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ORIGINAL ARTICLE

A membrane antibody receptor for noninvasive imaging of gene expression

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Monitoring gene expression is important to optimize gene therapy protocols and ensure that the proper tissue distribution is achieved in clinical practice. We developed a noninvasive imaging system based on the expression of artificial antibody receptors to trap hapten-labeled imaging probes. Functional membrane-bound anti-dansyl antibodies (DNS receptor) were stably expressed on melanoma cells in vitro *and* in vivo*. A bivalent $(DNS)_2$-diethylenetriaminepentaacetic ^{111}Indium probe specifically bound to cells that expressed DNS receptors but not control scFv receptors. Importantly, the ^{111}In probe preferentially localized to DNS receptors but not control receptors on tumors in mice as assessed by gamma camera imaging. By 48 h after intravenous injection, the uptake of the probe in tumors expressing DNS receptors was 72 times greater than the amount of probe in the blood. This targeting strategy may allow noninvasive assessment of the location, extent and persistence of gene expression in living animals and in the clinic.*
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Introduction

Reporter genes play critical roles in determining the mechanisms of gene expression in transgenic animals and in developing gene delivery systems for human gene therapy. Noninvasive imaging methods can allow definition of the location, magnitude and persistence of gene expression. Approaches using green fluorescent protein[1–3] and luciferase[4–6] as reporter genes in humans, however, are limited by the immunogenicity of the reporter gene products as well as by the poor spatial resolution of the detection system.[3] Herpes simplex type 1 virus thymidine kinase (*HSVtk*) allows selective retention of radiolabeled substrates that can be detected by autoradiography[7–9] and single photon emission computed tomography.[10–12] Unfortunately, the metabolites of the probes (acycloguanosine and uracil nucleoside derivatives) can incorporate into genes, cause DNA chain termination and act as mutagens.[13,14] Furthermore, the *HSVtk* gene product can induce cellular immunity which could result in tissue damage.[15,16] Similar problems were found in the bacterially derived cytosine deaminase reporter gene and 5-[^{18}F]-fluorocytosine system.[17,18]

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Endogenous cellular receptors are also being developed for *in vivo* imaging such as the dopamine D2 receptor (D2R) for selective uptake of the radiotracer-labeled antagonist 3-(2′-[^{18}F]-fluoroethyl)-spiperone.[19,20] Good correlations with image signal have been demonstrated *in vivo*, but D2R is also expressed in the brain stratum and in the pituitary glands,[21,22] limiting the specificity of D2R. In addition, D2R reporter gene expression can lead to initiation of intracellular signaling through ligand binding.[23,24] The endogenous transferrin (Tf) receptor is also under investigation as a reporter to trap Tf linked to monocrystalline iron oxide nanoparticles. Modest increases in receptor levels caused considerable changes in magnetic resonance (MR) images,[25] but poor specificity due to the widespread presence of the endogenous Tf receptor limits this strategy for clinical applications.

To overcome these problems, we developed a novel reporter gene that directs the expression to the plasma membrane of a nonimmunogenic receptor with specificity for a defined chemical hapten (Figure 1a). Specific hapten receptors were created by fusing a high-affinity anti-dansyl (5-dimethylamino-1-naphthalene sulfonic acid) single-chain antibody to the C-like extracellular, transmembrane and cytosolic domains of the murine B7-1 antigen to allow expression of the antibody on the plasma membrane of cells (Figure 1b). We also developed a divalent hapten-chelate that can avidly bind 111-indium (^{111}In) or gadolinium (Gd) metal ions to form radioactive or MR-imaging probes. Expression of the reporter gene results in accumulation of the receptor on

