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Journal

Cell transplantation, 17(8)

ISSN

0963-6897

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Publication Date

2008

DOI

10.3727/096368908786576471

Peer reviewed

Cell Transplantation, Vol. 17, pp. 899–909, 2008
Printed in the USA.

0963-6897/08 $90.00 + .00
E-ISSN 1555-3892
www.cognizantcommunication.com

Use of Bioluminescent Imaging to Assay the Transplantation of Immortalized Human Fetal Hepatocytes Into Mice

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Noninvasive serial monitoring of the fate of transplanted cells would be invaluable to evaluate the potential therapeutic use of human hepatocyte transplantation. Therefore, we assessed the feasibility of bioluminescent imaging using double or triple fusion lentiviral vectors in a NOD-SCID mouse model transplanted with immortalized human fetal hepatocytes. Lentiviral vectors driven by the CMV promoter were constructed carrying reporter genes: firefly luciferase and green fluorescence protein with or without herpes simplex virus type 1 thymidine kinase. Human fetal hepatocytes immortalized by telomerase reconstitution (FH-hTERT) were successfully transduced with either of these fusion vectors. Two million stably transduced cells selected by fluorescence-activated cell sorting were injected into the spleens of NOD-SCID mice pretreated with methylcholanthrene and monocrotaline. The transplanted mice were serially imaged with a bioluminescence charged-coupled device camera after D-luciferin injection. Bioluminescence signal intensity was highest on day 3 ($6.10 \pm 2.02 \times 10^5$ p/s/cm^2/sr, mean ± SEM), but decreased to $2.26 \pm 1.54 \times 10^5$ and $7.47 \pm 3.09 \times 10^4$ p/s/cm^2/sr on day 7 and 10, respectively ($p = 0.001$). ELISA for human albumin in mice sera showed that levels were similar to those of control mice on day 2 (3.25 ± 0.92 vs. 2.84 ± 0.59 ng/ml, mean ± SEM), peaked at 18.04 ± 3.11 ng/ml on day 7, and decreased to 8.93 ± 1.40 and 3.54 ± 0.87 ng/ml on day 14 and 21, respectively ($p = 0.02$). Real-time quantitative RT-PCR showed gene expression levels of human albumin, α1-antitrypsin, and transferrin in mouse liver were $60.7 \pm 6.5\%$, $26.0 \pm 1.4\%$, and $156.8 \pm 62.4\%$ of those of primary human adult hepatocytes, respectively, and immunohistochemistry revealed cells with human albumin and α1-antitrypsin expression in the mouse liver. In conclusion, our study demonstrated that bioluminescent imaging appears to be a sensitive, noninvasive modality for serial monitoring of transplanted hepatic stem cells.

Key words: Hepatocytes; Stem cells; Transplantation; Luciferase; Imaging techniques

INTRODUCTION

Liver transplantation is the mainstay of therapy for end-stage liver diseases, but its use is restricted by the shortage of donor organs as well as the considerable morbidity and mortality associated with the process. Liver-directed cell therapies, including hepatocyte transplantation and extracorporeal bioartificial liver support devices, have been suggested as alternatives (1,6,23). Obtaining sufficient numbers of functional hepatocytes for these cell-based therapies remains an unsolved problem (11,14).

Primary human cells are a preferred source of hepatocyte function for liver cell transplantation. Because the availability of primary human hepatocytes is severely limited, various efforts have been made to generate functional human hepatocytes from diverse sources, including embryonic stem cells (ESC) (7), bone marrow stem cells (20), and liver stem cells (17). However, previous studies to direct these cells to differentiate into hepatocytes have shown only limited success. Another potential source is our telomerase-immortalized fetal hepatocytes. We previously reported that human fetal he-

Received June 11, 2007; final acceptance January 25, 2008.
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patocytes that were transduced with the catalytic subunit of telomerase reverse transcriptase were immortalized, capable of expressing liver-specific gene products in vitro and in vivo, and were nontumorigenic (26).

To evaluate the potential therapeutic use of hepatocyte transplantation, it is essential to evaluate the effectiveness of transplanted cells to engraft and repopulate the recipient liver. However, conventional transplantation methods require many animals to be sacrificed in order to assess their fate in vivo, and they provide only one point of observation per animal. Hence, it would be invaluable to develop a noninvasive method to monitor the fate of these cells on a longitudinal basis. Molecular imaging is a rapidly emerging research field that may be useful for that purpose (18). Employing specific molecular probes as the source of imaging allows concomitant visual and analytical biological phenotyping of animals. In addition, this methodology eliminates the need to sacrifice each mouse for characterization, and the consequent repetitive imaging makes it possible to investigate biologic activities that are otherwise difficult to interpret with single time point data (18).

The triple fusion lentiviral vector, developed by one of us, is an important achievement in this field that allows the use of several imaging modalities to evaluate the same biological system (19). This vector contains three reporter genes: the firefly luciferase (*fl*) or *Renilla* luciferase (*rl*) gene for bioluminescent imaging, the green fluorescence protein (*gfp*) or red fluorescence protein (*rfp*) gene for fluorescence microscopy, and a mutant herpes simplex virus type 1 sr39 thymidine kinase (*ttk*) gene for positron emission tomography (PET). The feasibility of molecular imaging using this vector has been evaluated in various in vitro and in vivo studies. To track the fate of transplanted cells, the viral vectors have been injected directly into animals (29), or into a variety of cells [neuroblastoma cells (5), rat glioma cells (2,24), melanoma cells (19), human embryonic stem cells (3,8), pancreatic islet cells (10), and 293T cells (24)] that were then transplanted into animals, mainly by implantation into subcutaneous tissue (2,5,19,24) or by direct injection into a target organ (3,10,28). However, this vector system and novel imaging modality have not previously been employed to assess the engraftment of human liver stem cells in mice.

