Specific Activation of Sodium Iodide Symporter Gene in Hepatoma Using Alpha-fetoprotein Promoter Combined with Hepatitis B Virus Enhancer (EIIAPA)

REN-SHYAN LIU[1,2,5,6,7*], YA-JU HSIEH[4,7*], CHIEN-CHIH KE[3,7], FU-DU CHEN[4], LUEN HWU[7], FU-HUI WANG[7], JENG-JONG HWANG[2], CHIN-WEN CHI[3,6], CHEN-HSEN LEE[3,6] and SHIN-HWA YEH[1,5,6]

[1]*Department of Nuclear Medicine,* [2]*Institute of Radiological Sciences,* [3]*Department and Institute of Pharmacology, Faculty of Medicine, National Yang-Ming University, Taipei;*
[4]*Institute of Radiological Sciences, Central Taiwan University of Science and Technology, Taichung;*
[5]*National PET/Cyclotron Center,*
[6]*Taipei Veterans General Hospital,* [7]*Molecular and Genetic Imaging Core, NRPGM, Taipei, Taiwan, R.O.C.*

Abstract. *Background: This study aimed to develop a novel tumor-specific promoter gene linking sodium iodide symporter (NIS) gene to specifically target hepatocellular carcinoma in a mouse tumor model. Materials and Methods: A tumor-specific chimeric promoter for alpha-fetoprotein gene (AFP) was combined with hepatitis B virus (HBV) enhancer II to investigate radioiodine uptake in vitro and in vivo in hepatoma (HepG2) and nonhepatoma (ARO) cell lines after transfer of hNIS gene. A lentiviral vector carrying the hNIS gene was employed in vitro and in vivo. Radionuclide imaging was acquired for 30 min at 60 min after administration of ^{124}I to monitor hNIS gene expression in vivo using microPET. Results: The highest radioiodide uptake of ARO and HepG2 clones which stably expressed hNIS gene were 87- and 208-fold higher than that of parental cells, respectively. After infection of lentivirus, hNIS gene controlled by cytomegavirus (CMV) promoter was expressed in both ARO and HepG2 cells, and hNIS gene induction by EIIAPA promoter was higher than by CMV promoter in HepG2 cells but not in ARO cells. A similar result was observed in vivo, hNIS controlled by CMV promoter was highly expressed in both HepG2 and ARO tumors. The HepG2 tumor multi-infected with LV-EIIAPA-hNIS virus specifically, but the ARO tumor did not activate the EIIAPA promoter and further express the hNIS protein. Conclusion: Transduction of the hNIS gene controlled by the novel EIIAPA chimeric promoter successfully induces iodide transport in hepatoma.*

Hepatocellular carcinoma (HCC) is one of the major causes of cancer mortality worldwide (1). Various therapies such as cancer surgery (1), chemoembolization (2), alcohol injection (3), radiofrequency ablation (3) and focused ultrasound ablation (4) have been introduced in the last two to three decades. Complete cure of the disease is achieved only in selected patient subgroups (5). There has been no significant improvement in the treatment for HCC patients with large, multiple or recurrent tumors (5). The possible curative therapies are resection and liver transplantation (6, 7) but recurrence of HCC in either approach is common. More importantly, the vast majority of patients with HCC are not candidates for these therapies because HCC is practically always diagnosed late in an advanced and unresectable stage. In addition, most patients with HCC have underlying cirrhosis and do not tolerate cytotoxic therapy or extensive resection. Accordingly, new therapeutic procedures including gene therapy are urgently needed.

Sodium iodide symporter (NIS), a plasma membrane glycoprotein, is essential for active iodide uptake in the thyroid gland and other nonthyroidal tissues (8). The NIS-catalyzed active accumulation of iodide from the interstitium into the cell is achieved against an electrochemical gradient generated by sodium-potassium adenosine triphosphatase (Na^+/K^+-ATPase) and is stimulated by thyroid-stimulating hormone (9). Impaired iodide uptake observed in normal tissues and thyroid carcinomas of humans and animals was attributed to a reduced expression of the *NIS* gene associated with down-regulated expression of thyroid peroxidase thyroglobulin and thyroid-stimulating hormone receptor

*Both authors contributed equally to this work.

Correspondence to: Ren-Shyan Liu, MD, Molecular and Genetic Imaging Core, NRPGM; Department of Nuclear Medicine, Faculty of Medicine, National Yang Ming University, 155, Sec. 2, LiNong Street, Taipei, Taiwan 11221, R.O.C. Fax: +886 2 28749431, e-mail: rsliu@vghtpe.gov.tw

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genes (10, 11). Thyroid carcinoma iodide uptake might be predicted by the immunological quantification of NIS expression (12). Specific gene transfer of human sodium iodide symporter (*hNIS*) gene into nonthyroid and thyroid carcinoma cells increased the uptake of radioiodide and resulted in better tumor control by radioiodide of various types of tumor (13, 14). *NIS* gene transfer has been successfully performed in tumor animal models, such as in nude mice xenografted with human melanoma, glioma, breast cancer or prostate cancer, by either plasmid-mediated transfection or virus-mediated gene delivery (15-17). The accumulation of radioiodide in specific cells or tissues mediated by the *NIS* gene transfer shows its possibilities for molecular imaging (18). Furthermore, using *NIS* gene transfer to induce radioiodide accumulation by expression of functionally active NIS may extend the utility of radioiodide therapy to the treatment of cancer.

The aim of this study was to induce gene expression of *hNIS* in hepatoma cells by the tumor-specific alpha fetoprotein (AFP) promoter isolated from a human genomic library. It is able to induce HCC-selective expression of therapeutic genes in human HCC cell lines (19). Acute and chronic hepatitis caused by hepatitis B virus (HBV) is closely associated with the development of HCC (20). The HBV enhancer II is located immediately upstream from the core open-reading frame (ORF) and regulates the transcription of a set of viral RNAs of about 3.5 kb in length. These 3.5 kb RNAs occur in well-differentiated hepatocytes but not in undifferentiated hepatocytes nor cells of nonhepatic origin (21, 22). This liver-specific regulation is controlled by the binding of liver-specific transcription factors within the HBV enhancer II regulation region. HBV enhancer II provides cell type specificity in some highly differentiated human hepatoma cell lines, such as HepG2 and Huh 6, but not in nonhepatocyte-derived cell lines (23). It has been postulated that tissue-specific expression of HBV may be attributed largely to the activity of the enhancer II (24, 25). Linking the AFP promoter and the HBV enhancer (EIIAPA) could achieve more stringent transcriptional targeting of HCC. In this paper, we have demonstrated the specific transfection and expression of *hNIS* gene in HepG2 hepatoma *in vivo* as well as *in vitro*.

