Molecular Imaging of Biological Gene Delivery Vehicles for Targeted Cancer Therapy: Beyond Viral Vectors

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Abstract Cancer persists as one of the most devastating diseases in the world. Problems including metastasis and tumor resistance to chemotherapy and radiotherapy have seriously limited the therapeutic effects of present clinical treatments. To overcome these limitations, cancer gene therapy has been developed over the last two decades for a broad spectrum of applications, from gene replacement and knockdown to vaccination, each with different requirements for gene delivery. So far, a number of genes and delivery vectors have been investigated, and significant progress has been made with several gene therapy modalities in clinical trials. Viral vectors and synthetic liposomes have emerged as the vehicles of choice for many applications. However, both have limitations and risks that restrict gene therapy applications, including the complexity of production, limited packaging capacity, and unfavorable immunological features. While continuing to improve these vectors, it is important to investigate other options, particularly nonviral biological agents such as bacteria, bacteriophages, and bacteria-like particles. Recently, many molecular imaging techniques for safe, repeated, and high-resolution in vivo imaging of gene expression have been employed to assess vector-mediated gene expression in living subjects. In this review, molecular imaging techniques for monitoring biological gene delivery vehicles are described, and the

specific use of these methods at different steps is illustrated. Linking molecular imaging to gene therapy will eventually help to develop novel gene delivery vehicles for preclinical study and support the development of future human applications.

Keywords Molecular imaging · Gene therapy · Gene delivery vector · Cancer

Introduction

Nucleic acids and their analogs have many therapeutic applications in cancer that have been exploited to deliver genes as DNA plasmids, to mediate gene knockdown via RNA interference (RNAi) mechanisms, or to alter pre-mRNA splicing to ameliorate disease-causing mutations [1]. Naked therapeutic genetic molecules are generally difficult to deliver primarily due to rapid clearance [2] via nucleases, which limit the serum half-life of unmodified small interfering RNA to 5–60 min [3] and DNA to 10 min [4], a lack of organ-specificity, and a low efficiency of cellular uptake following systemic delivery. Although nucleic acid modifications, such as incorporation of targeting ligands and engineering using tissue-specific promoters, can overcome some of these limitations, specialized gene delivery vehicles (GDV) that improve delivery efficiency and cell-specificity are preferred.

Viral vectors and cationic liposomes are at the forefront of GDV technology with a large number already in clinical trials. Despite their potential, significant limitations remain, including immune recognition [5] of most viral GDVs, mutagenic integration [6] for some viruses, and inflammatory toxicity and rapid clearance of liposomes [7]. For example, immune activation can require the concomitant use of immunosup-

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pressive strategies to overcome readministration problems with current GDVs [8]. Furthermore, most current GDVs lack sufficient specificity for cancer tissue, which limits their gene delivery capability. This is particularly important given that optimizing the specific affinity of GDVs would improve the efficiency of gene/protein delivery and reduce unwanted transfection of noncancerous tissues [9].

Molecular imaging of reporters for specific genes could play a crucial role in optimizing gene delivery and expression by enabling quantitative monitoring of reporter gene expression and the therapeutic effect of transgenes in vivo [10]. Despite sustained efforts to improve the efficacy and safety of liposome formulations and viral vectors using imaging technologies, research into alternative GDVs may provide the least complex solution for novel therapeutic and diagnostic applications. This review will introduce imaging applications for biological delivery vector systems that have been studied so far with an emphasis on bacteria-mediated cancer gene therapy. Additionally, exemplary examples of combined imaging and therapy will be briefly reviewed.

Molecular Imaging Modalities and Imaging Reporter Genes

Several molecular imaging technologies have been used to try to monitor transgene expression for gene therapy, including MRI, optical imaging, and radionuclide imaging techniques such as PET and SPECT [10–14]. Reporter genes with optical signatures (e.g., fluorescence and bioluminescence) are low-cost alternatives for real-time analysis of gene expression in small-animal models. Fluorescence imaging uses a fluorescent protein, such as green fluorescent protein (GFP), that is excited with external illumination, and the emission is subsequently detected [15]. GFP encoding cDNA can easily be included in the myriad of therapeutic vectors and serve as a monitoring tool for gene therapy. Nevertheless, excitation and emission wavelengths in the range of 500 nm (e.g., GFP) have limited penetration in mammalian tissues (1–5 mm). Since mammalian tissues absorb light that is used to excite these fluorophores, the tissues also fluoresce when excited at these wavelengths. The combination of absorption of specific signal and autofluorescence of tissues can result in a poor signal-to-noise ratio [16]. Recently, red-shifted mutants of GFP (RFP) have been shown to have an advantage over GFP in that red light penetrates tissues more efficiently than green [17]. A newer approach to fluorescence imaging of deeper structures uses fluorescence-mediated tomography [18]. The subject is exposed to continuous wave or pulsed light from different sources in an imaging chamber and detectors arranged in a spatially defined order capture the emitted light. Mathematical processing

of this information results in a reconstructed tomographic image. Fluorescence mediated tomography is still in its infancy and requires extensive mathematical validation prior to routine implementation.

