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Radionuclide-based reporter gene imaging: pre-clinical and clinical implementation and application

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Inna Serganova[1], Vladimir Ponomarev[2, 3], Ronald G. Blasberg[1–3]

[1]Department of Neurology, Memorial Hospital, Memorial Sloan-Kettering Cancer Center, New York, New York
[2]Department of Radiology, Memorial Hospital, Memorial Sloan-Kettering Cancer Center, New York, New York
[3]Molecular Pharmacology and Chemistry Program, Sloan-Kettering Institute, Memorial Sloan-Kettering Cancer Center, New York, New York

Abstract

Noninvasive reporter gene imaging using radiolabeled probes was first described in 1995, and requires "pre-targeting" (delivery) of the reporter construct to the target tissue (by transfection/transduction). In each case, the reporter system involves a "complimentary pair"; a reporter gene construct and a complimentary radiolabeled probe or substrate. The most widely used reporter gene, HSV1-tk (and a variety of mutants, such as HSV1-tksr39), utilizes enzymatic amplification through phosphorylation and trapping of a specific radiolabeled probe (such as [124I]FIAU or [18F]FHBG), similar to imaging hexokinase activity with [18F]FDG. Recent attention as turned to "human" reporter genes to avoid a potential immunological response to a foreign protein; seven human reporter genes are briefly discussed.
Reporter gene imaging can provide non-invasive assessments of endogenous biological processes in living subjects. At least two different reporter constructs will be required in most future applications of reporter gene imaging. One will be a "constitutive" reporter that will be used to identify the site, extent and duration of vector delivery and tissue transduction or for identifying the cell distribution/trafficking, homing/targeting and persistence (the "normalizing" or denominator term). The second one will be an "inducible" reporter that is sensitive to endogenous transcription factors, signaling pathways or protein-protein interactions that monitor the biological activity and function of the transduced cells (the "sensor" or numerator term).
The initial applications of reporter gene imaging in patients will be developed within two different clinical disciplines: 1. gene therapy and 2. adoptive cell-based therapies. These studies will benefit from the availability of efficient human reporter systems that can provide critical monitoring information for adeno-, retro- and lenteviral-based gene therapy, oncolytic bacterial and viral therapy, and adoptive cell-based therapies. The translational applications of noninvasive in vivo reporter gene imaging are likely to include: 1. quantitative monitoring of the gene therapy vector and transduction efficacy in clinical protocols by imaging the location, extent and duration of transgene expression; 2. monitoring cell trafficking, targeting, replication and activation in adoptive T cell and stem/progenitor cell therapies; 3. assessments of endogenous molecular events using different inducible reporter gene imaging systems.
Key words: PET, gene imaging

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Correspondence to: Dr. Ronald G. Blasberg, MD
Departments of Neurology and Radiology,
MH (Box 52) Molecular Pharmacology & Chemistry Program, SKI
Memorial Sloan Kettering Cancer Center (MSKCC)
415 East 68th Street, New York, NY 10065
Tel: 646 888–2211
Fax: (646) 422-0408
E-mail: blasberg@neuro1.mskcc.org

Background

Reporter gene imaging is one component of "molecular imaging"; a term that is currently fashionable and describes the visualization of normal as well as abnormal cellular processes at a molecular-genetic or cellular level of function, in both space and time. Although the term "molecular imaging" was coined in the mid 1990's, it has its roots in molecular biology and cell biology as well as in imaging technology and chemistry. For example, for

many years researchers used reporter genes encoding enzymes, such as bacterial β-galactosidase (lacZ gene) [1] and chloramphenicol acetyltransferase (CAT gene) [2], to study various cellular processes. However, "visualization" of these reporter enzymes required post-mortem tissue sampling and processing for precise quantitative analysis. Advances in cell biology, especially the translation of these advances to clinical applications, stimulated the development of novel non-invasive visualization systems that would provide accurate and sensitive measurements, as well as allow visualization in living cells, live animals and human subjects. Molecular imaging is now being applied in several disciplines, including oncology, neuroscience, cardiology, gene therapy, cell tracking, and combined diagnosis and therapy (theranostics) [3]. Non-invasive molecular imaging in living animals is the direct result of significant developments in several imaging technologies, including: 1. magnetic resonance (MR) imaging [4, 5]; 2. radionuclide imaging (QAR, gamma camera and PET) [6, 7]; 3. optical imaging of small animals [8–11]. It should be noted that these developments occurred more or less in parallel to each other, and were largely independent of the advances that were occurring in genetics and in molecular and cell biology during the 1980's and early 1990's. However, each of these imaging technologies have also had important antecedents. For example, Polish born Marie Skłodowska-Curie shared a Nobel Prize in Physics in 1903 with her husband Pierre Curie and with the physicist Henri Becquerel for their initial studies and discovery of "radioactivity", a term she coined at the time. Eight years later, in 1911, she received a second Nobel Prize in Chemistry, for the discovery of the elements radium and polonium. Another important antecedent in the development radionuclide-based molecular imaging was the establishment of the radiotracer principle, first described by George de Hevesy. In 1935 he published a letter in Nature on the tracer principle using 32P for the study of phosphorus metabolism [12, 13] and he was awarded the Nobel Prize in Chemistry in 1943. The tracer technique was later adapted for many applications in physiology and biochemistry, as well as in functional diagnosis and use in nuclear medicine and molecular imaging.

The convergence of imaging technology and the disciplines of molecular/cell biology in the mid-1990's is at the heart of this success story, and was the wellspring for further advances in this new field. This development was originally stimulated by NIH funding programs in the US (In vivo and Cellular Molecular Imaging Centers, ICMICs), initiated by Rick Klausner, then director of the National Cancer Institute (NCI). This initiative has now spread globally throughout Europe and Asia, and new Molecular Imaging Societies and new journals have been developed to address this rapidly expanding discipline.

This review will focus on radionuclide-based reporter gene imaging, as developed and applied in pre-clinical and clinical studies. None-the-less, the reader should be aware that molecular imaging involves many different imaging modalities. The multi-modality structure facilitates the investigation of biology and disease in small animal models and provides the opportunity to perform similar studies in human subjects. The incorporation and use of multi-modality imaging allows rapid translation of preclinical studies (using optical reporter systems) to clinical investigations and specific applications (using radionuclide-based reporter systems). For example, optical imaging (fluorescence, bioluminescence, spectroscopy, optical coherence tomography, Raman) has specific advantages including "low-cost" applications in small animals, although fluorescence and Raman imaging in the near- and far-infrared spectrum will likely be selectively translated into clinical applications [14, 15]. Magnetic resonance imaging (spectroscopy, DCE-contrast, diffusion-weighted imaging) has distinct resolution and dynamic imaging advantages and can obtain tomographic information from deep structures in human subjects. Other imaging modalities, such as ultrasound and CT (targeted micro-bubbles, quantum dots and other nano-particles) are actively being explored [16, 17]. However, radionuclide-based imaging (PET, SPECT, gamma camera, quantitative autoradiography) provides a far greater and wider range of opportunities for molecular imaging in human subjects than other modalities [3, 18]. This is particularly the case for reporter gene imaging, as discussed below.

Molecular imaging strategies

Before discussing reporter gene imaging issues, it would be helpful to briefly note three currently used imaging strategies to non-invasively monitor and measure molecular events. They have been broadly defined as "biomarker", "direct" and "indirect" imaging. These strategies have been discussed previously in several reviews [6, 19–27] and in other perspectives on molecular imaging [3, 6, 7, 11, 24, 28–31].

Reporter gene imaging

In this review, we will focus on a subset of "indirect" imaging strategies, involving genetically encoded reporter constructs. Imaging genetically-encoded reporter constructs is more complex than direct or biomarker imaging, but provides the prospect for highly specific and detailed in vivo analysis of transcription factor activity, endogenous bio-molecular and protein-protein interactions, and endogenous cell signaling through specific pathways [32–34]. Reporter gene imaging requires "pre-targeting" (delivery) of the reporter gene to the target tissue by transfection, transduction or by cell delivery. To accomplish this, a genetically-encoded reporter construct is usually introduced into cells by viral vectors. Establishing animal models usually involves the implantation or injection of reporter cells or tumors, or through vectors that target specific tissues (e.g. tumors) or organs, and by constructing transgenic animals. The objective is to visualize and quantify molecular events in vivo. In small animals, optical reporter imaging has become dominant, due to the cost and relative simplicity of optical-based imaging systems, compared to radionuclide-based imaging. In human subjects, a limited number of clinical studies have all involved radionuclide-based reporter imaging.

A common feature of all reporter constructs (and their vectors) is that the cDNA expression cassette, containing the reporter transgene(s) of interest (e.g. HSV1-tk), can be placed under the control of specific promoter-enhancer control elements. The versatility of the reporter constructs (and their vectors) is due in part to their modular design, since arrangements in the expression cassette can be varied to some extent. For example, reporter genes can be constructed to be "always on" by using constitutive promoters (such as LTR, RSV, CMV, PGK,

EF1, etc.). These reporter constructs are used to monitor cell trafficking by identifying the location, migration, targeting and proliferation of stably transduced cells. Reporter gene labeling provides the opportunity for repetitive imaging and sequential monitoring of tumor growth rate and response to treatment [35, 36], as well as imaging metastases [37–39]. Alternatively, the promoter/enhancer elements can be constructed to be "inducible" and "sensitive" to activation and regulation by specific endogenous transcription factors and promoters (factors that bind to and activate specific enhancer elements in the promoter region of the reporter vector construct leading to the initiation of reporter gene transcription). These strategies have been widely applied in both optical [19, 32, 35, 40] and radionuclide-based imaging [38, 41–47] and to a lesser degree in magnetic resonance (MR) imaging [48, 49].

