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Non-Invasive Imaging of Reporter Genes

Harvey R. Herschman*

Departments of Biological Chemistry and Pharmacology, Director for Basic Research, UCLA Jonsson Comprehensive Cancer Center, David Geffen School of Medicine at UCLA, Los Angeles, California

Abstract Non-invasive, quantitative and repetitive imaging of biological processes in living animals is rapidly changing the way in which many experiments in models of human disease and normal physiological processes are conducted. This review summarizes the newest molecular imaging approaches to analyzing reporter gene expression, with particular emphasis on pre-clinical cancer research. Alternative modes of imaging are summarized, followed by descriptions of the major reporter gene systems now used for radionuclide imaging in vivo of gene expression. Several somatic delivery paradigms for co-ordinate expression of therapeutic and imaging genes are presented, and our own emphasis on the dopamine D2 receptor and Herpes Simplex Virus Type I thymidine kinase reporter genes are detailed. J. Cell. Biochem. Suppl. 39: 36–44, 2002. © 2002 Wiley-Liss, Inc.

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The use of molecular imaging technologies in basic biological research and pre-clinical translational studies has evolved in a substantially different fashion from many technologies shared by basic researchers and clinical practitioners. It is traditional for new technologies, new pharmacologic agents, and other medical advances to first be developed in the laboratory of basic scientists, explored subsequently in animal models by translational researchers, and finally reduced to clinical practice by physician-scientists in clinical trials that lead to advances in conventional medicine. In contrast, the imaging technologies used in medicine—technologies such as ultrasound imaging, magnetic resonance spectroscopy, gamma camera imaging, single photon emission spectroscopy (SPECT), and positron emission tomography (PET) have been much more extensively used in the clinic than in the research laboratory of the biologist studying cellular and molecular processes in laboratory models of disease.

Why have the imaging technologies used so widely in clinical practice been relatively slow to become used in the research laboratory? In my view—indeed, in my own research experience—the reasons have been: (1) a lack of knowledge and awareness of the technologies. Researchers trained in molecular and cell biology often begin to work with animal models to investigate molecular and cellular observations they made initially with cultured cells. This is likely to be the history of many of the readers of this dedicated issue of the Journal of Cellular Biochemistry. For many of us, our previous experience with non-invasive imaging is limited to the MRI we had as our aging bodies have suffered from our commitment to our tennis game or our passion for running, the nuclear medicine scans we or our colleagues have had to investigate cardiac blood flow, or the PET scan a friend or relative may have undergone to determine the nature of a sadly progressing neurodegenerative process or a search for occult metastases following diagnosis of a primary tumor. (2) The lack, until recently, of instrumentation to image small laboratory animals. The mouse has become the research platform for most of us interested

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*Correspondence to: Harvey R. Herschman, 341 Boyer Hall, UCLA, 611 Charles E. Young Drive East, Los Angeles, CA 90095. E-mail: hherschman@mednet.ucla.edu

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either in studying the processes of development or in modeling human disease. Because of the relatively low cost of mouse husbandry, the rapid generation time of mice, the vast store of knowledge of mouse genetics, the availability of a large "bank" of mutants, and—most importantly—our ability to manipulate the mouse genome, adding and subtracting sequences at will at the level of exact alterations in the genome, the mouse has become the premier platform for studies in development and disease. But it is only in the recent past that microCT, microSPECT, microPET, and related instruments that permit non-invasive imaging of mice have been developed and made commercially available. In addition, an enormous advance has been made in the development of optical techniques for non-invasive, in vivo imaging.

This combination—a relative lack of familiarity with non-invasive technologies and the lack of user-friendly imaging technologies for rodent studies—accounts for the lack of application of these technologies to research in murine developmental studies and murine models of human disease. However, as should be clear from the avalanche of research papers, review articles and dedicated compendia such as this one for the Journal of Cellular Biochemistry, molecular imaging research applications are coming on with a vengeance.