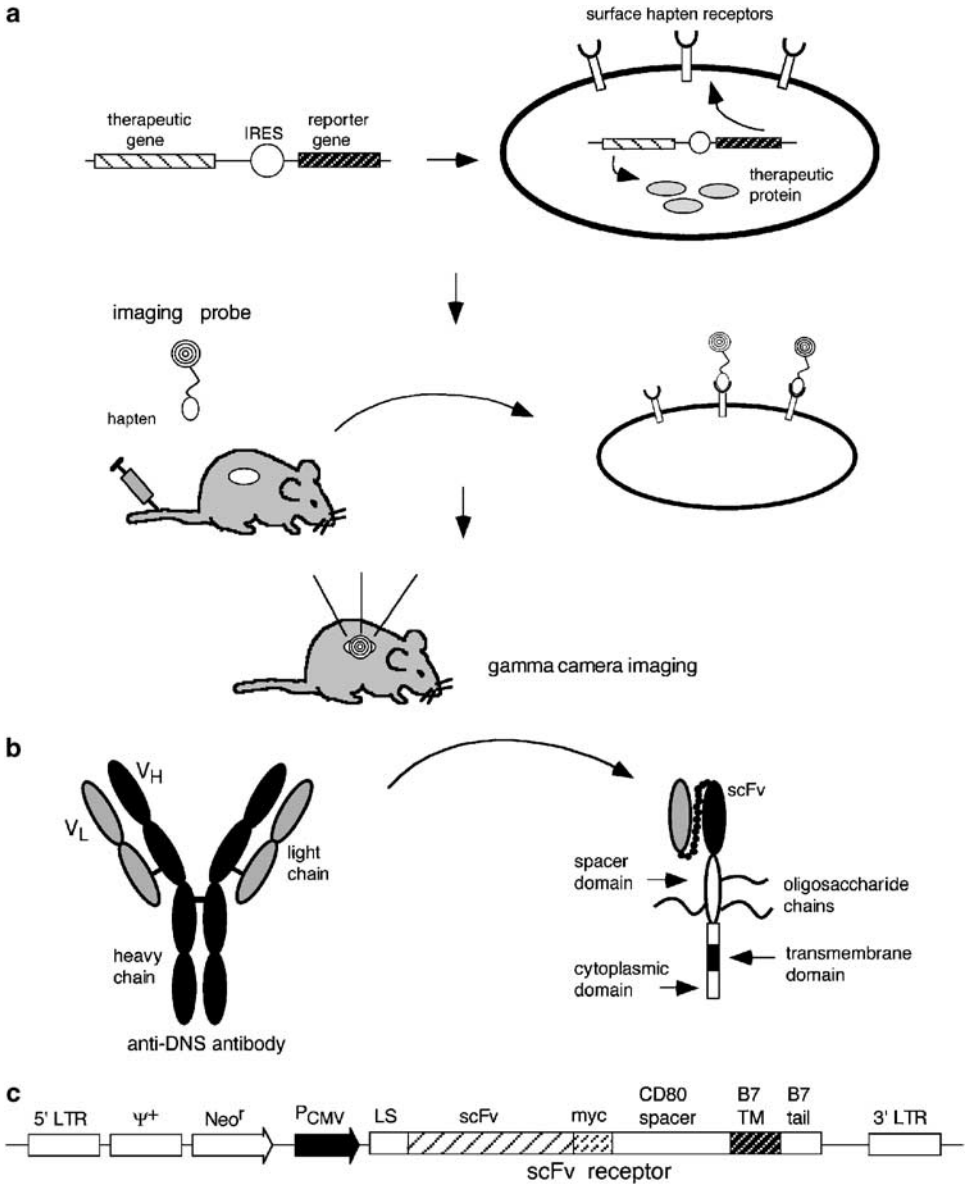

Figure 1 Noninvasive reporter gene imaging system. (**a**) A bicistronic vector containing a therapeutic gene and a reporter gene can be delivered to the desired tissue by viral or nonviral delivery technologies. The reporter gene encodes artificial surface receptors that can bind to a small chemical hapten. An imaging probe in which the hapten is chemically linked to a radioisotope is then administered. The imaging probe accumulates at sites of the hapten receptor expression which can then be imaged with a gamma camera. (**b**) The hapten receptor is constructed from a single-chain antibody (scFv) that was derived from a monoclonal antibody against DNS. The anti-DNS scFv was fused to a spacer domain that contains oligosaccharide chains to reduce receptor shedding. The receptor is anchored to the plasma membrane by a hydrophobic transmembrane domain. (**c**) Schematic of the retroviral DNS receptor vector. The DNS receptor is comprised of a leader sequence (LS), the DNS scFv, a myc epitope, a B7-1 immunoglobulin C2-type extracellular 'spacer' to reduce shedding, a B7-1 transmembrane domain and the intact B7-1 cytoplasmic tail. Expression of the transgene is under control of the CMV immediate early promoter.

cells which can bind and retain subsequently administered probe. Sites of gene expression can then be imaged by a gamma camera or by MR imaging (MRI). We show that ^{111}In-labeled hapten can specifically accumulate at sites of DNS receptor expression *in vitro* and *in vivo*, allowing noninvasive imaging of sites of receptor gene expression.

Results

Characterization of the anti-dansyl surface receptor

We constructed a retrovirus vector, pLNCX-DNS-eB7, to direct the expression of DNS scFv receptors to the plasma membrane of mammalian cells. This vector encodes a chimeric protein consisting of an anti-DNS scFv against the dansyl hapten, an 11 amino-acid myc epitope and the immunoglobulin C2-type extracellular, transmembrane and cytosolic domains of murine B7-1 (Figure 1c). The immunoglobulin C2-type extracellular domain of B7-1 contains three N-linked oligosaccharide chains that reduce the shedding of scFv receptors from the cell surface.[26] Expression of the chimeric receptor is under the control of the human cytomegalovirus (CMV) immediate early promoter. We employed the retroviral vector pLNCX-phOx-eB7, which encodes a similar chimeric receptor with specificity for 4-ethoxymethy-

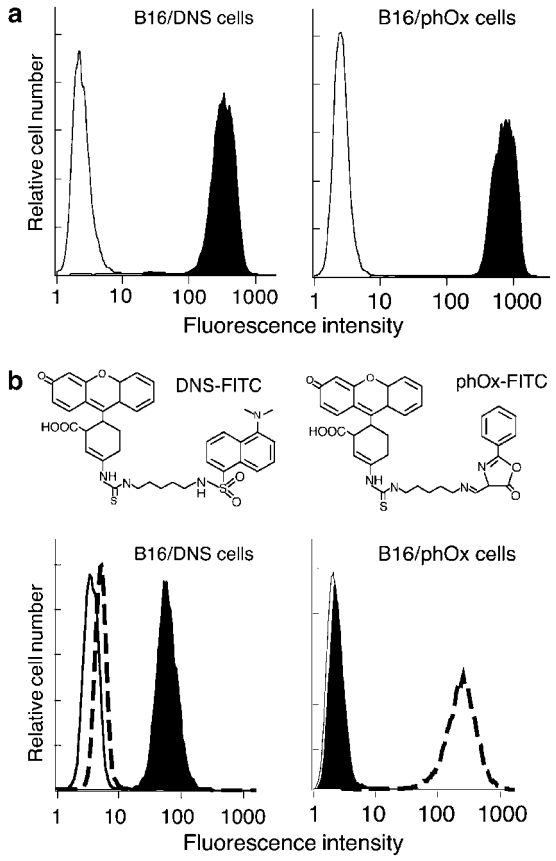

Figure 2 Cells surface display of functional scFv receptors. (**a**) The immunofluorescence of B16/DNS cells (left panel) and B16/phOx cells (right panel) is shown after staining with control antibody (open curves) or anti-myc antibody (solid curves) for the presence of the myc epitope in the DNS and phOx scFv receptors. (**b**) The immunofluorescence of B16/DNS cells (left panel) and B16/phOx cells (right panel) cells is shown after mock staining with PBS (open curves), or staining with 1 μM DNS-FITC (solid curves) or phOx-FITC (dashed line).

lene-2-phenyl-2-oxazoline-5-one (phOx), as a control receptor.[27,28]

B16-F1 murine melanoma cells were infected with recombinant retroviral particles, selected in G418 and sorted in a flow cytometer for high expression of the DNS or phOx scFv receptors to obtain stable B16/DNS and B16/phOx cell lines. The surface expression of the receptors was analyzed by immunofluorescence staining using an antibody with specificity for the c-myc epitope present in the receptors. Figure 2a shows that B16/DNS and B16/phOx cells expressed high levels of DNS and phOx receptors, respectively, on their surface ($\sim 3.8 \times 10^5$ DNS receptors per cell). To assess the functional antigen-binding activity of the receptors, we synthesized small fluorescent probes that possessed either a DNS or phOx hapten moiety (Figure 2b). DNS-FITC bound to B16/DNS cells but not to B16/phOx cells whereas phOx-FITC displayed the opposite cellular specificity (Figure 2b). These results demonstrate that the hapten receptors on the cell surface were functionally active and displayed hapten-binding specificity.

The location and functional stability of the DNS receptors was investigated by allowing B16/DNS or B16/phOx cells to bind DNS-FITC and then culturing the cells in complete medium at 37°C for 4 h. Examination of the cells under a fluorescence microscope demonstrated that the DNS-FITC probe was present on the surface of B16F1/DNS cells but not B16/phOx cells (Figure 3a), indicating that the DNS receptor remained on the cell surface after hapten binding. Measurement of cellular fluorescence intensity by flow cytometry after B16/DNS cells were exposed to DNS-FITC and then incubated at 37°C for up to 10 h revealed that similar amounts of DNS-FITC were associated with the cells at all times (Figure 3b), demonstrating that the probe was well retained by the cells. To determine the fraction of total DNS receptors that were present on the cell surface, we labeled surface proteins on B16/DNS cells with a membrane-impermanent form of biotin, solubilized the cells in detergent, separated biotin-labeled and -unlabeled proteins and performed immunoblot analysis for the presence of the myc epitope in the DNS receptors. Quantification of band intensities revealed that 84% of the DNS receptors was present on the plasma membrane of B16/DNS cells (data not shown), indicating that the receptor was efficiently transported to the cell surface.

