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Viral-based gene delivery and regulated gene expression for targeted cancer therapy

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Importance of the field: Cancer is both a major health concern and a care-cost issue in the US and the rest of the world. It is estimated that there will be a total of 1,479,350 new cancer cases and 562,340 cancer deaths in 2009 within the US alone. One of the major obstacles in cancer therapy is the ability to target specifically cancer cells. Most existing chemotherapies and other routine therapies (such as radiation therapy and hormonal manipulation) use indiscriminate approaches in which both cancer cells and non-cancerous surrounding cells are treated equally by the toxic treatment. As a result, either the cancer cell escapes the toxic dosage necessary for cell death and consequently resumes replication, or an adequate lethal dose that kills the cancer cell also causes the cancer patient to perish. Owing to this dilemma, cancer- or organ/tissue-specific targeting is greatly desired for effective cancer treatment and the reduction of side effect cytotoxicity within the patient.
Areas covered in this review: In this review, the strategies of targeted cancer therapy are discussed, with an emphasis on viral-based gene delivery and regulated gene expression.
What the reader will gain: Numerous approaches and updates in this field are presented for several common cancer types.
Take home message: A summary of existing challenges and future directions is also included.

Keywords: gene delivery, gene-directed enzyme prodrug therapy, tissue-specific promoter, tumor-specific targeting, viral-based gene therapy, virotherapy

Expert Opin. Drug Deliv. (2010) 7(1):19-35

1. Introduction

It is estimated that there will be a total of 1,479,350 new cancer cases and 562,340 deaths from cancer in 2009 in the US [1]. The therapeutic index from current mainstays in cancer treatment, including chemotherapy, radiation therapy and hormonal manipulation, is limited by lack of efficacy, tumor cell resistance, and toxicity. The key issue to improving the cancer treatment is to perfect the existing targeted cancer therapy strategy, that is, to achieve accurate and effective cytotoxic drug delivery that renders powerful killing specific to tumor cells.

In the line of conventional drug delivery, gene delivery (or gene therapy) is the delivery of a therapeutic gene, the 'drug' that works at the genetic level, into patients' diseased cells for the purpose of therapy. This direct transfer of DNA can be achieved by either non-viral or viral-based gene delivery. Non-viral gene delivery includes the use of liposomes, cationic polymers, and disruption of the cell membrane by physical methods such as electroporation [2]. In addition, modified bacterium *Salmonella typhimurium* has been reported to express prodrug-activation genes and have resultant antitumor effects [3]. Non-viral vectors are the major means and strategies for gene vaccine therapy and are used widely in *ex vivo* gene delivery, the transfer of

10.1517/17425240903419608 © 2010 Informa UK Ltd ISSN 1742-5247

Article highlights.

- The importance of cancer-specific targeting and advantage of viral vectors for gene delivery.
- Pros and cons of commonly used viral vectors.
- Comparison on the transgene specificity and activity by different delivery routes.
- Regulated gene expression by tissue-specific promoters.
- Tissue- and tumor-specific GDEPT strategies.
- Tumor-targeting and organ-specific oncolytic virotherapy.
- Summary, existing challenges and future directions in this field.

This box summarises key points contained in the article.

genes into cells growing outside the body in tissue culture. By contrast, viral-based gene delivery utilizes a genetically modified, replication-defective virus as the gene transfer vehicle to deliver the therapeutic transgene directly into the diseased cells. Viral-based gene delivery in general has a much higher gene transfer efficiency *in vivo* compared with non-viral gene therapy, so it is the preferable choice of gene delivery method for the *in vivo* therapeutic approach.

2. Choice of viral vectors for gene expression in cancer cells

With the advances of *in vivo* gene delivery and regulated gene expression technology, gene therapy has quickly emerged as an effective strategy for cancer treatment, especially for the localized disease. Viral vectors have emerged as the major means for *in vivo* gene delivery because of their superior *in vivo* transduction rate over non-viral vectors. As the gene is delivered directly to the defective cells *in situ*, the therapeutic strategies have expanded to include corrective gene therapy, suicide gene therapy, immune gene therapy, and oncolytic virotherapy. The commonly used viral vectors for gene therapy include retrovirus, adenovirus, vaccinia virus, adeno-associated virus and herpes simplex virus. The rapid advance in lentivirus and its abilities to transduce efficiently non-dividing cells and stably integrate in the host genome make it a promising newcomer as a gene transfer vector. In addition, some other viruses such as sendal virus [4] and reovirus [5,6] have also been added to the therapeutic viral vector list owing to their antitumor immunogenic and oncolytic abilities, respectively. The details of biology and infection mechanism of each viral vector can be referred to [7].

Each of these viral vectors has advantages and disadvantages (**Table 1**). Some viruses (i.e., adenovirus and vaccinia virus) can infect non-dividing cells but cannot integrate into the host cell chromosome, thus its mediated expression is not sustained; conversely, some viruses (i.e., retrovirus) can integrate into the host chromosome and sustain expression but cannot infect non-dividing cells; whereas some viruses (i.e., adeno-associated virus and lentivirus) can do both – infect non-dividing cells and integrate into the host genome in order to sustain the expression – but they may possess other drawbacks (discussed below). Some viruses (i.e., adenovirus and retrovirus) can infect a wide variety of cell types, whereas some viruses have certain organ/tissue tropism (i.e., herpes simplex virus has a neuronal tropism). Some viruses (i.e., adenovirus, vaccinia virus and herpes simplex virus) can accommodate a large inserted size of foreign DNA (transgene expression cassette, etc.), whereas some (retrovirus and adeno-associated virus) have small capacity of inserted foreign DNA. Some viruses (i.e., adenovirus and vaccinia virus) cause high host immune response, whereas some (i.e., adeno-associated virus) have a low level of immunogenicity. Some viruses (i.e., adenovirus and lentivirus) have much higher *in vivo* transduction efficiency than others (i.e., retrovirus). Moreover, some viruses have high-dose-related toxicity (i.e., herpes simplex virus) or require a helper virus to produce an infection (i.e., adeno-associated virus), and some (i.e., lentivirus, because of its derivative nature from HIV-1) raise concerns and have unresolved issues for clinical trials at the present time [8].