USES OF NON-INVASIVE IMAGING IN PRE-CLINICAL CANCER RESEARCH

Although molecular imaging in vivo is now being applied to a wide range of problems, including developmental studies, regulation of transcription, protein–protein interactions, and other questions, its greatest applications at the moment are in murine cancer models in general and cancer gene therapy in particular. The introduction of molecular imaging techniques into small animal based cancer research has taken three roads. First, therapeutic approaches to xenografted and experimentally induced tumors in mice have been monitored for tumor progression, vascularization, and metabolism by small animal adaptations of standard clinical procedures using microPET, microCT, microSPECT, etc. [Bogdanov and Weissleder, 2002]. Second, conventional imaging technologies have been adapted to study the response of xenografted, "marked" tumor cells that can be monitored by non-invasive imaging techniques for responses to chemotherapy, gene therapy, radiation therapy, etc. [Sweeney et al., 1999]. Finally, the development of reporter genes whose expression can be non-invasively imaged in living animals has been exploited to monitor the delivery and efficacy of therapeutic genes [Herschman et al., 2002]. My own laboratory has emphasized the development of non-invasive reporter gene technology to monitor somatic gene transfer; this will be the major topic of this article.

NON-INVASIVE IMAGING OF REPORTER GENE EXPRESSION IN LIVING ANIMALS

The use of reporter genes to monitor gene expression and cell trafficking is likely to be familiar to most Journal of Cellular Biochemistry readers. The ease of measurement of proteins such as chloramphenicol acetyl transferase, beta-galactosidase (β-gal), alkaline phosphatase, green fluorescent protein (GFP), firefly luciferase (ffluc), and *renilla* luciferase (rluc) has resulted in their use for studying subcellular trafficking of proteins and organelles, regulation of gene expression in development and in response to cellular stimuli, signal transduction mechanisms elicited by ligands and a host of other applications to cell and molecular biology.

Optical Imaging of Reporter Gene Expression In Vivo

Until recently, the exposure of most cell and molecular biologists to in vivo imaging at the whole animal level was restricted to optical measurements of proteins with intrinsic florescence. Analysis of xenografts of tumor cells stably expressing GFP has, most likely, been the introduction to reporter gene imaging in living animals for most molecular and cell biologists [Yang et al., 2000, 2002]. The development of sensitive, cooled charge coupled device (CCD) cameras has permitted the use of both ffluc [O'Connell-Rodwell et al., 2002] and rluc [Bhaumik and Gambhir, 2002] for non-invasive imaging of reporter gene expression in living mice. The ease and sensitivity of non-invasive, in vivo imaging of luciferase has resulted in explosive application of this technology in a wide range of experimental contexts. However, quantitation of luciferase imaging in vivo does suffer from several drawbacks; flurophore quenching, depth-dependent photon

attenuation, tissue scattering, and lack of tomographic resolution. Principally because of attenuation, application of luciferase imaging to the clinic is likely to be very limited.

Magnetic Resonance Imaging of Reporter Gene Expression In Vivo

In principle, the use of magnetic resonance to detect reporter genes in vivo should provide extraordinary resolution. However, the mass limitations required to obtain MR signals provides a substantial encumbrance to development of MR based reporter gene imaging technologies. Although a great deal of research is currently underway in this area, using, for example, superparamagnetic derivatives of transferrin to monitor expression of the transferrin receptor as a reporter gene [Allport and Weissleder, 2001], relatively few applications of MR techniques to reporter gene imaging have been described to date.

Radionuclide-Based Imaging of Reporter Gene Expression In Vivo

The development of imaging technologies such as microSPECT, microPET, and gamma camera imaging to detect radionuclides in small animals has spawned a new generation of reporter genes for use in in vivo imaging. Because these technologies are also used clinically, the reporter genes developed for these applications should be rapidly extrapolated to the clinic. The spectrum of new reporter genes that utilize radionuclide detection mechanisms include ectopically expressed enzymes that convert labeled, freely-diffusable substrates to sequestered products, ectopically expressed receptors that bind labeled ligands, and ectopically expressed transporters that result in accumulation of radiolabeled compounds.

