B. Terris, D. Franco, T. Poynard, *Hepatology* **21**, 760 (1995); S. Milani, H. Herbst, D. Schuppan, H. Stein, C. Surrenti, *Am. J. Pathol.* **139**, 1221 (1991).
5. T. Kitada, S. Seki, N. Kawakita, T. Kuroki, T. Monna, *Biochem. Biophys. Res. Commun.* **211**, 33 (1995); N. Miura *et al.*, *Cancer Genet. Cytogenet.* **93**, 56 (1997); Y. Urabe *et al.*, *Liver* **16**, 293 (1996).
6. A. G. Bodnar *et al.*, *Science* **279**, 349 (1998); H. Vaziri and S. Benchimol, *Curr. Biol.* **8**, 279 (1998); C. M. Counter, *Mutat. Res.* **366**, 45 (1996); T. Kiyono *et al.*, *Nature* **396**, 84 (1998).
7. M. A. Blasco *et al.*, *Cell* **91**, 25 (1997).
8. H. W. Lee *et al.*, *Nature* **392**, 569 (1998); K. L. Rudolph *et al.*, *Cell* **96**, 701 (1999).
9. K. L. Rudolph, S. Chang, M. Millard, N. Schreiber-Agus, R. A. DePinho, unpublished data.
10. J. L. Heckel, E. P. Sandgren, J. L. Degen, R. D. Palmiter, R. L. Brinster, *Cell* **62**, 447 (1990).
11. E. P. Sandgren *et al.*, *Cell* **66**, 245 (1991).
12. Mice homozygous null for the mouse telomerase RNA gene ($mTR^{-/-}$) were bred to transgenic mice hemizygous for the Alb-uPA transgene. Alb-uPA $mTR^{+/-}$ mice were crossed to $mTR^{+/-}$ mice, generating Alb-uPA $mTR^{-/-}$ mice (G1). Intercrosses of Alb-uPA $mTR^{-/-}$ mice according to previously published mating schemes (*7*) resulted in successive generations of Alb-uPA $mTR^{-/-}$ mice.
13. N. Rufer, W. Dragowska, G. Thornbury, E. Roosnek, P. M. Lansdorp, *Nature Biotechnol.* **16**, 743 (1998).
14. PH was performed as described (*15*). Briefly, mice were anesthetized by intraperitoneal (i.p.) injections of 2.5% Avertin. After ligation of the blood supply and resection of the left and the left-anterior lobes of the liver, the abdominal cavity was closed with peritoneal and dermal sutures. Regenerating livers were removed at the indicated time points, photographed with a Nikon 2000 camera, and fixed in 10% phosphate-buffered saline (PBS)–buffered formalin for histology.
15. K. L. Rudolph *et al.*, *Hepatology* **30**, 1159 (1999); A. M. Diehl and R. M. Rai, *FASEB J.* **10**, 215 (1996).
16. Single-cell suspensions of regenerating livers were produced by homogenizing the organs between two glass slides. Homogenates were then filtered through a nylon mesh (60-μm opening; Sefor America, Kansas City, MO). After several washes with ice-cold PBS, single-cell suspensions were fixed in 70% ethanol and stained with propidium iodide.
17. B. McClintock, *Genetics* **26**, 234 (1941); B. van Steensel, A. Smorgorzewska, T. de Lange, *Cell* **92**, 401 (1998); E. Blackburn *et al.*, *Ciba Found. Symp.* **211**, 2 (1997); K. E. Kirk, B. P. Harmon, I. K. Reichardt, J. W. Sedat, E. H. Blackburn, *Science* **275**, 1478 (1997).
18. R. P. Tamayo, *Hepatology* **3**, 112 (1983).
19. K. R. Prowse and C. W. Greider, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 4818 (1995).
20. D. K. Podolsky and K. J. Isselbacher, in *Harrison's Principles of Internal Medicine*, A. S. Fauci *et al.*, Eds. (McGraw-Hill, New York, ed. 14, 1998), p. 1704.
21. P. P. Anthony *et al.*, *Bull. WHO* **55**, 521 (1977).
22. Ten microliters of 10% $CCl_4$ in olive oil were injected into mice i.p. once per month starting with 6-week-old animals. Peripheral bleeds were performed 48 hours and 144 hours after $CCl_4$ injections. Liver biopsies were taken 1 month after the sixth injection of $CCl_4$. Formalin-fixed, paraffin-embedded liver sections were stained with Masson-trichrome, H&E, and reticulin.
23. A 5-kb Eco RI fragment of mTR including 4 kb of the upstream promoter region was cloned into the pAdTrack-Shuttle vector (*24*). Homologous recombination of Pme I–linearized pAdTrack-mTR DNA with the viral backbone vector pAd-Easy-1 (*24*) was performed in *Escherichia coli* BJ5183 cells, yielding a replication-deficient (lack of E1/3) Ad5-mTR-virus. Virus was grown in 293 cells and purified as in (*24*).
24. T. C. He *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 2509 (1998).
25. Six- to eight-week-old $mTR^{+/+}$ and G6 $mTR^{-/-}$ mice were infected with $10^{12}$ virus particles of Ad-mTR-GFP or Ad-GFP by tail-vein injections. Two days after injection, liver biopsies were performed and GFP expression was monitored on unstained, 5-μm-thick cryostat sections. Subsequent alternate-day $CCl_4$ i.p. injections were administered (10 μl of 10% $CCl_4$ per gram of body weight in olive oil). Liver biopsies for both GFP expression and histology were performed 14 and 28 days after initiation of the $CCl_4$ injections.
26. J. Korula, in N. Kaplowitz, Ed., *Liver and Biliary Diseases* (Williams & Wilkins, Baltimore, MD, 1992), pp. 529–541.
27. For immunostaining, 10-μm paraffin liver sections were incubated overnight at 4°C with 1:100 dilutions of the primary antibody (mouse anti-PCNA or mouse anti–TGF-β1) in the presence of 1.5% goat serum. The Elite Vectastain ABC kit (Vector Labs) was used for subsequent peroxidase substrate detection. Stained sections were dehydrated and mounted in Permount.
28. C. B. Harley, A. B. Futcher, C. W. Greider, *Nature* **345**, 458 (1990); C. Mondello, R. Riboni, A. Casati, T. Nardo, F. Nuzzo, *Exp. Cell Res.* **236**, 385 (1997); C. Pan, B. H. Xue, T. M. Ellis, D. J. Peace, M. O. Diaz, *Exp. Cell Res.* **231**, 346 (1997); R. C. Allsopp and C. B. Harley, *Exp. Cell Res.* **219**, 130 (1995).
29. H. Niida *et al.*, *Nature Genet.* **19**, 203 (1998); L. Chin *et al.*, *Cell* **97**, 527 (1999).
30. R. A. Greenberg *et al.*, *Cell* **97**, 515 (1999); W. C. Hahn *et al.*, *Nature* **400**, 464 (1999); J. W. Shay, *J. Cell. Physiol.* **173**, 266 (1997); C. B. Harley *et al.*, *Cold Spring Harbor Symp. Quant. Biol.* **59**, 307 (1994); T. de Lange, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 2882 (1994).
31. H. Tahara *et al.*, *Cancer Res.* **55**, 2734 (1995); G. T. Huang *et al.*, *Eur. J. Cancer* **34**, 1946 (1998); Y. M. Park *et al.*, *Exp. Mol. Med.* **30**, 35 (1998); R. Nakishio, M. Kitamoto, T. Nakaishi, H. Takahashi, G. Kajiyama, *Nippon Rinsho* **56**, 1239 (1998); T. Suda *et al.*, *Hepatology* **27**, 402 (1998); H. Kojima, O. Yokosuka, F. Imazeki, H. Saisho, M. Omata, *Gastroenterology* **112**, 493 (1997).
32. J. P. Pommier *et al.*, *Virology* **231**, 148 (1997), W. S. Nichols, S. Schneider, R. C. Chan, C. F. Farthing, E. S. Daar, *Scand. J. Immunol.* **49**, 302 (1999).
33. J. H. Ohyashiki *et al.*, *Clin. Cancer Res.* **5**, 1155 (1999); S. E. Ball *et al.*, *Blood* **91**, 3582 (1998); J. H. Ohyashiki *et al.*, *Cancer Res.* **54**, 3557 (1994).
34. We thank I. Arias, G. Merlino, M. Meyerson, N. Sharpless, and G. David for critical reading of the manuscript; B. Vogelstein for the Ad-Easy-Adenovirus-System; J. Furgiueli for serum chemistry analysis; and V. V. Lam for technical assistance. Supported by Deutsche Forschungsgemeinschaft grant Ru 745/1-1 (K.L.R.), Leukemia Society of America fellowship (N.S.-A.), and the Dana-Farber Cancer Institute Cancer Core Grant (R.A.D.). R.A.D. is funded by NIH grants (R01HD34880 and R01HD28317), the American Heart Association Grant-in-Aid, and the American Cancer Society.