Materials and Methods

Cell culture. Human HCC cell lines HepG2 (BCRC60025) and 293T (BCRC60019) were obtained from the Bioresource Collection and Research Center in Taiwan. The ARO thyroid cancer cell line was kindly provided by Dr. Yan-Hwa Wu Lee and Dr. Chin-Wen Chi (National Yang-Ming University, Taiwan). All media and tissue culture supplements were obtained from Gibco-BRL (Carlsbad, CA, USA) unless indicated otherwise. HepG2 and 293T cells were grown in minimum essential medium (MEM). ARO cells were maintained in RPMI-1640 medium. All complete media were supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were incubated in a humidified incubator at 37˚C and 5% CO_2:95% air.

Vector construction. The *hNIS* cDNA cloned in pcDNA3 (Invitrogen, San Diego, CA, USA) as a clone designated FL*-hNIS/pcDNA3 was kindly provided by Dr. Sissy Jhiang (Ohio State University). The full length hNIS cDNA was digested with BamHI and XhoI from FL*-hNIS/pcDNA3 and cloned into the BglII and SalI digested pIRES2-EGFP vector (BD Bioscience Clontech, USA) to create the CMV-hNIS-IRES-EGFP construct. The *hNIS* in pcDNA3 vector was under the control of CMV promoter and can be digested by Hind III which was filled-in by Klenow enzyme and EcoR I. The CMV-hNIS fragment was inserted into LentiLox 3.7 vector (pLL3.7) which was digested by NheI (fill-in) and EcoRI to create pLL3.7/CMV-hNIS for viral experiments. The HBV enhancer IIA (155 bp, named EIIA from hereon in) was cloned from clinically isolated HBV genome by polymerase chain reaction (PCR) using 5'-AGATCTCTGCCCAAGG TCTTACATA-3' as upper primer and 5'-GAATTCAAAGACCTTTAA CCTAATC-3' as lower primer. The PCR product of EIIA was then inserted into the Bgl II-EcoRI site to replace the AFP enhancer in the $AFPP_{AFP}$-GFP (AFPPA-GFP) plasmid of which the green fluorescence protein (GFP) gene was driven by AFP promoter and then generated the EIIAPA-GFP construct. The EIIAPA fragment was also used to replace the CMV promoter and enhancer in pLL3.7/CMV-hNIS to generate pLL3.7/EIIAPA-hNIS construct.

Virus production. The day before transfection, 6×10^6 293T cells were plated in a 100 mm tissue culture plate. The plating was such that there was 70-80% confluent on the day of transfection. Before transfection, the culture medium was removed from the 293T cells and replaced with 10 ml of growth medium containing serum. Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) was then gently mixed with DNA (12 μg) and incubated for 20 minutes at room temperature to form the DNA-Lipofectamine™ 2000 complexes. After adding the DNA-Lipofectamine™ 2000 complexes to each plate of cells, the cells were incubated overnight at 37˚C in a CO_2 incubator. On the next day, the medium containing the DNA-Lipofectamine™ 2000 complexes was removed and replaced with complete culture medium. At 72 hours post transfection, the virus-containing supernatants were harvested in a 15 ml sterile, capped conical tube and centrifuged at 3000 rpm for 15 minutes at 4˚C to pellet cell debris. The viral supernatants (LV-CMV-hNIS, LV-EIIAPA-hNIS and LV-CMV-EGFP) were then passed through a sterile, 0.45 μm low protein-binding filter (Cat# SLHVR25LS, Bedford; Millipore, MA, USA) to remove cellular debris by low speed centrifugation. The supernatants were transferred to Centricon plus–20 filters (Cat # UFC2BHK08; Millipore, Bedford, MA, USA) and centrifuged at 2,500 rpm for 15 minutes at 4˚C. By inverting the filter in the sterile cup and spinning at 1,000 rpm for 2-3 minutes the sample was collected and stored at –80˚C. The virus titer was assayed by the QuickTiter™ Lentivirus Quantitation Kit (Cell Biolabs, Inc., USA).

Cell transfection and selection of stable clones. Approximately 200,000 cells were seeded equally into six-well tissue culture plates (Corning, USA). The cells were approximately 60% confluent on day 1. For transfection, cells were treated with jetPEI™ (Polyplus, France) using a modification of the manufacturer's procedure. In