Genetically-encoded optical reporters, such as luciferase or fluorescent proteins, provide cost-effective strategies for pre-clinical small animal studies. The tagged molecules offer a direct linkage to cancer-associated molecules and the ability to observe molecular dynamics in real time in vivo. This approach provides molecule-specific pharmacodynamic information noninvasively in vivo, allowing a time course for response to treatment to determine specific dosage for a desired molecular action [32, 50–52]. As genetically encoded imaging reporters are engineered into mouse lines (e.g. [53–56]) preclinical pharmacodynamic studies will be made more accurate due to direct observation of drug action on specific targets over time. These observations will confirm the mechanism of disease and drug action, validate that the drug is affecting the expected target, and validate the drug's efficacy [57].

Indirect imaging strategies using reporter genes requires "pre-targeting" (delivery) of the reporter construct to the target tissue (by transfection/transduction). This strategy has been widely applied in optical- [8, 19, 35] as well as radionuclide-based imaging [41–45], and to a lesser degree for MR [48, 49] imaging. Early reporter gene imaging approaches required post-mortem tissue sampling and processing [1, 2], but more recent studies have emphasized non-invasive imaging techniques involving live animals and human subjects [58, 59].

A general paradigm for noninvasive reporter gene imaging using radiolabeled probes was initially described in 1995 [41] and is diagrammatically shown in Figure 1. In each case (except fluorescence) the reporter system involves a "complimentary pair"; a reporter gene construct and a complimentary probe or substrate. A simplified cartoon of reporter gene is shown in Figure 1A, and a representation of different reporter strategies (including enzyme-, transporter-, receptor- and antigen-based reporter systems) for imaging transduced cells is shown in Figure 1B. The reporter transgene can encode for a receptor (e.g. hD2R — human dopamine D2 receptor [60], hSSTR2 — human somatostatin receptors [61, 62], or a transporter (e.g. hNIS-human sodium iodide symporter [63–66], hNET-human norepinephrine transporter [67–69], or a fluorescent protein (e.g. enhanced green fluorescent protein, eGFP [37, 70]), in addition to enzymes, such as HSV1-tk [41] (Figure 1Aa,b) or luciferases [40, 71, 72] (Figure 1Aa,c) or cell surface antigen [73, 74] (Figure 1B).

Considerable progress in reporter gene imaging has been achieved. Important proof-of-principle experiments in small animals include the imaging of endogenous regulation of transcription [34, 38, 75–78], post-transcriptional modulation of translation [79], protein-protein interactions [46, 80–83], protein degradation and activity of the proteosomal ubiquination pathway [84], apoptosis [85], etc. Noninvasive imaging of viral [44, 86–88], bacterial [89–91] and cell trafficking [92–95], plus tissue-specific reporter gene imaging in prostate cancer [96], hepatocytes [97] and colorectal cancer cells have also been reported [98].

Radiotracer-based reporter gene imaging. The paradigm for noninvasive reporter gene imaging using radiolabeled probes was initially described in 1995 [41] and is diagrammatically shown in Figure 1. Thus, it will be helpful to consider the herpes simplex virus type 1 thymidine kinase (HSV1-tk) reporter imaging paradigm as an example of an in vivo radiotracer assay that reflects reporter gene expression (Figure 1Aa,b). Enzymatic amplification of the signal (e.g. level of radioactivity accumulation) facilitates imaging the location and magnitude of reporter gene expression. Viewed from this perspective, HSV1-tk reporter gene imaging with radiolabeled FIAU or FHBG is similar to imaging hexokinase activity with FDG. It is important to note that imaging transgene expression is largely independent of the vector used to shuttle the reporter gene into the cells of the target tissue; namely, any of several currently available vectors can be used (e.g., retrovirus, adenovirus, adeno-associated virus, lentivirus, liposomes, etc.).

HSV1-tk is the most widely used reporter gene for radiotracer-based molecular imaging, and has been used as a therapeutic "suicide" gene in clinical anti-cancer gene therapy trials as well as a research tool in gene targeting strategies. The HSV1-TK enzyme, like mammalian TKs, phosphorylates thymidine to thymidine-monophosphate (TdR). Unlike mammalian TK1, viral HSV1-TK can also phosphorylate modified thymidine analogs, including 2'-deoxy-2'-fluoro-5-iodo-1-[beta]--D-arabinofuranosyluracil (FIAU), 2'-fluoro-5-ethyl-1-[beta]--D-arabinofuranosyl-uracil (FEAU) as well as acycloguanosine analogs [e.g. acyclovir (ACV); ganciclovir (GCV); penciclovir (PCV)] that are not (or minimally) phosphorylated by eukaryotic thymidine kinases [41]. The resulting monophosphorylated compound is subsequently diphosphorylated and triphosphorylated by cellular kinases. The triphosphorylated compound can act as an inhibitor of DNA-polymerization resulting in chain termination during DNA replication leading to cell death. For this reason, HSV1-tk has been studied extensively as a therapeutic gene and used in gene therapy clinical trials that have been performed in the United States and Europe [99–101].

In the mid 1990's, a number of potential reporter probes for imaging HSV1-tk gene expression were studied in our laboratory [41–43]. After in vitro determinations of HSV1-TK sensitivity and selectivity for FIAU, this compound was found to have good imaging potential and can be radiolabeled with a variety of radionuclides (11C, 124I, 18F 131I, 123I). FIAU contains a 2'-fluoro substitution in the sugar that impedes cleavage of the N-glycosidic bond by nucleoside phosphorylases. This results in a significant prolongation of the nucleoside in plasma and an increase in delivery of non-degraded radiolabeled tracer to the target tissues. The first series of imaging experiments involving HSV1-tk transduced tissue and FIAU were performed in rats bearing intracerebral (i.c.) RG2 tumors using quantitative autoradiography (QAR)

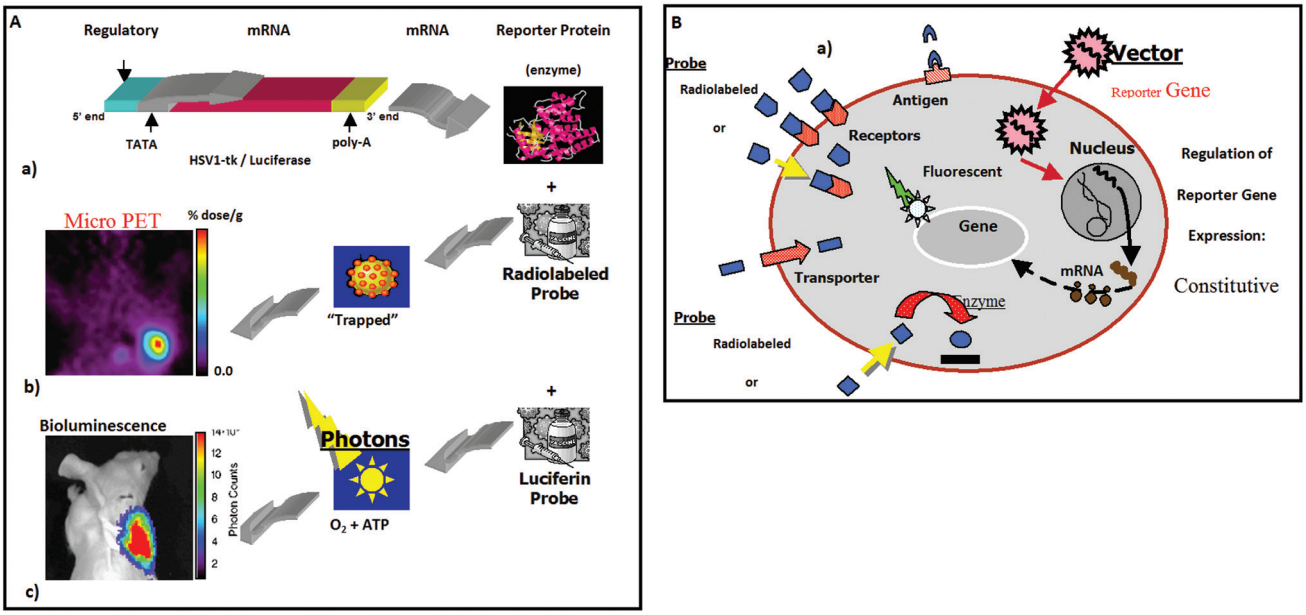

Figure 1. HSV1-tk reporter construct and the indirect reporter imaging paradigm (**A**). The basic structure of a reporter gene complex is shown, in this case herpes simplex virus type 1 thymidine kinase (HSV1-tk). The control and regulation of gene expression is performed through promoter and enhancer regions that are located at the 5' end ("up-stream") of the reporter gene. These promoter/enhancer elements can be "constitutive" and result in continuous gene expression ("always on"), or they can be "inducible" and sensitive to activation by endogenous transcription factors and promoters. Following the initiation of transcription and translation, the gene product — a protein — accumulates. In this case the reporter gene product is an enzyme (HSV1-TK). HSV1-TK will phosphorylate selected thymidine analogs (e.g. FIAU or FHBG), whereas these probes are not phosphorylated by endogenous mammalian TK1. The phosphorylated probe does not cross the cell membrane readily; it is effectively "trapped" and accumulates within transduced cells. Thus, the magnitude of probe accumulation in the cell (level of radioactivity) reflects the level of HSV1-TK enzyme activity and level of HSV1-tk gene expression.