REPORTER GENES THAT UTILIZE RADIONUCLIDE LABELED PROBES FOR IN VIVO MOLECULAR IMAGING

Dopamine D2 Receptor (D2R) Gene as a Reporter Gene

The D2R gene is expressed, in large quantities, primarily in the striatum. Radionuclide-labeled probes such as 3-(2-[^{18}F]fluoroethyl)spiperone (FESP) [Satyamurthy et al., 1990], [^{11}C]raclopride [Hume et al., 1992], and [^{123}] iodobenzamide derivatives [Kessler et al., 1991] for clinical imaging of striatal D2R were developed to monitor the dopaminergic system in a variety of neurodegenerative diseases. We reasoned that, if we could express the D2R ectopically as a reporter gene, we could subsequently image its expression by microPET, using the positron-emitting FESP molecule as a probe. We constructed stable tumor cell lines expressing the D2R from a cytomegalovirus (CMV) early promoter and, using tumor xenografts, demonstrated that D2R gene expression could be imaged in the microPET scanner, following systemic FESP injection [MacLaren et al., 1999].

Although we use tracer levels of ligand to detect the D2R by microPET, ectopic expression of the D2R gene might result in altered biochemistry in response to endogenous ligands. However, mutations in the D2R protein that uncouple ligand binding from activation of the G-protein coupled response system have been described [Neve et al., 1991; Cox et al., 1992]. We created adenoviral vectors expressing the wild-type D2R reporter gene and a mutant D2R in which binding is uncoupled from signaling, and demonstrated (1) that the D2R80A mutant is, indeed, unable to modulate intracellular cyclic AMP levels in response to dopamine and (2) that the D2R and D2R80A reporters are equivalent in sensitivity as reporter genes [Liang et al., 2001].

Somatostatin Receptor (SSTr) Gene as a Reporter Gene

Expression of the type 2 somatostatin receptor (SSTr2) occurs primarily in the pituitary gland. The SSTr2 receptor binds several naturally occurring peptides and a number of synthetic somatostatin analogues, including octretide, P829 and P2045. Several of these molecules have been labeled with radionuclides such as ^{111}In and ^{99m}Tc for clinically approved use in gamma camera imaging of endogenous SSTr2 expression in tumors. In addition, positron-emitting radionuclides such as [^{64}Cu] and [^{68}Ga] have been used to label probes for the SSTr2 [Zinn and Chaudhuri, 2002]. Like the D2R, the SSTr2 gene has been ectopically expressed in both stably transfected tumor cell xenografts and conditionally replicating adenoviruses, and its expression imaged following systemic administration of a radioactively-labeled ligand (e.g., [^{111}In]ocreotide, [^{99m}Tc]P829), using conventional gamma camera imaging [Zinn and Chaudhuri, 2002].

Sodium Iodide Symporter (NIS) Gene as a Reporter Gene

The NIS protein is expressed primarily in the thyroid gland, where it promotes transport of iodide into follicular cells. The transported iodide is organified in these cells, and used to iodinate thyroglobulin. The NIS protein facilitates both of several forms of radioactive iodine and [^{99m}Tc]O_4. As described above for the D2R and SSTr2 reporter genes, the NIS coding sequence has been placed into stably transfected tumor cells and into gene delivery vectors such as replication-defective adenoviruses. Even though cells ectopically expressing the NIS gene do not organify transported iodine, sufficient radioactivity can be accumulated as a result of NIS gene expression in tumor xenografts or virally infected tumors to permit imaging of the NIS reporter gene by gamma camera scintography, using ^{123}I or [^{99m}Tc]O_4. Chung [2002] has recently reviewed the use of the NIS gene as an in vivo reporter gene.