Therefore, we assessed the usefulness of bioluminescent imaging employing this triple fusion vector system in a NOD-SCID mouse model transplanted with immortalized fetal hepatocytes. Our results indicate that this imaging system appears to be a promising approach for repeatedly and noninvasively monitoring transplanted hepatic cells.

MATERIALS AND METHODS

Culture of Immortalized Fetal Hepatocytes (FH-hTERT)

Our experiments were approved by the Institutional Review Board at the University of California, Davis and were performed in accordance with their guidelines. Human fetal hepatocytes were procured by Prof. S. Gupta of Albert Einstein College of Medicine, Bronx, New York with the approval of the Institutional Committee of Clinical Investigations. Telemerase reconstitution was done in our lab by ectopic expression of telomerase reverse transcriptase using a retrovirus vector as described previously (26). These immortalized fetal hepatocytes (FH-hTERT) were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% inactivated fetal bovine serum (Invitrogen), 5 μg/ml insulin (Sigma-Aldrich, St. Louis, MO), 2.4 μg/ml hydrocortisone (Sigma-Aldrich), 4 μmol/ml L-glutamine (Invitrogen), and standard antibiotics in a humidified 5% CO_2 atmosphere (26).

Construction of Double/Triple Fusion Lentiviral Vectors

A self-inactivated lentivirus was prepared by transient transfection of 293T cells as described previously by De et al. (5). Briefly, the firefly luciferase gene and the green fluorescence protein gene, with or without a mutant herpes simplex virus type 1 sr39 thymidine kinase gene, driven by the cytomegalovirus promoter (pCMV) in the lentiviral backbone (CS) (CS-pCMV-fl-gfp or CS-pCMV-fl-gfp-ttk), were cotransfected into 293T cells with the HIV-1 packaging vector and vesicular stomatitis virus G glycoprotein-pseudotyped envelop vector (pVSVG). Fu-Gene®6 (Roche Molecular Biochemicals, Indianapolis, IN) was used for cotransfection according to the manufacturer. Lentivirus supernatant was harvested 48 and 72 h after transfection, centrifuged at a low speed (1500 rpm for 5 min), and filter purified by passing through a 0.45-μm filter. To make a high-titer concentrated stock of virus particles, the virus-containing medium was centrifuged for 3 h at $50{,}000 \times g$ using a SW28 (Beckman-Coulter Inc., Fullerton, CA) rotor. After centrifugation, the virus particles were dissolved in 300 μl serum-free media and stored at −70°C in aliquots. The viral titers of double/triple fusion vectors were assessed using the HIV-1 p24 Antigen EIA kit (Beckman Coulter Inc.). Viral titers of the double/triple fusion vectors as high as 10^{10} transducing units/ml were obtained.

Transduction of FH-hTERT With Double/Triple Fusion Lentiviral Vectors

FH-hTERT were transduced with either double or triple fusion lentiviral vectors at a multiplicity of infection

(MOI) 50 to 100 in the presence of 8 μg/ml hexadimethrine bromide (Sigma-Aldrich). Successfully transduced cells (FH-hTERT-DF/TF) were selected by fluorescence-activated cell sorting (FACS) using a Cytomation MoFlo Cell Sorter (Becton Dickinson, San Jose, CA) 3 days after transduction.

FH-hTERT-DF/TF Cell Transplantation

The protocols for animal experiments performed in the present study were approved by the UC Davis Institutional Animal Care and Use Committee, according to *Guideline for the Care and Use of Laboratory Animals* and *Principals for the Utilization and Care of Vertebrate Animals* by the National Institutes of Health. FH-hTERT-DF or FH-hTERT-TF were transplanted into groups of 7–8-week-old NOD-CB17-prkdc-scid mice (Jackson Labs, Bar Harbor, ME). These mice have a severe combined immunodeficient syndrome; they exhibit no T- or B-cell activity and demonstrate impaired natural killer cell and macrophage function. The mice were maintained in a rodent barrier facility providing a sterile environment and fed autoclaved chow and water ad libitum. Five days before transplantation, all animals were treated with methylcholanthrene (Sigma-Aldrich) 50 mg/kg for 4 consecutive days. Methylcholanthrene was dissolved in corn oil and each mouse received a volume of 200 μl. These animals received monocrotaline (200 mg/kg, Sigma-Aldrich) IP, 24 h before cell transplantation. Five hundred milligrams of monocrotaline was dissolved in HCl, pH 2.5, followed by neutralization by NaOH 0.1 N to a final pH of 7.4. Normal saline was added to a final volume of 10 ml and a final concentration of 50 mg/ml. A dose of 200 mg/kg was injected—the total volume depending on mouse body weight being less than 150 μl (mouse body weight was between 24 and 27 g). Two million FH-hTERT-DF/TF were transplanted via intrasplenic injection into NOD-SCID mice (9). Before midline laparotomy, general anesthesia was induced by IP injection of 2,2,2-tribromoethanol (250 mg /kg body weight, Sigma-Aldrich). The spleen was exposed and the tip of the spleen was ligated. Cell suspension (200 μl with 1.0×10^7 cells/ml) was injected into the spleen with a 27-gauge needle. Before transplantation, cell clumps were dispersed with the use of a 70-μm cell strainer (26).