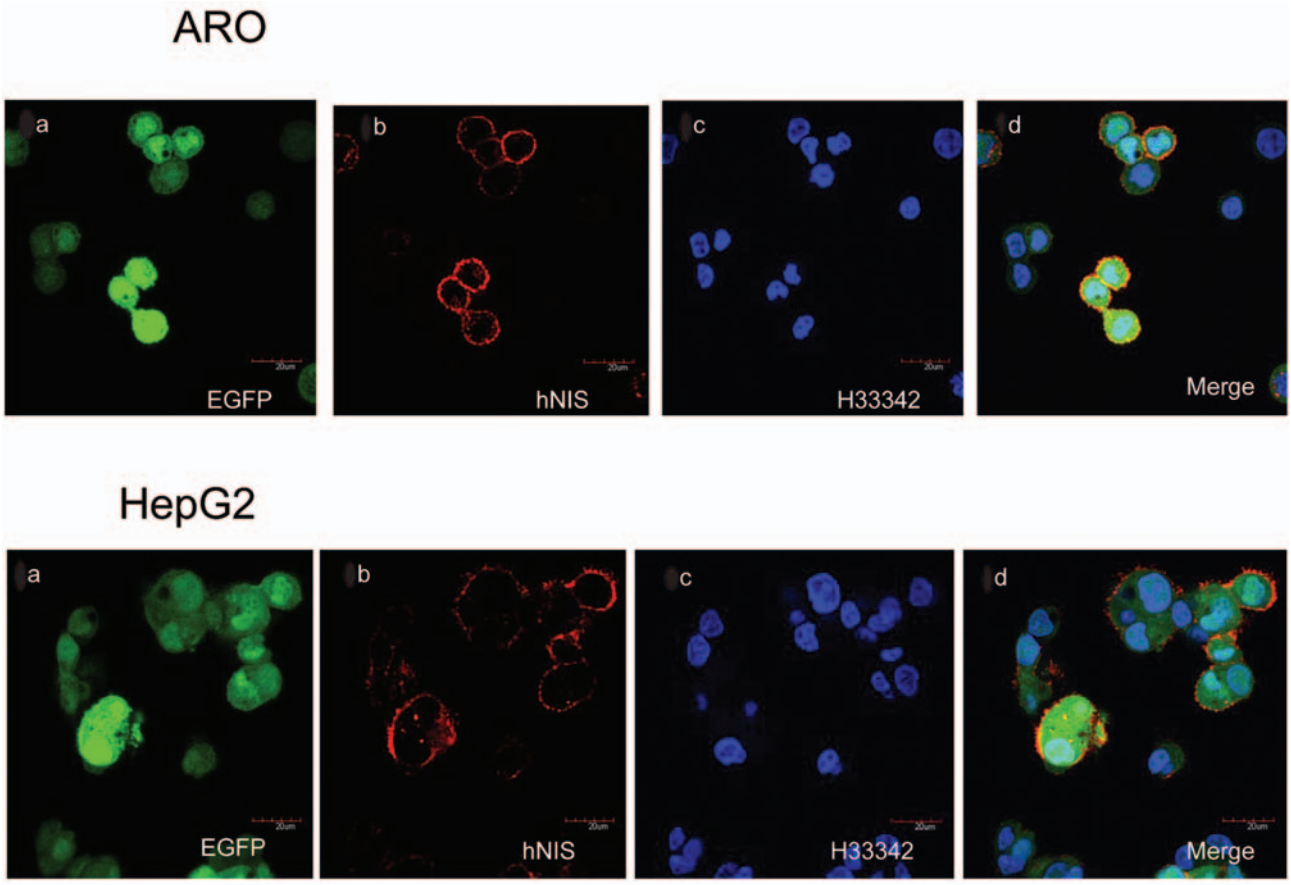

Figure 1. *Immunofluorescence of ARO (upper panel) and HepG2 (lower panel) cells stably expressing hNIS and EGFP. Being stably transfected with pCMV-hNIS-IRES2-EGFP, green fluorescence was observed in both ARO and HepG2 cells (a). hNIS protein was targeted by incubating cells with mouse anti-hNIS Ab followed by phycoerythrin-conjugated anti-mouse IgG Ab and is shown as a red color in the cell membrane (b). Nuclei were stained blue using H33342 as shown in (c). The 3 images were combined to produce (d).*

brief, 3 μg of plasmid DNA and 9.6 μL of jetPEI™ reagent were mixed and incubated for 15 to 30 minutes at room temperature; then the jetPEI™/DNA mixture was added drop-wise onto the serum-containing medium in each well and the mixture on the plate was gently swirled. The cells were incubated at 37˚C in a 5% CO_2 humidified air atmosphere. In this study, the CMV-hNIS-IRES-EGFP plasmid was transfected into HepG2 and ARO cells and after 48 h of gene expression the medium was replaced by fresh medium containing 900 and 1200 μg/ml G418 antibiotics (Sigma, USA), respectively, for selection of stably transfected cells.

Detection of integrated vectors in transduced cells using polymerase chain reaction (PCR). Transduced ARO and HepG2 cells were expanded in culture and total genomic DNA was extracted using the DNeasy kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. *hNIS* F, 5'-TCAGCACAGCATC CACCAG-3' and *hNIS* R, 5'-GGGCACCGTAATAGAGATAG-3' primers were used to detect the presence of vector integrants using 0.2 μg total genomic DNA. The reaction temperatures were 94˚C for 4 min, 94˚C for 30 s, 56˚C for 30 s and 72˚C for 30 s, with the reaction cycle repeated 30 times.

Lentivirus-mediated hNIS expression in transduced cells determined by reverse transcriptase-polymerase chain reaction (RT-PCR). ARO and HepG2 cells were transduced with LV-CMV-hNIS and LV-EIIAPA-hNIS lentivirus in the presence of polybrene (Sigma, USA). Two days after transduction, the cells were lysed and total RNA was prepared using RNeasy mini kit (Qiagen, Valencia, CA, USA). cDNA synthesis was carried out using MMLV reverse transcriptase (Epicentre Biotechnologies, Madison, WI, USA) according to the manufacturer's instructions. In the subsequent PCR reaction, the primers previously described were used to amplify the *hNIS* gene. As internal controls, primers that hybridize with a segment of the *β-actin* gene were included in the same reaction mixture (β-actin forward primer: 5'-TCAAGATCATTGCTCCTCCTGAGC-3'; β-actin reverse primer: 5'-GGGCACCGTAATAGAGATAG-3'). The annealing temperature for β-actin was 54˚C.

In vitro ^{125}I uptake. Uptake of radioiodide by cells was measured according to Weiss *et al*. (26) with little modification. The selected ARO and HepG2 stable clones (ARO-S and HepG2-S) underwent ^{125}I uptake assay, as determined by incubating the cells with 0.5 ml Hanks' balanced salt solution (HBSS) containing 10 μM of NaI and

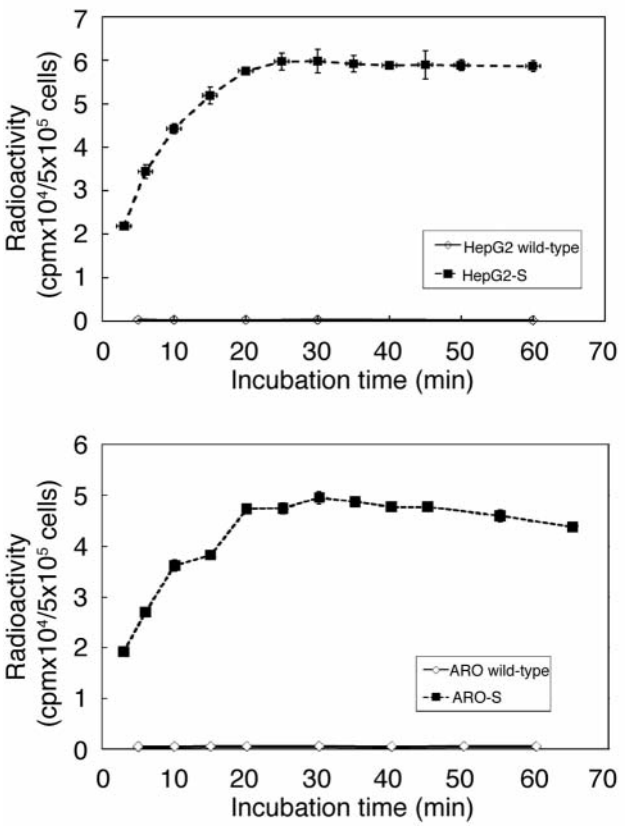

Figure 2. *Time course of ^{125}I uptake by HepG2 (upper panel) and ARO (lower panel) cells stably expressing hNIS gene (■) compared with that of HepG2 parental cells (◇). Values are the mean ± standard deviation (n=3). HepG2-S= HepG2 stable clone, ARO-S= ARO stable clone.*

3700 Bq of ^{125}I at 37˚C at various time points. The cells were then washed once as quickly as possible with 1 ml of iodide-free ice-cold HBSS buffer. Cells were detached with 500 μl of trypsin and resuspended in 1 ml HBSS. The radioactivity was measured using a Cobra II autogamma counter (Packard instrument, CT, USA). Parental ARO and HepG2 cells and cells infected with LV-CMV-EGFP, LV-CMV-hNIS, or LV-EIIAPA-hNIS (as described below) were inoculated into 12-well plates with 5×10^5 cells. Twenty-four hours later, cells were washed with HBSS buffer and then incubated with 3700 Bq of ^{125}I in pre-warmed HBSS containing 10 μM of NaI for 5, 10, 20, 30 and 60 minutes. Cells were then rapidly washed with HBSS, trypsinized and resuspended in 1 ml of HBSS. The radioactivity was measured thereafter.