The choice of viral vector depends on the characteristics of the cancer type, therapeutic strategy, and certain viral tissue tropism. For example, prostate cancer is a relatively slow-growing cancer. In a rat model, the prostate cells have a usual doubling time of > 150days and there are < 5% of cells actively dividing at any one time [9]. Therefore, an ideal gene transfer vehicle would be a viral vector that is able to transduce cells in high efficiency and independent of cell division. Adenoviral vector is a suitable vector for this purpose; because of its higher *in vivo* transduction rate and ability to infect non-dividing cells, adenoviral vector has an edge over the retroviral vector for *in vivo* prostate cancer gene therapy. However, retrovirus may have the advantage when the *ex vivo* approach (such as gene vaccine therapy) is applied owing to its sustained expression. On the other hand, most bladder tumors lack the coxsackievirus and adenovirus receptor (CAR), which is important for binding and internalizing the adenovirus [10]. Consequently, adenoviral vector may not be the ideal gene transfer vehicle for bladder tumor without other helper means, such as viral retargeting [11] and chemical treatment [12]. A comparative study of gene delivery efficiency on normal versus tumor lung cells demonstrated that lentivirus pseudotyped with the vesicular stomatitis virus (VSV) glycoprotein was the most efficient gene transfer method for normal mouse airway epithelial cells, whereas adeno-associated virus type 6 was the most efficient for MLE-12 adenocarcinoma cells (a murine lung SV40 transformed cell line) [13]. These results imply that various viral vectors have not only different infection preference for different cell types, but also discrimination among normal versus tumor cells with the same cell type. In general, but not exclusively, retrovirus are commonly used for *ex vivo* gene therapy on hematopoietic cells (such as leukemia) or immunotherapy (vaccine), and

Table 1. Commonly used viral vectors for cancer gene therapy.

Virus	Advantage	Disadvantage	Organ tropism
Adenovirus	Infects non-dividing cells, large size transgene, high *in vivo* transduction efficiency	Transcient transgene expression, high immune response	A wide range of cell types containing CAR receptor
Retrovirus	Stable transgene expression	Cannot infect non-dividing cells, low *in vivo* transduction efficiency, small capacity for transgene size	Hematopoietic cells, etc
Herps simplex virus	Large size transgene, cell-type tropism	High-dose-related toxicity	Neuron, brain
Adeno-associated virus	Low immunogenicity, stable transgene expression	Requires helper virus in viral production, small capacity for transgene size	Muscle, liver, etc
Vaccina virus	Infects non-dividing cells, large size transgene	High immune response	Various organs/tissues
Lentivirus	High *in vivo* transduction efficiency, stable transgene expression	Some unresolved issues for clinical trial	A large variety of cell types

adenovirus are preferred for *in vivo* gene therapy for a variety of cancers. Use of adeno-associated virus is intended more for applications involving muscle and liver, whereas herpes simplex virus is preferred for targeting neuron cells and brain tumors.

3. Study for the best gene delivery route to the target organ

One of the limiting factors in targeted gene therapy is the lack of a suitable delivery system/route to carry the therapeutic genes to the target tissue efficiently and safely. Theoretically, metastatic disease may be treated only by the systemic delivery of gene vectors. However, for locally advanced prostate cancer, variations in delivery strategy may help to target gene therapy vectors to the prostate. The authors have conducted a comparison study for the effectiveness of gene transfer by adenoviral vectors to the prostate by three different delivery routes in a canine model. An adenoviral vector, AdRSVlacZ, was constructed that expresses bacterial *Escherichia coli* β-galactosidase (*lacZ*) reporter gene under the control of a constitutively active Rous sarcoma virus (RSV) promoter. AdRSVlacZ was administrated into the dogs by intravenous (i.v.), intra-arterial (i.a.) (inferior vesical/prostatic artery) and intraprostatic (i.p.) injections. Seventy-two hours after injections, the expression of the *lacZ* gene was measured in canine prostate by X-gal staining of tissue cryosections and β-galactosidase enzymatic activity of tissue extracts. There was no detectable expression of *lacZ* in the prostate following i.v. injection (**Figure 1A**); low expression of *lacZ* (< 5% of cells) in the prostate by i.a. injection (**Figure 1B**); and higher expression of *(30% of cells) in the prostate by i.p. injection (***Figure 1C***). The enlarged image of* **Figure 1C** *(***Figure 1D***) demonstrates a nuclear localization of blue color, confirming that the detected lacZ* expression was a consequence of viral transduction of AdRSVlacZ because the designed AdRSVlacZ has a nuclear localization signal for *lacZ* transgene, which differentiates any potential endogenous cytoplasmic expression of β-galactosidase from some organs. Prostate extracts were also isolated and subjected to a colormetric β-gal assay to measure the level of β-gal enzymatic activity. The i.p. injection resulted in a 7.5-fold and 2.4-fold higher β-gal activity than either i.v. or i.a. injection, respectively (**Figure 1E**). These data together demonstrated that i.p. injection resulted in greatest expression of *lacZ* transgene among three different delivery routes.

The distribution of the disseminated adenoviral vector was also examined by PCR of genomic DNA extracted from various tissues and organs using primers specific for adenoviral genome. To increase the sensitivity of PCR analysis, the PCR products on the agarose gel were converted to a Southern blot hybridization. Interestingly, prostates from dogs that had i.v. and i.a. injections had an 860-bp adenoviral signal band on PCR Southern, which was not seen in agarose gel stained with ethidium bromide (not shown). Moreover, the autoradiographic signal was less intense in prostates from dogs that underwent i.v. injection than those that underwent i.a. injection. Both signals, however, were very weak when compared with that of i.p. injection (**Figure 2**). These results suggest that transduction of prostate cells with adenovirus was greatest by i.p. injection, followed by i.a. injection, and then i.v. injection. Dissemination to other organs was common regardless of the route of administration. The i.v. injection resulted in adenovirus infection to the lung and external iliac artery. The i.a. route resulted in virus dissemination to multiple organs: these