Different reporter systems (**B**). The reporter gene complex is transfected into target cells by a vector (e.g. a virus). Inside the transfected cell, the reporter gene may or may not be integrated into the host-cell genome; transcription of the reporter gene to mRNA is initiated by "constitutive" or "inducible" promoters, and translation of the mRNA to a protein occurs on the ribosomes. The reporter gene product can be a cytoplasmic or nuclear enzyme, a transporter in the cell membrane, a receptor at the cell surface or part of cytoplasmic or nuclear complex, an artificial cell surface antigen, or a fluorescent protein. Often, a complimentary reporter probe (e.g. a radiolabeled, magnetic or bioluminescent molecule) is given and the probe concentrates (or emits light) at the site of reporter gene expression. The level of probe concentration (or intensity of light) is usually proportional to the level reporter gene product and can reflect several processes, including the level of transcription, the modulation and regulation of translation, protein-protein interactions, and post-translational regulation of protein conformation and degradation.

techniques [102] (Figure 2A). This was subsequently followed by gamma camera, single photon emission computed tomography (SPECT) (Figure 2B) and positron emission tomography (PET) imaging studies (Figure 2C). Other pyrimidine nucleoside probes for imaging viral thymidine kinase activity have been proposed (Figure 3A).

Investigators from UCLA have used other radiolabeled compounds for PET imaging of HSV1-tk expression with goal to develop methods for repetitive imaging (every 6 to 8 hours) of the reporter protein. Their choice of acycloguanosine derivatives as reporter probes was based on the ability of these nucleosides to be radiolabeled with short-lived Fluorine-18 (t 1/2 = 110 min) and little or no affinity to the mammalian TK-1. A list of 18F-labeled acycloguanosine analogues is shown in Figure 3B. After several years of comparative studies [45, 103, 104], a new radiolabeled acycloguanine, 9-(4-[18F]fluoro-3-hydroxymethylbutyl)guanine or [18F]FHBG (FHBG) [105, 106] was developed at USC. In parallel, the UCLA investigators evaluated a mutant HSV1-TK enzyme (HSV1-sr39TK) with increased acyclovir and ganciclovir suicidal efficacy. They showed higher affinity and uptake of [18F]FHBG in HSV1-sr39tk transduced cells [107]. The mutant, HSV1-sr39TK, enhances [18F]FHBG uptake by two-fold compared to wild-type HSV1-TK, thus improving the imaging capabilities of the enzyme. However, differences exist between the sensitivity and specificity of [124I]FIAU and [18F]FHBG with respect to wild-type HSV1-tk [108] and HSV1-sr39TK [109]. Several additional mutant forms of HSV1-sr39tk were developed and described [110, 111]. HSV1-A167Ysr39tk mutant lost its ability to phosphorylate pyrimidine-base nucleoside derivatives, but retained activity to phosphorylate acycloguanosine analogs, including [18F]-FHBG. The HSV1-A167Ysr39tk - [18F]-FHBG pair is useful for PET-imaging and GCV is effective for "suicide" control for safety concerns. Additionally, another HSV1-R176Qtk mutant with an enhanced activity to pyrimidine-based radiotracers was developed and applied [110]. These two mutants can be used in combination for PET imaging of two independent reporters or cell populations or molecular biological processes in the same living subject, using consecutive administration of [18F]-FEAU and [18F]-FHBG.

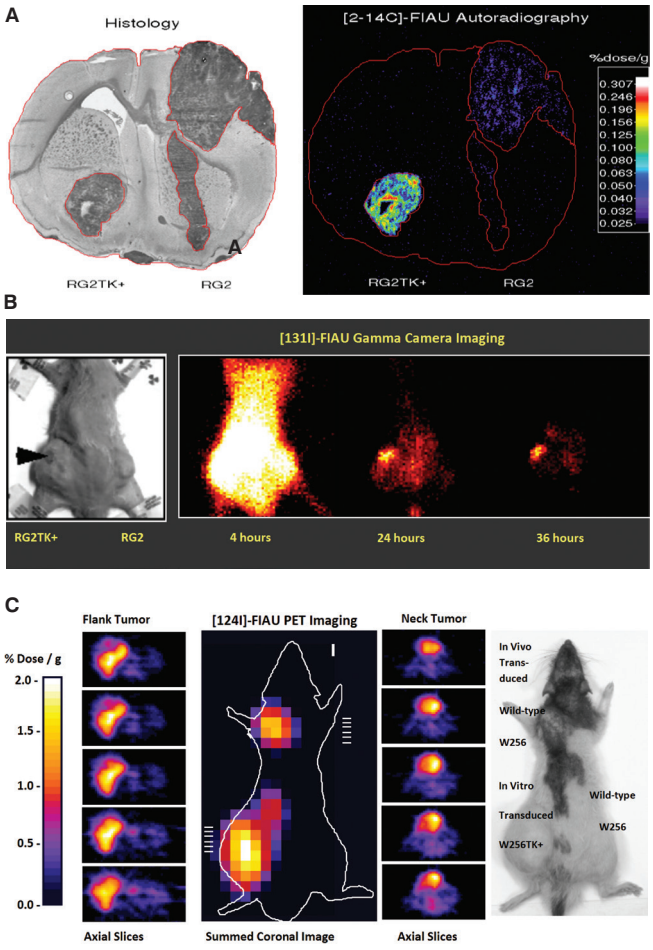

Figure 2. Autoradiographic imaging HSV1-tk expression (**A**). A rat brain with a stably transduced RG2TK+ brain tumor in the left hemisphere and a wild-type (non-transduced) RG2 tumor in the right hemisphere is shown. The histology and autoradiographic images were generated from the same tissue section. Both tumors are clearly seen in the toluidine blue stained histological section. Twenty four hours after i.v. administration of [14C]FIAU, the RG2TK+ tumor is clearly visualized in the autoradiographic image, whereas the RG2 tumor is barely detectable; the surrounding brain is at background levels. Figure adapted from Tjuvajev et al. 1995 [41].

Gamma camera imaging HSV1-tk expression (**B**). Gamma camera imaging was performed at 4, 24 and 36 hours after [131I]FIAU injection in an animals bearing bilateral RG2 flank xenografts; all images have been normalized to a reference standard (not shown in the field of view). The site of inoculation of HSV1-tk retroviral vector producer cells (gp-STK-A2) into the left flank xenograft is indicated by the arrow. The sequential images demonstrate washout of radioactivity from the body, with specific retention of activity in the area of gp-STK-A2 cell inoculation and transduction of RG2 tumor cells with the HSV1-tk reporter (see the 24 and 36 hour images; readjustment of the pseudocolor intensity scale demonstrated visualization or the gp-STK-A2 flank tumor at 4 hours although background activity was high). The non-transduced contralateral xenograft (right flank) and other tissues did not show any retention of radioactivity. This sequence of images demonstrates the advantages of a "washout strategy" and late imaging with [131I] — or [124I]-labeled FIAU. Figure adapted from Tjuvajev et al. 1996 [42].

PET imaging HSV1-tk expression (**C**). Three tumors were produced in rnu rats. A W256TK+ (positive control) tumor was produced from stably transduced W256TK+ cells and is located in the left flank, and two wild-type W256 tumors were produced in the dorsum of the neck (test) and in the right flank (negative control). The neck tumor was inoculated with 106 gp-STK-A2 vector-producer cells (retroviral titer: 106–107 cfu/ml) to induce HSV1-tk transduction of the tumor wild-type in vivo. Fourteen days after gp-STK-A2 cell inoculation, no carrier added [124I]FIAU (25 μCi) was injected i.v. and PET imaging was performed 30 hours later. Localization of radioactivity is clearly seen in left flank tumor (positive control) and in the in vivo transduced neck tumor (test), but only low background levels of radioactivity were observed in the right flank tumor wild-type (negative control). Figure adapted from Tjuvajev et al. 1998 [43]

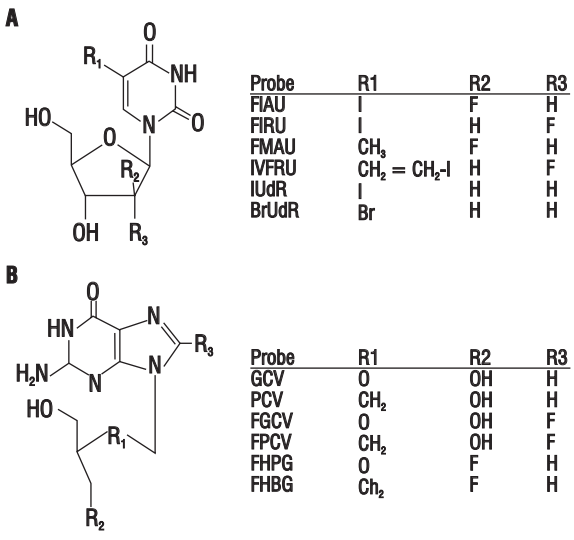

Figure 3. Substrates for HSV-1 thymidine kinase (HSV1-TK) phosphorylation. **A.** Pyrimidine nucleosides. **B.** Acycloguanosine analogues. Figure adapted from Tjuvajev et al. 2002 [108]