Bioluminescent photoproteins, such as luciferase, have been used as reporter proteins in living animals [10, 19, 20]. The most common bioluminescence reporter genes are firefly luciferase (*fluc*) and renilla luciferase (*rluc*). (Note, in general a lowercase abbreviation refers to the gene while an uppercase abbreviation refers to the protein.) The *fluc* (550 aa, 62 kDa) from the North American firefly, *Photinus pyralis*, is one of the best studied luciferases due to its high quantum yield (> 88%). Light is produced by catalyzing the oxidation of its small molecule substrate, beetle D-luciferin (benzothiazole), in an ATP-dependent process [21]. Another well-studied luciferase, *rluc*, is from the sea pansy, *Renilla reniformis* (311 aa, 34 kDa). RLuc utilizes coelenterazine as a substrate and emits light with a peak at 480 nm in a process that does not require ATP. RLuc has a low enzymatic turnover and quantum yield (6%) [22]. Gaussia luciferase (*gluc*) is from the marine copepod *Gaussia princeps*. GLuc (185 aa, 19.9 kDa) is the smallest luciferase known and is naturally secreted [23]. This luciferase emits light at a peak of 480 nm with a broad emission spectrum extending to 600 nm. RLuc and GLuc induce flash kinetics within the first 10 s that rapidly decay with time, whereas FLuc has glow kinetics [19, 23]. The bacterial luciferase gene (*lux*) operon encodes five gene clusters, *lux* C, D, A, B, and E. *Lux* A and B control luciferase enzyme expression, while *lux* C, D, and E control fatty aldehyde enzyme complex production, which synthesizes the substrates. Because the *lux* operon encodes all of the proteins necessary for light-emitting systems to function in bioluminescent bacteria, including luciferase, substrate, and substrate-regenerating enzymes, bacteria that express the *lux* operon only require oxygen and do not require an exogenous substrate to produce bioluminescence [9, 24]. The advantage of bioluminescence is its minimal background noise, since luciferase is not a natural constituent of mammalian organisms. Bioluminescence-based approaches currently lack detailed tomographic information and are limited to relatively small animals [9, 25, 26].

The advantage of MR for imaging gene expression is its excellent three-dimensional spatial resolution (tens of millimeter range). Owing to the indirect nature of the enhancement produced by MR contrast agents, much higher concentrations of injected material (10–100 μ M concentrations and higher) are generally necessary to produce sufficient image contrast [10, 13]. The low sensitivity often entails long imaging times, and consequently data acquisition is slow [13]. While magnetic resonance spectroscopy (MRS) does not usually produce three-dimensional images, this technique does provide

accurate measurements of gene expression over short time frames and may eventually be harnessed to produce true spatial images, although at a much poorer spatial resolution than MRI. Radionuclide imaging with PET and SPECT has been used to characterize enzyme activity, receptor/transporter status, and biodistribution of various radiolabeled substrates (tracers) [27]. For these reasons, the most significant progress has been made using these techniques to image gene therapy, allowing the monitoring of gene delivery and the identification of therapeutic and/or reporter gene expression in living subjects. While the sensitivity of PET imaging is high (as little as $10^{-11} \sim 10^{-12}$ M of tracer can be detected) and the speed of imaging is relatively rapid (minutes), these techniques lack micrometer spatial resolution (1–2 mm with micro-PET) [10, 12]. An alternative approach to PET is SPECT imaging. While the sensitivity of the single-photon system is intrinsically about one to two orders of magnitude less than the PET systems, the required radiopharmaceuticals and imaging systems are more readily available. Further details on the instrumentation available and the relative advantages of the various types of imaging instrumentation may be found elsewhere [10, 11, 28].

Biological Gene Delivery

Many forms of microorganisms have evolved to infect cells effectively and stably while evading host immune responses. Furthermore, many such microorganisms are tolerated by the immune system, including commensal bacteria in the gut and transfusion-transmitted viruses in the liver. The “ideal” biological GDV should have the appropriate packaging size for cargo, the ability to evade immune recognition, target cell-specificity, and efficient cargo delivery. The requirements at each of these steps differ between applications, and thus different GDVs are likely to be required. Although the most-developed GDVs in this class, such as viruses, show great promise for a wide range of disease applications, they have the aforementioned limitations. On the other hand, unconventional biological GDVs with unique properties might fill therapeutic niches poorly served by mainstream viral and liposomal GDVs (Table 1).

Any bacterium suitable for use as a GDV must possess several features. It must (1) be genetically tractable, (2) be nonpathogenic so it is well-tolerated by the host, (3) exhibit preferential replication and accumulation within tumors, (4) be motile and nonimmunogenic, and (5) be susceptible to antibiotics so it can be cleared from the body following gene delivery [29]. As bacterial vectors satisfy the above criteria, two strategies are emerging depending on whether the bacteria is used as a protein or gene delivery tool (Fig. 1a, b) [30].

The basic idea of gene delivery is that transformed bacterial strains deliver genes encoded on a eukaryotic plasmid into cells where they are expressed as therapeutic gene products. The delivery process might involve intracellular localization of the bacteria (Fig. 2b). In another approach, bacteria are not used for gene transfer, but rather persist in the target tissues and produce the therapeutic proteins *in situ*. The transgene can be expressed in the cytoplasm after the bacteria penetrate the cells by prokaryotic transcription and translation machinery. Bacteria that do not enter host cells express the transgene in the intercellular space. In this case, extracellularly expressed protein may need to be fused to a cell permeable peptide (CPP) in order to penetrate the cell membrane (Fig. 2a).

Bacteria

That bacteria colonize tumors and that there is some beneficial effect of bacterial infection in cancer cells was first recognized by Dr. William B. Coley over 100 years ago [31]. Since then, oncolytic strategies using various bacterial strains, such as *Salmonella typhimurium* (*S. typhimurium*) [32–36], *E. coli* [24, 37], *Clostridium* [38], *Bifidobacteria* [39], and *Listeria* [40–44], have been widely explored as alternatives to traditional cancer therapies such as radiotherapy or chemotherapy. These bacteria reportedly have a propensity to naturally accumulate and replicate in a wide variety of solid tumors [45]. For cancer therapy, tumor-targeting bacteria have been applied alone [36] or in combination with conventional therapeutics [46] and have also been manipulated to deliver therapeutic molecules [47]. In particular, *S. typhimurium* has been bioengineered to generate bioluminescence [9, 48] or fluorescence [49] signals and has been employed to monitor bacterial migration to tumors in living small-animal models. These data may facilitate the prediction of the therapeutic efficacy of bacterio-therapy through visualization of bacterial accumulation and replication in specific organs.