The expression of the DNS receptors *in vivo* was examined by recovering single cells from established B16/DNS and B16/phOx tumors and then measuring by flow cytometry the expression of the receptors on the recovered cells. Figure 3c shows that high levels of DNS and phOx receptors could be detected on the cells, demonstrating that receptor expression was stable *in vivo*. We also wished to examine the immunogenicity of the DNS receptors. The plasmids pLNCX, pLNCX-DNS-eB7 or pLNCX-LacZ-eB7, which codes for a membrane-bound form of LacZ, were intravenously (i.v.) injected into C57BL/6 mice by the hydrodynamic-based gene transfer technique. Serum samples were collected 10 days later and the presence of antibodies against DNS scFv or LacZ was examined by ELISA employing 293 cells that transiently expressed DNS receptors (293/DNS cells) or membrane-anchored LacZ (293/LacZ cells), as shown by the binding of anti-myc antibody to the cells (Figure 3d, black bars). Serum isolated from the mice that were injected with pLNCX-LacZ-eB7 contained antibodies that bound to 293/LacZ cells (striped bars). In contrast, serum from the mice that were i.v. injected with pLNCX-DNS-eB7 did not contain detectable antibodies to 293/DNS cells (dotted bars). Thus, the DNS receptors could be stably expressed on cells in an active form and did not induce a specific immune response, prerequisites for repetitive and persistent imaging in live animals.

Stability and specificity of the imaging probe

A divalent $(DNS)_2$-diethylenetriaminepentaacetic-imaging probe, $(DNS)_2$-DTPA, was synthesized (Figure 4a) because preliminary experiments revealed poor retention of a monovalent probe at DNS receptors (results not shown). $(DNS)_2$-DTPA was labeled with ^{111}In to form $(DNS)_2$-DTPA-^{111}In with a specific activity of 2.3×10^4 Ci/mmole. The binding and stability of $(DNS)_2$-DTPA-^{111}In was examined by incubating the probe in 20% mouse serum at 37°C for 0 or 24 h before addition to B16/DNS or B16/phOx cells. The cells were then washed and the radioactivity remaining bound to the cells was measured. Figure 4b shows that $(DNS)_2$-DTPA-^{111}In selectively bound to B16F1/DNS cells as compared to B16/phOx cells even after incubation in serum for 24 h,

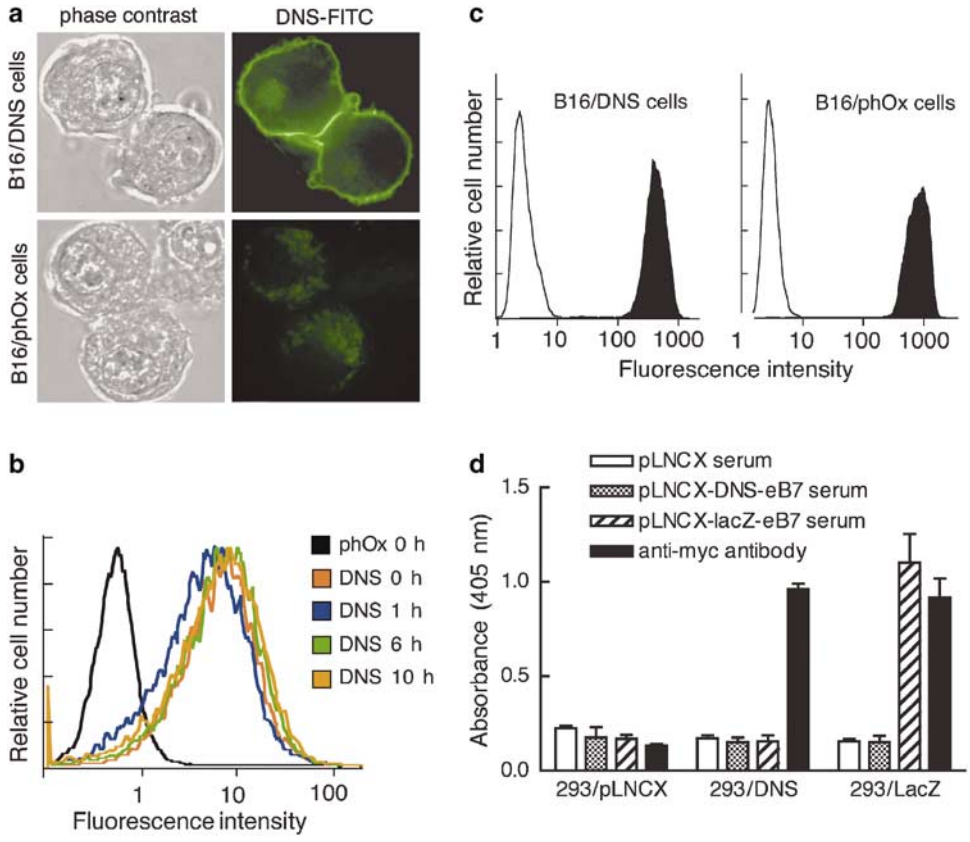

Figure 3 *In vivo* expression, stability and immunogenicity of hapten receptors. (**a**) B16/phOx and B16/DNS cells were stained with DNS-FITC and then incubated at 37°C for 4 h before the cells were observed under a fluorescence microscope. (**b**) B16/phOx and B16/DNS cells were stained with DNS-FITC and then incubated at 37°C for the indicated times before immunofluorescence was measured on a flow cytometer. (**c**) Established B16/DNS tumors (left panel) or B16/phOx tumors (right panel) were excised from C57BL/6 mice and disaggregated by collagenase treatment. The cells were cultured for 24 h before the immunofluorescence of the cells was measured on a flow cytometer after staining with control antibody (open curves) or anti-myc antibody (solid curves) for the presence of the myc epitope in the DNS and phOx scFv receptors. (**d**) Serum samples collected from C57BL/6 mice 10 days after i.v. hydrodynamic injection of pLNCX, pLNCX-DNS-eB7 or pLNCX-LacZ-eB7 plasmids were diluted 200-fold and then assayed for the presence of antibodies against 293/vector, 293/DNS or 293/LacZ cells. The binding of anti-myc antibody to the myc epitope present in the surface receptors was also assayed (black bars). Results show the mean absorbance values of triplicate determinations. Bars, s.d.

demonstrating that $(DNS)_2$-DTPA-^{111}In was stable. To investigate whether the amount of cell-bound radioactivity could reflect the number of DNS receptors on cells, we transiently transfected HeLa Tet-on cells with a tetracycline-responsive plasmid containing the DNS receptor transgene. The cells were exposed to graded concentrations of doxycycline to control the transcription of the receptor and 1 day later the receptor surface expression and binding of $(DNS)_2$-DTPA-^{111}In probe to the cells were determined. Figure 4c shows that cell-bound radioactivity closely mirrored DNS receptor density on the cells, indicating that cell-bound radioactivity depends on the level of DNS receptor transcription. We also examined the half-life of $(DNS)_2$-DTPA-^{111}In the circulation of C57BL/6 mice (Figure 4d). The probe was eliminated from the blood of mice with apparent two-phase exponential decay kinetics at an initial half-life of 0.18 ± 0.5 h and a terminal half-life of 2.7 ± 0.4 h.

In vivo *imaging of DNS receptors*

To investigate whether sites of DNS receptor expression could be noninvasively imaged, we i.v. injected 100 μCi $(DNS)_2$-DTPA-^{111}In into two mice that bore an established B16/DNS tumor on their right shoulder and a control B16/phOx tumor on their left shoulder (Figure 5a). The mice were then imaged with a gamma camera after 1, 4, 24 and 48 h. Figure 5b shows that $(DNS)_2$-DTPA-^{111}In was selectively retained in B16/DNS tumors. The B16/DNS tumors could be distinguished within 4 h, but elimination of the probe from normal organs allowed more obvious visualization after 24 h. Table 1 shows that the region of interest (ROI) ratio of B16/DNS to B16/phOx tumors increased with time.