Human reporter genes

Despite successful imaging in patients, HSV1-tk is a non-human gene and poses a small risk of generating an immune response against cells and tissue transduced with this gene. One approach to reduce this risk is to use selected human nucleoside kinase genes in reporter constructs that will be administered to patients. Several novel human-derived, enzyme-based reporter genes and reporter probes have been described. They include human mitochondrial thymidine kinase-2 (hmtk2) [112] and several mutants [113], as well as mutant of human deoxycytidine kinase (hdCK) utilizing clinically available pyrimidine-based radiotracers [114].

hmtk2/hΔTK2

A human delta mitochondrial thymidine kinase type 2 (hΔTK2) was developed and can be imaged with pyrimidine-based radiotracers. However, cells expressing hΔTK2 show relatively low levels of pyrimidine radiotracer accumulation compared to HSV1-tk (1/6th to 1/10th) [112]. Further enzyme engineering resulted in the development of a mutant hTK2-N93D/L109F with the ability to phosphorylate [F18]-FMAU [113].

hdCK

A mutant form of human deoxycytidine kinase (hdCK) was explored as a PET reporter gene in combination with clinically-approved pyrimidine-based drugs and radiotracers. hdCK is capable of converting deoxycytidine, deoxyadenosine, deoxyguanosine, and several pyrimidine-based cytotoxic drugs (including cytosine-arabinoside (Ara-C) and 2'-difluoro-cytosine) into their monophosphate forms. dCK along with 18F-2'-deoxy-2'-fluoroarabinofuranosylcytosine (18F-FAC) was developed as a new reporter system, and it was shown that the dCK mutants phosphorylate pyrimidine-based radiotracers at levels comparable with that of wild-type HSV1-tk [114]. The in vitro radiotracer accumulation and in vivo imaging studies demonstrated the advantage of this mutant reporter gene over its native predecessor, due to a thymidine-permissive conformation of this enzyme that allows phosphorylation of FIAU and FEAU.

Five other reporter systems will be described: hNIS (human sodium iodide symporter [63]), hNET (human norepinephrine transporter [67]), D2R (rat dopamine D2 receptor [60]), and hSSTR2 (human somatostatin receptors [61]), hCEA (human carcinoembryonic antigen [115]).

hNIS

hNIS is an intrinsic transmembrane glycoprotein and member of the sodium/solute symporter family [116]. Since cloning of the NIS gene in 1996 [116], NIS was considered an attractive imaging reporter gene [116, 117]. There are several distinct advantages for using NIS as a reporter gene. First, the distribution of endogenous NIS protein is limited in the body (thyroid and stomach are major exceptions); as a result, imaging of exogenous NIS gene expression can be performed in a variety of tissues due to low background activity. Second, NIS mediates the uptake of simple radiopharmaceuticals; therefore, complicated syntheses and labeling of substrate molecules are not required for imaging. Third, most of the radiotracers are specific only to NIS-expressing cells; therefore, background signal is significantly reduced. Fourth, NIS-mediated radiotracer uptake in target tissue is rapid, as is the clearance of radioactivity from both target and non-target tissues; this facilitates repetitive sequential imaging. Fifth, the human and murine genes of NIS have been cloned which provides a non-immunogenic reporter system for human as well as rodent imaging studies.

An application of hNIS as a reporter gene was demonstrated by gamma-camera imaging myocardial gene transfer in living rats using adenoviral vectors and radioiodide [118]. In this study, an adenovirus that expressed both NIS protein and enhanced green fluorescent protein (EGFP) (Ad.EGFP.NIS) was injected into the myocardium of living rats. Following 123I scintigraphy demonstrated clear focal myocardial uptake at the Ad.EGFP.NIS injection site. Histologic analysis confirmed the co-localization of 123I radioactivity, EGFP fluorescence and NIS staining. To develop a molecular imaging method suitable for monitoring viable cancer cells, another dual-imaging reporter gene system was constructed from 2 individual reporter genes, sodium iodide symporter (NIS) and luciferase [119]. In parallel, our group has developed a self-inactivating retroviral vector containing a dual-reporter gene cassette (hNIS-IRES2-GFP) with the hNIS and GFP genes, separated by an internal ribosomal entry site (IRES) element; the expression cassette was driven by a constitutive CMV promoter. A stably transduced rat glioma (RG2) cell line was generated with this construct and used for in vitro and in vivo imaging studies of 131I-iodide and 99mTcO4-pertechnetate accumulation, as well as GFP fluorescence. The experiments demonstrated a high correlation between the expression of hNIS and GFP. Gamma-camera imaging studies performed on RG2-hNIS-IRES2-GFP-tumor bearing mice revealed that the IRES-linked dual reporter gene is functional and stable [66].

In a recent paper we showed that the cell response to proteotoxic cell stresses is mediated primarily through activation of heat shock factor 1 (HSF1) and hNIS reporter gene can be used to image and monitor the activation of the HSF1/HSP70 transcription factor in response to drug treatment (17-allylamino-demethoxygeldanamycin [17-AAG]). Good correspondence between reporter expression and HSP70 protein levels were observed. MicroPET imaging based on [124I] iodide accumulation in pQH-NIG70-transduced RG2 xenografts showed a significant 6.2-fold reporter response to 17-AAG, with a corresponding increase in tumor HSP70 and in tumor human sodium iodide symporter and green fluorescent protein reporter proteins [64]. Other groups have used the hNIS gene under a constitutive CMV promoter to follow the effect of anticancer treatment by scintigraphic imaging of hepatomas [118], and assessed the effect of adriamycin on hNIS expression, placing this reporter gene under a p53 promoter [120].

hNET

hNET is a transmembrane protein and one of several monoamine transporters involved in the transport of norepinephrine, epinephrine and dopamine [121], and functions as a rapid reuptake system located at or near presynaptic terminals. Several radiolabeled probes are used clinically ([123 I], [131 I], [123/4I] MIBG and [11C]ephedrine), and the small size of the hNET reporter gene cassette allows it to be easily incorporated into the delivery vehicle [68]. The successful imaging of hNET expression in neuroendocrine tumors and altered sympathetic enervation of the heart have led to the suggested application of hNET as a human reporter gene [67, 122, 123]. Sequential imaging of animals bearing both hNET reporter-transduced and wild-type xenografts clearly showed the advantage of "late" imaging [68]. The background-corrected radioactivity profile of the hNET-transduced xenograft shows a peak value at 8 h and a slow exponential washout (t1/2 = 63 h), compared to the more rapid washout from the nontransduced xenograft (t1/2 = 12 h) [68].

D2R

hD2R is also a potential reporter gene due to the availability of [18F]fluoroethylspiperone (FESP), a well studied probe for imaging of the dopaminergic system in human subjects with PET [124]. However, D2R has potential problems, it is not a human gene (rat) and the occupancy of the receptor by endogenous natural ligands and adverse biological effects on transduced cells have been raised. To address this concern, the UCLA group developed a signaling-inert mutant variant (D2R80A) as a reporter gene and visualized gene expression with [18F]FESP [60, 124]. They showed that the D2R80A variant has similar in vitro [3H]spiperone binding parameters compared to wild-type D2R, but there

was complete uncoupling of ligand-binding from activation of G-protein-linked signaling. Unfortunately, the non-human origin of the Dopamine D2R80A receptors does not favor their use in clinical reporter gene imaging studies.

hSSTR2

The somatostatin receptors are G-protein-linked transmembrane pass receptors. The expression of one of the six SSTr genes, subtype 2, is largely restricted to the pituitary, although other tissues express low levels of this protein [125]. Although the hSSTr2 gene was originally described as a promising reporter gene in 1999 [125] and again in 2005 [126], image quality remained suboptimal using 111In-DTPA-octreotide, 99mTc-P829, 188Re-P829, 99mTc-P2045, or 94mTc-demotate-1 radioligands. This has now changed significantly with the availability of 68Ga-DOTATOC, 68Ga-DOTATATE and 111In-DOTA-BASS, due to high specific uptake and comparatively rapid body clearance and low background. These peptide-chelate conjugate were originally designed for radiotherapy of SSTr2-expressing tumors [127]. They have undergone extensive clinical testing in both Europe [128] and the United States [129] and are being developed as clinical imaging and targeted radiotherapeutic agents.

Since ligand binding to the hSSTr2 expressed on transduced cells could potentially perturb the biology of these cells through G-protein-coupled receptor signaling (as was shown for the D2R [60, 124]), this issue must be addressed. In vitro, activation of SSTr2 on tumor cells results in cell growth arrest, whereas activation of SSTr5 results in cell proliferation [130]. We observed that transduction with SSTr2 bearing vector had variable effects on xenograft growth rates; these effects varied with cell type used for a transduction, but not with the level of SSTr2 expression.

hCEA

A recombinant reporter gene based on the human carcinoembryonic antigen (CEA) was developed for imaging. It is well documented the hCEA expression increases during the development of cancer, with high percent of human colon cancers being CEA positive [74]. The CEA-based reporter gene can be detected by molecular imaging using a well-established radiolabeled anti-CEA antibody fragment as a reporter probe [115]. In vivo micro-PET imaging using an 124I-labeled anti-CEA scFv-Fc antibody visualized CEA-transfected Jurkat T cell xenografts. This study demonstrated the ability to generate and express an internalizing reporter gene on the surface of T cells, both in vitro and in vivo, supporting the potential use of the internalizing forms of CEA as PET reporter genes.