8 November 1999; accepted 22 December 1999

# Prevention of Acute Liver Failure in Rats with Reversibly Immortalized Human Hepatocytes

**Naoya Kobayashi,[1]* Toshiyoshi Fujiwara,[1] Karen A. Westerman,[2] Yusuke Inoue,[3] Masakiyo Sakaguchi,[3] Hirofumi Noguchi,[1] Masahiro Miyazaki,[3] Jin Cai,[4] Noriaki Tanaka,[1] Ira J. Fox,[4]* Philippe Leboulch[2,5]***

**Because of a critical shortage in suitable organs, many patients with terminal liver disease die each year before liver transplantation can be performed. Transplantation of isolated hepatocytes has been proposed for the temporary metabolic support of patients awaiting liver transplantation or spontaneous reversion of their liver disease. A major limitation of this form of therapy is the present inability to isolate an adequate number of transplantable hepatocytes. A highly differentiated cell line, NKNT-3, was generated by retroviral transfer in normal primary adult human hepatocytes of an immortalizing gene that can be subsequently and completely excised by Cre/Lox site-specific recombination. When transplanted into the spleen of rats under transient immunosuppression, reversibly immortalized NKNT-3 cells provided life-saving metabolic support during acute liver failure induced by 90% hepatectomy.**

Orthotopic allogeneic liver transplantation remains the only treatment option available to patients with terminal liver failure or inborn errors of liver metabolism. Because its application is limited by donor organ availability, considerable morbidity, mortality, and high cost, there is a need to develop bridging procedures to sustain patients with subacute or acute liver failure. Hepatocyte transplantation (HTX) has been used to correct metabolic defects and provide metabolic support in experimental animal models of hepatic failure (*1*). The hyperbilirubinemia of rats genetically deficient in uridine diphosphate glucuronosyltransferase was corrected by HTX (*1*). Substantial increases in plasma albumin levels were also documented after HTX into analbuminemic rats (*1*). Recently, intrasplenic transplantation of differentiated adult hepatocytes in human patients with severe encephalopathy and multisystem organ failure was able to control hyperammonemia and ensure short-term survival until orthotopic liver transplantation was successfully per-

formed (*2*). Unfortunately, the shortage of human livers available for hepatocyte isolation limits the clinical use of this procedure (*2*). A potential alternative source of human liver cells for transplantation would be a clonal cell line that could be expanded in vitro, would exhibit the characteristics of differentiated hepatocytes, and could provide metabolic support upon transplantation.