Additional tumor-bearing mice were i.v. injected with 60 μCi $(DNS)_2$-DTPA-^{111}In and the radioactivity of isolated tissues was measured after 4, 24 and 48 h to quantify the probe biodistribution. Figure 6a shows that the radioactivity associated with $(DNS)_2$-DTPA-^{111}In in the blood decreased more rapidly as compared to the radioactivity in B16/DNS tumors, indicating selective retention of the probe at B16/DNS cells. The uptake of probe in B16/DNS tumors was 72 times greater than the amount in blood at 48 h. The distribution of the probe at 48 h in several tissues is shown in Figure 6b. The mean radioactivity of B16/DNS tumors was about 15 times greater than the mean radioactivity of B16/phOx tumors at 48 h. Some radioactivity also remained in the liver and kidney at 48 h. These results show that $(DNS)_2$-

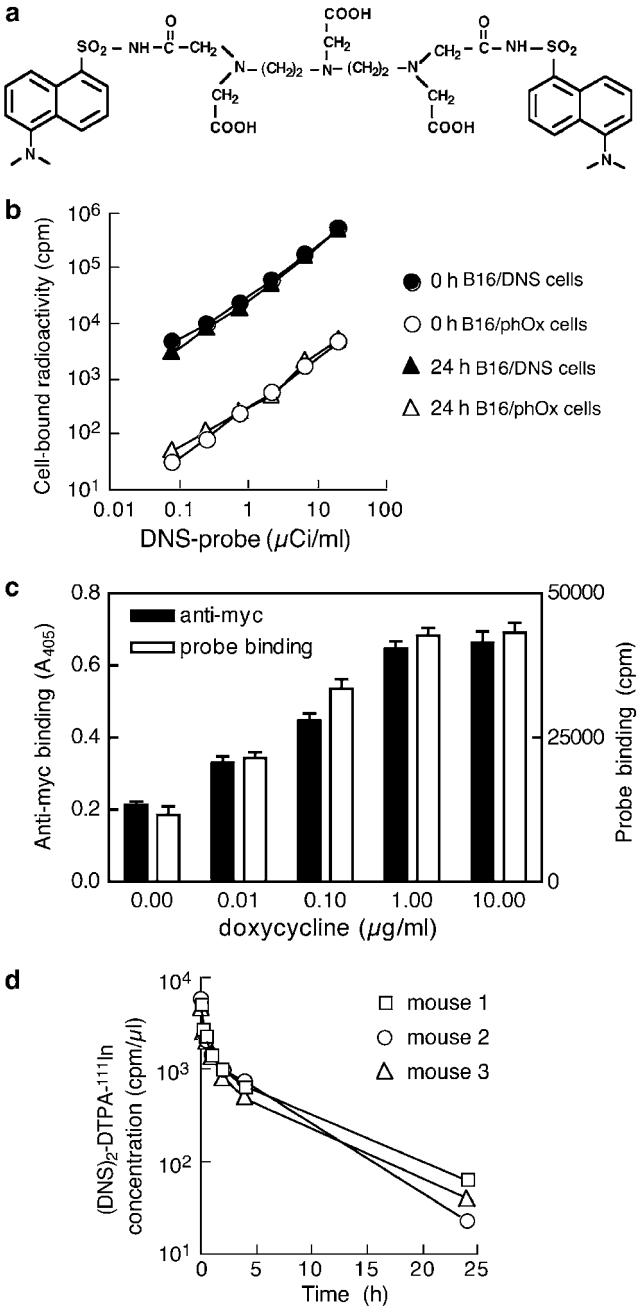

Figure 4 Specificity, stability and half-life of the imaging probe. (**a**) Chemical structure of the $(DNS)_2$-DTPA-^{111}In probe. (**b**) $(DNS)_2$-DTPA-^{111}In was freshly prepared (circles) or incubated in 20% serum at 37°C for 24 h (triangles) before graded concentrations of the probe were incubated in triplicate with B16/DNS cells (solid symbols) or B16F/phOx cells (open symbols). The plates were washed and bound radioactivity was measured in a gamma-counter. (**c**) HeLa tet-on cells were transfected with a tetracycline-responsive DNS reporter plasmid and the indicated concentrations of doxycycline were added to the cells after 24 h. After an additional 24 h, the relative expression of DNS scFv receptors on the cells was measured by ELISA with anti-myc antibody (solid bars). Excess $(DNS)_2$-DTPA-^{111}In was added to the cells, the cells were washed and cell-bound radioactivity was measured (empty bars). The results show mean values of triplicate determinations. Bars, s.d. (**d**) C57BL/6 mice were i.v. injected with 20 μCi $(DNS)_2$-DTPA-^{111}In. Blood samples were periodically collected and the radioactivity was measured in a gamma-counter.

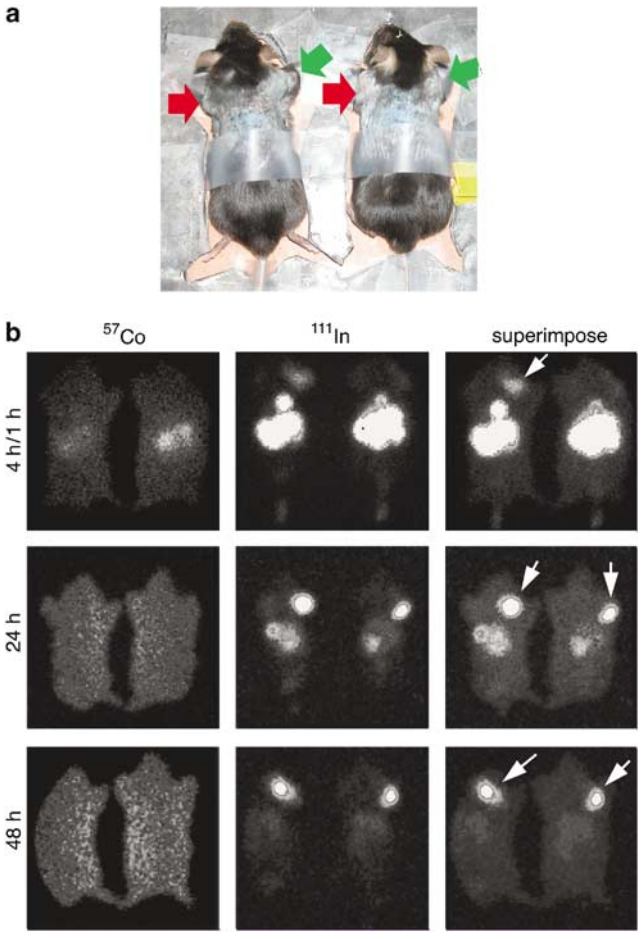

Figure 5 *In vivo* noninvasive imaging. (**a**) Photograph showing the locations of the B16/DNS tumors (green arrows) or the control B16/phOx tumors (red arrors). (**b**) 100 μCi $(DNS)_2$-DTPA-^{111}In was i.v. injected into the mice and images were acquired at the indicated times. The left panels show the mice positions, measured by the shielding of a ^{57}Co source, the middle panels show gamma radiation produced by the $(DNS)_2$-DTPA-^{111}In probe and the right panels show the superimposed images. The images in the top panels were acquired after 4 h (left mouse) or 1 h (right mouse). The white arrows indicate the position of B16/DNS tumors.