Hoffman and colleagues have exploited the nutritional needs of *S. typhimurium* to target and kill tumor cells [32, 35, 36] by selecting strains of *S. typhimurium* that are dependent on the presence of the amino acids leucine and arginine for growth. They also reisolated *S. typhimurium* from the tumor after infection of human colon cancer cells in nude mice and demonstrated that the reisolated bacteria had increased tumor-targeting capability *in vivo* as well as *in vitro* [32]. These *S. typhimurium* strains were used to treat many types of metastatic cancers in mouse models, and tumor regression was observed after intravenous (IV) administration. In these experiments, GFP-expressing, attenuated *S. typhimurium* strains were successfully monitored after IV injection into various mice tumor

Table 1 Comparison of selected parameters of the main vector types

	Viral vectors	Bacterial vectors	Bacteriophages	Minicells
Natural cancer targeting	±	+++	±	±
Safety	+	+	+++	+
Efficiency	+++	+	+	+
Low production costs	+	+++	+++	+
Simple production	+	+++	+++	+
Simple delivery	++	+++	+	+++
Amount of cargo molecule	++	+++	+	+++

models [35]. This approach had several advantages for imaging living animals [50]: (1) it produced a strong fluorescent signal that could be used to image unrestrained animals, and (2) the use of different types of fluorescent probes enabled multi-color imaging. However, because excitation is required, the applicability of this technique

depends on the number and location of the bacteria in the animal [50]. This limitation, however, can be overcome using skin-flap techniques. For example, the spatiotemporal dynamics of bacterial infection following oral administration have been reported in great detail using this approach [49].

Fig. 1a–g Production of biological gene delivery vehicles labeled with molecular imaging probes. **a** Strains of bacteria with desirable properties are transformed with the plasmid cargo. Imaging reporter genes such as the *lux* operon are transduced into the bacteria. **b** The bacteria are cotransformed with the plasmid encoding the therapeutic gene and the imaging reporter gene. **c** Docking of bacteria with functionalized multiple-sized nanoparticles through biotinylated antibodies and surface-antigen interactions (microbot). Streptavidin-coated nanoparticles can carry biotinylated cargo. **d** Minicells are derived from a minCDE-chromosomal deletion mutant of *Salmonella enterica* serovar Typhimurium (*S. typhimurium*). Target genes are

incubated with minicells overnight. Bispecific antibody (BsAb) is used to target recombinant minicells to tumor cells. One arm of these antibodies recognizes the O-polysaccharide component of the minicell surface lipopolysaccharide and the other a tumor-preferential cell surface-receptor, such as the epidermal growth factor receptor (EGFR), which is overexpressed in several cancers. **e** The coat proteins of the bacteriophages can be engineered to incorporate targeting ligands. Phage nanoparticles with multiple peptides engineered for different functions can then be produced with phagemid technology to enhance gene delivery efficiency. **f** Retrovirus. **g** Adenovirus

Fig. 2a–g Intracellular delivery of cargo by delivery vehicles. **a** Extracellular secretion of protein by bacteria, such as *E. coli* or *S. typhimurium*, which are defective in ppGpp synthesis. Proteins made by bacteria must be engineered with a leader secretory signal sequence (S) for extracellular (bacteria) secretion. For intracellular transport, bacterial proteins need to be fused to cell permeable peptide (CPP) sequences. **b** Intracellular delivery of genes/proteins by bacteria such as *S. typhimurium*, *S. typhi* or *E. coli* expressing the invasion of *Y. pseudotuberculosis*. The bacteria invade host cells and remain in the vacuole. There they die due to metabolic attenuation and release their expression plasmid or protein. By an unknown mechanism, the plasmids cross the vesicular membrane and reach the cell nucleus of the host cells where they are expressed. **c** Bacteria enter cells via induced phagocytosis. Bacterial toxin causes endosomal compart-

ments to disintegrate. The therapeutic cargo then separates from the bacterium and is delivered to the nucleus. **d** Minicells carrying the target gene, such as siRNA, bind biomarkers on the surface of cancer cells via BsAb and enter the cell by endocytosis. After endocytosis, the minicells traverse the well-established early and late endosomal pathways, terminating in acidified organelles, the lysosomes, where they are degraded and release their cargo. **e** Bacteriophage nanoparticles carrying target molecules bind biomarkers on the surface of cancer cells via a targeting peptide and enter the cell by endocytosis. After endocytosis, the bacteriophages traverse the well-established early and late endosomal pathways, terminating in acidified organelles, the lysosomes, where they are degraded and release their cargo. **f** Retroviral gene transfection. **g** Adenoviral gene transfection

In an interesting application, GFP-labeled bacteria have also been used to screen for tumor-expressing promoters from a huge library. A population of *Salmonella* containing a random library of 14,028 genomic DNA fragments cloned upstream of a promoterless gene for GFP was IV injected into tumor-free nude mice and into human PC3 prostate s.c. tumors in nude mice to screen for candidate promoters preferentially induced in bacteria growing in tumors [51]. This strategy led to the identification of the pflE and ansB promoters, which are known to be induced in hypoxic conditions, as well as many other candidate promoters not related to hypoxia.

Our laboratory developed bioluminescent *Salmonella* and *E. coli* using the bacterial *lux* operon to visualize bacterial location in infected mice. The *lux* operon was integrated into *Salmonella*'s chromosome using Phage22 transduction. The bioluminescence emitted from the bacteria was detected in various tumor models after IV bacterial injection using a cooled-CCD camera. The *lux* operon can also be cloned into a plasmid, and transformed bacteria will generate light [9, 24]. However, using this strategy, *E. coli* fails to maintain the pLux expression plasmid in the absence of selection, particularly in infected animals [48]. Therefore, we employed a balanced-lethal host-vector

system that coupled the expression of the *Lux* operon to the gene for aspartate β -semialdehyde dehydrogenase (*asd*), a key enzyme in the biosynthetic pathway of diaminopimelic acid (DAP), which is an essential component of the peptidoglycan of all Gram-negative and certain Gram-positive bacteria [52]. *E. coli asd* mutants require DAP and undergo lysis in its absence. In order to select bacteria that retained pLux in vivo, the chromosomal copy of *asd* was mutated in the host strain, resulting in viability only of strains that retained the Asd⁺pLux construct. The procedure for constructing the *asd* mutant of wild-type MG1655 was based on a method previously reported by Datsenko and Wanner [53]. In addition to strategies for maintaining exogenous genes, strides have also been made in increasing expression of these light emitting proteins and thus enhancing their detectability. For example, Riedel et al. have improved a system for luciferase tagging *Listeria monocytogenes* using a highly active, constitutive promoter that results in 100-fold higher luciferase activity in broth than any native promoter tested. This allowed for imaging of lux-tagged *L. monocytogenes* in food products, during murine infection, and in tumor-targeting studies [54].