Transfer of specific oncogenes can generate cell lines that propagate an intermediate stage of differentiation, a process known as cell immortalization (*3*). However, in vivo injection of immortalized cell lines would expose patients to an unacceptable tumorigenic risk (*3, 4*). Even with xenografts or mismatched allografts, ultimate rejection of implanted cells is not guaranteed, because stable xenogenic chimerism and cases of accidental engraftment of human lymphocyte antigen (HLA) mismatched allogeneic tumors have been documented in humans (*5*). In an effort to address this crucial safety issue, we investigated whether primary human hepatocytes can be successfully submitted to a reversible immortalization procedure that involves the retrovirus-mediated transfer of an oncogene that can be subsequently excised by site-specific recombination.

We constructed the polycistronic retroviral vector SSR#69 (*3*) to transfer and express the simian virus 40T (SV40T) immortalizing gene flanked by LoxP recombination targets (Fig. 1). A protein fusion, referred to as Hygro-TK, is concurrently expressed and confers both resistance to hygromycin and sensitivity to ganciclovir (Fig. 1). After transient expression of the Cre recombinase, precise recombination occurs between LoxP sites within the chromosomally integrated provirus (Fig. 1). As a consequence, both Hygro-TK and SV40T genes are permanently excised from the genome, whereas the neomycin resistance gene (NeoR), which confers resistance to G418, becomes activated (Fig. 1).

Primary adult human hepatocytes were submitted to the reversible immortalization procedure upon transduction with SSR#69 virions produced in an amphotropic packaging cell line free of replication-competent retrovirus (*6*). One of the resulting immortalized clones, referred to as the NKNT-3 cell line, was chosen for further analysis on the basis of growth characteristics and liver-specific functions. NKNT-3 cells became immortal without an obvious growth crisis, grow in monolayers in the chemically defined serum-free medium CS-C (*7*), and double in number about every 48 hours. NKNT-3 cells display morphological characteristics of liver parenchyma cells such as cytoplasmic granules and large nuclei with a few nucleoli. NKNT-3 cells express SV40T, as assessed by immunofluorescence staining (*8*), but were not tumorigenic after transplantation into severe combined immunodeficiency (SCID) mice (*9*). Sensitivity of NKNT-3 cells to either 5 μM ganciclovir or G418 (500 μg/ml) was confirmed by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl 2H-tetrazolium bromide (MTT) cytotoxic assays (*10*).

To determine whether excision of the transferred oncogene from the NKNT-3 genome could be achieved in the presence of Cre recombinase, we transduced NKNT-3 cells with a replication-deficient recombinant adenovirus (Ad) that expresses the Cre recombinase tagged with a nuclear localization signal (NLS) (*11*) (Fig. 1). As expected, transient expression of Cre recombinase triggered a switch in gene expression: Cells became resistant to G418 and no longer expressed SV40T, as assessed by Southern blot, reverse transcription polymerase chain reaction (RT-PCR), and Western blot analyses (Fig. 2A). After a 7-day selection with G418, complete elimination of cells expressing SV40T was achieved beyond the limit of detection (Fig. 2A). To ensure that SV40T had been permanently removed from virtually all cells, we performed a thorough examination of a large number of cells by immunofluorescence with a labeled monoclonal antibody to SV40T both in vitro and after intrasplenic transplantation in vivo (Fig. 3, B and D). After removal of SV40T, NKNT-3 cells looked more differentiated, with nucleus to cytoplasm ratios and number of cytogranules comparable to those of normal primary hepatocytes (Fig. 3C), and could no longer proliferate. On the basis of these data, we adopted for further studies a combination of infection with Ax-CANCre at multiplicity of infection (MOI) 10 and subsequent selection with G418 (500 μg/ml) for 7 days.

A candidate hepatocyte cell line must meet several biochemical requirements to alleviate acute liver failure: it must (i) reduce bilirubinemia and jaundice, (ii) improve he-