Reporter limitations

Every reporter gene has its advantages and disadvantages, as discussed above. Although HSV1-tk has a greater potential to elicit an immune reaction than the human reporter systems described above (hSSTr2, hNIS, hNET or hCEA), the expression of a transporter, a receptor or an antigen on the surface of all cells involves a complex process of protein trafficking and membrane integration. An enzyme-based reporter would be expected to produce a higher signal than a receptor-based reporter, due to enzymatic/catalytic trapping of the radiolabeled substrate resulting in signal amplification. On the other hand, intracellular enzyme-based reporters may be limited by transport of the radiolabeled substrate into transduced cells. For transporter-, receptor- and antigen-based reporter systems, the ligand binding domain is usually located on the external plasma membrane [68] and does not require radioligand permeation of the cell membrane to bind to the receptor.

Multi-modality imaging

The coupling of a nuclear reporter gene (e.g. HSV1-tk, hNIS) with optical reporter genes (e.g. luciferases and fluorescent proteins) has been reported [131, 132]. An example is shown in Figure 4. A series of HSV1-tk/eGFP mutants were developed with altered nuclear localization and better cellular enzymatic activity to optimize the sensitivity for imaging HSV1-tk/eGFP reporter gene expression [110, 111, 133]. The HSV1-tk/eGFP reporter gene has been introduced into several different reporter systems to assess different molecular pathways [75, 76]. Furthermore, mutant thymidine kinase (HSV1-sr39tk) — Renilla luciferase (RL) fusion reporter construct (tk20rl) was developed for both nuclear and optical imaging [134]. This study demonstrated the specificity and sensitivity of bioluminescence imaging and showed a good correlation between the nuclear (microPET) and optical (CCD camera) readouts of the dual reporter system.

Several triple-reporter constructs (e.g. HSV1-TK/eGFP/Luc or TGL), have been developed [134, 135]; representative images are shown in Figure 4. A single reporter construct (vector) with a gene product(s) that can be assayed by three different imaging technologies (nuclear, fluorescence and bioluminescence) combines the benefits of each modality. Such systems facilitate the development, validation and testing of new reporter systems in small animals, as well as provide preliminary data that will facilitate the translation of such studies into humans. Using dual or triple modality reporter constructs (PET, fluorescence and bioluminescence) overcomes many of the shortcomings of each modality alone. Although optical imaging does not yet provide optimal quantita-

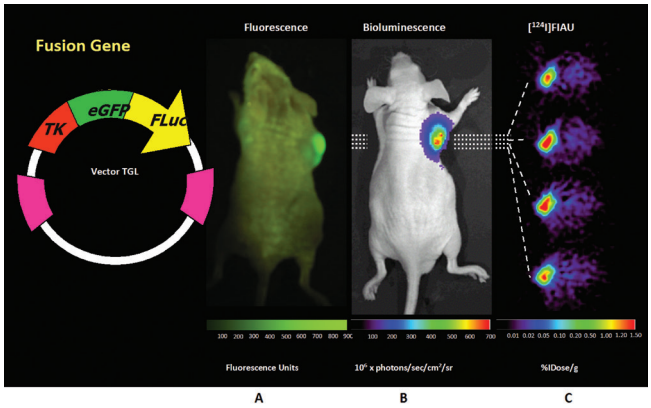

Figure 4. Noninvasive multimodality imaging. Noninvasive, multimodality imaging of mice bearing subcutaneous xenografts produced from nesHSV1-tk/eGFP-Fluc transduced U87 cells (right shoulder) and wild-type (non-transduced) U87 cells (left shoulder). Whole-body fluorescence imaging (**A**), whole-body bioluminescence imaging (**B**), and axial microPET images of [124I]FIAU accumulation obtained at the levels indicated by the dotted white lines (**C**) are shown for the same mouse. Figure adapted from Ponomarev et al. [135]

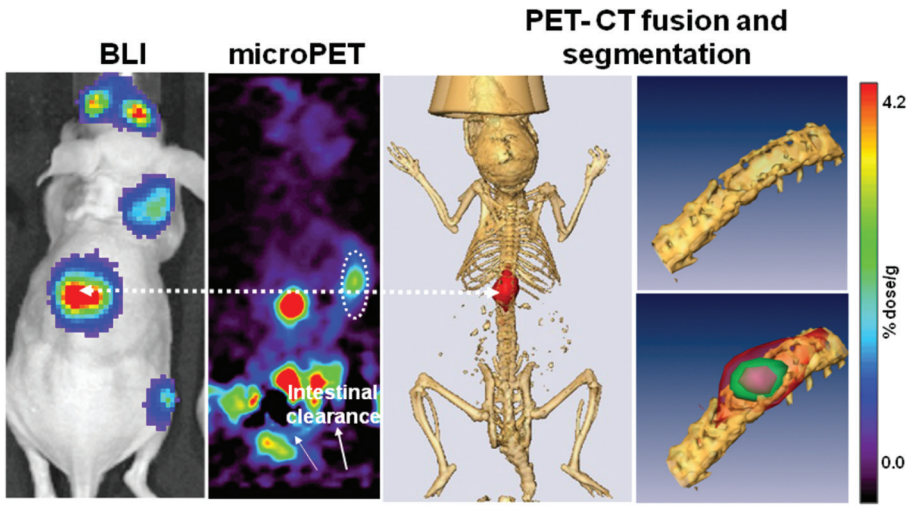

Figure 5. Micro-PET imaging of TGFβ-Response in metastatic lesions. Bioluminescence imaging (BLI) of the Firefly Luciferase reporter (SFG-tdRFP-cmvFLuc) bearing SCP3T metastatic cells at 89 d postinjection. BLI signal of FLuc was acquired for 1 min and identified the location of the reporter-transduced cells. [18F]FEAU microPET images of the same metastatic tumors (transduced with a second reporter, HSV1-TK/GFP) were acquired over 10 min, 2 h after i.v. administration of [18F]FEAU (100 μCi/animal). Note nonspecific accumulation of the radiotracer in the gastrointestinal tract and bladder (normal routes of tracer clearance). MicroCT shows focal bone resorption in the vertebral bodies and the skull. A segmented microPET image of the spine following volumetric coregistration with the corresponding microCT image is shown. A segmented volume-rendered microCT and microPET images demonstrating resorption of multiple vertebral bodies by the tumor metastasis, shows moderate TGFβ signaling activity, most pronounced in areas adjacent to bone. Figure adapted from Serganova et al. 2009 [38]

tive or tomographic information, these issues are not limiting for PET-based reporter systems and PET animal studies are more easily generalized to human applications. Multimodality reporters have been shown to facilitate the development, validation and testing of new reporter systems in small animals [29, 136], as well as provide preliminary data that will facilitate the translation of such studies into humans [137, 138].

Reporter gene imaging applications

Reporter gene imaging can provide non-invasive assessments of endogenous biological processes in living subjects. For example, imaging the transcriptional regulation of endogenous genes in living animals using noninvasive imaging techniques can provide a better understanding of normal and cancer-related biological processes. Our group demonstrated that p53 and HIF-1 (hypoxia inducible factor-1), Smad2/3/4/Runx dependent gene expression can be imaged in vivo with PET and by in situ fluorescence [38, 75, 78, 138] (Figure 5). Retroviral vectors were generated by placing the HSV1-tk/eGFP, a dual-reporter gene or HSV1-TK/eGFP/Luc or TGL, under control of a several repeats of the p53 protein (transcription factor) [75] or the hypoxia response element (HRE, a specific response element for HIF-1) [78, 138] or as in case of Smad2/3/4/Runx2 activity a specific from the mouse germline Igα promoter with Smad and Runx binding motifs.

At least two different reporter constructs will be required in most future applications of reporter gene imaging. One will be a "constitutive" reporter that will be used to identify the site, extent and duration of vector delivery and tissue transduction or for identifying the distribution/trafficking, homing/targeting and persistence of adoptively administered cells (the "normalizing" or denominator term). The second one will be an "inducible" reporter that is sensitive to endogenous transcription factors, signaling pathways or protein-protein interactions that monitor the biological activity and function of the transduced cells (the "sensor" or numerator term).

The initial application of such double-reporter systems in patients will most likely be performed as part of a gene therapy protocol or an adoptive therapy protocol where the patients own cells are harvested (e.g. lymphocytes, T cells or blood-derived progenitor cells), transduced with the reporter systems and expanded ex-vivo, and then adoptively re-administered to the patient. For example, adoptive T cell therapy could provide a venue for imaging T cell trafficking, targeting, activation, proliferation and persistence. These issues could be addressed in a quantitative manner by repetitive PET imaging of the double-reporter system described above in the same subject over time. In this review, we will focus on the application of reporter gene imaging in adoptive therapies.

Imaging adoptive therapies

A non-invasive method for repetitive evaluation of adoptively administered cells will benefit the assessment of current adoptive therapies in clinical use (e.g. bone marrow transplantation, immune cell and blood-derived progenitor cell-based therapies) as well as future adoptive therapies using stem cells. Individual patient monitoring would contribute to patient management by visualizing the trafficking, homing-targeting and persistence of adoptively administered cells, as well as assess their functional activation, proliferation and cytokine expression. Such studies would significantly aid in the clinical implementation and management of new therapeutic approaches based on the adoptive transfer of immune cells, progenitor cells and stem cells.