In addition to the optical reporter genes, a reporter gene for radionuclide imaging was also explored. The HSV1-tk gene has been utilized as an effective reporter gene for nuclear imaging using PET or a gamma camera. Bermudes and colleagues showed that VNP20009 *Salmonella* constitutively expressing the HSV1-tk gene could be detected noninvasively by [¹²⁴I]FIAU PET in xenograft tumor models. These data demonstrated the efficiency and duration of vector targeting as well as indicated the location of tumors. Moreover, the ability to detect *Salmonella* vectors non-invasively by PET imaging has potential for use in clinical settings and could aid in development of these vectors by demonstrating the efficiency and duration of targeting as well as indicating the locations of tumors [55, 56].

Recently, MRI has also been utilized to image bacteria in living small animals. Benoit et al. reported that the magnetotactic bacteria *Magnetospirillum magneticum* AMB-1 could produce positive MRI contrast and colonize mouse tumor xenografts, providing a potential tool for MRI visualization of bacteria in preclinical and translational studies to track cancer despite its lower sensitivity. Following IV injection of ⁶⁴Cu-labeled AMB-1, PET imaging also revealed increased bacterial colonization of tumors, but decreased colonization of other organs, such as liver and spleen, after 4 h [57].

For therapeutic applications, tumor-targeting bacteria have been transformed with plasmids encoding therapeutic genes [58, 59]. A major problem hindering full exploitation of therapeutic gene-expressing *Salmonella* in cancer treatment is its toxicity in nontumoral reticuloendothelial organs, mainly the liver and spleen. Studies have shown

that when injected through the tail vein, the majority of *Salmonella* initially localizes in those organs (Fig. 3a) [24, 48]. Therefore, it is necessary to establish an inducible system that allows complete, deliberate control over the expression of a gene of interest in the *Salmonella* transformants after host infection. Loessner et al. employed the inducible promoter *P_{BAD}* from the arabinose operon of *E. coli* to control expression of reporters such as GFP, FLuc, or Lux in vivo in the *S. typhimurium* strain SL7207 (*hisG*, Δ *aroA*), which can be activated by the sugar L-arabinose [60]. We also confirmed that RLuc8 expression was restricted in subcutaneously implanted tumors using *Salmonella* transformed with pBAD-rluc8 (Fig. 3b). To advance this system, we engineered attenuated *Salmonella typhimurium* that was defective in ppGpp synthesis (strain Δ ppGpp) to carry cytotoxic proteins (cytolysin A) and express reporter genes [61]. In this system, we transformed *Salmonella* with a plasmid encoding a cytotoxic protein (cytolysin A) under the control of *P_{BAD}*. Subsequently, the bacterial luciferase (*lux*) operon from *S. typhimurium*-Xen26 (Xenogen-Caliper) was transduced in the transformed *Salmonella*. Engineered anti-tumoral bacteria labeled with imaging molecules allowed determination of bacterial fate in a simple, noninvasive manner that was amenable to repeated observation.

Nanoparticles and bacteria have also been used independently to deliver genes and proteins into mammalian cells to monitor or alter gene expression and protein production [62]. In addition, Akin et al. reported the simultaneous use of nanoparticles and bacteria to deliver DNA-based model drug molecules in vivo and in vitro. In this approach, cargo (in this case, a fluorescent or a bioluminescent gene) is loaded onto the nanoparticles, which are carried on the bacterial surface (*Listeria monocytogenes*) (Fig. 1c). When incubated with cells, the cargo-carrying bacteria (“microbots”) were internalized, and the genes released from the nanoparticles were expressed (Fig. 2c). Mice injected with microbots also successfully expressed the genes, as seen by luminescence in different organs. This new approach may be used to deliver different types of cargo into live animals and a variety of cells in culture without the need for complicated genetic manipulation.

Bacteria-like Particles

Minicells, or bacteria-like particles, have been produced by derepressing cryptic polar sites of cell fission through inactivating genes controlling normal bacterial cell division [63]. These minicells can be packaged with therapeutically relevant concentrations of a range of therapeutics and selectively targeted to cancer cells via bispecific antibodies (BsAbs) (Fig. 1d) [64, 65]. A two-wave cancer treatment

Fig. 3a, b Specific gene expression in tumors by bacterial vectors. **a** A bacterial expression plasmid was constructed in which a *Renilla* luciferase variant (RLuc8) was placed under the control of a constitutive promoter (pLac-RLuc8). *S. typhimurium* defective in ppGpp synthesis (strain Δ ppGpp) was transformed with the pLac-RLuc8 plasmid. The transformed bacteria were IV administered into immunocompetent BALB/c mice bearing CT-26. Due to early distribution of bacteria in the liver and spleen, bacterial bioluminescence was produced not only in the tumor, but also in the liver and spleen. **b** To verify that remote control of the bacterial gene expression is possible, a bacterial expression plasmid was constructed in which a

Renilla luciferase variant (RLuc8) was placed under the control of the P_{BAD} promoter (pBAD-RLuc8). The mutant *S. typhimurium* Δ ppGpp strain was electrotransformed with the pBAD-RLuc8 vector. The transformed bacteria were IV administered into immunocompetent BALB/c mice bearing CT-26. RLuc8 expression was induced with L-arabinose (60 mg) at 4 dpi. Bioluminescence was detected in the tumors after L-arabinose administration, but not in its absence. Bioluminescence was observed only in the tumor and not in the liver or spleen because the bacteria were cleared from these organs (liver, spleen) at 4 dpi

has been explored in which minicells are specifically and sequentially delivered to tumor xenografts. First, siRNA- or shRNA-encoding plasmids are delivered to compromise drug resistance by knocking down a multidrug resistance protein. Subsequently, targeted minicells containing cytotoxic drugs are administered, which then eliminate the formerly drug-resistant tumors (Fig. 2d) [66]. Monoclonal BsAbs or anti-O-polysaccharide was labeled with Alexa Fluor-488 or Fluor-647 to visualize minicells invasion into cells in vitro. In vivo visualization may also be possible by conjugating the minicells with a near-infrared fluorophore.