Multi-infection of cells by lentivirus in vitro and establishment of animal models. Appropriate numbers of HepG2 hepatoma and ARO cells were seeded in 150 mm culture dishes. On the second day, virus with a titer of 10^8 viral particles per ml (10^8 VP/mL) was added to the culture media for infection. Forty-eight hours after infection, the culture media were replaced with media containing fresh viral particles to infect the cells again. The same procedure was repeated six times and the infected cells were collected and then implanted into the right shoulder of NOD/SCID mice from National Experimental Animal Center, average weight 28 g, age 6 weeks using the protocol described elsewhere (27). On the left shoulder of the same animal, HepG2 or ARO parental tumors were grown for comparison with the multi-infected tumors. Cells which stably expressed *hNIS* and *GFP* genes were also inoculated on the right shoulder of other NOD/SCID mice, and likewise, parental tumors were induced on the opposite shoulders.

Immunofluorescence staining of stable clones. HepG2 or ARO stable clones were seeded onto poly-(lysine) coated coverslips. When cells reached 40-50% confluence they were fixed with 4% paraformaldehyde for 15 min and then washed three times with 1× PBS. Cells were permeabilized with 0.1% Triton®X-100 in phosphate-buffered saline (PBS) for 5 min and washed three times with PBS, then the cells were blocked with 4% bovine serum albumin (BSA)/PBS and subsequently incubated with mouse anti-hNIS Ab (Chemicon, USA) at 1:100 dilution in 4% BSA/PBS for 1 h. Cells were washed 3 times for 5 min with 0.2% Tween-20 in BSA/PBS. Coverslips were then transferred into BSA/PBS containing phycoerythrin-conjugated anti-mouse IgG at 1:100 dilution (Santa Cruz, USA) for 1 h in the dark and then washed as above. *Bis*benzimide H33342 was dropped onto the coverslips for 5 min to stain the nuclei. The coverslips were washed 3 times with PBS and then mounted onto slides containing 80% glycerol in PBS with phenylenediamine. Coverslips were sealed with quick-dry nail polish and allowed to dry in the dark for 2 h at room temperature, then stored at 4˚C. Fluorescence images of cells were obtained using confocal microscopy (Leica TCS SP5, Germany).

In vivo ^{124}I imaging and quantitative image analysis. Xenotransplants derived from ARO and HepG2 stable clones (on the right shoulders) and ARO and HepG2 parental cells (on the left shoulders) were established in male NOD/SCID mice by subcutaneous injection of 1×10^7 cells suspended in 0.25 ml of RPMI-1640 and 0.25 ml of MEM medium, respectively. Another group of SCID mice bearing HepG2 hepatoma in the right shoulder was derived from the cells multi-infected *in vitro* with letivirus carrying pLL3.7/CMV-hNIS or pLL3.7/EIIAPA-hNIS gene constructs. After the tumors reached 8 mm in diameter, the mice were injected intravenously with ^{124}I (1.85 MBq). One hour after injection of the ^{124}I, the animals were anesthetized with 2% isoflurane/98% oxygen mixture and secured on an imaging table in the prone position. Static images were performed on a microPET R4 scanner (Concorde Microsystems, Tennessee, USA) for 30 min. Fully 3-dimensional listmode data were collected for 30 min using an energy window of 350-750 keV and a timing window of 6 ns. For maximum sensitivity, coincidence data were rebinned into 3-dimensional sinograms using the full axial acceptance angle of the scanner (max. ring difference=31). To preserve axial resolution, high sampling of the polar angle was used (span=3). The resulting 3D sinograms were then rebinned into 2D sinograms using Fourier rebinning (FORE) and reconstructed with 2-dimensional filtered back projection (FBP) using a ramp filter with cutoff at Nyquist. The image matrix size was 256×256. Data manipulation was carried out *via* the MicroPET Manager software provided by the manufacturer. No attenuation correction was performed. The images were interpreted qualitatively. All images were expressed in the ordinary rainbow color scale with standard visual spectrum installed in the computer of the microPET system. Tumors with high uptake of radioiodide

as shown in red *versus* background uptake shown in blue were defined to have significant uptake of radioiodide and consequently significant expression of NIS. For quantitative image analysis, the comparison of tumor to background was carried out by a region of interest (ROI) approach. ROIs were manually defined on each section of the entire tumor. Volumes of interest (VOIs = sum of ROIs from each coronal image) of each tumor with various coronal sections were measured and calculated by ASI Pro software (Siemens/CTI Concorde Microsystems, LLC, Tennesse, USA). The thickness of each coronal plane was 0.423 mm and the number of coronal sections was dependent on the size of the tumor. The tracer uptakes of all sections of each tumor were summed to obtain the total tumor uptake. The tumor uptake ratio was calculated by dividing the total uptake of the *hNIS*-transduced tumor by that of the parental tumor.

Results

Assessment of dual gene expression in vitro by immunofluorescent technique. HepG2 and ARO cells stably transfected with CMV-hNIS-IRES-EGFP reporter construct were monitored by fluorescence microscopy to assess the function of *hNIS* and *GFP* genes. The GFP was excited by 488 nm excitation light and then emitted 500~600 nm fluorescence (Figure 1a). Secondary antibody conjugated with phycoerythrin (PE) recognized these membrane proteins (hNIS) and then the red light (excitation wavelength: 561 nm; emission wavelength: 570-620 nm) was observed on the plasma membrane using fluorescence microscopy (Figure 1b). The blue fluorescence indicates the locations of cell nuclei which were stained by *bis*benzimide H33342 dye (Figure 1c). Figure 1d is of the merged images. These results indicate that the HepG2 and ARO stable clones were well established and simultaneously expressed both hNIS and GFP proteins *in vitro*.