Table 1 The region of interest (ROI) ratio of B16/DNS to B16/phOx tumor was determined at the indicated times

Time (h)	*ROI (DNS tumor/phOx tumor)*	
	Mouse 1	*Mouse 2*
1	4.5	1.8
4	7.8	ND
24	16.6	12.5
48	32.3	25.0

ND = not determined.

DTPA-^{111}In specifically accumulated at sites of DNS scFv receptors *in vivo*.

Discussion

We have successfully developed a novel reporter gene/probe-imaging system based on the expression of anti-

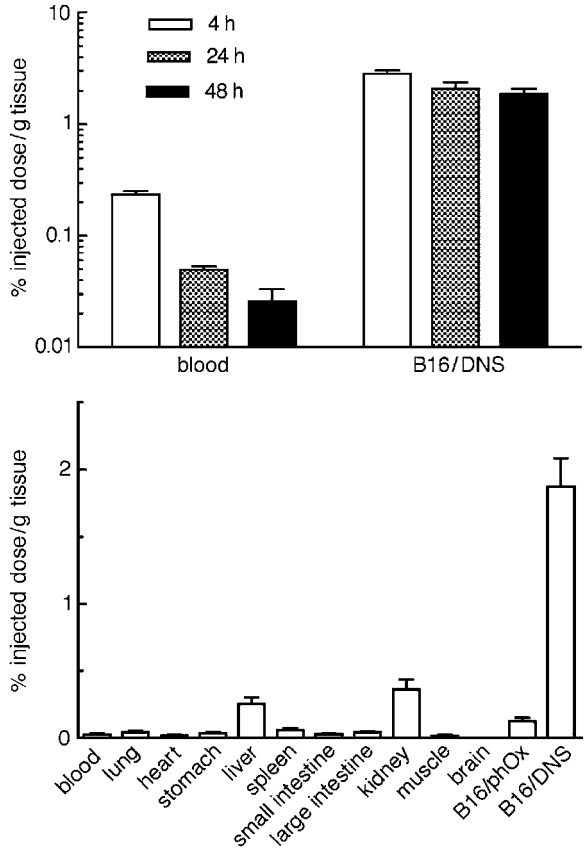

Figure 6 Biodistribution of $(DNS)_2$-DTPA-^{111}In in tumor-bearing mice. The uptake of $(DNS)_2$-DTPA-^{111}In in mice was measured 4 h ($n=3$), 24 h ($n=3$) and 48 h ($n=5$) after i.v. injection of the probe. Mice were killed and tumors, blood and organs were weighed and assayed for radioactivity in a gamma-counter. (**a**) The mean radioactivity of the probe in blood and B16/DNS tumors at 4, 24 and 48 h is shown. (**b**) The mean radioactivity of the probe in each tissue at 48 h is shown. Data are presented as percent injected dose per gram tissue. Bars, s.d.

DNS single-chain antibody receptors on cells to trap a DNS-imaging probe. Selective retention of the probe at sites of gene expression may allow assessment of the delivery and expression of genes in living animals by gamma camera imaging. Proportional expression of the gene of interest and the reporter gene (DNS receptor) can be achieved by introduction of an internal ribosomal entry site between the genes.[29–31] For example, Yu *et al*.[31] demonstrated that the relative magnitude of the expression of the first cistron (*D2R*) can be quantified by measuring the expression of the second cistron (*HSV-tk*).

Successful development of a noninvasive imaging method based on the expression of artificial receptors on cells required that several prerequisites be fulfilled. First, the receptors must be efficiently transported in the cell to the plasma membrane. Second, the receptor must be retained on the cell surface in a functional state for prolonged periods. Third, the receptor should display low immunogenicity. Fourth, the imaging probe must be stable *in vivo*. Finally, the probe should be efficiently captured and retained by the receptor to allow imaging. The antibody receptor/hapten-probe system described in our study appears to meet these criteria.

Efficient transport of anti-DNS receptors to the cell surface is important to achieve high levels of the receptors on cells. We previously performed a series of studies to determine the optimal conditions for the expression of proteins[32] and scFv[26,28,33] on mammalian cells. The transmembrane domain and cytoplasmic tail derived from the B7-1 antigen was found to facilitate rapid intracellular transport of chimeric proteins to the cell surface.[32,33] We also demonstrated that the introduction of 'spacer' domains containing oligosaccharide chains between the scFv and B7-1 transmembrane domain dramatically enhanced the expression of scFv on cells by reducing the shedding of antibody from the plasma membrane.[26] Based on these studies, the anti-DNS scFv was fused to the immunoglobulin C2-type extracellular domain of B7-1 to introduce three oligosaccharide chains between the scFv and the B7 transmembrane domain. Our results demonstrate that this construct allowed functionally active DNS scFv to be highly expressed on the cell surface (3.8×10^5 receptors per cell) *in vitro* and *in vivo*.

The reporter gene product should display low immunogenicity to prevent tissue damage by cellular immune responses and allow repeated and persistent imaging of gene expression. Exogenous reporter gene products such as thymidine kinase or cytosine deaminase are immunogenic,[15–17] limiting their applications in both animals and humans. Endogenous reporter genes display low immunogenicity but may suffer from poor specificity. In contrast, antibody receptors can be designed to exhibit low immunogenicity by using scFv derived from the species of interest. The DNS scFv receptor employed in our study was derived from a murine immunoglobulin and therefore did not induce a detectable antibody response in mice. Prolonged expression *in vivo* also suggests that cellular immunity was not induced by the DNS receptors. Likewise, a humanized or human scFv can be employed to minimize immune responses in humans.

The stability of the DNS-DTPA/^{111}In probe was critical for the successful localization of receptors *in vivo*. We previously developed an analogous imaging probe based on the phOx hapten (phOx-DTPA-^{111}In). Although this probe was retained by B16/phOx cells *in vitro*, we were unable to obtain good images of B16/phOx tumors *in vivo* (results not shown). We subsequently discovered that phOx is unstable in serum, resulting in loss of binding to antibody.[27] $(DNS)_2$-DTPA-^{111}In, in contrast, could be specifically bound by DNS scFv receptors even after 24 h in the presence of 20% mouse serum. The high thermodynamic stability of ^{111}In for DTPA[34] argues that the $(DNS)_2$-DTPA-^{111}In complex should be very stable for *in vivo* imaging.