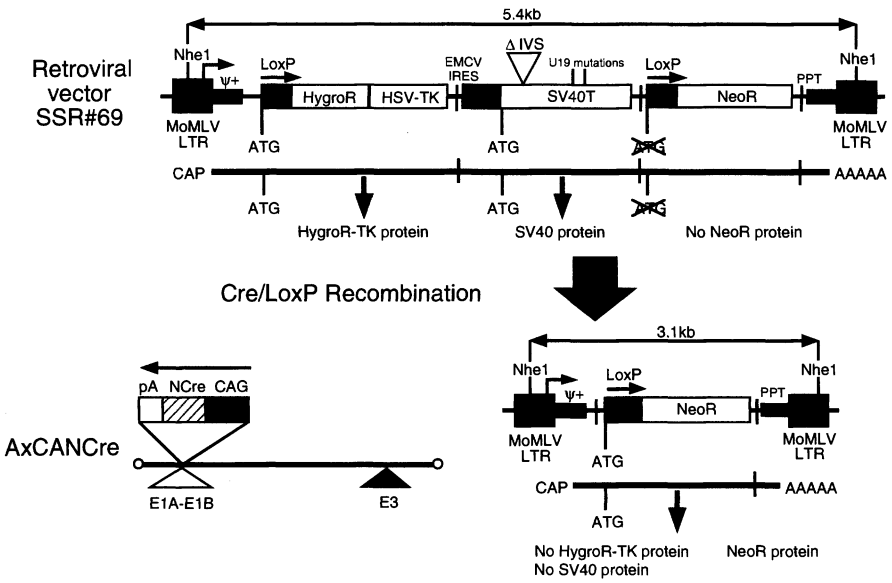


**Fig. 1.** Principles of the procedure of reversible immortalization. The retroviral vector SSR#69 (*3*) (**top**) comprises the following elements from 5′ to 3′: (i) Moloney murine leukemia virus (MoMLV) long terminal repeat (LTR) with packaging signal (Ψ+), (ii) an initiation codon followed by a LoxP recombination target, whose overlapping open reading frame was fused to a hygromycin resistance/herpes simplex virus thymidine kinase (HygroR/HSV-TK) fusion gene, (iii) the encephalomyocarditis virus internal ribosomal entry site (EMCV-IRES), which allows internal initiation of translation, (iv) the supertransforming U19 mutant of SV40T from which the intron was deleted to avoid splicing of the viral transcript and prevent expression of SV40 small t, (v) a second LoxP in direct orientation followed in frame by the neomycin resistance (NeoR) gene, but, importantly, lacking an initiation codon, and (vi) another LTR preceded by its polypurine track. Only HygroR/HSV-TK and SV40T are expressed in transduced cells in the absence of Cre recombinase. After Cre/LoxP recombination, the intervening DNA segment between the two recombination targets is excised (**bottom right**), so that only cells having excised SV40T become simultaneously G418 and ganciclovir resistant. (**bottom left**) Schematic representation of AxCANCre, the adenoviral vector expressing Cre recombinase (*11*).

[1]First Department of Surgery and [3]Department of Cell Biology, Okayama University Medical School, 2-5-1 Shikata-cho, Okayama 700-8558, Japan. [2]Massachusetts Institute of Technology, Division of Health Sciences and Technology, 77 Massachusetts Avenue, Cambridge, MA 02139, USA, and Genetix Pharmaceuticals, 840 Memorial Drive, Cambridge, MA 02139, USA. [4]Department of Surgery, University of Nebraska Medical Center, Omaha, NE 68198, USA. [5]Harvard Medical School and Division of Hematology, Department of Medicine, Brigham and Women's Hospital, 75 Francis Street, Boston, MA 02115, USA.

*To whom correspondence should be addressed. E-mail: ntanaka@med.okayama-u.ac.jp (N.K.); ifox@surgery.unmc.edu (I.J.F.); paulvw@mit.edu (P.L.).

patic encephalopathy due to hyperammonemia, (iii) eliminate other toxic substances, and (iv) produce clotting factors (*4*). Decreasing hyperammonemia seems especially important to prevent the development of hepatic encephalopathy and brain death (*2*). As glutamine synthetase (GS) is the main contributing enzyme responsible for ammonia clearance, high expression of GS appears essential (*12*). Accordingly, we assessed the expression of key genes of liver metabolism by Northern blot analysis: albumin, GS, hepatic bilirubin–uridine diphosphate–glucuronosyltransferase (Bil-UDT), glutathione *S*-transferase π (GST-π), and human blood coagulation factor X (HBCF-X); the housekeeping mRNA glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control (Fig. 2C). We found that Bil-UDT, GS, and GST-π mRNAs were expressed in NKNT-3 cells before reversal of immortalization but that their levels increased substantially after excision of the SV40T gene. Furthermore, albumin and HBCF-X mRNAs could only be detected after removal of the SV40T gene (Fig. 2C). This was not observed with a control Ad expressing LacZ (*13*).

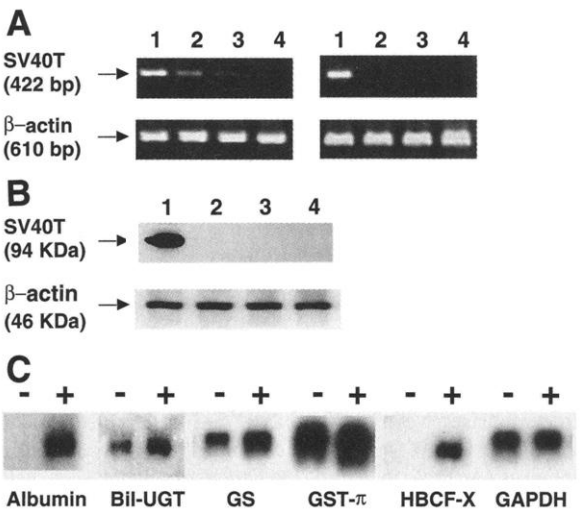


**Fig. 2.** (**A**) Expression of proviral RNA encoding SV40T in NKNT-3 cells before and after Cre recombination and without (*21*) or with (*22*) G418 selection. (top left) RT-PCR analysis with primers specific for SV40T (*23*) without G418 selection of cells (*21*). (Top right) RT-PCR analysis with primers specific for SV40T (*23*) after 7-day G418 selection of cells (*22*). (Bottom) β-actin loading controls for RT-PCR. Lane 1, no AxCANCre; lane 2, AxCANCre at MOI 5; lane 3, AxCANCre at MOI 10; and lane 4, AxCANCre at MOI 25. SV40T mRNA was markedly decreased after infection with AxCANCre at MOI 25 but could still be detected. However, SV40T mRNA could no longer be detected after AxCANCre infection and G418 selection. bp, base pairs. (**B**) (Top) Western blot analysis with SV40T monoclonal antibody (*24*) after 7-day G418 selection of cells (*22*). (Bottom) β-actin loading controls for Western blot analysis; lanes 1, 2, 3, and 4 are as in (A). SV40T protein could no longer be detected after AxCANCre infection and G418 selection. (**C**) Northern blot analysis of liver-specific mRNAs before (−) and after (+) Cre-mediated recombination of NKNT-3 cells; GAPDH was used as "housekeeping" mRNA control (*25*). Data in (A), (B), and (C) were obtained from three independent experiments.