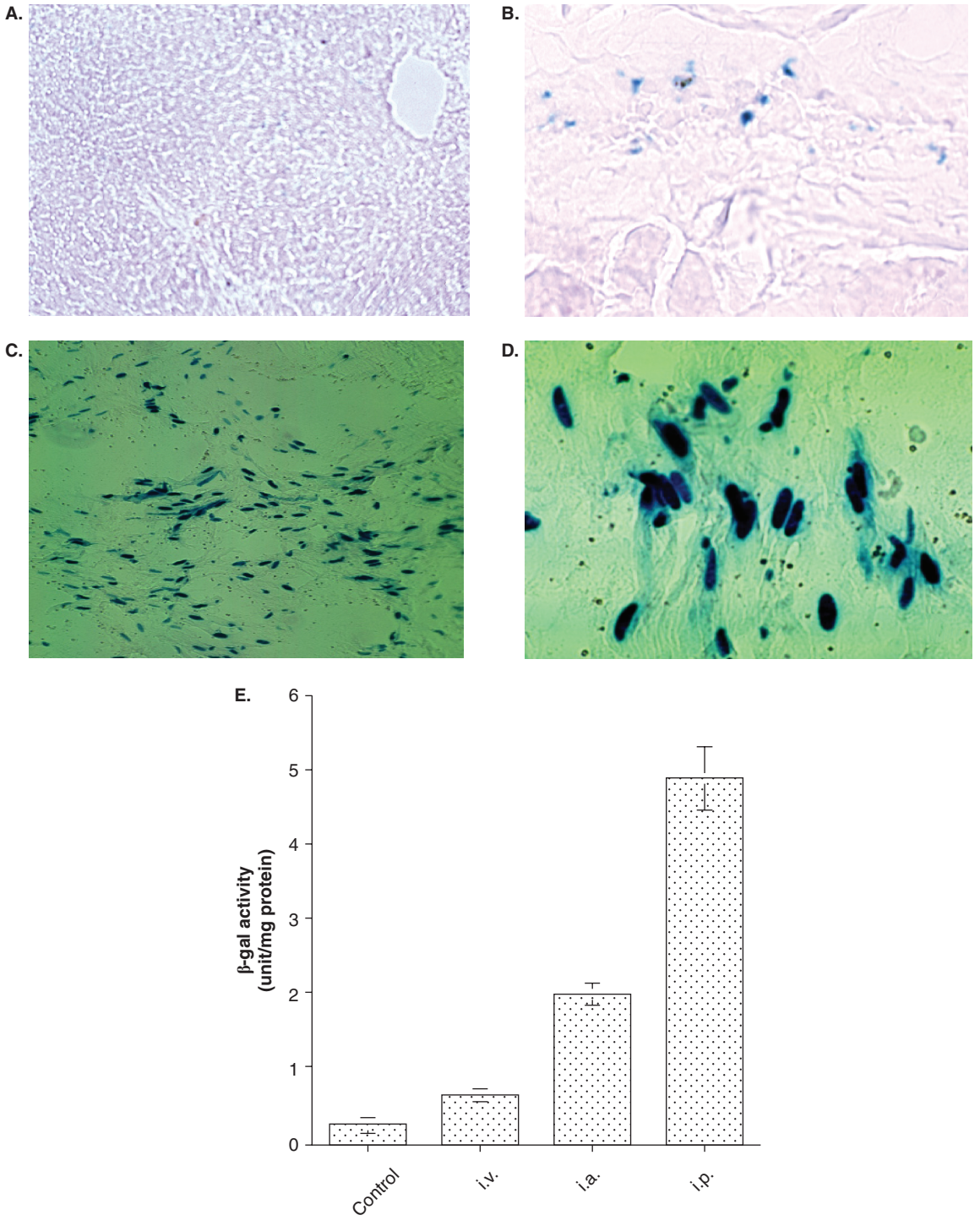

Figure 1. Expression of transgene *lacZ* in canine prostate. Dogs were injected with AdRSVlacZ (4.8×10^9 plaque-forming units per dog), and prostate tissues were collected at 72h. **A – D** are X-gal staining of dog prostate cryosections from (**A**) i.v. injection, (**B**) i.a. injection and (**C**) i.p. injection. Original magnification, ×33. **D.** A high-power image of prostate injected by i.p. Original magnification, ×132. **E.** The enzymatic activity of β-galactosidase in dog prostates by various delivery routes.