Preliminary Evaluation for Feasibility of Bioluminescent Imaging

To assess the detection threshold for luciferase, transduced cells (from 10^6 to 10^2 per well) were evaluated using a bioluminescence optical charged-coupled device (CCD) camera. Serial cell dilutions of FH-hTERT-TF were done in triplicate and plated on 96-well plates for bioluminescent imaging. After adding reporter probe 1 μl D-luciferin potassium salt (30 mg/ml in PBS; Xenogen, Hopkinton, MA), bioluminescent imaging was performed with the in vivo Imaging System (IVIS, Xenogen) (2). Bioluminescence was quantified in units of maximum photons per second per centimeter squared per steradian (p/s/cm^2/sr) (27).

To evaluate the feasibility of bioluminescent imaging in mice transplanted with FH-hTERT-TF, 2×10^6 cells sorted by FACS for GFP were injected into the spleens of pretreated NOD-SCID mice. One week following the cell transplantation, the mice were imaged with the CCD camera after D-luciferin potassium salt (375 mg/kg body weight) injection under isoflurane anesthesia. The mice were sacrificed and tissue evaluated for luciferase activity. Mice were sacrificed 3 weeks after cell transplantation and liver tissue was removed to evaluate the engraftment of transplanted cells. The expression level of human glyceraldehyde phosphate dehydrogenase (hGAPDH) was evaluated in mouse liver tissue and serially diluted cultured FH-hTERT by real-time quantitative RT-PCR using mouse β-actin (mBAC) as a reference. Samples were amplified on the same plate with standards consisting of mouse liver tissue with 10^2 to 10^6 spiked human cells (FH). All amplifications were performed in duplicate and repeated twice to control for Poisson errors. Following amplification, the threshold of specific amplification was set within the log-linear region of product generation to determine the fractional cycle number at which the reaction passes the threshold of positive amplification (C_T). hGAPDH amplification was normalized to mBAC by subtraction: $\Delta C_T = C_T(\text{hGAPDH}) - C_T(\text{mBAC})$. A standard curve was established with the ΔC_T values of standards and the number of the engrafted cells was calculated from 30 mg of initial liver tissue. An engraftment rate (%, number of FH-hTERT-TF after 3 weeks/number of transplanted FH-hTERT-TFtimx100) was calculated to estimate cell survival after 3 weeks (25).

RNA extracts from liver tissue of transplanted mice were used for real-time quantitative RT-PCR assessment of intrahepatic maturation of engrafted FH cells. Human-specific amplification was performed for albumin. Amplification curves were compared to the human specific endogenous control (hGAPDH). RNA extracts from FH cells in vitro were used as calibrators to determine relative expression levels.

Assessment of Bioluminescent Imaging for Mice Transplanted With FH-hTERT-DF/TF

NOD-SCID mice transplanted with 2×10^6 FH-hTERT-DF/TF cells were serially imaged as described above. The first imaging was performed 3 days after transplantation (day 3), then on day 7 and day 10 (Fig. 3). After various intervals, animals were sacrificed by exsanguination under general anesthesia. Blood was collected

from the inferior vena cava for serological analysis and liver samples were obtained for further analysis. Freshly isolated primary human adult hepatocytes, cultured FH-hTERT, or liver tissue samples from control mice (pretreated mice not undergoing transplantation) served as controls (26). Human albumin secretion of the transplanted cells was evaluated by ELISA and liver sections were stained with antibodies specific for human cells and used for RNA extraction to assess the human liver-specific gene expression of these cells.

Measurement of Luciferase Activity

Luciferase activity was measured using the Dual-Luciferase® Reporter Assay kit (Promega, Madison, WI). In brief, 30 mg of liver tissue or each well of cultured FH-hTERT-DF or FH-hTERT-TF cultured in six-well plates was dispersed in 500 µl of 1× passive lysis buffer for 15 min at room temperature. A mixture of 100 µl LAR II and 20 µl of lysate was used to measure the firefly luciferase activity using the Lumat LB 9507 (Berthold Technologies GmbH & Co. KG, Germany). The protein content of the cell lysates was determined and the luminescence results were described as relative light units per milligram of protein (RLU/mg).

PCR Amplification of GFP Sequences in Liver DNAs

Genomic DNA was extracted from liver of NOD-SCID mouse transplanted with FH-hTERT-DF cells using the DNeasy Tissue Kit® (Qiagen, Valencia, CA). PCR proliferation of the GFP sequence was done as follows. Cultured FH-hTERT transduced with lentiviral vectors carrying the GFP gene (FH-hTERT-GFP) was used as a positive control and the PCR mix as a negative control. The sequence of the forward primer was AGA ACGGCATCAAGGTGAAC and that of the reverse primer was TGCTCAGGTAGTGGTTGTCG. PCR was done in a final volume of 50 µl containing 45 µl of Platinum PCR Supermix (Invitrogen), 1.5 µl of each primer (15 mM), and 200 ng genomic DNA. The conditions were 94°C for 5 min, followed by 40 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, and final elongation at 72°C for 7 min. PCR products were electrophoresed in 1.5% agarose gels containing ethidium bromide. A PCR product of 125 bp size was expected.

ELISA for Human Albumin

Sera from recipient mice were analyzed for human albumin by ELISA using the Human Albumin ELISA Quantitiation Kit (Bethyl Laboratories Inc., Montgomery, TX). Affinity-purified goat anti-human albumin antibody was used as coating antibody and goat anti-human albumin-HRP conjugate as the HRP detection antibody. Human reference serum was used as a calibrator.

Quantitative Gene Expression Analysis by Real-Time RT-PCR

RNA extracts were obtained from 30 mg of mouse liver tissue. Total RNA (1 µg) was extracted with RNeasy Mini Kit (Qiagen). Following digestion with DNase I, complementary DNA (cDNA) was generated employing Thermoscript RT-PCR Systems and oligo $(dT)_{20}$ to prime first-strand synthesis. Digestion with DNase I was performed before RT-PCR because not all the primer–probe sets were intron sparing and the existence of pseudogenes was not excluded for all target sequences. The generated cDNA was diluted to 10 or 20 ng RNA-derived cDNA/µl, and aliquots of 5 µl were used for various PCR amplifications (21,26,30).