Herpes Virus 1 Thymidine Kinase (HSV1-tk) Gene as a Reporter Gene

The HSV1-TK enzyme, like mammalian thymidine kinase, converts thymidine to its phosphorylated derivative, thymidine monophosphate. However, the viral TK enzyme can also convert uracil analogues such as 5-iodo-2′-fluoro-2′deoxy-1-β-D-arabino-furanosyl-uracil (FIAU) and acycloguanosines (e.g., acyclovir, ACV; ganciclovir, GCV; penciclovir, PCV) to their phosphorylated forms. In contrast, these molecules are not nearly as effective as substrates for mammalian TK. The HSV1-TK acycloguanosine products can be converted by cellular enzymes to the di and triphosphate derivatives, which can inhibit DNA polymerase and/or be incorporated into DNA to cause chain termination. These properties are responsible for the anti-viral effects of the acycloguanosine drugs. Radionuclide-labeled HSV1-TK substrates have been prepared and used for in vivo imaging of the HSV1-tk reporter gene using gamma camera imaging, SPECT and PET. When these radiolabeled substrates are phosphorylated they are trapped within cells; the trapped products can subsequently be detected using the appropriate imaging technologies. Several articles have recently compared the efficacy of ^{124}I labeled uracil derivatives and ^{18}F labeled acycloguanosine derivatives [Brust et al., 2001; Tjuvajev et al., 2002] as probes for HSV1-TK expression. Currently, the HSV1-tk gene has been the most extensively utilized for in vivo reporter gene imaging employing radionuclide detection; a number of reviews describing this system in more detail are available [e.g., Gambhir et al., 2000; Ray et al., 2001; Herschman et al., 2002].

Site-directed mutation of the HSV1-tk gene has been performed to increase the efficacy of HSV1-TK as a suicide gene for gene therapy with the acycloguanosine prodrugs [Black et al., 1996]. The most effective of these mutants, HSV1-sr39tk, has been used in conjunction with ^{18}F-acycloguanosines to increase the sensitivity of in vivo imaging assays using these fluorinated probes [Herschman et al., 2002]. Both reporter proteins and reporter probes will continue to be the subject of experimental enhancement for all of the radionuclide-based in vivo reporter gene imaging systems.

INDIRECT MONITORING OF THERAPEUTIC GENE DELIVERY BY MEASUREMENT OF REPORTER GENE EXPRESSION

One of the major problems in advancing application of gene therapy in general and cancer gene therapy in particular has been the inability to monitor (1) where therapeutic genes are being expressed, (2) to what level the therapeutic gene is expressed following gene transfer, and (3) how long expression continues. Our laboratory, like many others, is interested in developing imaging paradigms that allow investigators to monitor gene delivery and expression. In some cases, therapeutic genes can be imaged directly; for example, the HSV1-TK enzyme is used to convert the acycloguanosine prodrugs to toxic compounds, but can also be imaged with the radionuclide agents described above. Similarly, both SSTR2 and NIS can be used both as imaging genes and as therapeutic genes. However, for the great majority of therapeutic genes, no specific imaging probes are available. While, in principle, it should be possible to develop radiolabeled imaging probes that bind to any protein, this is not usually a practical solution.

We have been developing gene delivery paradigms in which the correlated expression of a reporter gene in vivo permits inferential measurement of the expression of a second gene. Our goal has been to develop procedures that