Bacteriophages

Bacteriophages are natural viruses that exclusively infect bacteria and are ingested without serious side effects through the consumption of fermented food, such as yoghurt. Development of phagemid vectors, plasmids with a bacteriophage origin of replication that are packaged into phage particles upon the addition of helper phages, has made the genetic manipulation of bacteriophages as easy as manipulating plasmids used in the production of viral vectors [67]. The phagemid system allows for precise control over the relative composition of wild-type and modified coat proteins [68] while eliminating potentially immunogenic genes encoding bacteriophage proteins. The

bacteriophage coat proteins can be engineered to incorporate targeting ligands without significantly affecting structure. Phage nanoparticles with multiple peptides engineered for different functions can then be produced with phagemid technology to enhance gene delivery efficiency [47]. Based on these technologies, disease-specific phage library-driven fluorescent probes could be developed for use in the early detection of cancers by optical imaging systems (Figs. 1e and 2e). For example, Newton et al. selected bacteriophages in vivo labeled with a near-infrared fluorophore (Alexa-Fluor 680) that displayed affinity to prostate cancer [69]. A tumor-specific bacterial phage was also generated by displaying up to five copies of melanocyte-stimulating hormone (-MSH) peptide analogs on the phage surface. To visualize these phages, streptavidin was conjugated to diethylenetriaminepenta acetic acid, which enabled radiolabeling with ^{111}In [70] and hence radionuclide imaging.

Viruses

Over the last century, many viral types, including adenovirus, herpes simplex virus, Newcastle disease virus, myxoma virus, vaccinia virus, and vesicular stomatitis virus, have been studied intensively as novel agents for cancer treatment [71]. Targeted gene expression can be obtained via different strategies, such as exploiting tissue/

tumor-specific promoters that only activate the gene in certain tissues or conditions [72, 73], the use of antibody against tumor-specific antigen to modify tropism and redirect the virus to cancer cells [74–77], and natural or artificial modification of conditionally replicating viruses [78–81]. These techniques are largely thought to limit undesired spreading of the virus to normal organs. The tropism of viral vectors can be visualized by encoding imaging reporter genes in the viral genome. Recombinant vaccinia virus-mediated expression of the human norepinephrine transporter (hNET) allowed deep tissue imaging by radiolabeled metaiodobenzylguanidine (MIBG) [82]. In addition to hNET, the Na/I symporter (hNIS) has also been commonly used in adenovirus [83] or in measles virus Edmonston [84] as an imaging reporter gene to monitor viral gene therapy.

Tumor-specific promoters have been actively investigated as a means to confine gene expression to tumors and limit undesired gene expression in other normal organs. A combination of transcriptional targeting and noninvasive imaging could improve the safety of viral-based therapeutic approaches in cancer. For example, prostate-specific adenoviral vectors encoding firefly luciferase produced a robust signal in prostate cancers. Repetitive imaging over a 3-week period after IV injection into tumor-bearing mice revealed that the virus could locate and illuminate metastases in the lung and spine [85]. To enhance the expression of a prostate-specific promoter, Rubinchik et al. placed the tetracycline transactivator gene under the control of a prostate-specific ARR2PB promoter, and a mouse *Tnfsf6* (encoding FASL)-GFP fusion gene under the control of the tetracycline responsive promoter. A single construct with both expression cassettes carried by adenovirus showed that GFP expression was restricted to prostate cancer cells [86]. Sato et al. developed a two-step transcriptional amplification (TSTA) method using an adenoviral vector by utilizing the prostate specific promoter to drive a potent GAL4VP16 transcriptional activator, which in turn binds to tandem repeats of the GAL4 binding site that activates the secondary reporter or therapeutic gene. Analysis of real-time gene expression by both optical imaging and the combined modality of PET/CT showed that this approach enhanced the weak native PSA promoter up to 1,000-fold and was even stronger than the robust expression driven by the viral cytomegalovirus promoter [87].

Conclusions

The biological GDVs discussed above, although less well established than viral vectors and liposomal delivery agents, present tantalizing opportunities for niche therapeutic applications and provide a rich opportunity for further research and

development. Besides their intrinsic advantages, biological GDVs can also benefit tremendously from the development of tangential technologies. For example, bacteria and bacteriophage production can benefit from the research and industrial experience of the vaccine production and food probiotic industries. Given the state-of-art molecular imaging techniques that are available, the barrier between bench and bedside applications is likely not as high as is perceived; hence the development of biological GDVs through molecular imaging techniques probably deserves greater attention from the gene therapy community.