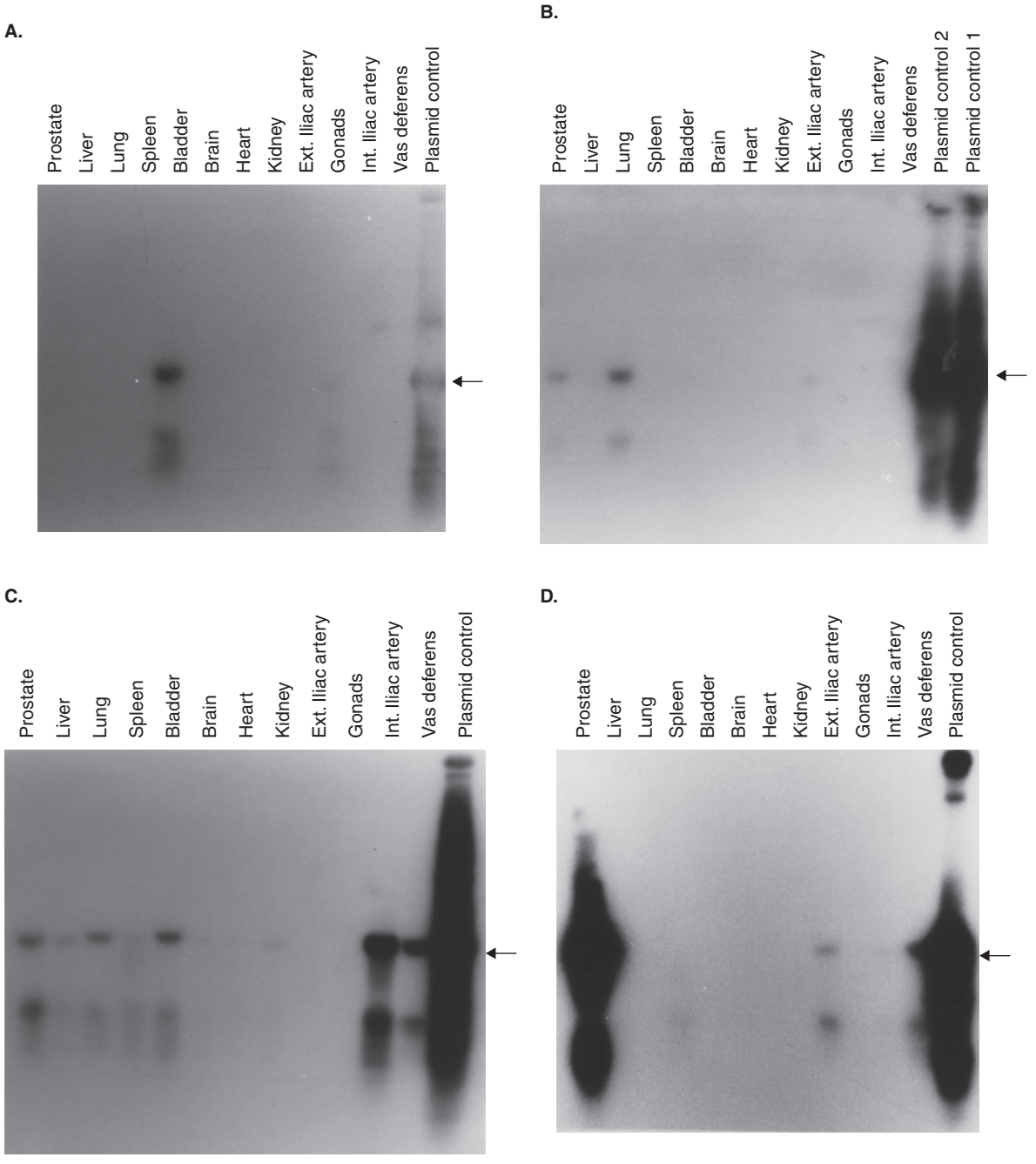

Figure 2. PCR Southern blots for detection of adenoviral vectors in various tissues. PCR were performed on genomic DNA isolated from various dog tissues using primers specific for adenoviral genome which showed a 860-bp signal band of the expected PCR product. The PCR electrophoretic gel was transferred to a Nylon membrane by Southern blot, and the blot was hybridized with ^{32}P-labeled 860-bp PCR product from control plasmid. Arrows show the 860-bp PCR signal band. Shown are the PCR Southern blots of control (**A**), AdRSVlacZ by i.v. injection (**B**), AdRSVlacZ by i.a. injection (**C**), and AdRSVlacZ by i.p. injection (**D**). To demonstrate the integrity of genomic DNA and a comparable amount of DNA was used for PCR from various organs, the same amount of DNA used for PCR of adenoviral genome was amplified for house-keeping gene β-actin as a control. A 314-bp signal band of β-actin PCR product was observed in all organs at an equal levels (not shown).

were represented by stronger signals in the lung, bladder, vas deferens and internal iliac artery (the site where AdRSVlacZ was injected), and weaker bands in liver, spleen and kidney. By contrast, following i.p. injection, most adenovirus was seen in the prostate, with only a minor spread of virus, as evidenced by weak bands, to the vas deferens and external iliac artery (**Figure 2**). This study also confirms that adenoviral dissemination does occur and the pattern of dissemination is consistent, with those organs receiving a high percentage of the cardiac output more likely to be infected by adenovirus, such as the lung, liver, kidney, spleen and endothelium (artery). Interestingly, PCR Southern also showed a band in the DNA extracted from the bladder of untreated control dog (**Figure 2A**). One explanation is that this dog may have been exposed previously to adenovirus. Although the i.v. viral delivery in the authors' experiment only gave lung dissemination, not liver dissemination (**Figure 2B**), several studies by others have shown that i.v. injection of adenovirus showed dissemination of the virus primarily to the lung and liver in mouse model [14,15]. The study showed that intraprostatic injection of adenoviral vector resulted in a greater transduction rate and expression level of *lacZ* in the prostate than either i.v. or i.a. injection. Thus, intraprostatic (or intratumoral) injection seems to be the best route to treat local regional prostate cancer by viral-based gene therapy. It is not surprising, therefore, that most gene therapy clinical trials for patients with prostate cancer have administrated vectors into the prostates/tumors [16-24].

With a similar principle, a comparison study on the route of vector transfer (direct injection, systemic, intraperitoneal, gastric serosal surface and oral administration) for the efficacy and safety in gene therapy against gastric cancer was discussed, and stomach local injection appears to be best delivery route [25]. Likewise, the local delivery of viral vectors remains the main approach to most solid tumors, with special consideration in each case. For example, for gene delivery to the bladder, the glycosaminoglycan layer on the surface of the bladder urothelium, which acts as a nonspecific antiadherence barrier, needs to be 'softened' to allow an enhanced viral infection of bladder urothelium. The pretreatment of the bladder with chemical agents dodecyl-β-D-maltoside or sodium dodecyl sulfate before intravesicular adenoviral injection resulted in > 90% transduction of the urothelial layer compared with < 5% transduction in untreated bladders [12].

For highly infiltrative tumors such as glioma, transgene delivery remains a challenge. Indeed, viral vehicles tested in current clinical trials often target only those tumor cells that are adjacent to the injection site (such as prostate cancer). In a recent study that examined feasibility of using human mesenchymal stem cells (hMSC) to deliver a therapeutic adenovirus in a mouse model of intracranial malignant glioma, hMSC was shown to be able to migrate and effectively deliver adenovirus to distant glioma cells. When injected away from the tumor site *in vivo*, virus-loaded hMSC migrated to the tumor and delivered 46-fold more viral copies than injection of the same adenovirus alone [26]. This study implies that the hMSC approach may improve the therapeutic viral vector delivery for glioma gene therapy.

4. Tissue/organ-specific regulated gene expression

The early viral-based vectors for gene therapy are mostly tissue nonspecific because of constitutively active viral promoters such as RSV and cytomegalovirus (CMV). Consequently, the expression of the delivered therapeutic gene may occur in unintended, non-targeted tissues that may potentially cause systemic toxic effects, especially if a toxic gene is the expressed gene. As it was shown in the previous section that even local prostatic injection of gene expression vectors might lead to an unwanted viral dissemination to other organs and tissues (**Figure 1**), the systemic therapy would require a tissue- or tumor-specific targeting or regulated gene expression in order to minimize side effects from systemic toxicity. Various approaches have been developed to try to permit the exclusive expression of the therapeutic gene in the target organ or cell type. For example, prostate-specific promoters have been used in adenoviral-mediated toxic gene therapy for prostate cancer treatment. Several prostate tissue-specific promoters have been defined over the years, which include promoters of prostate-specific antigen (PSA) [27,28], probasin (PB) [29,30], mouse mammary tumor virus (MMTV LTR) [27], prostate-specific membrane antigen (PSMA) [31], human glandular kallikrein-2 (hK2) [32,33] and prostatic steroid-binding protein C3 [34,35]. Recently, the promoter of PCAN1, a newly discovered gene with remarkable prostate tissue specificity, has been demonstrated to be a promising candidate for regulating prostate-specific gene expression with high-level activity [36]. In addition, prostate cancer has a predictable spreading pattern as it always metastasizes to the bones; another tactical approach for targeting metastatic prostate cancer is to use the promoter of osteocalcin, a major non-collagenous bone protein that is coexpressed by prostate cancer and bone stroma [37,38].