We then set out to evaluate NKNT-3 cells after their transplantation in a rat model of acute liver failure (ALF) (*14*). NKNT-3 cells, before or after recombination, were transplanted into the spleen of rats (*15*) with ALF induced by 90% hepatectomy. We adopted this surgical procedure because of its superior reproducibility when compared with ALF induced by hepatotoxins or liver ischemia (*1*, *4*). In this model, 100% of rats having undergone 90% hepatectomy die of ALF within 3 days (*1*, *4*). Lewis rats weighing 350 g were used in transplant experiments and divided into the following groups: group 1 (G1: $n$ = 10), intrasplenic injection of 0.5 ml of medium; group 2 (G1: $n$ = 10), intrasplenic transplantation (Isp-Tx) of $5 \times 10^7$ nonreverted NKNT-3 cells; and group 3 (G3: $n$ = 10), Isp-Tx of $5 \times 10^7$ NKNT-3 cells treated with AxCANCre at MOI 10 and subsequent G418 (500 μg/ml) selection for 7 days. All animals underwent 90% hepatectomy 1 day after transplantation and received a daily intramuscular administration of FK506 (1mg/kg) to prevent rejection of human xenotransplanted cells. Transplantation of normal rat hepatocytes was not performed as a control in the present experiments, because the beneficial effect of transplanting primary hepatocytes into the spleen has been shown by many investigators in the rat model of ALF (*1*, *4*). Rats were followed for 4 weeks after hepatectomy, at which time liver regeneration sufficient to ensure long-term survival was achieved if the animals did not die of ALF during the first 3 days after hepatectomy (*1*, *4*). Postoperatively, biochemical parameters that include total bilirubin (T.Bil), prothrombin time (PT), and blood ammonia ($NH_3$) were measured in the plasma of transplanted and hepatectomized rats at various time points (*16*) (Fig. 4, A, B, and C). Ninety percent of hepatectomized rats having undergone transplantation of NKNT-3 or reverted NKNT-3 cells showed significant improvement in all parameters (Fig. 4, A, B, and C) and significantly better survival rate when compared with G1 rats (Fig. 4D). G3 rats having received reverted NKNT-3 cells showed better improvement in survival compared with G2 rats, but this was not statisti-

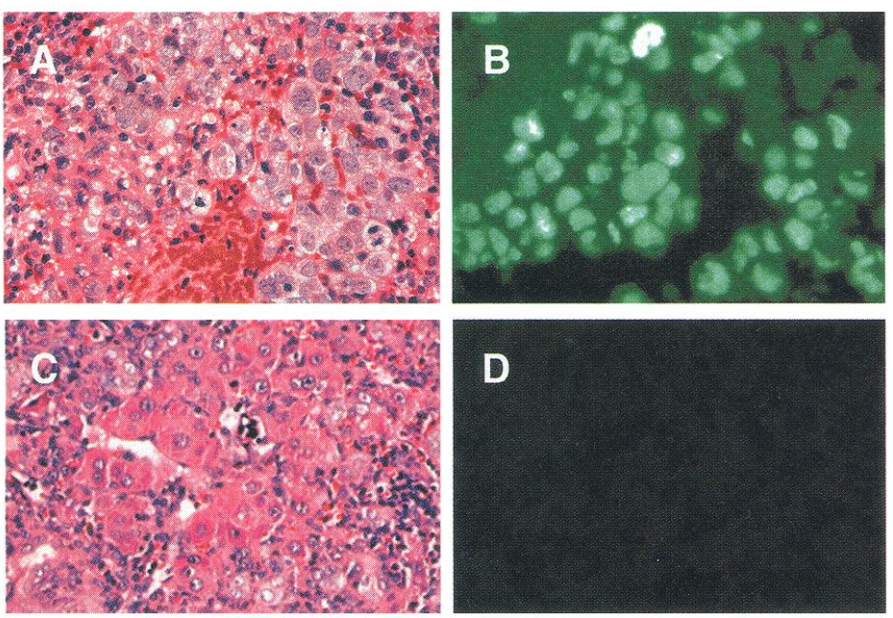


**Fig. 3.** Microphotographs of spleen sections 4 days after 90% hepatectomy and intrasplenic implantation of NKNT-3 cells (*18*). (**A** and **C**) Hematoxylin and eosin staining. Magnification, ×200. (**B** and **D**) Immunofluorescent staining with SV40T-specific monoclonal antibody. Magnification, ×200. (A and B) Nonreverted NKNT-3 cells; (C and D) Cre-reverted NKNT-3 cells. Clusters of implanted NKNT-3 cells with hepatocellular morphology are evident in the splenic red pulp. In contrast to nonreverted NKNT-3 cells (A), reverted NKNT-3 cells have a single nucleus and normal nucleus to cytoplasm ratios and are organized in a trabecular pattern in the spleen (C). Extracellular bile accumulation is seen around transplanted cells. No SV40T nuclear immunofluorescence was observed in reverted NKNT-3 cells transplanted into the spleen (D versus B).

cally significant (Fig. 4D). In another cohort, rats that survived were killed 4 days after transplantation for anatomical and histopathological examinations. In the surviving rats, the remnant caudate lobes of the liver was markedly enlarged, a characteristic sign of ongoing liver regeneration (*17*). NKNT-3 cells and reverted NKNT-3 cells were found in the spleen on histological sections (*18*) (Fig. 3). Islands of splenic "hepatization" were also apparent with reverted NKNT-3 cells, and extracellular bile accumulation was observed around transplanted cells because of the lack of drainage system in the spleen (Fig. 3C). These findings demonstrate the feasibility of controlling the expansion of primary human hepatocytes by Cre/Lox-based reversible immortalization with adequate preservation of metabolic functions, which become in fact further enhanced after removal of the transferred oncogene. These data also indicate that transplanted NKNT-3, whether reverted or not, survive in the spleen of rats and provide adequate metabolic support during ALF until the native liver recovers.