Relative human gene expression analysis was performed by real-time quantitative RT-PCR with the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) and TaqMan PCR Master Mix (Applied Biosystems). The concentration of all primers and probes, as listed in Table 1, was optimized for specific annealing and primer–probe sets were validated to ensure equal PCR efficiencies over a two-log range (26). Expression levels of hepatocyte-specific genes were normalized to the housekeeping control (hGAPDH or mBAC) and compared with those of primary human adult hepatocytes or cultured FH-hTERT.

Immunohistology of Mouse Liver Tissue

To detect expression of human liver specific proteins, tissue was fixed for 2 h with 4% paraformaldehyde (PFA) in PBS at room temperature, for 1–2 h in 4% PFA-5% sucrose in PBS, and finally overnight in 20% sucrose in PBS. The fixed tissue was embedded in Tissue-Tek® Cryostat and OCT compound (Sakura Finetek USA, Torrance, CA) and quickly frozen in methylbutane at –70°C and stored at –70°C. Sections (5–7 µm thick) were cut with Cryostat CM3050 (Leica, Germany) and collected on microscope slides. Then slides were fixed with 1% PFA for 10 min at room temperature and post-fixed with ethyl alcohol/acetic acid (2:1) for 5 min at –20°C. The fixed tissue was incubated sequentially overnight with primary goat antibodies against human albumin and α1-antitrypsin (α1-AT, Sigma-Aldrich) diluted 1:1000 and with secondary rabbit anti-goat IgG-fluorescein isothiocyanate conjugates (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:1000 at room temperature for 1 h to visualize human albumin and α1-AT under a fluorescent microscope (21).

Statistical Analysis

Data are presented as means ± SEM. The Friedman test and the Kruskal-Wallis test with Dunn's multiple

Table 1. Primer Pairs and Hybridization Probes for Quantitative RT-PCR

Gene	Primer–Probe Sequence 5′–3′	5′-Label	Concentration
Human albumin	F: AGTTTGCAGAAGTTTCCAAGTTAGTG		300 nmol/L
	T: ACATTCAAGCAGATCTCCATGGCAGCA	FAM	300 nmol/L
	R: AGGTCCGCCCTGTCATCAG		50 nmol/L
Human α1-antitrypsin	F: TCGCTACAGCCTTTGCAATG		900 nmol/L
	T: AGCCTTCATGGATCTGAGCCTCCGG	FAM	300 nmol/L
	R: TTGAGGGTACGGAGGAGTTCC		300 nmol/L
Human transferrin	F: GTGTATCAGCAGAGACCACCGA		300 nmol/L
	T: TCTCCATTCATGATCTTGGCGATGCA	FAM	300 nmol/L
	R: CATCCAAGCTCATGGCATCA		300 nmol/L

F, forward primer; R, reverse primer; T, hybridization probe; FAM, 6-carboxyfluorescein.

comparison test were used for statistical analysis, and a value of $p < 0.05$ was considered significant.

RESULTS

Transduction of Immortalized Fetal Hepatocytes With Double/Triple Fusion Lentiviral Vectors

FH-hTERT were cultured and successfully transduced with these double or triple fusion lentivirus vectors showing a transduction efficiency of 40–50%, and stably transduced cells were selected by FACS (Fig. 1).

Preliminary Evaluation of Suitability of Bioluminescent Imaging

The detection threshold for luciferase in vitro was assessed from 96-well plates containing 10^2 to 10^6 FH-hTERT-TF cells by bioluminescent imaging. As few as 10^2 cells could be detected under CCD camera (Fig. 2).

Two million sorted FH-hTERT-TF cells were injected into the spleens of NOD-SCID mice that were treated with monocrotaline and methylcholanthrene. One week after cell transplantation, CCD camera imaging showed the signal in both mice. Luciferase activity of the tissue was high (2.7×10^6 RLU/mg protein) confirming the CCD camera images. Three weeks after cell transplantation, the number of surviving cells was estimated from the expression level of hGAPDH as determined by quantitative RT-PCR. An engraftment rate of 7.19% was observed in the mouse with the best levels of repopulation and engraftment.

Liver tissue extracts from transplanted NOD-SCID mice were used for real-time quantitative RT-PCR assessment of the intrahepatic maturation of engrafted FH cells. Human-specific amplification was performed for albumin. Amplification curves were compared to the human specific endogenous control (hGAPDH). RNA extracts from passage 34 FH-hTERT cells (FH-hTERT P34) in vitro were used as calibrators to determine relative expression levels. The increase in expression compared to FH-hTERT P34 was graphed on a log scale. Replicate RNA extracts ($n = 3$) were analyzed for each transplanted mouse. Three weeks after cell transplantation, expression of the human albumin gene increased by more than 5 log levels compared to cultured FH-hTERT P34.

Assessment of Bioluminescent Imaging for Mice Transplanted With FH-hTERT-DF/TF

Two million sorted transduced cells were injected into the spleens of another set of nine NOD-SCID mice pretreated with methycholanthrene and monocrotaline. Two mice died early after transplantation. Three days, 7 days, and 10 days after the cell transplantation, CCD camera imaging was obtained. Bioluminescence signal intensity was highest on day 3 ($6.10 \pm 2.02 \times 10^5$ p/s/cm^2/sr, mean ± SEM), but showed a decreasing tendency thereafter ($2.26 \pm 1.54 \times 10^5$ p/s/cm^2/sr on day 7 and $7.47 \pm 3.09 \times 10^4$ p/s/cm^2/sr on day 10, $p = 0.001$) (Fig. 3). A control mouse showed only background signal (data not shown).