Effective localization of DNS receptors requires prolonged retention of the probe at cells. Construction of receptors with high-affinity antibodies can slow the release of the DNS probes. Morrison reported that the anti-DNS scFv possessed high affinity ($K_D = 1.4 \times 10^{-8}\ M^{-1}$) for DNS hapten.[35,36] Our result showing that DNS-FITC was retained by B16/DNS cells for at least 10 h indicates that the DNS hapten is stably attached to the DNS receptors. The valency of the DNS-imaging probe was also important for *in vivo* imaging. We previously synthesized and tested a monovalent DNS-DTPA-^{111}In probe (1.1×10^4 Ci/mmole). Although this

probe specifically targeted to B16/DNS tumors at 1.5 h as shown by gamma camera imaging, the monomeric DNS-DTPA-^{111}In probe was poorly retained in the tumors and poor tumor images were obtained after 5 h (data not shown). The ROI ratio of the divalent $(DNS)_2$-DTPA-^{111}In probe in B16/DNS tumors increased with time, demonstrating that the divalent probe was selectively retained by DNS receptors. We attribute the improved retention of $(DNS)_2$-DTPA-^{111}In to the higher avidity of the divalent probe. Northrop *et al*.[37] showed that a monovalent fluorescein-DPTA probe could localize at tumors expressing an antifluorescein scFv anchored on their surface via a phosphatidylinositide glycan (GPI) linker. It is unclear, however, how well the probe was retained because tumor localization was measured 60 min after injection when kidney accumulation was also very high.

In summary, the power of our approach is based on several factors including: (1) the high affinity and selectivity of antibody–antigen interactions allows stable retention of the probe without interference from cellular factors, (2) the ability to employ a human or humanized scFv to minimize both humoral and cellular immune responses and allow repeated and persistent imaging of gene expression in humans, (3) hydrophilic probes can be designed to reach areas of interest without the need for transport across the cell membrane, (4) the small size of scFv antibody fragment genes (0.75 kb) should facilitate its use in a wide range of delivery vectors and (5) the DNS hapten can be linked to any suitable imaging probe to extend its utility to other isotopes as well as PET or MRI probes. Based on these advantages, we believe that the novel membrane-scFv/DNS-probe system possesses attractive characteristics for monitoring gene expression in animals and humans.

Materials and methods

Reagents and cells

Dansyl, dansylcadaverine, DTPA dianhydride and phOx were obtained from Sigma-Aldrich (St Louis, MO, USA). Dansyl and phOx were linked to fluorescein via a diaminopentane spacer to generate DNS-FITC and phOx-FITC as described.[38] B16-F1 melanoma cells, 293 cells and an anti-myc hybridoma (CRL-1729) were obtained from the American Type Culture Collection (Manassas, VA, USA). Mycoplasma-free cell cultures were maintained as described.[26]

Construction and transduction of reporter genes

A scFv was constructed as described[28] from plasmids encoding the V_k and V_H genes of the 27–44 mouse anti-dansyl hybridoma.[36] The anti-dansyl scFv was fused to the eB7 domain present in p2C11-e-B7[26] and then inserted into the retroviral vector pLNCX (BD Biosciences, San Diego, CA, USA) to generate pLNCX-DNS-eB7. A plasmid (pLNCX-phOx-eB7) that encodes a membrane scFv with specificity for phOx was constructed in an analogous fashion. Recombinant retroviral particles were packaged by transfection of pLNCX-DNS-eB7 or pLNCX-phOx-eB7 in Phoenix-Eco cells (generously provided by Dr Garry Nolan, Stanford University, CA, USA). B16-F1 cells were infected with recombinant retroviral particles, selected in G418 and sorted on a flow cytometer for high surface expression to generate B16/DNS and B16/phOx cell lines.

Flow cytometer analysis

Receptor surface expression was measured by staining cells with 1 μg/ml mouse anti-myc antibody or isotype-matched control antibody followed by 1 μg/ml FITC-conjugated anti-mouse IgG $(Fab')_2$ (Jackson ImmunoResearch Laboratories, Westgrove, PA, USA) at 4°C. Functional antigen-binding activity of the surface receptors was determined by incubating the cells with 1 μM phOx-FITC or DNS-FITC at 4°C. After removing unbound antibodies or probe by extensive washing, the surface immunofluorescence of viable cells was measured with a FACScaliber flow cytometer (Becton Dickinson, Mountain View, CA, USA) and fluorescence intensities were analyzed with Flowjo V3.2 (Tree Star, Inc., San Carlos, CA, USA).

Relative surface expression of the scFv receptor

B16/DNS cells (5×10^6) were washed three times with cold phosphate-buffered saline (PBS) and reacted with sulfo-NHS-biotin (Pierce, Rockford, IL, USA) (0.5 mg/ml in PBS, pH 8) for 30 min at room temperature. The reaction was stopped by addition of one-tenth volume of 0.1 M glycine. The cells were washed three times with cold PBS and then the cells were lysed in lysis buffer (1% Triton X-100, 50 mM Tris-HCl pH 7.4, 300 mM NaCl, 5 mM EDTA, 0.02% sodium azide, 10 mM iodoacetamide, 1 mM phenylmethylsulphonylfluoride, 2 μg/ml leupeptin). The biotinylated (extracellular) fraction was separated from the unlabeled (intracellular) fraction by incubation of the lysate with 20 μl streptavidin beads (Promega, Madison, WI, USA) for 2 h at 4°C. The fractions were electrophoresed on a reducing SDS-PAGE, transferred to a PVDF membrane and immunoblotted with 1 μg/ml anti-myc Ab and 1 μg/ml goat anti-mouse Ig-HRP (Jackson ImmunoResearch Laboratories). Blots were washed and specific bands were visualized by ECL detection according to the manufacturer's instructions (Pierce). AlphaEaseFC™ software was employed to analyze the density of the bands.

DNS scFv receptor internalization

Five million B16/DNS or B16/phOx cells were incubated in 2 ml PBS containing 1 μM DNS-FITC for 1 h at 4°C. The cells were washed with cold PBS to remove unbound DNS-FITC and then incubated in Dulbecco's modified Eagle's medium containing 10% bovine serum at 37°C in a CO_2 incubator. Samples of 10^5 B16/DNS cells were transferred to an ice bath after 0, 1, 4, 6 and 10 h. All samples were then washed with cold PBS and the surface fluorescence of 10 000 cells was measured with a FACSCalibur flow cytometer. Some of the cells were also observed under a fluorescence microscope to localize cellular fluorescence.

Transcriptional regulation of membrane antibody expression

HeLa Tet-on cells (Clontech, Mountain View, CA, USA) were transiently transfected with pRevTRE (Clontech) containing the DNS scFv reporter gene. After 1 day, the cells were incubated with graded concentrations of doxycycline for 24 h. The expression of the DNS scFv

on the cells was measured by ELISA using 1 μg/ml anti-myc Ab. Antibody binding to the myc epitope was detected by serial addition of horse-radish peroxidase-conjugated goat anti-mouse antibody and ABTS substrate (0.4 mg/ml 2,2′-azino-di(3-ethylbenzthiazoline-6-sulfonic acid), 0.003% H_2O_2, 100 mM phosphate-citrate, pH 4) for 30 min at room temperature. The absorbance (405 nm) of the wells was measured in a microplate reader (Molecular Device, Menlo Park, CA, USA). To measure probe binding to the cells, $(DNS)_2$-DTPA-^{111}In (10 μCi/ml) was added to cells for 1 h. The wells were washed to remove unbound probe, the cells were collected by treatment with trypsin and the radioactivity of the cells was measured in a gamma-counter.

