore, compared with the adenovirus approach, hydrodynamic gene delivery of Cre recombinase more organ-specific.

We found that the ALT and AST values fell into a normal range 5 days after injection. Animals injected with either Ringer's solution or Ringer's solution containing alb-Cre plasmid DNA had an identical pattern of ALT changes, suggesting that

the increased ALT value is not caused by plasmid DNA or gene products. Compared with normal mice that were not injected, there were no obvious histological changes seen in liver sections of animals injected with either Ringer's solution or Ringer's solution containing different amounts of alb-Cre plasmid DNA. These results are consistent with previous reports [19–23]. Importantly, these animals appear to recover from such minor liver damage quickly, and thus hydrodynamic Cre gene delivery appears to be safe.

However, recently in vitro and in vivo toxicity from Cre recombinase has been reported [13,14,29,30]. The toxicity seems to result from aberrant activity of Cre at "pseudo" loxP sites. Loonstra et al. [14] demonstrated that this Cre toxicity depends on expression levels. Cre was not toxic at low levels, suggesting that transient expression of Cre should minimize the chances of recombination at the endogenous pseudo-lox sites and/or give the target cell time to repair the DNA damage efficiently. Thus, compared with Cre transgenic mice, transient expression of Cre may minimize its potential toxicity in mice by hydrodynamic injection. In addition, Herweijer et al. [31] have reported that a liver-specific albumin promoter is expressed at a much lower level of transgene product than are viral promoters such as the CMV promoter. Consequently, a Cre expression vector driven by the mouse albumin enhancer and promoter also may minimize its potential toxicity.

One of the greatest advantages of this technique, compared with other vector-mediated gene delivery systems such as viral systems and transgenic technology, is that this simple technique does not require long and laborious preparation of virus and does not require any special devices for gene transfer. It eliminates time-consuming and expensive breeding strategies, as well as eliminating potentially complex genetic backgrounds that result from transgenic approaches. With the progression of human genome project, numerous hepatic genes with liver-specific expression have been found, but their exact functions are not clear. So far, many strains of mice with loxP-flanked genes of interest have been generated (see http://www.mshri.on.ca/nagy/cre.htm). We believe that the use of hydrodynamic gene delivery of Cre recombinase, in conjunction with mice with loxP-flanked genes of interest, may be a powerful tool to rapidly study genes of interest in liver function and to create mouse models for metabolic and liver diseases.

In summary, we have demonstrated that Cre recombinase can be transferred to mouse livers via a simple hydrodynamic gene-delivery approach and can mediate efficient recombination in hepatocytes in mice. These results show that hydrodynamic Cre gene delivery provides a simple, efficient, and inexpensive way of delivering Cre genes in vivo, as well as an inexpensive way to study liver-related gene functions in vivo.

References

[1] Sauer, B. and Henderson, N. (1988) Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. Proc. Natl. Acad. Sci. USA 85, 5166–5170.

[2] Rajewsky, K., Gu, H., Kuhn, R., Betz, U.A., Muller, W., Roes, J. and Schwenk, F. (1996) Conditional Gene Targeting. J .Clin. Invest. 98, 600–603.

[3] Lewandoski, M. (2001) Conditional control of gene expression in the mouse. Nat. Rev. Genet. 2, 743–755.

[4] Gu, H., Marth, J.D., Orban, P.C., Mossmann, H. and Rajewsky, K. (1994) Deletion of a DNA polymerase beta gene segment in T cells using cell type-specific gene targeting. Science 265, 103–106.

[5] Sinnayah, P., Lindley, T.E., Staber, P.D., Davidson, B.L., Cassell, M.D. and Davisson, R.L. (2004) Targeted viral delivery of Cre recombinase induces conditional gene deletion in cardiovascular circuits of the mouse brain. Physiol. Genomics 18, 25–32.

[6] Xia, C.H., Roberts, E.A., Her, L.S., Liu, X., Williams, D.S., Cleveland, D.W. and Goldstein, L.S. (2003) Abnormal neurofilament transport caused by targeted disruption of neuronal kinesin heavy chain KIF5A. J. Cell Biol. 161, 55–66.

[7] Kellendonk, C., Opherk, C., Anlag, K., Schutz, G. and Tronche, F. (2000) Hepatocyte-specific expression of Cre recombinase. Genesis 26, 151–153.

[8] Postic, C. and Magnuson, M.A. (2000) DNA excision in liver by albumin-Cre transgene occurs progressively with age. Genesis 26, 149–150.

[9] Tannour-Louet, M., Porteu, A., Vaulont, S., Kahn, A. and Vasseur-Cognet, M. (2002) A tamoxifen-inducible chimeric Cre recombinase specifically effective in the fetal and adult mouse liver. Hepatology 35, 1072–1081.

[10] Schuler, M., Dierich, A., Chambon, P. and Metzger, D. (2004) Efficient temporally controlled targeted somatic mutagenesis in hepatocytes of the mouse. Genesis 39, 167–172.

[11] Croquelois, A., Blindenbacher, A., Terracciano, L., Wang, X., Langer, I., Radtke, F. and Heim, M.H. (2005) Inducible inactivation of *Notch1* causes nodular regenerative hyperplasia in mice. Hepatology 41, 487–496.