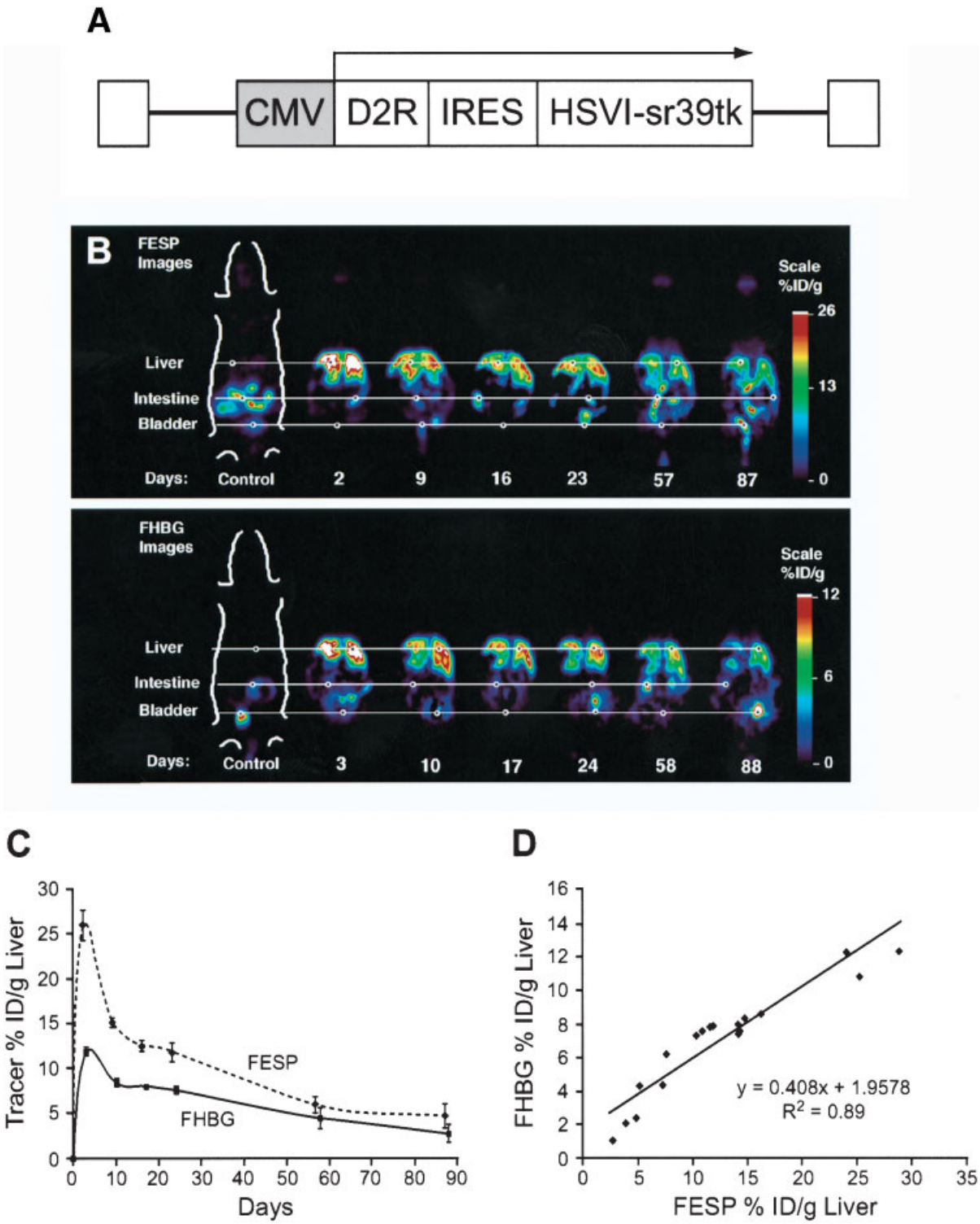
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Fig. 1

permit the non-invasive, quantitative and repeated measurement of the location, magnitude and duration of reporter gene expression, to reflect the expression of a coordinately expressed (therapeutic) gene. Because we have developed two reporter genes, D2R and HSV1-tk, that can be imaged by microPET, we have used microPET imaging to demonstrate co-ordinate, quantitative gene expression in vivo. Our proof-of-principle experiments use replication-defective adenoviruses and/or stably transfected, xenografted tumor cells to demonstrate co-ordinate gene expression in vivo.

Co-Administered Vectors to Monitor Therapeutic Gene Delivery In Vivo

In this application, two gene delivery vectors that are identical, except for the presence of the reporter gene in one vector and the therapeutic gene in the other, are administered. Although at a cellular level the two viruses may infect distinct target cells, at the macroscopic level of PET analysis we expect that exclusion between the two delivery systems would not occur.