Among these prostate-specific promoters, PSA, PB and MMTV LTR have been compared by the authors' group for their promoter activity and tissue specificity side-by-side. The promoters of PSA (-636/+8 bp), PB (-426/+28 bp) and Cla I truncated MMTV LTR (1.1 kb upstream) were fused separately to *E. coli lacZ* gene to generate type 5 adenoviral vectors (Ad-lacZ) containing the corresponding prostate-specific promoter, respectively (**Figure 3**). The activity and expression of reporter gene *lacZ* driven by these prostate-specific promoters were compared *in vivo* using a canine model by direct intraprostatic injection of these prostate-specific Ad-lacZ vectors, along with control viral vector AdRSVlacZ, where *lacZ* gene is under the control of a constitutively active RSV promoter. The prostate and various other organs were harvested at 72h post-injection. The PCR analysis of adenoviral sequences in various tissues indicated that, regardless of the promoter type, most adenoviral transduction occurred in the

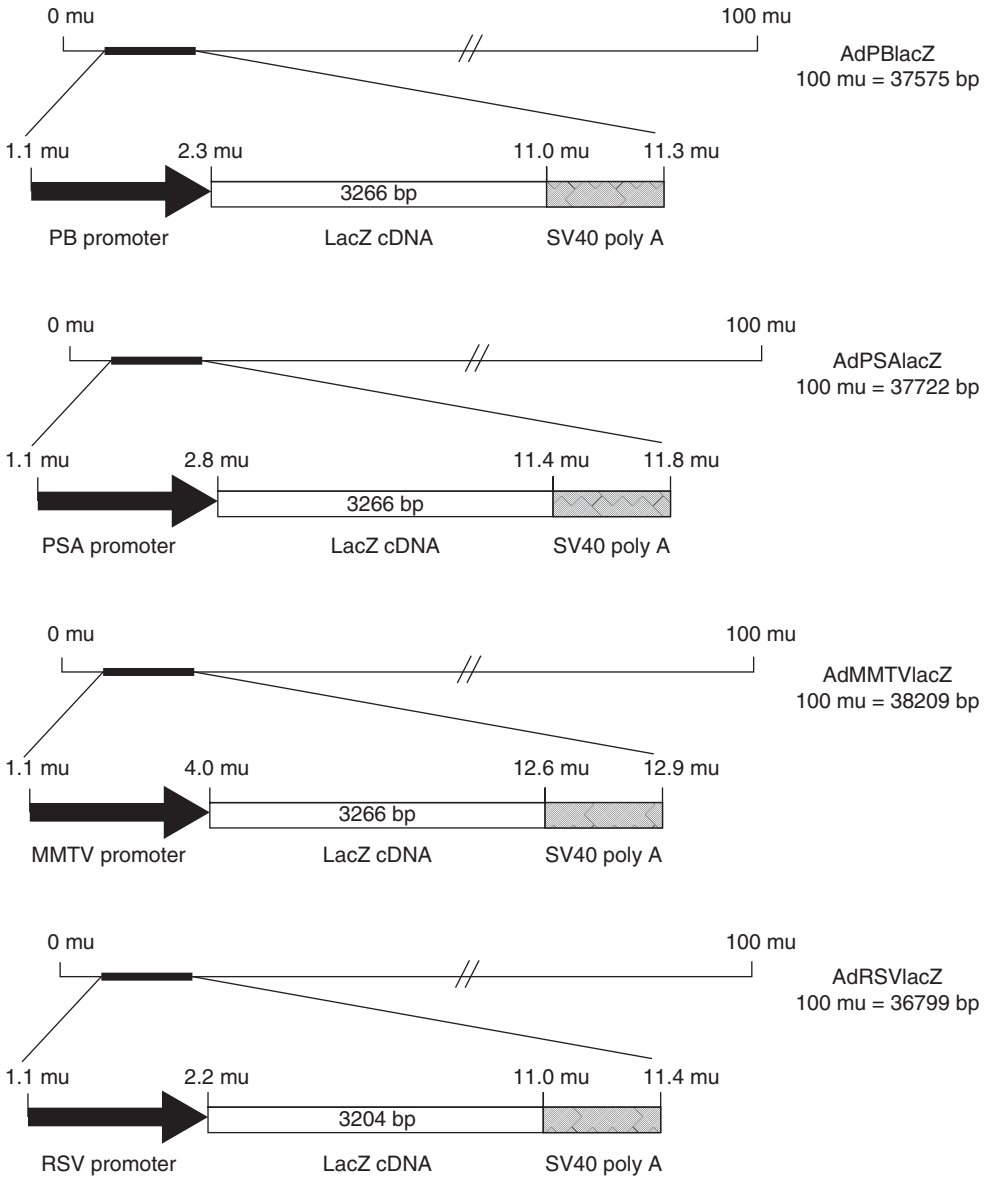

Figure 3. Schematic diagrams of various recombinant adenoviral vectors Ad-lacZ. The frame structures of adenoviral vectors expressing β-galactosidase (lacZ) under the control of prostate-specific promoters (AdPBlacZ, AdPSAlacZ and AdMMTVlacZ) and constitutive promoter (AdRSVlacZ) are shown.

prostate by all viruses. However, adenoviral sequences were found in other organs and tissues besides the prostate, primarily the bladder and vas deferens, and scarcely in heart, blood vessels (external iliac artery) and liver tissues [39]. To determine further whether transgene *lacZ* was expressed in tissues containing adenoviral DNA (an indication where Ad-lacZ has disseminated to), RNA was extracted from various canine tissues and subjected to RT-PCR analysis using a set of primers specific for *E. coli lacZ*. Southern hybridization of RT-PCR gel showed the 1036-bp signal band in the prostates by all Ad-LacZ vectors. However, only control viral vector AdRSVlacZ had *lacZ* mRNA expression in other tissues where adenoviral vectors had disseminated; this was expected as RSV has nonspecific promoter activity. Conversely, all three prostate-specific Ad-lacZs (AdPSAlacZ, AdPBlacZ and AdMMTVlacZ) had no expression of *lacZ* in the organs and tissues (except prostate) where the adenoviral sequences were detected, including bladder, vas deferens, bladder, heart and liver. Taken together, these results demonstrate that although prostate-specific adenoviral vectors do disseminate following intraprostatic injection, the adenoviral *lacZ* transgene was expressed only in the prostate [39]. Xenograft tumors grown in nude mice were also used to examine the

specificity of prostate-specific promoters. AdPSAlacZ or AdPBlacZ was injected into subcutaneous xenograft tumors derived from prostate cancer PPC-1 cells or bladder cancer RT4 cells. Both PPC-1 tumors transduced by AdPSAlacZ and AdPBlacZ showed X-gal-positive (blue stained) cells, whereas AdPSAlacZ and AdPBlacZ transduced RT4 tumors did not. By contrast, both PPC-1 and RT4 tumors transduced by AdRSVlacZ showed significantly higher X-gal-positive cells [40,41]. These results demonstrate that expression of the *lacZ* transgene driven by the prostate-specific promoters occurred only in xenograft prostate tumors, not in xenograft bladder tumors.