Intrasplenic HTX of $5 \times 10^7$ reversibly immortalized human hepatocytes, which are equivalent to about 5% of the total number of hepatocytes per adult rat, was able to protect animals from ALF. Why could such a small number of hepatocytes enable the rats to recover from ALF? Considering that all rats submitted to 90% hepatectomy without transplantation (G1 rats) die within 36 hours after surgery and that most rats survive after 80% hepatectomy with no special treatment, 10% additional liver mass equivalent appears sufficient to bridge life until spontaneous regeneration of the liver occurs. One can therefore surmise that intrasplenic injection of $5 \times 10^7$ cells (5% liver mass equivalent) may indeed suffice to ensure short-term survival.

For clinical applications in humans, preintegration in the NKNT-3 cell line of an inducible Cre expression cassette (e.g., by tetracycline-mediated induction) (*19*) may allow considerable expansion of the cell population before reversion without the need for adenoviral superinfection. In addition, replacing the NeoR gene with that encoding the green fluorescent protein should allow efficient and rapid isolation of cells having undergone Cre-mediated recombination (*20*). As alternatives to direct intrasplenic implantation, NKNT-3 cells may be placed in immunoprotective microcapsules or used to generate bioartifical liver support. Three levels of safeguards make it unlikely that transplanted and reverted NKNT-3 cells would expose patients to any oncogenic risk: (i) efficient elimination of the transferred oncogene by site-specific recombination followed by differential selection, (ii) allogeneic transplantation requiring temporary immunosuppression, and (iii) incorporation of a gene "suicide" (Hygro-TK) in the presence of ganciclovir. This approach may prove to be a valuable therapeutic strategy to surmount the problem of organ shortage that currently limits the use of liver or hepatocyte transplantation. In the future, reversible immortalization procedures may be extended to other somatic cells with potential applications in various medical conditions.

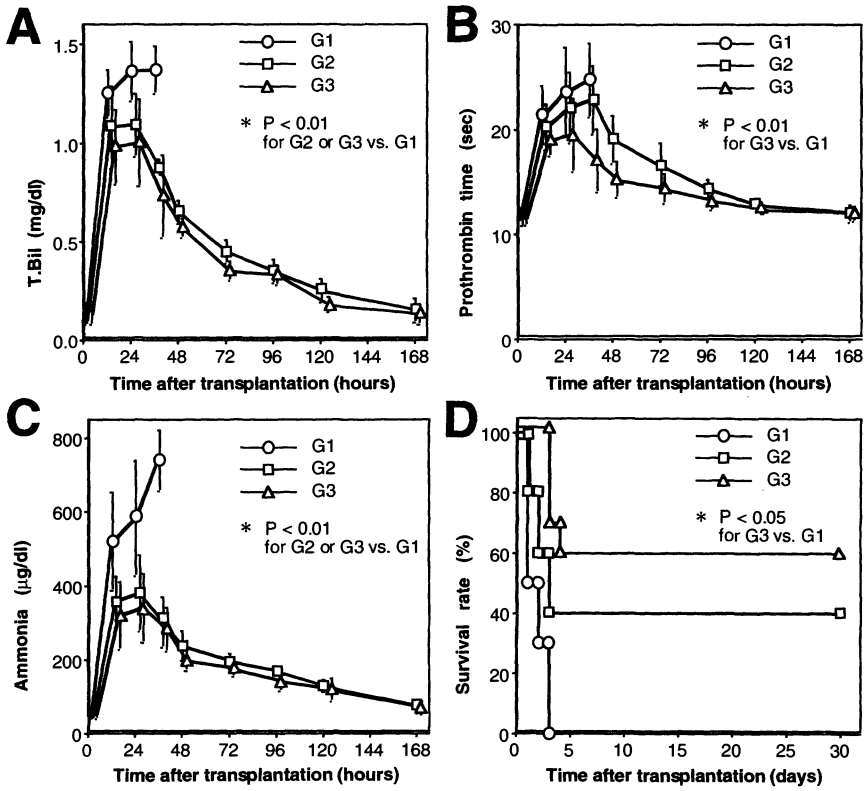


**Fig. 4.** Postoperative levels of total bilirubin (**A**), prothrombin time (**B**), and ammonia (**C**) and survival rate (**D**) in 90% hepatectomized rats (*16*). Group 1 (G1), no cell transplantation; group 2 (G2), intrasplenic transplantation of nonreverted NKNT-3 cells; and group 3 (G3), intrasplenic transplantation of reverted NKNT-3 cells (10 rats per group). Error bars indicate standard deviations. Statistical differences were determined by the Mann-Whitney U test, followed by the two-tailed Student's *t* test. The statistical analysis of survival time was done by the Kaplan-Meier survival test.