PCR Amplification of GFP Sequences in Liver DNAs

GFP sequences were amplified by PCR in genomic DNA extracted from livers of another six transplanted mice that were sacrificed 3, 7, and 10 days after transplantation. Electrophoresis of the PCR products showed GFP bands of 125 bp size in all six mice (Fig. 4).

ELISA for Human Albumin

Human albumin concentration was measured by ELISA in sera of mice transplanted with FH-hTERT-DF on day 2, 7, 14, and 21 and nontransplanted control mice. Whereas serum levels of human albumin on day 2 were similar to those of control mice (3.25 ± 0.92 vs. 2.84 ± 0.59 ng/ml, mean ± SEM), levels increased to reach a peak concentration of 18.04 ± 3.11 ng/ml on day 7 ($p = 0.02$). Then human albumin concentrations de-

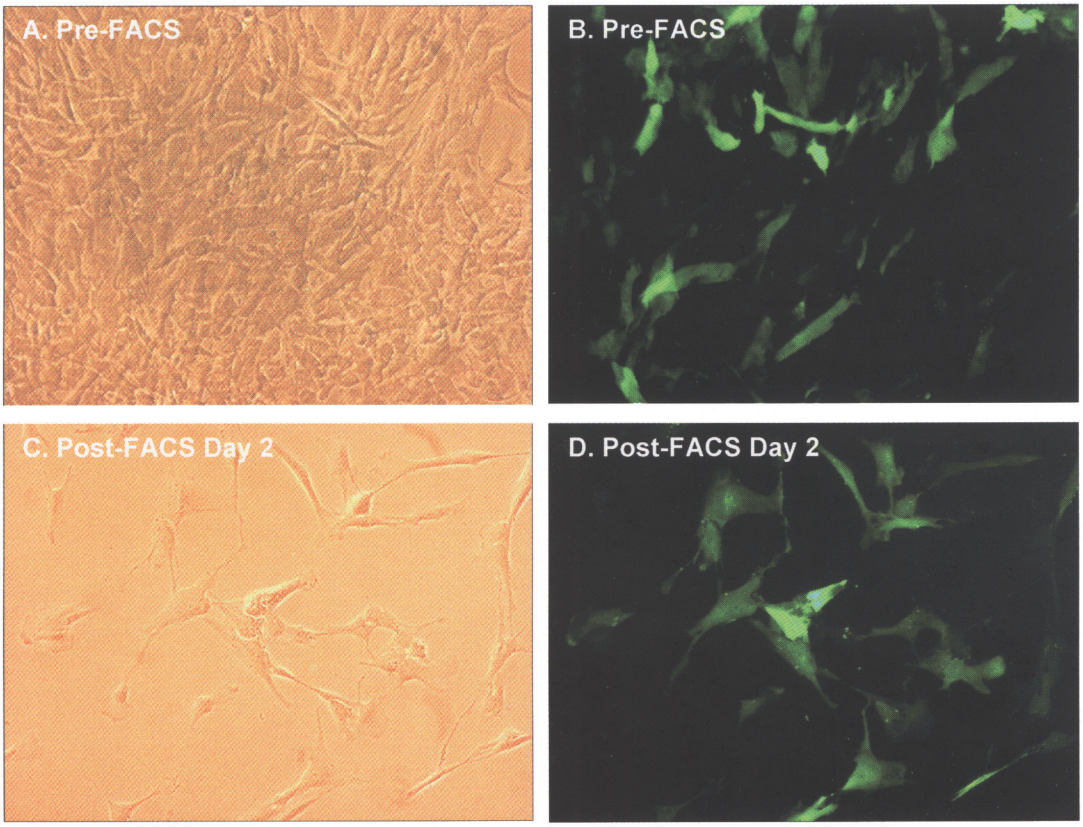

Figure 1. GFP imaging of transduced FH-hTERT before and after FACS. FH-hTERT were transduced with the double or triple fusion lentiviral vectors. While 40–50% of cells showed green fluorescence before FACS (A, B), the fluorescence was observed in nearly all cells 2 days after sorting (C, D) (original magnification 100×).

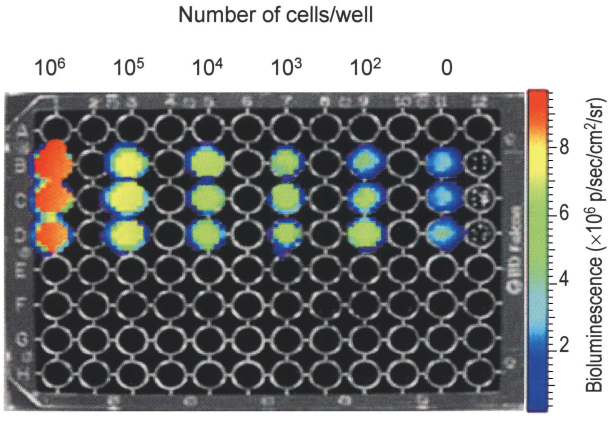

Figure 2. Detection threshold for luciferase activity in vitro. Serial cell dilutions of FH-hTERT-TF (from 10^6 to 10^2 per well) were done in triplicate and plated on 96-well plates. Bioluminescent imaging showed the detection threshold for luciferase of 100 cells per well.

creased to 8.93 ± 1.40 ng/ml on day 14 and to as low as those of control mice on day 21 (Fig. 5).