In this section we will focus on adoptive T-cell monitoring, although the methods for noninvasive monitoring can be readily transferred other systems (e.g. bone marrow stromal cells or en-

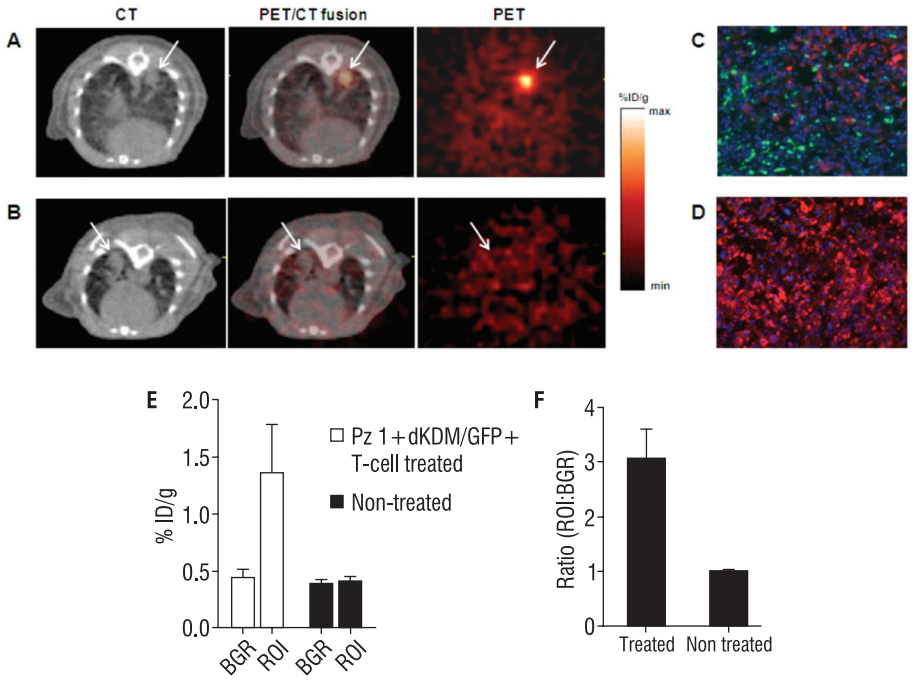

Figure 6. MicroPET/microCT imaging of dCKDM/GFP-expressing PSMA-targeted Pz1 T-cells. Analysis of microPET 18F-FEAU co-localization with tumor regions on microCT images in representative tumor-bearing animals; (**A**) Pz1+dCKDM/GFP+-treated and (**B**) control non-treated. High 18F-FEAU accumulation was detected on the day of T-cell administration in regions corresponding to tumor foci on microCT images of the Pz1+dCKDM/GFP+-treated animal. No 18F-FEAU accumulation was observed in regions corresponding to tumor foci in the non-treated control animal. Heart and blood vessels were enhanced by the vascular contrast agent FenestraTM VC. Immunofluorescent analysis revealed GFP+ (green, stained with anti-GFP-antibody) T-cells infiltrating hPSMA positive tumor (red, stained with anti-hPSMA-antibody) in the lung of a representative (**C**) Pz1+dCKDM/GFP+ T-cell-treated and (**D**) a non-treated tumor-bearing animal. Nuclei were stained with 4,6-diamidino-2-phenylindole (blue). Magnification X10. Quantitative analysis of (**E**) 18F-FEAU-accumulation and (**F**) ROI-to-BGR ratios in treated and non-treated groups is presented. BGR — background, ROI — region of interest, marked with arrow. Data expressed as % injected dose/gram of tissue (%ID/g). Figure adapted from Likar et al. 2010 [114]

dothelial precursor cell). Noninvasive imaging of lymphocyte trafficking started early 70s, when the first experiments were performed with extracorporeal labeling of lymphocytes using various metallic radioisotopes and chelation attachments to the cell surface (e.g. 111In, 67Co, 64Cu, 51Cr, 99mTc) [139–143]. A major limitation of ex vivo labeling of lymphocytes with radionuclides is the relatively low level of radioactivity per cell that can be attained by labeling cells. The exposure of cells to higher doses of radioactivity during labeling is also limited by radiotoxicity. Another shortcoming of ex vivo radiolabeling is the short period for cell monitoring, which is limited by radioactivity decay and biological clearance.

The genetic labeling of cells for adoptive therapy monitoring provides substantial advantages for long-term monitoring and for assessing the functional status of the adoptively transferred cells. Retroviral-mediated transduction has proven to be one of the most effective means to deliver transgenes into T-cells and results in high levels of sustained transgene expression [144, 145]. Genetic labeling of lymphocytes with the luciferase (FLuc) reporter gene and non-invasive bioluminescence imaging (BLI) of mice has been reported [146–148]. Costa et al. showed the migration of myelin basic protein specific, Luc-transduced CD4+ T-cells in the central nervous system [149]. The distribution of cytotoxic T-lymphocytes (CTL) can also be followed throughout the organism and monitored over time using BLI of Luc-expressing CTLs. The variable optical characteristics of tissue at different depths from the surface on the emitted photons must be recognized in any BLI assessment of cell trafficking. Nevertheless, BLI based on Luc expression has great potential in preclinical mouse-model studies, where high sensitivity, low cost, and technical simplicity are important for rapid screening.

Imaging T-lymphocyte trafficking, targeting and activation in tumor

The long-term trafficking and localization of T-lymphocytes is an important component of the immune response, and in the elimination of abnormal cells and infectious agents from the body. Passive (ex vivo) labeling of T-cells with radioisotopes or magnetic labels can be unstable, is limited in long-term assessments and does not account for proliferation of activated T-cells in the body. Our group demonstrated the feasibility of long-term in vivo monitoring of adoptively transferred antigen-specific T cells that were transduced to express a radiotracer-based reporter gene for noninvasive in vivo PET imaging [150].

The expression of the mutant hdCK-based reporter in primary human T-lymphocytes did not affect their cytolytic activity against target cells and allowed successful PET imaging of systemically administered T-cells targeting lung tumors in a previously described prostate metastatic cancer model (Figure 6). These findings suggest that this mutant of hdCK is an attractive candidate for pre-clinical and clinical PET imaging of transduced cells and tissues using arabinouracil-based radiotracers labeled with either

short-lived (18F) or long-lived (124I) radioisotopes. In addition we have demonstrated an increased sensitivity of the mutant hdCK-transduced cells to Ara-C and gemcitabine of ~10-fold, when compared with the sensitivity of non-transduced, wild-type cells.

The potential of PET imaging for quantifying cell signals in regions of anatomic interest exists. However, little is known about the constraints and parameters for using PET signal detection to establish cell numbers in different regions of interest. Su et al. determined the correlation of PET signal to cell number, and characterized the cellular limit of detection for PET imaging. These studies using human T-cells transduced with HSV1-tk reporter gene, revealed a cell number-dependent signal, with a limit of detection calculated as 106 cells in a region of interest of 0.1 mL volume. Quantitatively similar parameters were observed with stably transduced N2a glioma cells and primary T lymphocytes [151].

An essential component of the immune response in many normal and disease states is T-cell activation. Our group demonstrated and assessed T-cell receptor (TCR)-dependent activation in vivo using non-invasive PET imaging [76]. Recently the combined a constitutive and NFAT-activated inducible bioluminescence imaging reporters in a single system was developed and applied. In vivo studies of primary cells bearing a new system demonstrated for the first time that the combination of NFAT-inducible and constitutive reporters is feasible. This system allowed noninvasively monitor the behavior of donor T cells during graft-versus-host disease (GVHD) and T-cell precursors during T-cell development and studying their trafficking, expansion, and activation status [93].

Imaging the trafficking of bone marrow-derived cells

Imaging the trafficking of bone marrow-derived cells has also been performed using optical-, MR- and PET-based imaging studies. The use of PET for monitoring bone marrow and progenitor (stem) cell transplantation has lagged behind optical and MR techniques [152, 153]. In most cases, PET imaging has been applied to monitoring bone marrow transplantation (BMT), to assess for residual disease [154], or BMT conditioning regimen related toxicities [155]. Monitoring the fate of bone marrow stem cells with PET following direct labeling with [18F]fluorobenzoate and transplantation was first reported by Olasz et al. [156]. Direct labeling of bone marrow-derived cells is limited by the half-life and quantity of isotope used in the labeling. Reporter gene technology precludes this limitation, and allows for extended monitoring of stem cell engraftment [157]. Recently, Cao et al. reported luciferase bioluminescence imaging of hematopoietic stem cells following transplantation into irradiated recipient mice [158]. Donor stem cells were derived either from a luciferase or luciferase/GFP transgenic mouse and purified through cell sorting. After systemic administration, repeated optical imaging was used to detect the sites and kinetics of hematopoietic stem cell engraftment. The data suggests that the stem cells initially home to the bone marrow or spleen, while little specificity for a particular bone marrow compartment exists. Interestingly, different subsets of progenitor cells, such as short or long term repopulating cells, showed comparable homing profiles but differences in their proliferative potential. The potential of bioluminescence imaging to monitor engraftment of hematopoietic progenitor cells was previously shown in a mouse model of xenotransplantation of human hematopoietic stem cell populations [153]. We have applied reporter gene technology to image the trafficking and distribution of bone marrow cells using a multiple-modality reporter gene approach [159]. Co-registration of microPET and microCT images facilitated interpretation of the PET signal and allowed localization radioactive foci to specific anatomical structures (Figure 6). Others have studied effects of the bone marrow transplanted cells on the reconstruction of the ischemic myocardium [160, 161].