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References

1. Cao S, Cripps A, Wei MQ (2009) New strategies for cancer gene therapy: progresses and opportunities. *Clin Exp Pharmacol Physiol*. doi:10.1111/j.1440-1681.2009.05268.x
2. Takakura Y, Nishikawa M, Yamashita F, Hashida M (2001) Development of gene drug delivery systems based on pharmacokinetic studies. *Eur J Pharm Sci* 13:71–76
3. Soutschek J, Akinc A, Bramlage B, Charisse K, Constien R, Donoghue M et al (2004) Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. *Nature* 432:173–178
4. Kawabata K, Takakura Y, Hashida M (1995) The fate of plasmid DNA after intravenous injection in mice: involvement of scavenger receptors in its hepatic uptake. *Pharm Res* 12:825–830
5. Lowenstein PR, Mandel RJ, Xiong WD, Kroeger K, Castro MG (2007) Immune responses to adenovirus and adeno-associated vectors used for gene therapy of brain diseases: the role of immunological synapses in understanding the cell biology of neuroimmune interactions. *Curr Gene Ther* 7:347–360
6. Daniel R, Smith JA (2008) Integration site selection by retroviral vectors: molecular mechanism and clinical consequences. *Hum Gene Ther* 19:557–568
7. Zhang JS, Liu F, Huang L (2005) Implications of pharmacokinetic behavior of lipoplex for its inflammatory toxicity. *Adv Drug Deliv Rev* 57:689–698
8. Jiang H, Couto LB, Patarroyo-White S, Liu T, Nagy D, Vargas JA et al (2006) Effects of transient immunosuppression on adeno-associated, virus-mediated, liver-directed gene transfer in rhesus macaques and implications for human gene therapy. *Blood* 108:3321–3328
9. Min JJ, Nguyen VH, Kim HJ, Hong YJ, Choy HE (2008) Quantitative bioluminescence imaging of tumor-targeting bacteria in living animals. *Nat Protoc* 3:629–636
10. Massoud TF, Gambhir SS (2003) Molecular imaging in living subjects: seeing fundamental biological processes in a new light. *Genes Dev* 17:545–580
11. Gambhir SS (2002) Molecular imaging of cancer with positron emission tomography. *Nat Rev Cancer* 2:683–693
12. Min JJ, Gambhir SS (2004) Gene therapy progress and prospects: noninvasive imaging of gene therapy in living subjects. *Gene Ther* 11:115–125
13. Weissleder R, Mahmood U (2001) Molecular imaging. *Radiology* 219:316–333