Quantitative Gene Expression Analysis

We analyzed mRNA levels of human hepatocyte-specific genes from the liver tissue of mice transplanted with the transduced cells by real-time quantitative RT-PCR using hGAPDH as a housekeeping gene control. Primary human adult hepatocytes were used as a reference. A total of six mice were used for quantitative gene expression analysis. Three mice were sacrificed on day 7 and another three mice were sacrificed on day 14. Gene expression levels of human albumin, α1-AT, and transferrin of transplanted FH-hTERT-DF in mouse liver were 60.7 ± 6.5%, 26.0 ± 1.4%, and 156.8 ± 62.4% (mean ± SEM) of those of primary human adult hepatocytes, respectively. There was no difference according to the sacrificed time point.

Immunohistology of Mouse Liver Tissue

Immunohistochemistry using specific antibodies to human albumin and α1-AT showed cells with positive signals in the mouse liver specimen (Fig. 6).

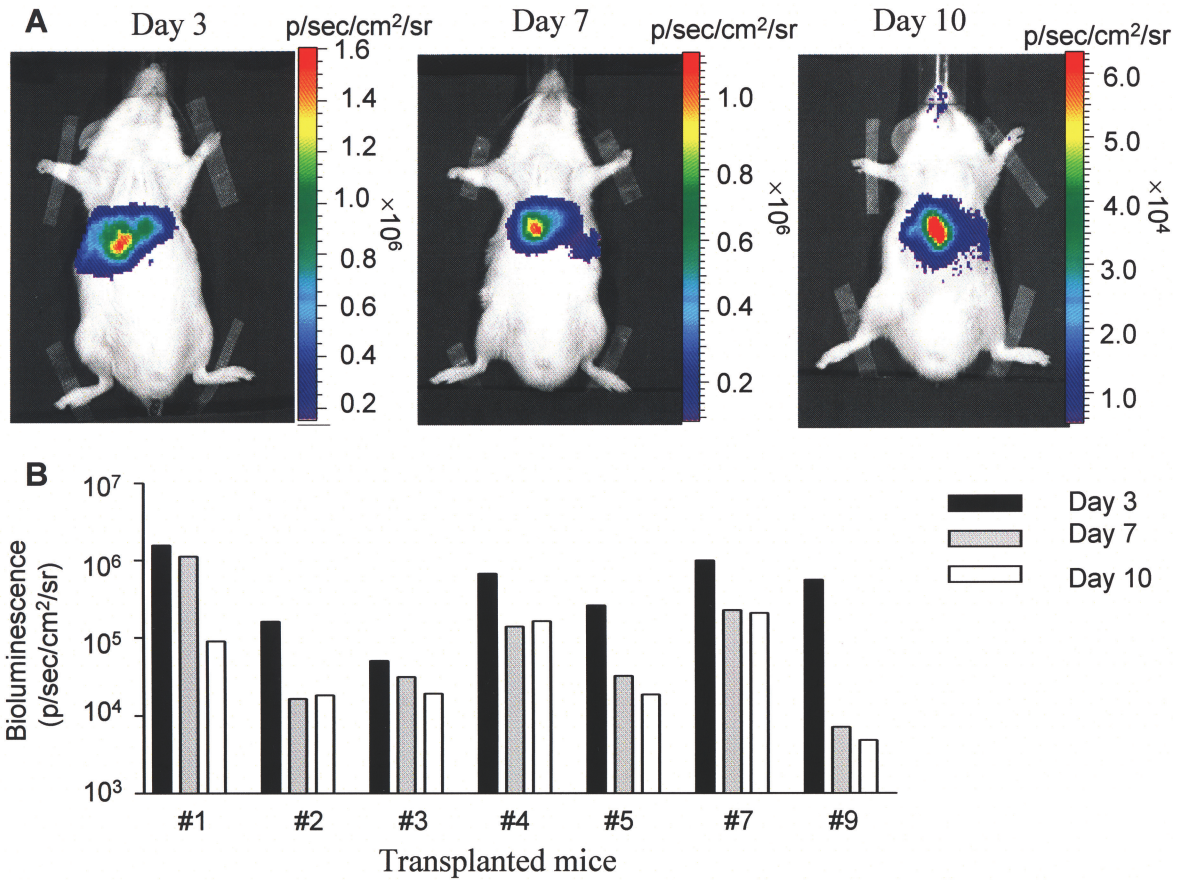

Figure 3. Bioluminescent imaging and bioluminescence signal intensity over time in mice transplanted with FH-hTERT-TF. (A) Under cooled CCD camera, a bioluminescence signal was observed from a location compatible with the liver sites in transplanted mouse #1. The signal was detected until day 10, although signal intensity was highest on day 3 and decreased thereafter. This mouse is representative of all mice that were transplanted. (B) Bioluminescence signal intensity reached peak level of $6.10 \times 10^5 \pm 2.02 \times 10^5$ p/s/cm²/sr, mean ± SEM) on day 3, but showed a decreasing tendency thereafter ($2.26 \times 10^5 \pm 1.54 \times 10^5$ p/s/cm²/sr on day 7 and $7.47 \times 10^4 \pm 3.09 \times 10^4$ p/s/cm²/sr on day 10, $p = 0.001$).

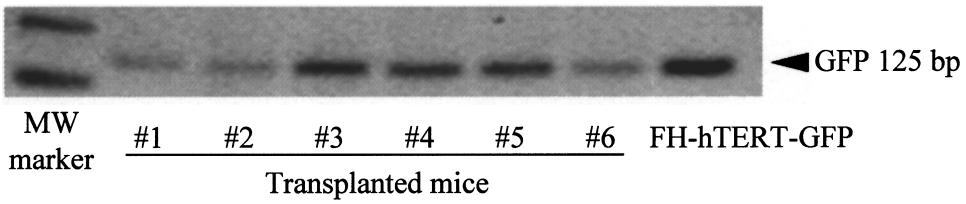

Figure 4. PCR amplification of GFP sequences in DNAs from livers of transplanted mice. Mice were sacrificed 3 days (#1, #2), 7 days (#3, #4), and 10 days (#5, #6) after transplantation. GFP sequences were amplified by PCR in genomic DNA extracted from livers of transplanted mice using cultured FH-hTERT transduced with lentiviral vectors carrying GFP gene (FH-hTERT-GFP) as a positive control and the PCR mix as a negative control. Electrophoresis of the PCR products showed GFP bands of 125 bp size in all six mice.