As a common observation, most tissue-specific promoters tend to have less activity compared with the constitutively active viral promoter such as CMV or RSV. For example, the activity of the PSA and PB promoter is much lower (at least 10-fold lower) than that of the constitutively active RSV promoter [41]. However, it has been argued that the relatively low degree of tissue-specific promoter activity may be sufficient for expression of a toxic transgene at a level that achieves a clinically significant cytoablative effect. Nevertheless, attempts to increase the activity of prostate-specific promoters have been made. A mutated PSA promoter (620-bp upstream) from a prostate cancer patient who had a high serum PSA was cloned and this promoter has a higher activity and specificity than the wild-type PSA promoter of the same length [28,42]. In another study, an 822-bp upstream regulatory sequence of the PSA promoter, referred to as prostate-specific enhancer (PSE), was cloned from the same patient's cancerous prostate tissue [43]. *In vitro*, this PSE increased PSA promoter activity 1000-fold, whereas PSE-isolated normal prostate tissue increased PSA promoter activity only 72-fold. It was speculated that the mutated promoter sequences from the cancer patient might have a higher binding affinity for the androgen receptor [43,44].

The thyroid transcription factor 1 (TTF1) and human surfactant protein A (hSPA) have been shown to be tissue-specific proteins that are expressed in normal lung respiratory epithelium [45], and the promoters of them have been used to target and regulate gene expression in lung [46]. Other identified tissue-specific promoters include human calcitonin gene (CALC-I) promoter for thyroid tissue [47,48], and murine muscle creatine kinase promoter for skeletal and cardiac muscle [49-51].

5. Gene-directed enzyme prodrug therapy

One approach effectively to kill the cancer cells with no or minimal damage to the host body is systemic delivery of the non-toxic prodrug and the local or targeted delivery of prodrug enzyme into the cancer cells. Gene-directed enzyme prodrug therapy (GDEPT), one of the suicide gene therapy strategies, catalyzes the prodrug into a cytotoxic metabolite or active drug by the product of the gene (prodrug enzyme) that has been introduced into the cancer cells by means of viral vectors. Consequently, the lethal activated drug kills not only the transduced cancer cells that produce the toxic drug, but also its neighboring untransduced cancer cells (the bystander effect) and even the distant cancer cells (the distant bystander effect, or the natural killer cell-mediated host immune responses induced by the dying cells in the region). There are several prodrug enzymes in the GDEPT system that are now being used, including herpes simplex virus-thymidine kinase (HSV-TK), cytosine deaminase (CD), purine nucleoside phosphorylase (PNP), xanthine guanine phosphoribosyltransferase (XGPRT), cytochrome P450 enzymes (2B1, 2C9, and 3A4), β-glucosidase, β-lactamase, β-glucuronidase, peroxidase and carboxylesterase (CES). The two most widely used GDEPT systems in cancer gene therapy are HSV-TK with prodrug ganciclovir (GCV) or its analogue acyclovir, and CD with prodrug 5-fluorocytosine (5-FC). HSV-TK enzyme converts inert prodrug GCV to ganciclovir monophosphate; normal cellular thymidine kinase further transforms ganciclovir monophosphate to ganciclovir triphosphate, resulting in DNA chain termination, inhibition of DNA replication and death of dividing cells. The HSV-TK enzyme is almost 1000-fold more efficient at monophosphorylating GCV than the cellular thymidine kinase [52]; thus, GCV is virtually non-toxic to uninfected cells at the therapeutic concentration of the drug (1 – 10 μM) [53]. The preclinical studies showed that the viral-mediated HSV-TK/GCV system significantly inhibited the growth of mouse and human cancer cells both *in vitro* and *in vivo* [54,55]. The limitation of the HSV-TK/GCV system is that it only selectively kills dividing cells. A relatively slow-growing cancer such as prostate cancer (only < 5% cell population at any one time is in the S phase) may require more effective GDEPT systems to improve the therapeutic index. Bacterial CD enzyme can deaminate the relatively non-toxic 5-FC to the highly toxic cytotoxic 5-fluorouracil (5-FU), the active anticancer drug whose nucleotide metabolites can affect both RNA and DNA metabolism by resulting in dysfunctional RNA and inhibiting DNA synthesis, thus causing cell death [56]. Another GDEPT system uses *E. coli* enzyme PNP to convert prodrug 6-methyl-9-(2 deoxy-β-D erythro-pentofuranosyl)purine (6 MPDR) into non-phosphorylated purine, a toxic compound that is capable of killing both quiescent and proliferating cells [57]. The carboxylesterase (CES) converts prodrug, 2′-ethylcarbonate-linked paclitaxel, into paclitaxel, one of the most important anticancer chemotherapy drugs. The potential of this relatively new GDEPT system is now being examined for its efficacy against ovarian cancer [58]. In another recent study, a new GDEPT system, which uses deoxycytidine kinase/uridine monophosphate kinase fusion gene (dCK/UMK) to convert gemcitabine to its active triphosphate form, demonstrated markedly improved therapeutic activity for pancreatic cancer in the animal model when combined with a small interference RNA silencing strategy against ribonucleotide reductase and thymidylate synthase [59].

The tissue-specific promoters have been combined with the GDEPT strategy to target organ/cancer killing. For example,

transfection of a 1648-bp PMSA enhancer/promoter-driven CD construct enhanced toxicity of 5-FC specifically in PMSA-expressing prostate cancer cells, whereas cells that did not express the PSMA were not significantly sensitized [60]. An inducible heat shock protein (hsp70) promoter was incorporated in an adenoviral vector to drive the CD/HSV-TK fusion gene to target localized expression of the therapeutic gene. Strong expression of the CD/HSV-TK fusion gene product was induced by heating at 41°C and significantly reduced the survival of PC-3 cells in the presence of both 5-FC and GCV [61]. Adenovirus carrying the CD/HSV-TK fusion gene was also used for combined gene therapy and radiation sensitization for prostate cancer [62]. Treatment of adenovirus containing PSA promoter-driven PNP and 6 MPDR prodrug significantly suppressed the growth of established PC-3 tumors and increased host survival [57]. Similarly, an efficacy study using an adenovirus containing PSA promoter and *HSV-TK* gene has shown effective inhibition of prostate cancer with prodrug acyclovir [63]. Moreover, by using an adenoviral vector composed of an *HSV-TK* gene driven by the osteocalcin promoter and acyclovir, growth of osteosarcoma was abolished [37,64]. Similarly, strategies using adenoviral-mediated expression of HSV-TK under control of a thyroglobulin promoter have been shown to be effective in animal models [47,48]. A recombinant adenovirus expressing HSV-TK driven by a human calcitonin gene promoter was used to drive therapeutic genes against thyroid cancer [48]. The combination of this adenovirus plus GCV treatment with administration of interleukin-12 (also under the control of the CALC-I promoter) resulted in effective growth suppression of tumor at the treated site and also at a distant untreated site. Moreover, intravenous injection of the virus in the animal model followed by administration of GCV did not cause evident toxicity after administration of GCV. These results indicate that this combined system can provide an effective therapy for metastatic medullary thyroid carcinoma with minimal toxicity [48].