14. Gambhir SS, Herschman HR, Cherry SR, Barrio JR, Satyamurthy N, Toyokuni T et al (2000) Imaging transgene expression with radionuclide imaging technologies. *Neoplasia* 2:118–138
15. Yang M, Baranov E, Moossa AR, Penman S, Hoffman M (2000) Visualizing gene expression by whole-body fluorescence imaging. *Proc Natl Acad Sci USA* 97:12278–12282
16. Ntziachristos V, Ripoll J, Wang LV, Weissleder R (2005) Looking and listening to light: the evolution of whole-body photonic imaging. *Nat Biotech* 23:313–320
17. Shaner NC, Steinbach PA, Tsien RY (2005) A guide to choosing fluorescent proteins. *Nat Meth* 2:905–909
18. Ntziachristos V, Tung CH, Bremer C, Weissleder R (2002) Fluorescence molecular tomography resolves protease activity in vivo. *Nat Med* 8:757–761
19. Bhaumik S, Gambhir SS (2002) Optical imaging of *Renilla* luciferase reporter gene expression in living mice. *Proc Natl Acad Sci USA* 99:377–382
20. Contag CH, Bachmann MH (2002) Advances in in vivo bioluminescence imaging of gene expression. *Annu Rev Biomed Eng* 4:235–260
21. Edinger M, Hoffmann P, Contag CH, Negrin RS (2003) Evaluation of effector cell fate and function by in vivo bioluminescence imaging. *Methods* 31:172–179
22. Matthews JC, Hori K, Cormier MJ (1977) Purification and properties of *Renilla reniformis* luciferase. *Biochemistry* 16:85–91
23. Tannous BA, Kim DE, Fernandez JL, Weissleder R, Breakefield XO (2005) Codon-optimized *Gaussia* luciferase cDNA for mammalian gene expression in culture and in vivo. *Mol Ther* 11:435–443
24. Min JJ, Kim HJ, Park JH, Moon SM, Jeong J, Hong YJ et al (2008) Noninvasive real-time imaging of tumors and metastases using tumor-targeting light-emitting *Escherichia coli*. *Mol Imaging Biol* 10:54–61
25. Allport JR, Weissleder R (2001) In vivo imaging of gene and cell therapies. *Exp Hematol* 29:1237–1246
26. Wu JC, Sundaresan G, Iyer M, Gambhir SS (2001) Noninvasive optical imaging of firefly luciferase reporter gene expression in skeletal muscles of living mice. *Mol Ther* 4:297–306
27. Nichol C, Kim EE (2001) Molecular imaging and gene therapy. *J Nucl Med* 42:1368–1374
28. Weissleder R (2002) Scaling down imaging: molecular mapping of cancer in mice. *Nat Rev Cancer* 2:11–18
29. Ryan RM, Green J, Lewis CE (2006) Use of bacteria in anti-cancer therapies. *BioEssays* 28:84–94
30. Palffy R, Gardlik R, Hodossy J, Behuliak M, Resko P, Radvansky J et al (2006) Bacteria in gene therapy: bactofection versus alternative gene therapy. *Gene Ther* 13:101–105
31. McCarthy EF (2006) The toxins of William B. Coley and the treatment of bone and soft-tissue sarcomas. *Iowa Orthop J* 26:154–158
32. Zhao M, Yang M, Ma H, Li XM, Tan X, Li S et al (2006) Targeted therapy with a *Salmonella typhimurium* leucine-arginine auxotroph cures orthotopic human breast tumors in nude mice. *Cancer Res* 66:7647–7652
33. Pawelek JM, Low KB, Bermudes D (1997) Tumor-targeted *Salmonella* as a novel anticancer vector. *Cancer Res* 57:4537–4544
34. Toso JF, Gill VJ, Hwu P, Marincola FM, Restifo NP, Schwartzentruber DJ et al (2002) Phase I study of the intravenous administration of attenuated *Salmonella typhimurium* to patients with metastatic melanoma. *J Clin Oncol* 20:142–152
35. Zhao M, Yang M, Li XM, Jiang P, Baranov E, Li S et al (2005) Tumor-targeting bacterial therapy with amino acid auxotrophs of GFP-expressing *Salmonella typhimurium*. *Proc Natl Acad Sci USA* 102:755–760
36. Zhao M, Geller J, Ma H, Yang M, Penman S, Hoffman RM (2007) Monotherapy with a tumor-targeting mutant of *Salmonella typhimurium* cures orthotopic metastatic mouse models of human prostate cancer. *Proc Natl Acad Sci USA* 104:10170–10174
37. Brader P, Stritzker J, Riedl CC, Zanzonico P, Cai S, Burnazi EM et al (2008) *Escherichia coli* Nissle 1917 facilitates tumor detection by positron emission tomography and optical imaging. *Clin Cancer Res* 14:2295–2302
38. Lemmon MJ, van Zijl P, Fox ME, Mauchline ML, Giaccia AJ, Minton NP et al (1997) Anaerobic bacteria as a gene delivery system that is controlled by the tumor microenvironment. *Gene Ther* 4:791–796
39. Kimura NT, Taniguchi SI, Aoki K, Baba T (1980) Selective localization and growth of *Bifidobacterium bifidum* in mouse tumors following intravenous administration. *Cancer Res* 40:2061–2068
40. Craft N, Bruhn KW, Nguyen BD, Prins R, Liao LM, Collisson EA et al (2005) Bioluminescent imaging of melanoma in live mice. *J Invest Dermatol* 125:159–165
41. Shahabi V, Reyes-Reyes M, Wallecha A, Rivera S, Paterson Y, Maciag P (2008) Development of a *Listeria monocytogenes* based vaccine against prostate cancer. *Cancer Immunol Immunother* 57:1301–1313
42. Shen H, Kanoh M, Maruyama S, Matsumoto A, Zhang W, Asano Y (2008) Attenuated *Listeria* infection activates natural killer cell cytotoxicity to regress melanoma growth in vivo. *Microbiol Immunol* 52:107–117
43. Wood L, Guirnalda P, Seavey M, Paterson Y (2008) Cancer immunotherapy using *Listeria monocytogenes* and listerial virulence factors. *Immunol Res* 42:233–245
44. Maciag PC, Radulovic S, Rothman J (2009) The first clinical use of a live-attenuated *Listeria monocytogenes* vaccine: a phase I safety study of Lm-LLO-E7 in patients with advanced carcinoma of the cervix. *Vaccine* 27:3975–3983
45. Pawelek JM, Low KB, Bermudes D (2003) Bacteria as tumour-targeting vectors. *Lancet Oncol* 4:548–556
46. Platt J, Sodi S, Kelley M, Rockwell S, Bermudes D, Low KB et al (2000) Antitumour effects of genetically engineered *Salmonella* in combination with radiation. *Eur J Cancer* 36:2397–2402
47. Seow Y, Wood MJ (2009) Biological gene delivery vehicles: beyond viral vectors. *Mol Ther* 17:767–777
48. Yu YA, Shabahang S, Timiryasova TM, Zhang Q, Beltz R, Gentschev I et al (2004) Visualization of tumors and metastases in live animals with bacteria and vaccinia virus encoding light-emitting proteins. *Nat Biotechnol* 22:313–320
49. Hoffman RM, Zhao M (2007) Whole-body imaging of bacterial infection and antibiotic response. *Nat Protoc* 1:2988–2994
50. Hoffman RM, Yang M (2006) Whole-body imaging with fluorescent proteins. *Nat Protoc* 1:1429–438
51. Arrach N, Zhao M, Porwollik S, Hoffman RM, McClelland M (2008) *Salmonella* promoters preferentially activated inside tumors. *Cancer Res* 68:4827–4832
52. Curtiss R, Kelly SM, Gulig PA, Nakayama K (1989) Stable recombinant avirulent *Salmonella* vaccine strains. *Adv Exp Med Biol* 251:33–47
53. Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci USA* 97:6640–6645
54. Riedel CU, Monk IR, Casey PG, Morrissey D, O'Sullivan GC, Tangney M et al (2007) Improved luciferase tagging system for *Listeria monocytogenes* allows real-time monitoring in vivo and in vitro. *Appl Environ Microbiol* 73:3091–3094
55. Soghomonyan SA, Doubrovina M, Pike J, Luo X, Ittensohn M, Runyan JD et al (2004) Positron emission tomography (PET) imaging of tumor-localized *Salmonella* expressing HSV1-TK. *Cancer Gene Ther* 12:101–108