[12] Streetz, K.L., Tacke, F., Leifeld, L., Wustefeld, T., Graw, A., Klein, C., Kamino, K., Spengler, U., Kreipe, H., Kubicka, S., Muller, W., Manns, M.P. and Trautwein, C. (2003) Interleukin 6/gp130-dependent pathways are protective during chronic liver diseases. Hepatology 38, 218–229.

[13] Kaspar, B.K., Vissel, B., Bengoechea, T., Crone, S., Randolph-Moore, L., Muller, R., Brandon, E.P., Schaffer, D., Verma, I.M., Lee, K.F., Heinemann, S.F. and Gage, F.H. (2002) Adeno-associated virus effectively mediates conditional gene modification in the brain. Proc. Natl. Acad. Sci. USA 99, 2320–2325.

[14] Loonstra, A., Vooijs, M., Beverloo, H.B., Allak, B.A., van Drunen, E., Kanaar, R., Berns, A. and Jonkers, J. (2001) Growth inhibition and DNA damage induced by Cre recombinase in mammalian cells. Proc. Natl. Acad. Sci. USA 98, 9209–9214.

[15] Schnutgen, F., Doerflinger, N., Calleja, C., Wendling, O., Chambon, P. and Ghyselinck, N.B. (2003) A directional strategy for monitoring Cre-mediated recombination at the cellular level in the mouse. Nat. Biotechnol. 21, 562–565.

[16] Wang, Y., Krushel, L.A. and Edelman, G.M. (1996) Targeted DNA recombination in vivo using an adenovirus carrying the Cre recombinase gene. Proc. Natl. Acad. Sci. USA 93, 3932–3936.

[17] Akagi, K., Sandig, V., Vooijs, M., Van der Valk, M., Giovannini, M., Strauss, M. and Berns, A. (1997) Cre-mediated somatic site specific recombination in mice. Nucleic Acids Res. 25, 1766–1773.

[18] Badorf, M., Edenhofer, F., Dries, V., Kochanek, S. and Schiedner, G. (2002) Efficient in vitro and in vivo excision of floxed sequences with a high-capacity adenoviral vector expressing Cre recombinase. Genesis 33, 119–124.

[19] Liu, F., Song, Y. and Liu, D. (1999) Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. Gene. Ther. 6, 1258–1266.

[20] Zhang, G., Budker, V. and Wolff, J.A. (1999) High levels of foreign gene expression in hepatocytes after tail vein injections of naked plasmid DNA. Hum. Gene. Ther. 10, 1735–1737.

[21] Lecocq, M., Andrianaivo, F., Warnier, M.T., Wattiaux-De, Coninck. S., Wattiaux, R. and Jadot, M. (2003) Uptake by mouse liver and intracellular fate of plasmid DNA after a rapid tail vein injection of a small or a large volume. J. Gene. Med. 5, 142–156.

[22] Maruyama, H., Higuchi, N., Nishikawa, Y., Kameda, S., Iino, N. and Kazama, J.J., et al. (2002) High-level expression of naked

DNA delivered to rat liver via tail vein injection. J. Gene. Med. 4, 333–341.

[23] Zhang, G., Song, Y.K. and Liu, D. (2000) Long-term expression of human alpha1-antitrypsin gene in mouse liver achieved by intravenous administration of plasmid DNA using a hydrodynamics-based procedure. Gene. Ther. 7, 1344–1349.

[24] Song, E., Lee, S.K., Wang, J., Ince, N., Ouyang, N., Min, J. and Chen, J., et al. (2003) RNA interference targeting Fas protects mice from fulminant hepatitis. Nat. Med. 9, 347–351.

[25] Mao, X., Fujiwara, Y., Chapdelaine, A., Yang, H. and Orkin, S.H. (2001) Activation of EGFP expression by Cre-mediated excision in a new ROSA26 reporter mouse strain. Blood 97, 324–326.

[26] Brocard, J., Warot, X., Wendling, O., Messaddeq, N., Vonesch, J.L., Chambon, P. and Metzger, D. (1997) Spatio-temporally controlled site-specific somatic mutagenesis in the mouse. Proc. Natl. Acad. Sci. USA 94, 14559–14563.

[27] Novak, A., Guo, C., Yang, W., Nagy, A. and Lobe, C.G. (2000) Z/EG, a double reporter mouse line that expresses enhanced green fluorescent protein upon Cre-mediated excision. Genesis 28, 147–155.

[28] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York, pp. 9.31–9.44.

[29] Schmidt, E.E., Taylor, D.S., Prigge, J.R., Barnett, S. and Capecchi, M.R. (2000) Illegitimate Cre-dependent chromosome rearrangements in transgenic mouse spermatids. Proc. Natl. Acad. Sci. USA 97, 13702–13707.

[30] Pfeifer, A., Brandon, E.P., Kootstra, N., Gage, F.H. and Verma, I.M. (2001) Delivery of the Cre recombinase by a self-deleting lentiviral vector: efficient gene targeting in vivo. Proc. Natl. Acad. Sci. USA 98, 11450–11455.

[31] Herweijer, H., Zhang, G., Subbotin, V.M., Budker, V., Williams, P. and Wolff, J.A. (2000) Time course of gene expression after plasmid DNA gene transfer to the liver. J. Gene. Med. 3, 280–291.