Beside tissue-specific promoters, tumor-specific promoters can also be used to control the GDEPT toxicity specific to tumors. Carcinoembryonic antigen (CEA) promoter is active only in CEA-positive cells, an exclusive characteristic of vast tumor cells; the CEA promoter-controlled CD strategy confers toxic specificity for CEA-producing colorectal cancer cells while preserving activity [54]. Furthermore, by taking advantage of the knowledge that nuclear transcriptional factor NF-kB is often highly transcribed in cancer cells, the addition of NF-kB enhancer to the CEA promoter significantly enhanced the efficacy of its driven CD/5-FC system in colon cancer cells [65]. In a study to select the optimal promoter to drive CD in a GDEPT strategy against gastric cancer, promoters of SEL1L, mucin-1 and KRT19 displayed the highest activity levels among several genes that are significantly highly expressed in gastric tumors [66]. In another study, adenovirus-mediated expression of HSV-TK by an α-fetoprotein (AFP) enhancer/promoter element selectively eliminates AFP-positive gastric tumors when treated with ganciclovir [55]. High mobility group Box2 (HMGB2) promoter-controlled HSV-TK expression specifically induced cell death in glioblastoma cells in the presence of GCV with no damage to the normal brain cells [67].

Another approach used hypoxia- and radiation-activated Cre/loxP 'molecular switch' to turn on the otherwise muted *HSV-TK* gene for specific targeting of breast cancer and glioma cells under hypoxic conditions and/or radiation therapy [68]. Therefore, by combining tissue/tumor-specific promoters and GDEPT strategies as described above, the transcriptionally regulated, cancer-targeted gene therapy should provide an effective and specific therapy against cancer.

6. Tumor-specific targeting and cancer virotherapy

Most of the earlier gene therapy vectors were engineered in such a way that viral self-replication was prevented, so that they could be used as safe gene transfer vehicles to deliver therapeutic transgenes without exposing host cells to the danger of viral lysis. The *in vivo* transduction of these replication-defective viral vectors confines the transgene expression in the transduced cells along the injected needle track owing to their inability to pass the transgenes to the neighboring cell. While bystander effect in GDEPT strategy may increase some therapeutic index, the effect is limited and tumors cannot be eradicated.

Virotherapy is a bold yet logical strategy in which the conditional oncolytic virus is used for cancer gene therapy. As oncolytic virus replicates in the transduced cells and enters the lysis cycle (oncolytic killing), the lysed virus infects the neighboring cells and continues this cycle until all the cells are eliminated; theoretically, a major portion of the tumor can be effectively eradicated by a small dose of oncolytic virus. The key issue in using this strategy is the 'conditional' oncolytic virus, that is, the virus is altered to target specifically the desired cell type or attenuated in a way that the desired target cells are more highly sensitive (in several magnitude differences) to its oncolytic cell lysis than non-targeted cells. By taking advantage of prostate-specific promoters that drive transgene specifically in the prostate, an *Ad5 E1a* (adenoviral type 5 early E1a) gene, whose product allows adenovirus to replicate and to enter the lytic cycle, was reintroduced to E1a/E3-deleted adenovirus under the control of PSA enhancer/promoter (PSE). The resultant adenovirus, CN706, specifically replicates in and thus kills PSA-producing cells such as LNCaP, but not PSA-non-producing cells such as DU145 [69]. Likewise, adenoviral vector CN764, which contains PSE to drive the *Ad5 E1a* gene and the hK2 enhancer/promoter to drive the *Ad5 E1b* gene, significantly attenuates in the primary human microvascular endothelial cells. It has a high therapeutic index with a cell specificity of 10,000:1 for prostate cancer LNCaP cells, compared with ovarian cancer

OVCAR-3, SK-OV-3 and PA-1 cells [70]. In the study of another prostate-specific replication-competent adenovirus, a single tail vein injection of CV787, which contains PB promoter-driven *E1a* gene and PSE-driven *E1b* gene, eliminates distant LNCaP xenograft tumors [71]. This result indicates that CV787 may have the potential to be developed as a powerful therapeutic vector for metastatic prostate cancer. By a similar approach, a hepatoma-specific promoter of progression elevated gene-3 was used to drive E1a expression in a virotherapy against hepatoma [72].

In addition to a tissue-specific promoter-driven oncolytic approach, tumor-specific promoters including survivin, CEA and telomerase have also been used in regulated E1A expression in oncolytic adenoviral approach. Survivin overexpresses in several human tumors, including gliomas [73] and prostate cancer [74], therefore, survivin promoter is a good candidate for tumor-specific regulated E1a expression in gliomas [75,76], and both PSA-producing and non-producing prostate cancers. Likewise, a CEA promoter-driven E1a replication-competent adenovirus, OV798, preferentially replicates in and kills CEA-producing colorectal cancer cells, but its replication is attenuated 1000-fold in the CEA-negative cell lines [77]. The telomerase (hTR and hTERT) promoters are active in most cancer cells but not in normal cells, thus making the telomerase promoter-controlled E1a replication-competent adenovirus an attractive approach for tumor-specific oncolytic targeting [78]. Studies using telomerase-specific oncolytic viruses demonstrated their effective anti-tumor abilities both *in vitro* and *in vivo* [79-82]. In addition, the promoter of the CREBBP/EP300 inhibitory protein 1 (CRI1), a gene specifically expressed in malignant pleural mesothelioma, was used to drive E1a-mediated virotherapy that specifically kills malignant mesothelioma cells but not normal cells [83]. Other tumor-specific promoters used to drive E1-mediated virotherapy against various cancers include mucin-1 promoter [84], osteocalcin promoter [85], AFP promoter [55], midkine promoter [86] and COX-2 promoter [87,88]. In general, regulation of therapeutic transgene expression (such as E1a) by a tumor-specific promoter is preferred over tissue-specific promoter strategy for several reasons: i) not all tissue-specific promoters are available for every organ/tissue; ii) only a few of organs/tissues are dispensable, such as the prostate, while most serve critical life-sustaining functions; and iii) tumor-specific promoters that express only in cancer cells, not (or express at a much lower level) in normal cells, such as telomerase and survivin, provide an ideal tumor targeting with minimal toxicity and maximal anticancer effect.