Radioiodide uptake studies. Sequential radioiodide uptake experiments by *hNIS* gene expression were performed on ARO and HepG2 stable clones which were stably transfected with CMV-hNIS-IRES-EGFP construct. As shown in Figure 2, the hNIS-expressing ARO and HepG2 stable clones showed rapid ^{125}I accumulation with a plateau being reached within 20-30 minutes after ^{125}I was added. The plateau uptakes of ARO stable and HepG2 stable clones were 87- and 208-fold higher than that of parental cells. Without hNIS-expression, neither ARO nor HepG2 wild-type cells exhibited any radioiodide uptake, as expected. Kinetic study of ^{125}I uptake was also performed on lentiviral-infected cells (Figure 3). With LV-CMV-hNIS and LV-EIIAPA-hNIS infection (10^8 VP/ml), HepG2 cells significantly accumulated ^{125}I, reaching a maximum level within 20-30 minutes, which was similar to that observed in the HepG2 stable clone. HepG2 wild-type cells and HepG2 cells infected with LV-CMV-EGFP (as a mock control) did not accumulate ^{125}I. In the ARO cell group, only LV-CMV-hNIS-infected cells showed accumulation of ^{125}I. LV-CMV-EGFP and LV-EIIAPA-hNIS-infected ARO cells, as well as wild-type cells exhibited different low background levels of ^{125}I uptake. These results suggested that *hNIS* gene transfer by the lentiviral system is feasible in HepG2 and ARO cells. HepG2 cells infected with LV-EIIAPA-hNIS accumulated more ^{125}I than cells infected with LV-CMV-hNIS, while LV-EIIAPA-hNIS-infected ARO cells exhibited only the background level of radioactivity. The results demonstrate the specificity of EIIAPA chimeric promoter for human hepatoma cells. With an equal number of ARO and HepG2 cells infected with an equal amount of LV-CMV-hNIS, HepG2 showed greater accumulation of ^{125}I than ARO. This difference was probably due to the different capacity of these different cells for being infected by lentivirus.

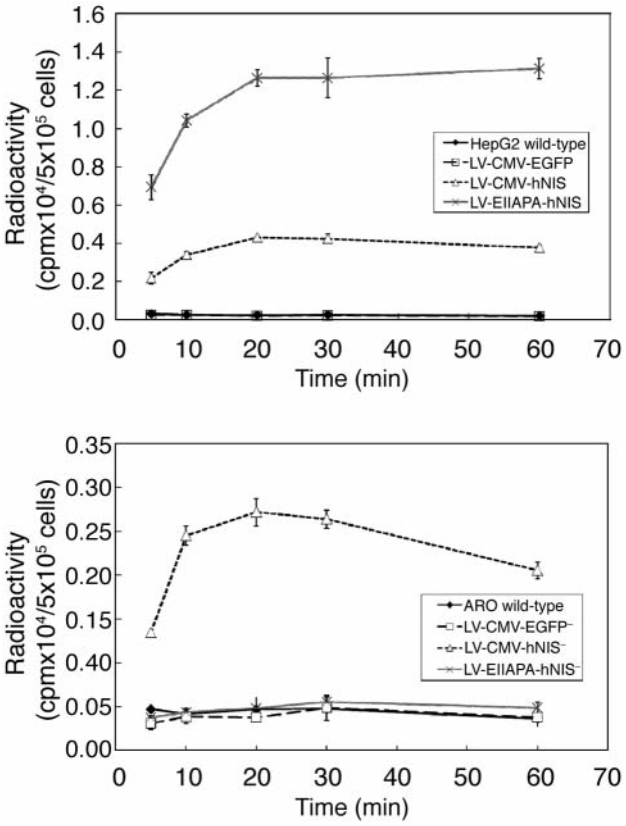

Figure 3. *Time course of ^{125}I uptake by HepG2 (upper panel) and ARO (lower panel) cells infected with different lentivirus including LV-CMV-EGFP (□), LV-CMV-hNIS (△) and LV-EIIAPA-hNIS (×). Parental cells were used as control group (◆). Values are the mean±standard deviations (n=3).*

The specificity of EIIAPA promoter mediated by lentivirus. To demonstrate whether the exogenous *hNIS* gene expression was induced in ARO and HepG2 cells which were transduced with LV-CMV-hNIS or LV-EIIAPA-hNIS,

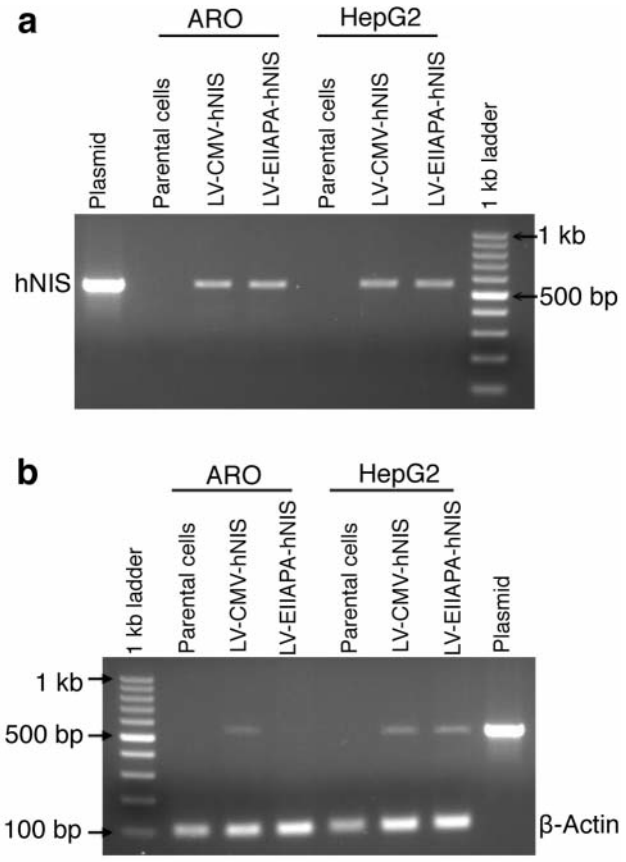

Figure 4. *PCR amplification of hNIS gene integrated into ARO and HepG2 genome. To determine that all vectors were integrated, the genomic DNA of ARO and HepG2 cells transduced with LV-CMV-hNIS or LV-EIIAPA-hNIS was extracted for PCR (a). Plasmid carrying HSV-tk gene was used as control. The total RNA extracted from transduced cells was analyzed by RT-PCR to demonstrate the hNIS gene expression mediated by lentivirus and the specificity of EIIAPA promoter to hepatoma. The internal control (β-actin) was used to ensure that equal amounts of cDNA were used for the subsequent PCR step (b).*