56. Tjuvajev J, Blasberg R, Luo X, Zheng LM, King I, Bermudes D (2001) *Salmonella*-based tumor-targeted cancer therapy: tumor amplified protein expression therapy (TAPET) for diagnostic imaging. *J Control Release* 74:313–315
57. Benoit MR, Mayer D, Barak Y, Chen IY, Hu W, Cheng Z et al (2009) Visualizing implanted tumors in mice with magnetic resonance imaging using magnetotactic bacteria. *Clin Cancer Res* 15:5170–5177
58. Bermudes D, Low B, Pawelek J (2000) Tumor-targeted salmonella. Highly selective delivery vectors. *Adv Exp Med Biol* 465:57–63
59. Ryan RM, Green J, Williams PJ, Tazzyman S, Hunt S, Harney JH et al (2009) Bacterial delivery of a novel cytotoxin to hypoxic areas of solid tumors. *Gene Ther* 16:329–339
60. Loessner H, Endmann A, Leschner S, Westphal K, Rohde M, Miloud T et al (2007) Remote control of tumour-targeted *Salmonella enterica* serovar Typhimurium by the use of L-arabinose as inducer of bacterial gene expression in vivo. *Cell Microbiol* 9:1529–1537
61. Nguyen VH, Kim HS, Ha JM, Hong YJ, Choy HE, Min JJ (2010) Genetically engineered *Salmonella typhimurium* as an imageable therapeutic probe for cancer. *Cancer Res* 70:18–23
62. Akin D, Sturgis J, Ragheb K, Sherman D, Burkholder K, Robinson JP et al (2007) Bacteria-mediated delivery of nanoparticles and cargo into cells. *Nat Nanotechnol* 2:441–449
63. de Boer PA, Crossley RE, Rothfield LI (1989) A division inhibitor and a topological specificity factor coded for by the minicell locus determine proper placement of the division septum in *E. coli*. *Cell* 56:641–649
64. MacDiarmid JA, Madrid-Weiss J, Amaro-Mugridge NB, Phillips L, Brahmabhatt H (2007) Bacterially-derived nanocells for tumor-targeted delivery of chemotherapeutics and cell cycle inhibitors. *Cell Cycle* 6:2099–2105
65. MacDiarmid JA, Mugridge NB, Weiss JC, Phillips L, Burn AL, Paulin RP et al (2007) Bacterially derived 400 nm particles for encapsulation and cancer cell targeting of chemotherapeutics. *Cancer Cell* 11:431–445
66. MacDiarmid JA, Amaro-Mugridge NB, Madrid-Weiss J, Sedliarou I, Wetzel S, Kocher K et al (2009) Sequential treatment of drug-resistant tumors with targeted minicells containing siRNA or a cytotoxic drug. *Nat Biotechnol* 27:643–651
67. Larocca D, Jensen-Pergakes K, Burg MA, Baird A (2001) Receptor-targeted gene delivery using multivalent phagemid particles. *Mol Ther* 3:476–484
68. Chatel JM, Pothelune L, Ah-Leung S, Corthier G, Wal JM, Langella P (2008) In vivo transfer of plasmid from food-grade transiting lactococci to murine epithelial cells. *Gene Ther* 15:1184–1190
69. Newton JR, Kelly KA, Mahmood U, Weissleder R, Deutscher SL (2006) In vivo selection of phage for the optical imaging of PC-3 human prostate carcinoma in mice. *Neoplasia* 8:772–780
70. Newton JR, Miao Y, Deutscher SL, Quinn TP (2007) Melanoma imaging with pretargeted bivalent bacteriophage. *J Nucl Med* 48:429–436
71. Woo Y, Adusumilli PS, Fong Y (2006) Advances in oncolytic viral therapy. *Curr Opin Investig Drugs* 7:549–559
72. Nettelbeck DM, Jérôme V, Müller R (2000) Gene therapy: designer promoters for tumour targeting. *Trends Genet* 16:174–181
73. Dachs GU, Dougherty GJ, Stratford IJ, Chaplin DJ (1997) Targeting gene therapy to cancer: a review. *Oncol Res* 9:313–325
74. Kashentseva EA, Douglas J, Zinn KR, Curiel DT, Dmitriev IP (2009) Targeting of adenovirus serotype 5 pseudotyped with short fiber from serotype 41 to c-erbB2-positive cells using bispecific single-chain diabody. *J Mol Biol* 388:443–461
75. Bachtarzi H, Stevenson M, Fisher K (2008) Cancer gene therapy with targeted adenoviruses. *Exp Opin Drug Deliv* 5:1231–1240
76. Douglas JT, Rogers BE, Rosenfeld ME, Michael SI, Feng M, Curiel DT (1996) Targeted gene delivery by tropism-modified adenoviral vectors. *Nat Biotechnol* 14:1574–1578
77. Watkins SJ, Mesyanzhinov VV, Kurochkina LP, Hawkins RE (1997) The 'adenobody' approach to viral targeting: specific and enhanced adenoviral gene delivery. *Gene Ther* 4:1004–1012
78. Greco O, Scott S (2007) Tumor hypoxia and targeted gene therapy. *Int Rev Cytol* 257:181–212
79. Jounaidi Y, Doloff JC, Waxman DJ (2007) Conditionally replicating adenoviruses for cancer treatment. *Curr Cancer Drug Targets* 7:285–301
80. Chang E, Chalikhonda S, Friedl J, Xu H, Phan GQ, Marincola FM et al (2005) Targeting vaccinia to solid tumors with local hyperthermia. *Hum Gene Ther* 16:435–444
81. Foloppe J, Kintz J, Futin N, Findeli A, Cordier P, Schlesinger Y et al (2008) Targeted delivery of a suicide gene to human colorectal tumors by a conditionally replicating vaccinia virus. *Gene Ther* 15:1361–1371
82. Brader P, Kelly KJ, Chen N, Yu YA, Zhang Q, Zanzonico P et al (2009) Imaging a genetically engineered oncolytic vaccinia virus (GLV-1h99) using a human norepinephrine transporter reporter gene. *Clin Cancer Res* 15:3791–3801
83. Peerlinck I, Merron A, Baril P, Conchon S, Martin-Duque P, Hindorf C et al (2009) Targeted radionuclide therapy using a Wnt-targeted replicating adenovirus encoding the Na/I symporter. *Clin Cancer Res* 15:6595–6601
84. Msaouel P, Iankov ID, Allen C, Aderca I, Federspiel MJ, Tindall DJ et al (2009) Noninvasive imaging and radiovirotherapy of prostate cancer using an oncolytic measles virus expressing the sodium iodide symporter. *Mol Ther* 17:2041–2048
85. Adams JY, Johnson M, Sato M, Berger F, Gambhir SS, Carey M et al (2002) Visualization of advanced human prostate cancer lesions in living mice by a targeted gene transfer vector and optical imaging. *Nat Med* 8:891–896
86. Rubinchik S, Wang D, Yu H, Fan F, Luo M, Norris JS et al (2001) A complex adenovirus vector that delivers FASL-GFP with combined prostate-specific and tetracycline-regulated expression. *Mol Ther* 4:416–426
87. Sato M, Johnson M, Zhang L, Gambhir SS, Carey M, Wu L (2005) Functionality of androgen receptor-based gene expression imaging in hormone refractory prostate cancer. *Clin Cancer Res* 11:3743–3749