### References and Notes


1. A. A. Demetriou, *Science* **233**, 1190 (1986); N. Arkadopoulos *et al.*, *Hepatology* **28**, 1365 (1998).
2. S. C. Strom *et al.*, *Transplantation* **63**, 559 (1997); I. J. Fox *et al.*, *N. Engl. J. Med.* **338**, 1422 (1998); J. R. Chowdhury *et al.*, *Pediatrics* **102**, 647 (1998); S. C. Strom, J. R. Chowdhury, I. J. Fox, *Semin. Liver Dis.* **19**, 39 (1999).
3. K. A. Westerman and P. Leboulch, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 8971 (1996).
4. I. K. Schumacher *et al.*, *Hepatology* **24**, 337 (1996); J. Nakamura *et al.*, *Transplantation* **63**, 1541 (1997).
5. H. V. Gartner *et al.*, *N. Engl. J. Med.* **335**, 1494 (1996); K. Paradis *et al.*, *Science* **285**, 1236 (1999).
6. The amphotropic ΨCrip packaging cell line producing SSR#69 virus was grown as described in (*3*). Viral titers were $5 \times 10^4$ plaque-forming units (PFU)/ml as assessed on NIH3T3 cells after selection with hygromycin as described in (*3*).
7. Adult human hepatocytes and the serum-free culture medium CS-C were purchased from Cells Systems (Seattle, WA). Cells were tranduced with 2 ml of ΨCrip cell supernatant per T75 flask in the presence of polybrene (12 μg/ml) at 37°C two times for 4 hours. Two days after transduction, selection was applied with CS-C medium containing hygromycin (320 μg/ml). Hygromycin-resistant hepatocyte clones were isolated with cloning rings 5 weeks after the start of selection.
8. For the detection of SV40T, indirect immunofluorescent staining was carried out. NKNT-3 cells ($5 \times 10^4$) were grown overnight on sterile slides at 37°C, rinsed with phosphate-buffered saline (PBS) solution, and fixed in cold acetone. Samples were blocked with 10% fetal bovine serum in PBS for 1 hour at room temperature. Primary antibody was mouse monoclonal immunoglobulin G2a (IgG2a) antibody to SV40T (Santa Cruz Biotechnology, Santa Cruz, CA), used at a concentration of 100 μg/ml overnight at 4°C. IgG–fluorescein isothiocyanate rabbit polyclonal antibody to mouse IgG (Santa Cruz Biotechnology; Santa Cruz, CA) was subsequently used at a concentration of 10 μg/ml for 1 hour at 37°C.
9. NKNT-3 cells suspended in 0.5 ml of ASF-104 medium were subcutaneously injected into the dorsal midline of five 6- to 8-week-old SCID mice. Mice were observed for 2 months after injection.
10. NKNT-3 cells were plated in a 96-well microplate at $5 \times 10^3$ cells per well to obtain a growth curve. On days 1, 3, 5, and 7 MTT (20 μg/ml) was added to a different well, incubated for 4 hours, and then reacted with 150 μl of isopropanol for 10 min. Relative percentage of viability was determined by the ratio of absorbance at 570 and 630 nm with Bio-Rad EIA reader (Richmond, CA).
11. Y. Kanegae *et al.*, *Nucleic Acids Res.* **23**, 3816 (1995). AxCANCre was obtained from Riken Gene Bank (Ibaragi, Japan). It expresses an NLS-tagged Cre recombinase under control of the CAG promoter with viral titers of $3 \times 10^8$ PFU/ml.
12. R. Gebhardt *et al.*, *Histochemistry* **92**, 337 (1989).
13. An Ad-expressing lacZ gene with the same promoter

as AxCANCre, AxCALacZ, was also purchased from Riken Gene Bank as a control vector.
14. Ninety percent hepatectomy was done by removing the median, left lateral, right upper, and lower lobes by ligation, leaving only the caudate lobe. After surgery, animals were allowed free access to tap water supplemented with 10% dextrose and received a daily intravenous injection of 2 ml of saline.
15. For intrasplenic transplantation, a small surgical incision was made in the animal's flank, and the spleen was exposed under anesthesia and surgical care approved by the animal committee of Okayama University Medical School. Cells ($50 \times 10^6$) suspended in 0.5 ml of ASF-104 were injected into the inferior pole of the spleen. The blood flow in the splenic artery and vein was temporarily occluded to avoid immediate passage of cells into the portal vein during transplantation. The injection site was also ligated to prevent cell leakage and bleeding.
16. Blood samples were obtained from tail veins, and the levels of T.Bil, PT, and NH3 were measured with Fuji Dry Chem (Tokyo, Japan).
17. N. Kobayashi *et al.*, data not shown.
18. Spleen specimens were compound embedded and frozen at −80°C. Cryostat sections of the spleen (5-μm thick) were fixed in ice-cold acetone. Immunofluorescence for SV40T of NKNT-3 cells transplanted into the spleen was performed as the same procedure as described in (*11*).
19. M. Gossen *et al.*, *Science* **268**, 1766 (1995).
20. R. Pawliuk *et al.*, *Nature Med.* **5**, 768 (1999).
21. NKNT-3 cells ($6 \times 10^6$) were plated in T75 flasks and infected 1 day later with AxCANCre at various MOI for 1 hour. Cells were subsequently cultured in the chemically defined serum-free medium ASF-104 (Ajinomoto, Tokyo, Japan) for 2 days and then harvested for RT-PCR, Western blot, and Northern blot analyses.
22. Infection of AxCANCre was performed as described in (*21*). After adenoviral infection, NKNT-3 cells were cultured in ASF-104 medium containing G418 (500 μg/ml) for 7 days and then harvested for RT-PCR, Western blot, and Northern blot analyses.
23. Total RNA was isolated by the RNAzol procedure (Cinna/BioTecx, Friendswood, TX). RT was performed at 22°C for 10 min and then 42°C for 20 min with 1 μg of RNA per reaction. PCR was performed with specific primers in volumes of 50 μl and according to the manufacturer's instructions (PCR kit; Perkin-Elmer/Cetus, Norwalk, CT). Primers used were as follows: for SV40T, 5′ primer CAGGCATAGAGTGTCTGC and 3′ primer CAACAGCCTGTTGGCATATG; and for β-actin, 5′ primer TGACGGGGTCACCCACACTGTGCCCATCTA and 3′ primer CTAGAAGCATTTGCGGTGGACGATGGAGGG. PCR conditions were as follows: denaturation at 92°C for 1 min, annealing at 58°C for 1 min, and elongation at 72°C for 1 min with a thermal cycler (Perkin-Elmer, Foster City, CA). PCR products were resolved on 2% agarose gels and visualized by ethidium bromide staining.
24. Proteins (30 μg per lane) from NKNT-3 cells and reverted NKNT-3 cells were separated by electrophoresis on SDS-polyacrylamide gels, transferred to hybond–polyvinylidene difluoride transfer membranes, and treated with mouse monoclonal antibody to SV40T (Santa Cruz Biotechnology) (1:100) followed by peroxidase-linked secondary antibody (1:2500). Labeled protein bands were stained with ECL kit (Amersham, Japan). Human β-actin protein served as an internal control.
25. Northern blot analysis was performed as described (*3*). Specific DNA probes were obtained by PCR of genomic DNA and then radiolabeled.
26. We thank J. Miyazaki for providing the CAG promoter and H. Lodish and I. M. London for comments on the manuscript. Supported by NIH grants HL55435 to P.L. and DK48794 to I.J.F.