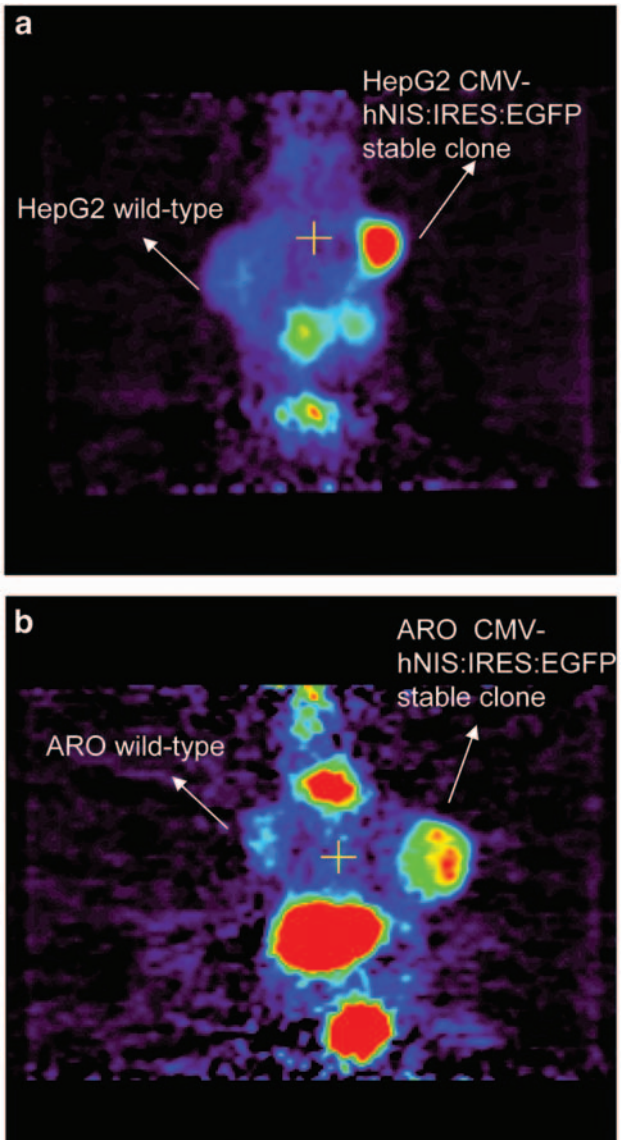

Figure 5. *^{124}I microPET imaging of HepG2 (a) and ARO (b) tumor-bearing mice. Tumors on the right shoulders of mice were grown from ARO or HepG2 cells stably expressing the hNIS gene. Parental tumors were inoculated on the opposite shoulders. Note a high tumorous uptake of ^{124}I in the right shoulder (a and b).*

PCR and RT-PCR techniques were performed. The result shown in Figure 4a indicates that both ARO and HepG2 cells could be transduced by lentivirus and that the hNIS gene was integrated into genomic DNA. To further determine the lentivirus-mediated *hNIS* gene expression, total RNA extracted from transduced cells was reverse-transcribed into cDNA and then measured by PCR. As seen in Figure 4b, after integration of lentiviral vectors, the *hNIS* controlled by CMV promoter was expressed in both ARO and HepG2 cells. In contrast, the EIIAPA promoter only induced *hNIS* gene expression in HepG2 cells, not in ARO cells. *β-Actin* acted as an internal control to ensure that equal amounts of cDNA were used. These results were indicative of EIIAPA promoter specific to hepatoma but not to nonhepatoma cells.

Demonstration of the function of hNIS gene in vivo by radionuclide imaging. In order to demonstrate that HepG2 and ARO stable clones could express the *hNIS* gene not only *in vitro* but also *in vivo*, radionuclide imaging was used to monitor the *hNIS* gene in animal models. Accumulation of ^{124}I reporter probes, as shown in Figure 5, was observed at one hour after ^{124}I administration in the tumors established from HepG2 and ARO CMV-hNIS-IRES-EGFP-transfected cells, respectively, in the right shoulders of mice. In contrast, the uptakes of tumors from wild-type ARO and HepG2 cells in the left