Potential limitations

Imaging endogenous gene expression may be hampered when weak promoters, in their usual Cis-configuration, are used to activate transcription of the reporter gene. This results in insufficient transcription and insufficient gene product for the reporter assay. To address this limitation, a "two-step transcriptional amplification" (TSTA) approach can be used to enhance transcriptional activity. Adenoviruses (Ad)-mediated vectors for molecular imaging and gene therapy have been intensively studied to detect and cure castration-resistant prostate cancer. In these Ad vectors, prostate-specific promoters or enhancers such as prostate-specific antigen (PSA), probasin, and human glandular kallikrein 2 (hK2) have been mostly used [162]. TSTA was used to image activation of the androgen-responsive prostate-specific antigen promoter (PSE) with Fluc and HSV1-sr39tkreporter genes in a prostate cancer cell line (LNCaP) [96]. Further improvements of the androgen-responsive TSTA system for reporter gene expression were made using a "chimeric" TSTA system that uses duplicated variants of the prostate-specific antigen (PSA) gene enhancer to express GAL4 derivatives fused to one, two, or four VP16 activation domains. A very encouraging result was the demonstration that the TSTA system was androgen concentration sensitive, suggesting a continuous rather than binary reporter response. Another study [98] validated methods to enhance the transcriptional activity of the carcinoembryonic antigen (CEA) promoter using the TSTA principle. To increase promoter strength while maintaining tissue specificity, a recombinant adenovirus was constructed which contained a TSTA system with a tumor-specific CEA promoter driving a transcription transactivator, which then activates a minimal promoter to drive expression of the HSV1-tk suicide/reporter gene. This ADV/CEA-binary-HSV1-tk system resulted in equal or greater cell killing of transduced cells by ganciclovir in a CEA-specific manner, compared with ganciclovir killing of all cells transduced with a CEA-independent vector containing a constitutive viral promoter driving HSV1-tk expression (ADV/RSV-tk). However, as observed with the PSE-TSTA reporter system above, the in vivo imaging comparison of the TSTA and cis-reporter systems showed substantially less dramatic differences than that obtained by the in vitro analyses.

Recently, the group from UCLA [163] took advantage of the 2-step transcriptional amplification (TSTA) system to boost the transgene expression level of PSES (prostate-specific enhancing sequence). Two Ad vectors were generated with PSES–TSTA system regulating the expression of firefly luciferase (FLuc) or a variant of herpes simplex virus-thymidine kinase (HSV-sr39tk) as bioluminescent and PET imaging reporter gene, respectively. The benefits of prostate specificity and androgen independency of

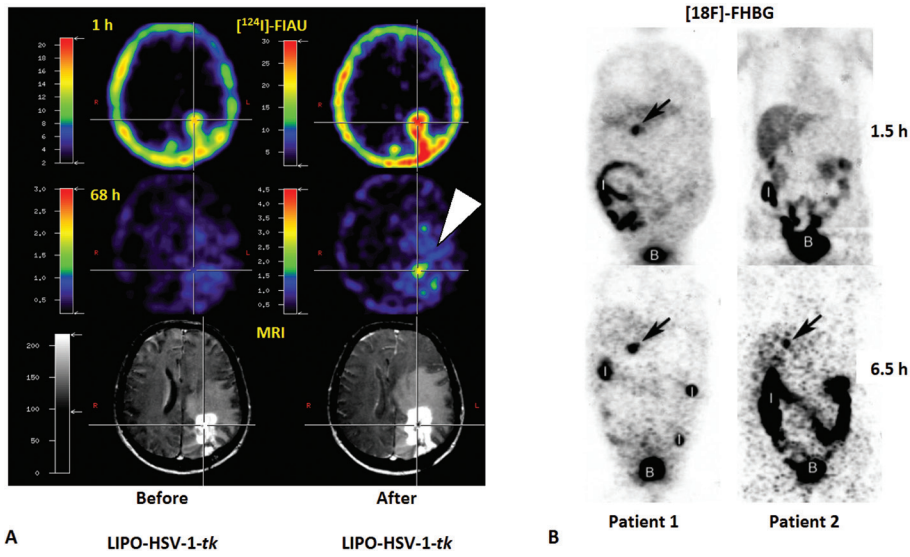

Figure 7. HSV1-tk reporter gene imaging in patients after Liposome-HSV-1-tk-complex transduction (**A**). Coregistration of [124I]FIAU-PET and MRI before (left column) and after (right column) HSV1-tk vector application. A region of specific [124I]FIAU retention (at 68 h) within the tumor is visualized (white arrow). This tumor region showed signs of necrosis (cross hairs, right column after ganciclovir treatment. Figure adapted from Jacobs et al. (2001) [100].

Adenoviral transgene (HSV1-tksr39) expression in patients with liver cancer (**B**). Coronal PET images 1.5 and 6.5 hours after injection of [18F]--FHBG (48 h after 2 x1012 Adv-tk). Localization of [18F]-FHBG in the treated lesion was variable in the early images, but could be seen at 6.5 hours in all patients (see arrow). Figure adapted from Penuelas et al. (2005) [101]

the PSES-TSTA vectors to achieve sensitive and specific imaging in the challenging clinical scenario of metastatic castration-resistant prostate cancer (CRPC) is discussed.

Gene expression levels are also regulated by post-transcriptional modulation, including the translation of mRNA. A recent study demonstrated that imaging post-transcriptional regulation of gene expression is feasible. This was shown by exposing cells to antifolates and inducing a rapid increase in the levels of the enzyme dihydrofolate reductase (DHFR). Several studies indicated that the DHFR binds to its own mRNA in the coding region, and that inhibition of DHFR by methotrexate (MTX) releases the DHFR enzyme from its mRNA. Consequently, this release results in an increase in translation of DHFR protein. In addition to the described translational regulation of DHFR in cancer cells exposed to MTX, increased levels of DHFR also result through DHFR gene amplification, a common mechanism of acquired resistance to this drug. In contrast to rapid translational modulation of DHFR, gene amplification occurs in response to chronic exposure to antifolates, and elevated cellular levels of DHFR result from transcription of multiple DHFR gene copies. Mayer-Kuckuk et al. utilized imaging to show that the antifolate-mediated regulation of DHFR indeed occurs in vivo [79]. For this study, a mutant DHFR was tagged with the reporter gene HSV1-TK; a modification that neither abolished the DHFR response to methotrexate or trimetrexate, nor compromises the activity of the robust HSV1-TK reporter gene. Regulation of the DHFR-HSV1-TK fusion protein could be visualized in PET imaging studies that were performed on nude rats bearing DHFR-HSV1-TK-transduced HCT-8 xenografts. In this model, systemic administration of antifolate results in increased accumulation of the DHFR-HSV1-TK fusion protein in tumor tissue. Positron emission tomography of this increase was achieved after injection of the HSV1-TK substrate [124I] FIAU and tracer clearance. The results of this in vivo imaging were consistent with complementing in vitro experiments and indicated that the increase in the fusion reporter protein DHFR-HSV1-TK was occurring at a translational level, rather than at the transcriptional level [164].

Clinical studies

The first successful reporter gene imaging study in patients was performed in Cologne using a HSV1-tk liposomal vector, [124I]FIAU and PET to monitor HSV1-tk suicide gene therapy of high-grade brain tumors [100]. HSV1-tk gene expression was visualized in only one of six patients who received an intratumoral injection of the vector (Figure 7A). Later on [18F]FHBG has been studied in normal human volunteers and the biodistribution, bio-safety, and dosimetry has been determined; it was found to be safe and potentially useful for human applications [165]. More recently, the HSV1-sr39tk/[18F]FHBG PET imaging system has been used to monitor thymidine kinase gene expression after intratumoral injection of the first-generation recombinant adenovirus in patients with hepatocellular carcinoma [101]. Transgene expression in the tumor was dependent on the injected dose of the adenovirus and was detectable by PET during the first hours after administration of the radiotracer in all patients, who received ≥ 1012 viral particles (Figure 7B). Non-specific expression of the transgene was not detected in any distant organs, or in the surrounding liver tissue in any of these studied cases. These results illustrate that PET imaging may help in the design of gene-therapy strategies and in the clinical assessment of