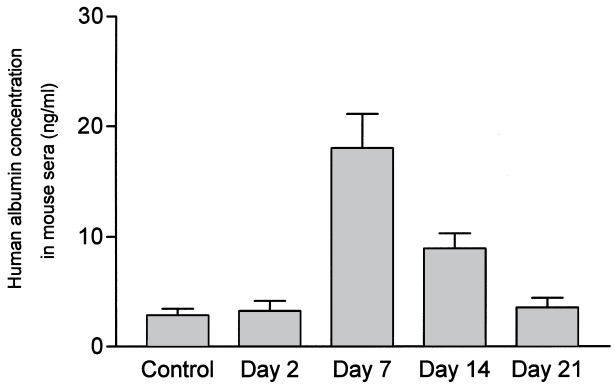

Figure 5. ELISA for human albumin in sera from transplanted mice. Sera from recipient mice ($n = 2$ for day 2, $n = 4$ for day 7, day 14, and day 21, respectively) and control mice ($n = 2$) were analyzed for human albumin by ELISA. Serum levels of human albumin on day 2 were not different form those of control mice (3.25 ± 0.92 vs. 2.84 ± 0.59 ng/ml, mean ± SEM), but levels increased to reach a peak (18.04 ± 3.11 ng/ml, $p = 0.02$) on day 7. Then the concentrations decreased to 8.93 ± 1.40 ng/ml on day 14 and to as low as those of control mice on day 21 ($p = 0.02$).

DISCUSSION

To assess the effectiveness of human hepatocyte transplantation, noninvasive serial monitoring of the fate of transplanted cells would be extremely useful. Hence, we evaluated the feasibility of bioluminescent imaging using double or triple fusion lentiviral vectors in a NOD-SCID mouse model transplanted with immortalized fetal hepatocytes. This represents the first time that human hepatic progenitor cells have been imaged in a longitudinal in vivo model of cell transplantation in rodents.

Bioluminescent imaging using *fl* reporter gene expression was first shown by Wu et al. (29). A cooled CCD camera was shown to provide consistent and reproducible results within ±8% standard deviation from mean values, and a detection sensitivity of 1×10^6 plaque-forming units of E1-deleted adenovirus expressing *fl* driven by a CMV promoter. After injecting this vector into the skeletal muscle of living mice, it was determined that a cooled CCD camera can sensitively and noninvasively track the location, intensity, and persistence of *fl* gene expression (29). Lentiviral vectors, which are self-inactivated and biologically safe for humans, can deliver genetic material into cells regardless of their proliferation status (12). De et al. constructed a lentiviral vector carrying two reporter genes: *ttk* for microPET and *fl* for CCD camera imaging. Neuroblastoma (N2a) cells were stably transfected by this virus showing high correlation ($R^2 = 0.91$) between the expression of the two reporter genes in cell culture. MicroPET and bioluminescent imaging in living mice implanted with the N2a cells was feasible and showed a high correlation ($R^2 = 0.86$) between them, with optical imaging being more sensitive (5). Ray et al. reported the use of a triple fusion lentiviral vector containing the *rl* gene, the *rfp* gene, and the *ttk* gene for bioluminescence, fluorescence, and PET imaging in several cell lines. For example, metastases of a human melanoma cell line (A375M) stably expressing the triple fusion were imaged by microPET and optical technologies over 40–50 days in mice (19).

In most previous bioluminescent imaging studies for cell transplantation, a tumor cell line was implanted subcutaneously (2,5,19,24) or directly injected into a target organ (3,10,28); for example, C6 rat glioma cells expressing *rl* were implanted subcutaneously in mice (2), N2a murine neuroblastoma cells transfected with *ttk* and *fl* or *rl* were implanted subcutaneously in nude mice (24), or embryonic stem cells expressing the triple fusion vector were injected into the myocardium (3). However, bioluminescence imaging employing this vector system has not yet been studied in living animals transplanted with human hepatic cells. Moreover, it is difficult to obtain CCD camera imaging in highly vascular organs such as liver and spleen because the efficiency of light transmission is low due to absorption of light by oxyhemoglobin and deoxyhemoglobin (4).

To our knowledge, our study is the first one to show the feasibility of this novel imaging system to monitor the engraftment of transplanted human hepatic cells into mice. Bioluminescence imaging using double or triple fusion lentiviral vectors was evaluated in a NOD-SCID mouse model transplanted with FH-hTERT. FH-hTERT were transduced with the double or triple fusion lentivirus with a high transduction efficiency (40–50%) and the transduced cells were successfully transplanted into the spleen of mice. After FH-hTERT were injected into the spleens of the mice, CCD camera imaging demonstrated the bioluminescence signal in their livers, consistent with high luciferase activity of the tissue, and successful engraftment and maturation of transplanted FH-hTERT was demonstrated by evaluation of human-specific gene expression. The detection of the human cell engraftment in transplanted mice was confirmed by PCR amplification of GFP sequences in liver DNAs, ELISA for human albumin in sera, quantitative RT-PCR for human hepatocyte-specific genes from the liver tissue, and immunohistochemistry of the mouse liver specimen.