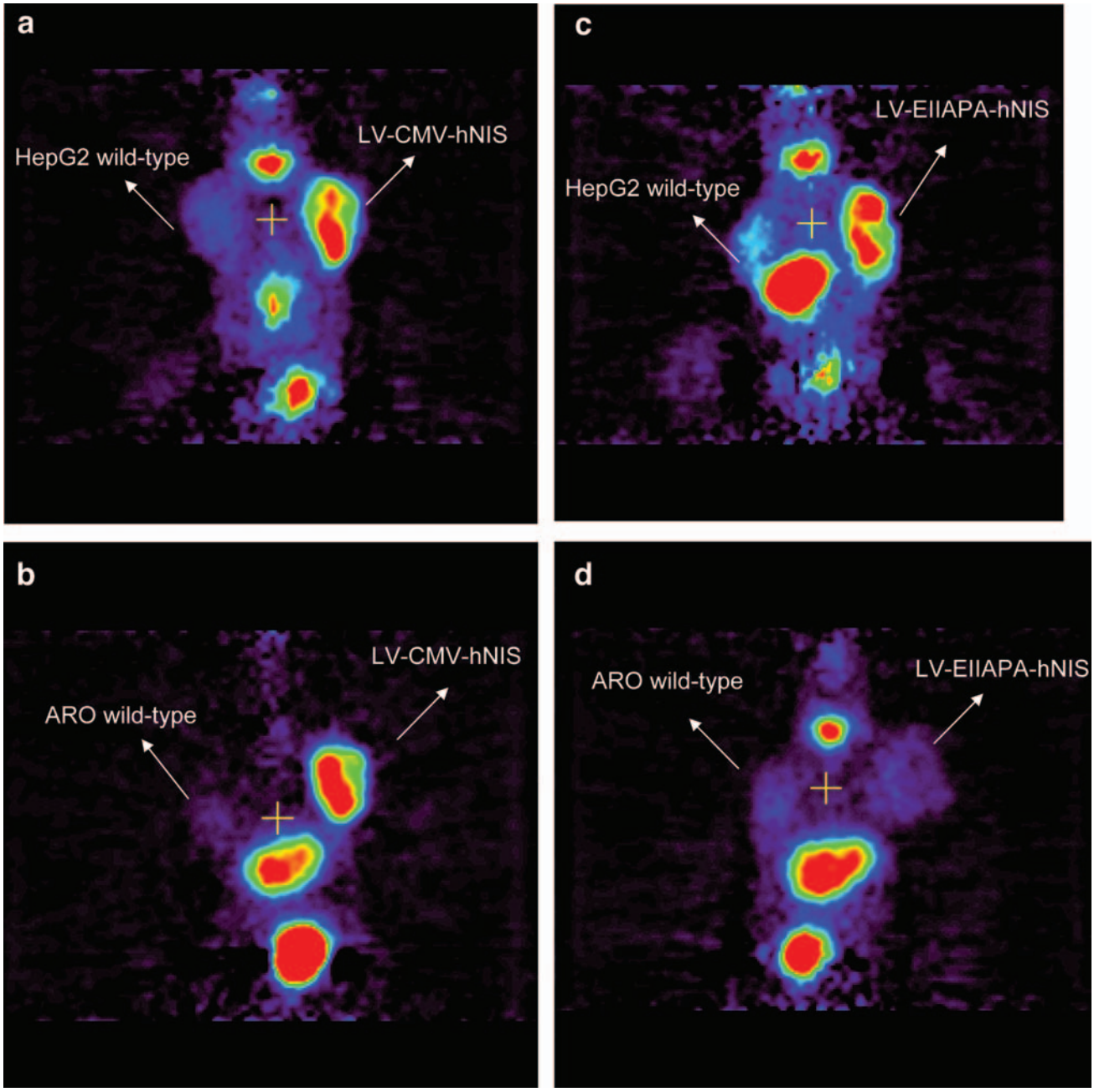

Figure 6. *^{124}I MicroPET imaging of HepG2 and ARO tumor-bearing mice. Both HepG2 (a, c) and ARO (b, d) cells were infected with pLL3.7/CMV-hNIS and pLL3.7/EIIAPA-hNIS viral particles, respectively, in vitro. pLL3.7/CMV-hNIS and pLL3.7/EIIAPA-hNIS infected cells were then implanted into the right shoulders of the mice. The parental ARO and HepG2 tumors were grown in the opposite shoulders as control. When tumors reached 8 mm in size, images were acquired for 30 min at 60 min post administration of 1.85 MBq of ^{124}I. Note the preferential localization of ^{124}I in hepatoma infected with pLL3.7/EIIAPA-hNIS virus (c).*

shoulders of the same mice showed less accumulation than the tumors in the right shoulders. The results of radionuclide imaging indicate the successful transfer and integration of the *hNIS* gene into different kinds of tumors, functioning well in both *in vitro* and *in vivo* systems.

Demonstration of specificity of EIIAPA promoter to hepatoma by in vitro multi-infection. In order to demonstrate that the EIIAPA chimeric promoter is specific to hepatoma, the HepG2 hepatoma and ARO cells were infected by lentivirus *in vitro* six times and were then implanted into the

shoulder of NOD/SCID mice. In the mice bearing HepG2 tumors, tumor transduced with LV-CMV-hNIS accumulated ^{124}I with a mean uptake ratio of 3.16, while tumor transduced with LV-EIIAPA-hNIS had an uptake ratio of 2.27 (Figure 6a and c). In the mice bearing ARO tumors, tumor transduced with LV-CMV-hNIS accumulated ^{124}I with a mean uptake ratio of 5.03 while tumor transduced with LV-EIIAPA-hNIS had an uptake ratio of 0.92 (Figure 6b and d). The ^{124}I uptake of transduced ARO tumor showed similar background levels as parental tumors. It was clearly observed that CMV promoter strongly, but non-selectively, induced iodide accumulation in the transduced tumor, whereas EIIAPA chimeric promoter specifically induced iodide accumulation only in HepG2 tumor. This result indicates the capability of EIIAPA promoter to selectively transcribe the downstream gene in hepatoma.

Discussion

The ability of the thyroid gland to concentrate iodide forms the basis for diagnosis and therapeutic management of benign thyroid diseases and thyroid cancer. In 1996, *NIS* was first cloned from rats (28) and then from humans (29). Research using *NIS* has improved our understanding of thyroid pathophysiology tremendously and has provided a promising field in cancer research. Cloning and characterization of the *NIS* gene offers the capability for its transfer into nonthyroidal cancer cells, including hepatoma, thereby inducing radioiodide accumulation. In turn, imaging and therapy in nonthyroidal tissue could thus be achieved (28, 30).

To investigate the possibility of radioiodide therapy by radionuclide imaging, we established two different human stable clones in ARO thyroid cancer and HepG2 hepatoma cells, which stably expressed *hNIS* and *GFP* bicistronic construct. Because NIS cell surface trafficking is quite susceptible to disruption and result in failure to retain NIS at the cell surface (31), the function and location of NIS protein in our established stable clones should be confirmed. In our immunofluorescence study, we have shown that NIS proteins controlled by CMV promoter were efficiently trafficked to the cell surface in both ARO and HepG2 stable clones which stably expressed exogenous genes. Moreover, the *GFP* gene following the *NIS* gene was also well expressed, attributed to the internal ribosome entry site (IRES) element, and allows the gene expression in ARO and HepG2 stable clones to be monitored by both fluorescence microscopy and radionuclide imaging by this dual-reporter system.

In the radioiodide uptake study, the radioiodide accumulation in ARO and HepG2 stable clones was 87- and 208-fold higher, respectively, than that of parental cells. Several studies have reported *NIS* gene transfer to various cancer cells such as undifferentiated thyroid carcinoma cells and nonthyroid cells. The latter include breast and prostate cancer, and glioma cells with increased iodine uptake (15, 16). In addition, NIS-expressing tumors were also found to accumulate ^{188}Re-perrhenate and ^{99m}Tc-pertechnetate efficiently (30).

An attractive strategy in gene therapy is using tissue-specific promoter to directly express therapeutic transgenes in specific tissues or tumors, thereby maximizing tissue-specific cytotoxity and also reducing extratumoral side-effects. Specific expression of transgenes in hepatoma cells has been obtained by the use of AFP promoter (32). Willhauck *et al*. used a mouse alpha-fetoprotein (AFP) promoter construct to target HCC cells. The *in vitro* and *in vivo* results showed a sufficient therapeutic effect of ^{131}I for the treatment of liver tumor (33). Some studies have linked the AFP enhancer directly to its promoter resulting in an improvement of herpes simplex virus 1-thymidine kinase (HSV1-tk) transgene expression in low-AFP expressing hepatoma cells as evidenced by increased sensitivity of transfected cells to gancyclovir (GCV) (34, 35). However, the improvement in cytotoxicity in hepatomas was accompanied by the increased sensitization of hepatic stem cells, which also express AFP, causing serious liver damage (34, 35). Our previous studies demonstrated that the combination of HBV enhancer II and AFP promoter is also specific to HepG2 cells but not to other nonhepatoma cancer cell lines and normal fibroblast cells and subsequently enhances the transcriptional activity of hepatoma-specific AFP promoter alone (27).