In vivo *expression of scFv chimeric protein*

C57BL/6 mice were subcutaneously injected with 2×10^6 B16/DNS or B16/phOx cells. After 14 days, resected tumors (200–300 mm^3) were cut into small fragments and digested with 0.5 mg/ml collagenase in Hank's Balanced Saline Solution containing Ca^{2+} and Mg^{2+} for 1 h at room temperature. The cells were cultured in complete medium for 24 h before the surface expression of scFv receptors was measured by flow cytometry as described above.

Reporter gene immune response

Groups of three C57BL/6 mice, anesthetized by pentobarbital (65 mg/kg), were i.v. injected with 10 μg pLNCX (vector control), pLNCX-DNS-eB7 or pLNCX-LacZ-eB7, which codes for a membrane form of *Escherichia coli* β-galactosidase, generated by replacement of the gene for the DNS scFv cDNA in pLNCX-DNS-eB7 with the *LacZ* gene. The vectors were injected in 2 ml PBS in less than 8 s to promote hydrodynamic-based gene transfer.[39,40] Serum samples were collected 10 days later. Preimmune and immune serum samples (diluted 1:200 in PBS) were added to the wells of microtiter plates coated with 293 cells transiently transfected with pLNCX, pLNCX-DNS-eB7 or pLNCX-LacZ-eB7. Binding of the serum antibodies to the cells was detected by ELISA as described above.

Synthesis of $(DNS)_2$-DTPA-^{111}In

A 200 mg portion of dansylcadaverine in DMF was reacted overnight at room temperature with 120 mg DTPA dianhydride (2:1 molar ratio). The mixture was then chromatographed on silica gel with a mobile phase of MeOH/CH_2Cl_2 (2:8 vol/vol) to obtain $(DNS)_2$-DTPA as a white power. ^{1}H NMR (400 mHz, DMSO-d_6) δ 1.09–1.27 (m, 12H, CH_2); 2.80 (s, 12H, N-CH_3); 2.97–4.34 (m, 26H, CH_2); 7.26 (d, $J = 7.6$ Hz, 2H, ArH); 7.55–7.62 (m, 4H, ArH); 7.90–7.95 (m, 2H, NH); 8.07 (d, $J = 7.2$ Hz, 2H, ArH); 8.28 (d, $J = 8.6$ Hz, 2H, ArH); 8.43 (d, $J = 8.6$ Hz, 2H, ArH) 9.8–10.0 (br s, COOH). $(DNS)_2$-DTPA and ^{111}InCl solution were mixed at a 2:1 molar ratio for 30 min at 70°C. The radiochemical purity of $(DNS)_2$-DTPA-^{111}In was 98.67% as measured by radio-TLC and the specificity activity was 2.34×10^4 Ci/mmole.

Specificity and stability of $(DNS)_2$-DTPA-^{111}In

$(DNS)_2$-DTPA-^{111}In was incubated in 20% mouse serum at 37°C for 0 or 24 h. Graded amounts of the $(DNS)_2$-DTPA-^{111}In were then added to 96-well microtiter plates coated with B16/DNS or B16/phOx cells for 1 h. The wells were washed to remove unbound probe and the cells were collected by treatment with trypsin. The radioactivity of the cells was then measured in a gamma-counter.

Serum half-live of $(DNS)_2$-DTPA-^{111}In

C57BL/6 mice were i.v. injected with 20 μCi $(DNS)_2$-DTPA-^{111}In and blood samples were periodically removed from the tail vein of the mice. The blood was weighed on an analytical balance and assayed for radioactivity in a multichannel gamma-counter. The initial and terminal half-lives of the probe were estimated by fitting the data to a two-phase exponential decay model with Prism 4 software (Graphpad Software, San Diego, CA, USA).

In vivo *imaging and biodistribution*

C57BL/6 mice bearing established B16/DNS and B16/phOx tumors (200 mm^3) in their right and left shoulder regions, respectively, were i.v. injected with 100 μCi $(DNS)_2$-DTPA-^{111}In. The whole-body scintigraphy of pentobarbital-anesthetized mice was performed at 1, 4, 24 and 48 h with a gamma camera (Siemens ECAM+DHC) using a pinhole collimator. The mouse contour was first imaged using a ^{57}Co flood disk source for 2 min. The contour images and the mice images were then superimposed for better localization of the tumors. Mice bearing established B16/DNS and B16/phOx tumors (200 mm^3) on the right and left shoulders, respectively, were i.v. injected with 60 μCi $(DNS)_2$-DTPA-^{111}In. These mice were killed after 4, 24 and 48 h and the radioactivity in isolated tissues was measured in a multichannel gamma-counter. The biodistribution of the probe was expressed as percent injected dose per gram tissue.

Statistical significance

Statistical significance of differences between mean values was estimated with Excel (Microsoft, Redmond, WA, USA) using the independent *t*-test for unequal variances. *P*-values of less than 0.05 were considered to be statistically significant.

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References

1 Hoffman RM. *In vivo* imaging with fluorescent proteins: the new cell biology. *Acta Histochem* 2004; **106**: 77–87.
2 Misteli T, Spector DL. Applications of the green fluorescent protein in cell biology and biotechnology. *Nat Biotechnol* 1997; **15**: 961–964.
3 Yang M, Baranov E, Jiang P, Sun FX, Li XM, Li L *et al*. Whole-body optical imaging of green fluorescent protein-expressing tumors and metastases. *Proc Natl Acad Sci USA* 2000; **97**: 1206–1211.
4 Jacobs Jr WR, Barletta RG, Udani R, Chan J, Kalkut G, Sosne G *et al*. Rapid assessment of drug susceptibilities of Mycobacter-