Two of our observations are worth additional attention. First, *fl* gene expression and bioluminescence signal intensity was highest at the earliest assessment; however, albumin synthesis (shown by ELISA) was not

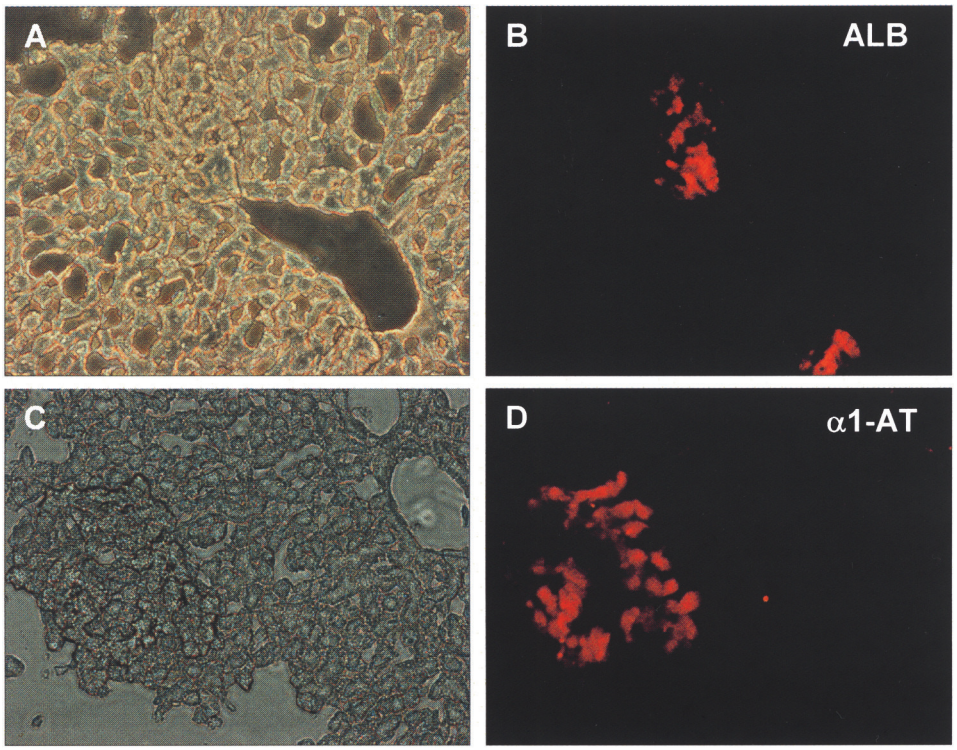

Figure 6. Immunohistochemistry of mouse liver specimen. Immunohistochemistry showed expression of human liver specific proteins in liver tissue from transplanted mice. Slides prepared from liver tissue were stained with primary goat antibodies against human albumin (ALB) and α1-antitrypsin (α1-AT) and with secondary rabbit anti-goat IgG-fluorescein isothiocyanate conjugates. Fluorescence microscopy showed some cells with positive signals for human albumin (A, B) and α1-AT (C, D) in the mouse liver specimen (original magnification 200×).

active at that time. This discrepancy between *fl* gene expression and liver-specific protein expression may be explained by the speculation that *fl* gene expression may be driven by the CMV promoter even at an immature stage of the immortalized fetal hepatocytes. On the other hand, albumin synthesis may not be fully activated until these transplanted fetal hepatocytes mature into more functional hepatocytes in mouse liver. This is consistent with our previous report showing maturation of transplanted FH-hTERT and subsequent increase in albumin gene expression in the mouse liver with time (26), and the fact that albumin mRNA expression increased by 5 logs in transplanted cells in comparison to their expression in the cells in culture. Epithelial-mesenchymal transition might be a possible explanation for this observation (22). Second, liver-specific gene and protein expression as well as bioluminescence signal intensity decreased instead of increasing over time, even though we induced injury in the endogenous mouse organ. This result reflects the general finding that transplanted human cells seldom proliferate in the mouse liver (13). This result occurred despite our effort to facilitate cell transplantation and endogenous cell proliferation by pretreating mice with monocrotaline, which promotes transplanted cell engraftment and advances liver repopulation by causing endothelial and hepatocyte injury (9), and methycholanthrene, a hepatic P450 enzyme inducer that enhances monocrotaline hepatic toxicity. Thus, our results highlight a major issue in human hepatic cell transplantation studies in rodent models: the difficulty in demonstrating exogenous human cell transplantation when human cells appear to have a selective proliferative disadvantage in rodent livers. This critical problem may lend itself to a series of alternative approaches in the future: better injury models in these fragile immunosuppressed animals, the use of non-human primates whose livers may well be more welcoming to human cells, and the use of human growth factors or other factors, such as activation of growth factor signaling [e.g., through c-Met agonistic antibody as shown by Kay and colleagues to enhance survival and proliferation of human hepatocytes growth in mice (15,16)]. Despite these peripheral

issues, our study demonstrated the feasibility of bioluminescent imaging using double or triple fusion lentiviral vectors as a sensitive, noninvasive, and serial monitoring modality for transplanted hepatocytes. The imaging results were confirmed by a series of biochemical and histologic modalities. This should provide investigators interested in hepatic cell transplantation with a new research tool to evaluate transplanted cell engraftment and repopulation in the liver.

ACKNOWLEDGMENTS: This work was supported in part by the GlaxoSmithKline Research Fund of the Korean Association for the Study of the Liver (M.S.C.), NIH grants AA014173, DK073901, DK075415 and California Institute Regenerative Medicine (CIRM) grant RC1-00359 (M.A.Z.), NIH grant DK46952 (S.G.), and NIH grants PAR-04-069, HL078632, and CA082214 (S.S.G.).

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