We constructed two adenoviral vectors, one in which the CMV promoter drives the HSV1-sr39tk PET reporter gene and the second in which the CMV promoter drives the D2R PET reporter gene. Systemically delivered adenoviruses infect liver cells far more extensively than any other tissue, because of the lack of an endothelial barrier between the blood and hepatic cells (in contrast to nearly all other tissues) and because of the elevated expression of the adenovirus receptor on hepatocytes relative to other cell types. When equivalent titers of Ad.D2R and Ad.HSV1-sr39tk were injected intravenously and the mice were subsequently imaged by microPET, using FESP to monitor D2R expression and FHBG to monitor HSV1-sr39tk expression, the correlation coefficient for hepatic expression of these two reporter genes was $r^2 = 0.76$, over one log of viral titers (from 4×10^7 to 4×10^8 pfu per mouse). Similar correlations between D2R and HSV1-sr39tk gene expression following co-administration of these two viruses were observed by microPET analysis of mice injected both intramuscularly and into xenografted tumors. When expression was serially followed over time in mice injected intravenously with both viruses, co-ordinated hepatic expression was observed, despite the expected decline in expression for both genes in Swiss-Webster mice. The correlation, over a 1 month period, for D2R and HSV1-sr39tk expression was $r^2 = 0.99$ [Yaghoubi et al., 2001]. These data validate perhaps the simplest approach repeatedly and non-invasively infer the expression, in vivo, of a therapeutic gene by measurement of a reporter gene.

Bidirectional Vectors to Monitor Therapeutic Gene Delivery In Vivo

There exist naturally occurring promoters that can drive transcription both "upstream" and "downstream" of these *cis*-acting elements. Baron et al. [1995] created an artificial bidirectional reporter in which polymerized tetracycline response elements separate two minimal CMV promoters. When a tetracycline-dependent transcription factor binds to this construct, gene expression is stimulated in both directions. Sun et al. [2001] adapted this expression system to co-ordinate expression of two PET reporter genes by placing the HSV1-sr39tk and the D2R coding regions proximal and distal to this tet-responsive promoter element, and creating a tumor cell line in which this reporter system and a tetracycline-activated transcription factor are co-expressed. Nude mice carrying

Fig. 1. Dopamine D2 receptor (D2R) positron emission tomography (PET) reporter gene expression and HSV1-sr39TK PET reporter gene expression measured by microPET, following intravenous administration of an adenoviral vector expressing these genes from a bicistronic vector. Three Swiss-Webster mice were injected via their tail-veins with 2×10^9 pfu of Ad.DTm. Each mouse received an i.v. injection of FESP and was then subjected to microPET scanning on days 2, 9, 16, 23, 57, and 87 after viral injection, to monitor D2R reporter gene expression. Each mouse also received an i.v. injection of FHBG and was then subjected to microPET scanning on days 3, 10, 17, 24, 58, and 88 after viral injection, to monitor HSV1-sr39TK PET gene expression. **Panel A**: Structure of Ad.DTm. The shaded area indicates the CMV promoter used to drive transcription of the bicistronic message. IRES, internal ribosomal entry site. **Panel B**: MicroPET scans of the same mouse, following injection of FESP or FHBG. **Panel C**: Retention of FESP and FHBG, determined by microPET analysis, at each time point. Data are means ± the standard errors for each FHBG or FESP measurement of the three mice. **Panel D**: The correlation between D2R PET reporter gene expression, measured as FESP retention, and HSV1-sr39TK PET reporter gene expression, measured as FHBG retention, for the six time points measured (2/3, 9/10, 16/17, 23/24, 57/58, and 87/88 days after virus injection), each point represents a value for an individual mouse.