ARO and HepG2 cells were transduced with lentiviral particles containing pLL3.7/CMV-EGFP, pLL3.7/CMV-hNIS and pLL3.7/EIIAPA-hNIS transfer vectors. According to the result of *in vitro* ^{125}I uptake and *in vivo* ^{124}I microPET imaging, hNIS controlled by EIIAPA promoter induced significant radioiodide accumulation only in HepG2 hepatoma cells/tumor but not in ARO thyroid cells/tumor, nor in the parental cells/tumor, demonstrating the specificity of EIIAPA-mediated *hNIS* gene transcription in hepatoma. LV-CMV-hNIS-infected cells/tumors were used as positive control of which the *NIS* gene expression was non-selectively induced by CMV promoter in both HepG2 and ARO cancer cells/tumors. These results indicate that combination of AFP promoter with HBV enhancer II not only maintains the basal transcriptional activity of the AFP promoter but also increases its specificity for transfection of the *hNIS* gene into hepatoma cells.

Some studies reported that fetoprotein transcription factor (FTF), which responds to particular hormonal signals, is a specific regulator of the *AFP* gene during early liver development. It not only regulates the *AFP* gene expression but also is involved in HBV replication by transcriptional up-regulation of HBV nucleocapsid proteins *via* two high-affinity binding sites located in the EIIA region of the HBV nucleocapsid promoter (36, 37). Another factor, HNF-4α, was shown to very efficiently co-stimulate the transcriptional activity of FTF from the HBV core promoter. These two factors are highly expressed together only in hepatocytes (36, 37). According to our results, the specificity of EIIAPA promoter to hepatoma but not to nonhepatoma cells may result from the

lack of FTF and HNF-4α transcription factors in the ARO thyroid cancer cells.

Various methods, such as native DNA delivery and recombinant viruses carrying exogenous genes, were studied in gene therapy but all have limitations (38). Development of lentiviral vectors has led to efficient delivery and long-term expression of transgenes to hepatocytes without observable toxicity and with no apparent requirement for cell proliferation (39, 40). In our study, both ARO thyroid cancer cells and HepG2 hepatoma cells were repeatedly infected with lentiviral vector containing pLL3.7/CMV-hNIS and pLL3.7/EIIAPA-hNIS vector *in vitro*. Up to eight weeks after inoculation of these transduced cells, *NIS* gene expression was demonstrated by radionuclide imaging, indicative of the stable integration of the exogenous gene by lentiviral-infection and durable *in vivo* expression.

To further demonstrate the integration that could be mediated by lentivirus, PCR was performed to detect the *hNIS* gene in the genomic DNA of transduced cells. According to our results, both ARO and HepG2 cells were successfully infected by lentivirus and *hNIS* gene expression induced by CMV promoter using a RT-PCR technique. Although the CMV-hNIS and EIIAPA-hNIS fragments were integrated into the genome by LV-CMV-hNIS and LV-EIIAPA-hNIS, *hNIS* transcripts were induced by EIIAPA promoter only in HepG2 cells, not in ARO cells. These results show that our lentiviral system can successfully deliver the exogenous *hNIS* into cells and the transcriptional targeting to hepatoma can be carried out using EIIAPA chimeric promoter.

Using lentiviral vector as a gene transfer modality has been extensively adapted in recent years. Its advantages, including gene stable integration and capability for infecting dividing and non-dividing cells, offer a practical way to transfer genes, with long-term gene expression. However, titers of produced lentivirus are still not comparable to titers routinely obtained with adenovirus, and such virus titer is not sufficient for *in vivo* purposes (41). In addition, different types of cell lines have different gene transfer efficiencies. Dingli *et al*. observed 30-90% efficiency in myeloma and nonmyleloma cell lines (42). Pellinen *et al*. (41) have shown 9-94% transduction efficeincy of 42 cell lines including these of bladder, bone, brain, breast, cervix, colon, lung, melanoma, pancreatic and prostate cancer. Even different cell lines representing the same cancer type have great differences in gene transfer efficiency. For example, bone cancer cell lines MG-63 and U-2-OS have efficiencies of 18.9% and 92.4% , respectively. In our study, titers of produced lentivirus were around 10^8 to 10^9 VP/ml. After 6 repeated infections, transduced ARO and HepG2 cells had 5.8- and 17.7-fold radioiodide accumulation, respectively, as compared to parental cells. Even infected with such high titer of viral particles, repeated infections were still needed for demonstration of the differences in accumulation of radioiodide between tranduced and parental cells. These results suggest that both hepatoma and anaplastic thyroid cancer cell lines do not have a high efficiency of gene transfer by lentivirus. Moreover, the differential restoration of the ability of radioiodide accumulation of ARO and HepG2 cells suggests that the transducing efficiency by lentivirus to these two cell types is quite different. We also performed intratumoral injection of lentivirus in the tumor-bearing mouse model. No significant difference in radioiodide accumulation was observed between tumors injected with viruses and parental tumors assessed by ^{124}I microPET imaging (data not shown). This result is to be expected because *in vivo* infection was a more complicated task than *in vitro* infection. To briefly sum up, the efficiency of gene transfer on certain types of cancer cell lines remains to be overcome by either developing a more effective production method or increasing the virus titer, although lentivirus-mediated gene transfer does have potential in gene therapy.

The EIIAPA promoter is a novel chimeric promoter by which a gene can be selectively transcribed in HepG2 cell lines but not in nonhepatoma cell lines. In a previous study we had shown the effectiveness in targeting HepG2 cells (27). However such speificity cannot be further enhanced due to the present limitation of lentiviral delivery. Another way in which specificity can be enhanced is to increase the cell targeting ability of lentivirus. In recent studies, specific peptides that target hepatocarcinoma were proposed. Zhang *et al*. (43) reported a synthetic peptide HCBP1 (sequence FQHPSFI) that binds to the tumor surfaces of two hepatoma cell lines. With this finding, lentiviral vector can be produced with selective targeting ability by adding the specific peptide-coding DNA sequence into the packaging plasmid pVSVG that encodes the envelope protein VSVG. This strategy is under study.

In conclusion, the results of the present study indicate that the *NIS* gene transferred by lentivirus successfully induces radioiodide uptake in HCC in our study and that the novel chimeric EIIAPA promoter has a high specificity to hepatoma as compared to nonhepatoma cells. Thus, such an approach may provide a promising strategy for tumor-specific *hNIS*-mediated radiogene therapy.

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