ium tuberculosis by means of luciferase reporter phages. *Science* 1993; **260**: 819–822.
5 Leo BM, Li X, Balian G, Anderson DG. *In vivo* bioluminescent imaging of virus-mediated gene transfer and transduced cell transplantation in the intervertebral disc. *Spine* 2004; **29**: 838–844.
6 Ray P, De A, Min JJ, Tsien RY, Gambhir SS. Imaging tri-fusion multimodality reporter gene expression in living subjects. *Cancer Res* 2004; **64**: 1323–1330.
7 de Vries EF, van Dillen IJ, van Waarde A, Willemsen AT, Vaalburg W, Mulder NH *et al*. Evaluation of [18F]FHPG as PET tracer for HSVtk gene expression. *Nucl Med Biol* 2003; **30**: 651–660.
8 Gambhir SS, Barrio JR, Wu L, Iyer M, Namavari M, Satyamurthy N *et al*. Imaging of adenoviral-directed herpes simplex virus type 1 thymidine kinase reporter gene expression in mice with radiolabeled ganciclovir. *J Nucl Med* 1998; **39**: 2003–2011.
9 Tjuvajev JG, Finn R, Watanabe K, Joshi R, Oku T, Kennedy J *et al*. Noninvasive imaging of herpes virus thymidine kinase gene transfer and expression: a potential method for monitoring clinical gene therapy. *Cancer Res* 1996; **56**: 4087–4095.
10 Gambhir SS, Bauer E, Black ME, Liang Q, Kokoris MS, Barrio JR *et al*. A mutant herpes simplex virus type 1 thymidine kinase reporter gene shows improved sensitivity for imaging reporter gene expression with positron emission tomography. *Proc Natl Acad Sci USA* 2000; **97**: 2785–2790.
11 Richard JC, Zhou Z, Ponde DE, Dence CS, Factor P, Reynolds PN *et al*. Imaging pulmonary gene expression with positron emission tomography. *Am J Respir Crit Care Med* 2003; **167**: 1257–1263.
12 Tjuvajev JG, Avril N, Oku T, Sasajima T, Miyagawa T, Joshi R *et al*. Imaging herpes virus thymidine kinase gene transfer and expression by positron emission tomography. *Cancer Res* 1998; **58**: 4333–4341.
13 Johnson AA, Ray AS, Hanes J, Suo Z, Colacino JM, Anderson KS *et al*. Toxicity of antiviral nucleoside analogs and the human mitochondrial DNA polymerase. *J Biol Chem* 2001; **276**: 40847–40857.
14 Morin KW, Duan W, Xu L, Zhou A, Moharram S, Knaus EE *et al*. Cytotoxicity and cellular uptake of pyrimidine nucleosides for imaging herpes simplex type-1 thymidine kinase (HSV-1 TK) expression in mammalian cells. *Nucl Med Biol* 2004; **31**: 623–630.
15 Bonini C, Ferrari G, Verzeletti S, Servida P, Zappone E, Ruggieri L *et al*. HSV-TK gene transfer into donor lymphocytes for control of allogeneic graft-versus-leukemia. *Science* 1997; **276**: 1719–1724.
16 Russo V, Tanzarella S, Dalerba P, Rigatti D, Rovere P, Villa A *et al*. Dendritic cells acquire the MAGE-3 human tumor antigen from apoptotic cells and induce a class I-restricted T cell response. *Proc Natl Acad Sci USA* 2000; **97**: 2185–2190.
17 Haack K, Linnebacher M, Eisold S, Zoller M, von Knebel Doeberitz M, Gebert J. Induction of protective immunity against syngeneic rat cancer cells by expression of the cytosine deaminase suicide gene. *Cancer Gene Ther* 2000; **7**: 1357–1364.
18 Haberkorn U, Oberdorfer F, Gebert J, Morr I, Haack K, Weber K *et al*. Monitoring gene therapy with cytosine deaminase: *in vitro* studies using tritiated-5-fluorocytosine. *J Nucl Med* 1996; **37**: 87–94.
19 Barrio JR, Satyamurthy N, Huang SC, Keen RE, Nissenson CH, Hoffman JM *et al*. 3-(2′-[^{18}F]fluoroethyl)spiperone: *in vivo* biochemical and kinetic characterization in rodents, nonhuman primates, and humans. *J Cereb Blood Flow Metab* 1989; **9**: 830–839.
20 MacLaren DC, Gambhir SS, Satyamurthy N, Barrio JR, Sharfstein S, Toyokuni T *et al*. Repetitive, non-invasive imaging of the dopamine D2 receptor as a reporter gene in living animals. *Gene Therapy* 1999; **6**: 785–791.
21 Bunzow JR, Van Tol HH, Grandy DK, Albert P, Salon J, Christie M *et al*. Cloning and expression of a rat D2 dopamine receptor cDNA. *Nature* 1988; **336**: 783–787.
22 Wolf G. Vitamin A functions in the regulation of the dopaminergic system in the brain and pituitary gland. *Nutr Rev* 1998; **56**: 354–355.
23 Beom S, Cheong D, Torres G, Caron MG, Kim KM. Comparative studies of molecular mechanisms of dopamine D2 and D3 receptors for the activation of extracellular signal regulated kinase. *J Biol Chem* 2004; **279**: 28304–28314.
24 Strange PG. Aspects of the structure of the D2 dopamine receptor. *Trends Neurosci* 1990; **13**: 373–378.
25 Weissleder R, Moore A, Mahmood U, Bhorade R, Benveniste H, Chiocca EA *et al*. *In vivo* magnetic resonance imaging of transgene expression. *Nat Med* 2000; **6**: 351–355.
26 Liao KW, Chen BM, Liu TB, Tzou SC, Lin YM, Lin KF *et al*. Stable expression of chimeric anti-CD3 receptors on mammalian cells for stimulation of antitumor immunity. *Cancer Gene Ther* 2003; **10**: 779–790.
27 Cheng TL, Liao KW, Tzou SC, Cheng CM, Chen BM, Roffler SR. Hapten-directed targeting to single-chain antibody receptors. *Cancer Gene Ther* 2004; **11**: 380–388.
28 Liao KW, Lo YC, Roffler SR. Activation of lymphocytes by anti-CD3 single-chain antibody dimers expressed on the plasma membrane of tumor cells. *Gene Therapy* 2000; **7**: 339–347.
29 Chen IY, Wu JC, Min JJ, Sundaresan G, Lewis X, Liang Q *et al*. Micro-positron emission tomography imaging of cardiac gene expression in rats using bicistronic adenoviral vector-mediated gene delivery. *Circulation* 2004; **109**: 1415–1420.
30 Levenson VV, Transue ED, Roninson IB. Internal ribosomal entry site-containing retroviral vectors with green fluorescent protein and drug resistance markers. *Hum Gene Ther* 1998; **9**: 1233–1236.
31 Yu Y, Annala AJ, Barrio JR, Toyokuni T, Satyamurthy N, Namavari M *et al*. Quantification of target gene expression by imaging reporter gene expression in living animals. *Nat Med* 2000; **6**: 933–937.
32 Chou WC, Liao KW, Lo YC, Jiang SY, Yeh MY, Roffler SR *et al*. Expression of chimeric monomer and dimer proteins on the plasma membrane of mammalian cells. *Biotechnol Bioeng* 1999; **65**: 160–169.
33 Liao KW, Chou WC, Lo YC, Roffler SR. Design of transgenes for efficient expression of active chimeric proteins on mammalian cells. *Biotechnol Bioeng* 2001; **73**: 313–323.
34 Martell A, Smith RM. *Critical Stability Constants*, vol. 6. Plenum Press: New York, 1986.
35 Coloma MJ, Morrison SL. Design and production of novel tetravalent bispecific antibodies. *Nat Biotechnol* 1997; **15**: 159–163.
36 Morrison SL, Porter SB, Trinh KR, Wims LA, Denham J, Oi VT. Variable region domain exchange influences the functional properties of IgG. *J Immunol* 1998; **160**: 2802–2808.
37 Northrop JP, Bednarski M, Barbieri SO, Lu AT, Nguyen D, Varadarajan J *et al*. Cell surface expression of single chain antibodies with applications to imaging of gene expression *in vivo*. *Eur J Nucl Med Mol Imaging* 2003; **30**: 1292–1298.
38 Farinas J, Verkman AS. Receptor-mediated targeting of fluorescent probes in living cells. *J Biol Chem* 1999; **274**: 7603–7606.
39 Andrianaivo F, Lecocq M, Wattiaux-De Coninck S, Wattiaux R, Jadot M. Hydrodynamics-based transfection of the liver: entrance into hepatocytes of DNA that causes expression takes place very early after injection. *J Gene Med* 2004; **6**: 877–883.
40 Wang CH, Jawan B, Lee TH, Hung KS, Chou WY, Lu CN *et al*. Single injection of naked plasmid encoding alpha-melanocyte-stimulating hormone protects against thioacetamide-induced acute liver failure in mice. *Biochem Biophys Res Commun* 2004; **322**: 153–161.