this transplanted tumor were examined, by microPET analysis following FHBG and FESP administration, for expression of the two reporter genes in the presence and absence of doxycycline. In the absence of inducer, little or no retention of FESP or FHBG could be detected by microPET analysis in the tumors. When inducer was present in the water for 7 days prior to microPET imaging, substantial expression of both reporter genes occurred, as demonstrated by accumulation of both FESP and FHBG in the tumors. Seven days after withdrawal of the inducer from the water, microPET analysis of these same animals demonstrated a substantial decline of both D2R and HSV1-sr39tk gene expression as measured by FESP and FHBG accumulation. This experiment was performed with four mice. When the expression of the D2R and HSV1-sr39tk reporter genes was compared for the four mice prior to induction, after 7 days of induction, and 7 days after removal of the inducer, the correlation coefficient for expression of the two reporter genes was $r^2 = 0.98$.

Bicistronic Vectors to Monitor Therapeutic Gene Delivery In Vivo

A number of DNA viruses, including polio virus and encephalomyocarditis virus, encode polycistronic messages in which several proteins are translated from a single transcript. Proteins that are translated from internal sites on these polycistronic messages are initiated by a cap-independent mechanism in which ribosomes are bound at an internal ribosomal entry site, or internal ribosomal entry site (IRES) [Martinez-Salas, 1999]. Molecular and cell biological studies in which reporter genes are used to monitor the co-expression of a gene of interest have most frequently used constructs in which the reporter gene is expressed distal to an IRES. Several laboratories, in addition to our own, have utilized bi-cistronic vectors in molecular imaging experiments [e.g., Tjuvajev et al., 1999; Zinn et al., 2002].

We created a plasmid vector in which the D2R coding region is proximal to an encephalomyocarditis virus IRES and the HSV1-sr39tk gene is distal to the IRES. Transcription of the bicistronic message is driven by a CMV promoter. Stable tumor cell lines expressing varying levels of this bicistronic message were isolated following transfection. When a series of tumors expressing varying levels of this construct were analyzed by microPET for D2R-dependent FESP accumulation and HSV1-sr39tk-dependent FHBG accumulation, a linear correlation ($r^2 = 0.99$) was observed [Yu et al., 2000].

More recently, we created a replication-deficient adenovirus, Ad.DTm, in which the CMV promoter drives this same bicistronic vector expressing D2R and HSV1-sr39TK (Fig. 1A). Three mice were injected with this vector, then imaged sequentially over a 3 months period for D2R-dependent hepatic FESP retention and HSV1-sr39tk-dependent FHBG retention. Each mouse was imaged 12 times, six imaging sessions with FHBG as the probe, and six imaging sessions with FESP as the probe. Images acquired from one of these mice are shown in Figure 1B. All the mice survived the entire process. When region of interest values for FESP and FHBG retention are analyzed, excellent reproducibility at each time point for both probes is evident (Fig. 1C). The correlation coefficient for the co-ordinated expression of the D2R and HSV1-sr39tk genes is $r^2 = 0.89$ (Fig. 1D), despite substantial extinction of the expression of this bicistronic message over the course of the experiment [Liang et al., 2002]. These results demonstrate, using only non-invasive, in vivo imaging techniques, that the location, magnitude, and duration of expression of a gene of interest, for example, a therapeutic gene, can be accurately inferred, following somatic gene transfer, by non-invasive, quantitative, and repeated measurement of a reporter gene expressed in a bicistronic message.

WHAT DOES THE FUTURE HOLD?

This is a very opportune time for this set of reviews and Prospects articles discussing molecular imaging. Clearly, non-invasive molecular imaging is a very rapidly growing technology, with extensive research activity and great promise for translation to clinical applications. However, at the time the editors requested submission of these articles, only one clinical application of in vivo reporter gene analysis in cancer biology has appeared [Jacobs et al., 2001]. The use of luciferase vectors and optical imaging should increase the pace of progress in pre-clinical studies. The use of radionuclide-based, non-invasive imaging systems, and small animal imaging devices should speed the translation of technological advances in vector design, administration, redirection, etc to the clinic. We are poised on the precipice.

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