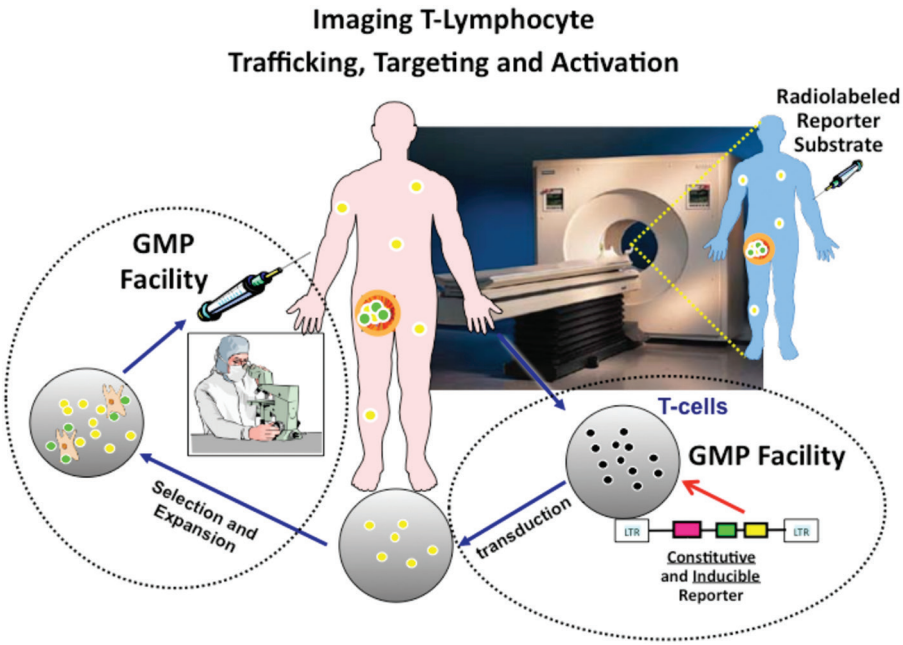

Figure 8. Imaging T-Lymphocyte Trafficking, Targeting and Activation. Therapeutic antitumor immunity depends on highly migratory T-cell populations capable of trafficking between lymphoid and tumor tissue sites as well as infiltration and activation within the micro-anatomic structure of tumors. Stable genetic labeling of adoptively transferred cells with various reporter genes can be used to circumvent the temporal limitations of ex vivo radiolabeling. Peripheral human T-cell can be harvested from individual patients, transduced in a GMP facility with vectors bearing both constitutive and activation-inducible reporter genes, followed by a round of selection and expansion. Then, these autologous genetically-labeled tumor-specific T-lymphocytes can be re-introduced into the same donor patient. Following the administration of radiolabeled reporter-specific substrates, the adoptively administered T cells can be monitored with respect to their trafficking through the body, their targeting and persistence in the tumor, as well as their activation and functional status in the tumor. In vivo monitoring of T-cell trafficking and activation can be performed non-invasively and sequentially with PET on daily or weekly basis, depending on the half-life of the radionuclide use to label the reporter substrate. Thus, reporter gene imaging allows for reliable assessments of adoptive immunotherapy through repetitive visualization of cellular trafficking, persistence, proliferation and function at the target site

new-generation vectors. Non-invasive monitoring of the distribution of transgene expression over time is highly desirable and will have a critical impact on the development of standardized gene therapy protocols and on efficient and safe vector applications in human beings. It is most likely that [124I]FIAU and [18F]FHBG will be the radiolabeled probes that will be introduced into the clinic for the imaging of HSV1-tk gene expression. Yaghoubi and colleagues presented studies in a patient with grade IV glioblastoma multiforme and demonstrated that 18F-FHBG PET can detect HSV1-tk bearing T lymphocytes infused intracranially into the site of tumor resection [99]. Additionally repetitive 18F-FEAU PET-CT imaging in two nonhuman primates demonstrated that activated autologous T lymphocytes transduced with a retroviral vector encoding for HSV1-sr39tk can be detected after intravenous infusion in lymphoid organs and in the sites of inflammation [166].

Infrastructure: GMP facilities for clinical studies

Good manufacturing practices (GMP) for clinical cell engineering and cell-based therapy of patients requires an institutional infrastructure that conforms with city, state and federal regulations. The operational structure for cell-based GMP and clinical activities include: 1. ex-vivo expansion and transduction of patient's cells, 2. the production of ancillary cell lines, as well as retroviral and/or lentiviral packaging cell clones with the creation of master cell banks (MCB), 3. the production of viral stocks for subsequent cell transduction of cell targeting, therapeutic and/or reporter genes, and 4. the production of clinical grade plasmid DNA for vaccines and to generate vector stocks. A schematic outline is shown in Figure 8. A Quality Control Unit is essential to oversee: 1. the development, maintenance and implementation of all standard operating procedures (SOPs) and batch records, 2. to continually monitor the Facility's environment, 3. to review and release results of assays carried out on critical raw materials (e.g. culture media and serum, and manufactured products such as viral stocks, packaging cells and the MCB, as well as the expanded and genetically engineered patient cells, plasmid DNA and vaccines), 4. to maintain the calibration programs of all pieces of equipment, and 5. to maintain personnel training files. The batch records and manufacture records should be independently reviewed by the institutional Office of Clinical Research.

Similarly, for the preparation of radiopharmaceuticals for clinical trials and for the routine practice of medicine, requires an institutional infrastructure that conforms with city, state and federal regulations. In the USA this governed by a combination of United States Pharmacopeia chapters (notably, USP<823> Radiopharmaceuticals for Positron Emission Tomography – Compounding,

and USP<797> Pharmaceutical Compounding — Sterile Preparations), USP drug monographs, FDA GMP regulations (21CFR 212 for radiopharmaceuticals), and the manufacturing procedures and QA controls that are claimed in IND applications. For clinical trials of investigational radiopharmaceuticals, strict compliance with USP<823> is required. USP<823> refers extensively to USP<797> which considers radiopharmaceuticals as "Low Risk Level CSPs" that should be compounded in a certified Class 5 PEC located in a Class 8 or better air environment. A quality control (QC) area adjacent to the radiochemistry GMP facility is necessary in order to provide the required pre- and post-release quality testing for each drug that has been manufactured. Batch records and manufacture records should be independently reviewed by the institutional Office of Clinical Research. Although not required, rapid transport between the radiochemistry facility and the clinical and preclinical imaging suites is important and can be facilitated by establishing a network of pneumatic tubes.

Conclusions

Molecular-genetic studies of disease and our understanding of the multiple and converging pathways that are involved in disease development (e.g. oncogenesis and tumor progression) have expanded rapidly over the past two decades. The era of molecular medicine has begun and benefits to individual patients are expected in the near future. Non-invasive molecular imaging is becoming an integral part of current patient-individualized medical care. Biomarker or surrogate imaging that reflects endogenous molecular/genetic processes is particularly attractive for expansion and translation into clinical studies in the near-term. This is because existing radiopharmaceuticals and imaging paradigms may be useful for monitoring down-stream changes of specific molecular/genetic pathways in diseases such as cancer (e.g. FDG PET). Although biomarker imaging is very likely to be less specific and more limited with respect to the number of molecular-genetic processes that can be imaged, it benefits from the use of radiopharmaceuticals that have already been developed and are currently being used in human subjects. Thus, the translation and application of biomarker imaging paradigms into patient studies will be far easier than either the direct imaging or reporter transgene imaging paradigms.

In contrast, the "direct" molecular imaging motif builds on established biology, chemistry and radiochemistry relationships. Bioconjugate chemistry linking specific binding motifs and bioactive molecules to paramagnetic particles for MR imaging or to radionuclides for PET and gamma camera imaging is rapidly expanding. Such approaches are now being pursued with the objective to develop and evaluate new compounds for imaging; compounds that target specific molecules (e.g. DNA, mRNA, proteins) or activated enzyme systems in specific signal transduction pathways. However, a constraint limiting direct imaging strategies is the necessity to develop a specific probe for each molecular target, and then to validate the sensitivity, specificity and safety of each probe for specific applications prior to their introduction into the clinic.

Molecular imaging paradigms involving reporter genes, although first applied to noninvasive in vivo imaging of small animals, are now being translated into clinical imaging paradigms [99–101]. This translation to clinical studies will rapidly expand and can be expected to establish new standards of medical practice in the future. However, reporter gene imaging studies will be more limited in patients compared to that in animals, due to the necessity of transducing the target tissue or cells with specific reporter constructs, or the production of transgenic animals bearing the reporter constructs. Nevertheless, reporter gene imaging, particularly the genetic labeling of cells with reporter constructs, has several advantages. For example, it is possible to develop and validate reporter imaging strategies more rapidly and at considerably lower cost than "direct" imaging strategies. This is because only a small number of well characterized and validated reporter gene — reporter probe pairs need to be established. For example, there are now seven well-defined human genes (hΔTK2, dCKDM, hNIS, hNET, D2R, hSSTR2 and hCEA) with complimentary, clinically approved, radiopharmaceuticals for PET or gamma camera imaging in patients. These seven complimentary pairs (gene + probe) are excellent candidates for future reporter gene imaging in patients. Importantly, these human genes are less likely to be immunogenic compared to the reporter genes currently used in animals (e.g. viral thymidine kinases, luciferases, fluorescent proteins). It should also be noted that a single reporter gene — reporter probe pair can be used in different reporter constructs to image many different biological and molecular-genetic processes. Once a complimentary reporter-pair (gene + probe) has been approved for human studies, the major regulatory focus will shift to the particular backbone and regulatory sequence of the reporter construct and to the vector used to target reporter transduction to specific cells or tissue, both ex vivo and in vivo.

We should be able to perform limited gene imaging studies in patients in the near future, since the tools and resources exist today. The advantages and benefits of noninvasive imaging to monitor transgene expression in gene therapy protocols and in adoptive cell therapies (as discussed above) are obvious. The major factor limiting translation of reporter gene imaging studies to patients is the "transduction requirement"; target tissue or adoptively administered cells must be transduced with reporter constructs for reporter gene imaging studies. Transduction is usually performed with viral vectors to achieve high transduction efficiency and stability. Reporter gene labeled cells allow for reliable and consistent visualization of cellular trafficking, proliferation and function, as well as persistence at the target site. Only genetically labeled cells can be imaged repetitively and observed over long periods of time (months).

The ability to visualize transcriptional and post-transcriptional regulation of endogenous target gene expression, as well as specific intracellular protein-protein interactions in patients will provide the opportunity for new experimental venues in patients. They include the potential to image the malignant phenotype of an individual patient's tumor at a molecular level and to monitor changes in the phenotype over time. In addition, the potential to image a drug's effect on a specific signal transduction pathway will provide the opportunity for monitoring treatment response at the molecular level and for adjusting drug dose and timing of administration. Although these studies are currently being performed in animal models, they are technically challenging and not likely to be performed in patients in the near-term. Ideal vectors for tar-

geting specific organs or tissue (tumors) do not exist at this time, although this is a very active area of human gene therapy research.

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