4 November 1999; accepted 23 December 1999

# The Glucocorticoid Receptor: Rapid Exchange with Regulatory Sites in Living Cells

**James G. McNally,* Waltraud G. Müller,* Dawn Walker, Ronald Wolford, Gordon L. Hager†**

**Steroid receptors bind to site-specific response elements in chromatin and modulate gene expression in a hormone-dependent fashion. With the use of a tandem array of mouse mammary tumor virus reporter elements and a form of glucocorticoid receptor labeled with green fluorescent protein, targeting of the receptor to response elements in live mouse cells was observed. Photobleaching experiments provide direct evidence that the hormone-occupied receptor undergoes rapid exchange between chromatin and the nucleoplasmic compartment. Thus, the interaction of regulatory proteins with target sites in chromatin is a more dynamic process than previously believed.**

Steroid receptors modulate rates of transcription at target genes through protein-protein interactions with basal transcription factors and through the recruitment of a variety of coactivators or corepressors (*1*). Some of these interacting proteins serve as bridging factors to other components of the soluble transcription apparatus, and others either harbor intrinsic chromatin-modifying activities [such as acetylation or methylation (*2*)] or interact with other chromatin-remodeling activities [including the swi/snf family of nucleosome-remodeling proteins (*3*)]. The dynamic process by which the receptors recruit these factors to activate transcription is poorly understood. The classic view is that the receptor binds to a recognition site and remains at that site for as long as the ligand is present in the cellular milieu (*4*). Alternatively, the receptor may interact transiently with a response element, recruiting a secondary set of factors that in turn form a stable complex at the regulatory site. This type of mechanism has been referred to as "hit and run" and has been proposed both for the glucocorticoid receptor (GR) (*5*, *6*) and for enhancer function in general (*7*) (Fig. 1). These issues could not be addressed by the indirect methods traditionally used to detect transcription factor– DNA binding and function. We report here the direct observation of GR interaction with hormone response elements in living cells. Using photobleaching techniques, we show that the receptor undergoes continuous exchange between chromatin regulatory elements and the nucleoplasmic compartment when ligand is constantly available.

The cell line 3134 contains a large tandem array of a mouse mammary tumor virus/Harvey viral ras (MMTV/v-Ha-ras) reporter (*8*). The repeat structure arose from the spontaneous chromosomal integration of a 9-kb bovine papilloma virus (BPV) multicopy episome, creating a head-to-tail array of $1.8 \times 10^6$ base pairs (bp) (Fig. 2A). This structure contains about 200 copies of the long terminal repeat (LTR) and thus includes 800 to 1200 binding sites for GR. Derivatives of this cell line express a green fluorescent protein (GFP)–tagged version of GR (GFP-GR) from a chromosomal locus under control of the tetracycline-repressible promoter (*8*). The GFP-GR that is expressed in these cell lines after removal of tetracycline is resident in the cytoplasm in the absence of ligand but translocates to the nucleus within 10 min of hormone addition, as detected by live-cell epifluorescence (*8*).

The MMTV array in these cell lines was large enough to be observed by light microscopy. Fluorescence in situ hybridization analysis (FISH) of DNA was performed on metaphase chromosomes from cell line 3617 with a probe to the ras insert in the tandem array (Fig. 2). The cell line is aneuploid, as expected for a ras-transformed murine carcinoma cell maintained in culture for many generations. Hybridization with the ras probe alone revealed the presence of a large ras-specific structure near the centromere of one chromosome (Fig. 2C). Subsequent analysis with a chromosome 4 telomere-specific probe showed that the array was located on this chromosome (Fig. 2B). The array thus exists as a unique amplified element near the centromere in the A region of chromosome 4 (Fig. 2A).

When the distribution of GFP-GR is examined in living cells (Fig. 3), multiple nuclear structures are observed after ligand activation (*9*). The receptor is located in a series of bright focal structures, overlayed against a fine reticular pattern that is present throughout the nucleus but not including the nucleoli. In cell line 3617, however, the receptor also accumulates in one large structure that is present as one copy

Laboratory of Receptor Biology and Gene Expression, Building 41, Room B602, National Cancer Institute, Bethesda, MD 20892–5055, USA.

*These authors contributed equally to this work.
†To whom correspondence should be addressed. E-mail: hagerg@exchange.nih.gov