Besides promoter-driven E1 expression-mediated virotherapy, strategies have also been developed in which the oncolytic adenoviruses specifically target cancer cells to lead a tumor cell-specific oncolysis. By taking advantage of the fact that most cancer cells have dysfunctional p53 owing to mutation or deletion of the *p53* gene, a mutant adenovirus that selectively kills p53-deficient cells has been designed to target cancer cells. p53 is a multiple-function transcriptional factor that plays a pivotal role in the regulation of several cellular functions, including DNA repair, growth arrest, apoptosis and tumorigenesis [89-94]. In addition, p53 also serves to protect the normal cell from viral replication if the cells should become infected by any of a number of viruses [95-97]. The wild-type adenovirus can inactivate this p53-mediated cell cycle arrest and protection from viral replication by binding p53 with one of its E1b proteins, E1b-55kD, and the complex subsequently leads to a ubiquitin-mediated proteolysis of p53 [98]. The viruses are therefore replicated in the cells and viral-mediated oncolysis would occur. However, ONYX-015, a mutant adenovirus lacking the *E1b-55kD* gene, would not be able to replicate in the p53-positive (functional) cells, as the E1b-55kD protein is necessary for inhibition of wild-type p53 function [95,96], including protection from viral replication [97], but selectively replicates the p53-deficient cells. As > 50% of human cancers are p53 dysfunctional/deficient [94], ONYX-015 is thought to have a great potential to specifically target cancer cells. The clinical trial Phase I and II of ONYX-015 showed no adverse effect and an encouraging initial response in terms of tumor size reduction in patients with head and neck tumors and sarcomas after intratumoral injection of ONYX-015 and combined systemic chemotherapy [99,100,56].

A comparative study was conducted to analyze the transgene delivery and expression in tumors between an oncolytic (replication-conditional) HSV expressing *lacZ* transgene and its replication-defective counterpart. By direct inoculation of similar size subcutaneous human xenograft tumors with the same viral dose, *lacZ* expression was observed in ~ 40% of the tumor area 3, 7 and 14days after injection of the oncolytic vector, whereas only 10% of the tumor area expressed the *lacZ* transgene 3days after injection of the replication-defective vector, with a rapid decline in expression thereafter [101]. This result indicates that the oncolytic virus gave much higher *in vivo* transgene delivery than its replication-defective counterpart; however, it is not a 100% transduction as theoretically expected, implying that other factors such as immune response may a play role in preventing the further transgene delivery and expression mediated by oncolytic virus.

In addition to transcriptional regulated gene expression, tumor-specific targeting can also be regulated at the post-translational level. One general characteristic of fast-growing solid tumors is the development of intratumoral hypoxia, whose existence correlates to a more malignant tumor phenotype and worse diagnosis. Adaptation to the hypoxic environment is critical for tumor cell survival and growth. The hypoxic cells in tumors modify gene expression in order to obtain a blood supply and prevent cellular damage; the main mediator of the hypoxia response is hypoxia-inducible factor-1, or HIF-1. HIF-1 is composed of an inducible subunit, HIF-1α and a constitutively expressed subunit, HIF-1β [102,103]. HIF-1 is an important transcriptional factor

that stimulates a group of downstream genes for tumor progression and metastasis [104-106]. HIF-1α is inducible and stabilized under hypoxic conditions [61]; however, under normoxic conditions, HIF-1α is degraded by the 26S proteasome as a result of oxygen-dependent prolyl hydroxylation and subsequent ubiquitination of the oxygen-dependent degradation (ODD) domain [107] that locates in the C-terminal half of HIF-1α protein. By taking advantage of this unique property of the ODD domain, which stabilizes the protein fused to ODD only in hypoxic conditions, ODD fused cytotoxic genes can be used specifically to target the hypoxic area, a major feature of most fast-growing tumors. To facilitate the *in vivo* delivery of ODD-fusion protein, a protein transduction domain of the transcriptional activator of transcription (Tat) of HIV-1 [108,109] was used. Tat is one of the protein transduction domain (PTD) family members that possess the unique capability to penetrate cells and transduce biologically active macromolecules into living cells [110,111]. The Tat-ODD peptide can be fused to a proapoptotic protein for hypoxic targeting [112-114]. A precursor of caspase-3, procaspase-3, was chosen to result in the fusion protein Tat-ODD-Procaspase-3 (TOP3) [112]. The systemic administration with TOP3 was tested for its antitumor effect as well as any potential side effects in the well-oxygenated normal tissues. The intraperitoneal injection of TOP3 reduced the tumor mass and suppressed tumor growth without any obvious side effects in the animal model, resulting in a significant increase in the lifespan of animals with malignant ascites [115].

One of the determining factors for cell-type specificity of viruses is the distribution of the specific cellular receptors for the virus. For example, cell-surface CAR receptor is important for binding and internalizing the adenovirus into the cells. Thus, specific targeting of tumor cells can also be achieved by means of viral retargeting using tumor-specific characteristics. By taking advantage of the knowledge that i) bladder cells express low or no CAR receptor for adenovirus; and ii) epidermal growth factor receptor (EGFR) is frequently overexpressed in bladder cancer, but has only low expression in normal urothelium basal layers [116,117], a bispecific single chain Fv fragment recognizing both EGFR and the adenoviral fiber has been constructed [118]. By premixing the bispecific single chain Fv fragments with the Ad5-d55K virus, a replication-competent attenuated adenovirus (lacking the *E1b-55kD* gene, an analogue of ONYX-015) that specifically replicates in p53-deficient cells, the viral transduction strategy was shown to retarget and replicate in bladder cancer cells that lack p53 expression but not in normal cells expressing p53 [11]. By a similar principle, EGF-polymer coated wild-type adenovirus has been used for retargeting ovarian cancer for virotherapy [119].

Other oncolytic viral vectors have also been developed to specifically target tumor cells with no or minimal toxicity to the proximal normal cells. A multi-mutated herpes simplex virus, G207, that was genetically modified and has deletion of both *ICP34.5* genes and an insertion inactivation of the *ICP6* gene, permits replication within cancer cells but limits replication in normal cells [120]. One injection of G207 significantly inhibited growth of malignant brain tumors in mouse model without harming surrounding normal brain cells [121]. In another study, intravenous injection of G207 suppressed growth of distant subcutaneous prostate tumor in mice [122]. Replication-competent retrovirus (RCR) has recently emerged as another promising approach for cancer oncolytic virotherapy. RCR vectors based on murine leukemia virus show intrinsic tumor selectivity owing to its inability to infect quiescent cells; it can achieve highly selective and stable gene transfer throughout entire solid tumors *in vivo* at extremely high efficiency by low-dose viral inoculation [123]. In addition, replication-selective oncolytic vaccinia virus and herpes simplex virus have also been designed to target ovarian cancer [124] and HER2-positive cells (i.e., breast cancer and ovarian cancer) [125], respectively. Oncolytic measles has also been attempted in virotherapy for cancer [126]. Recently, a wild-type common-cold-producing enterovirus, coxsackievirus A21 (CVA21), has been tested for its therapeutic efficacy as an effective oncolytic agent against human breast cancer. CVA21 specifically targets and lyses cells expressing the CVA21 cellular receptors, intercellular adhesion molecule-1 (ICAM-1) and/or decay-accelerating factor, both of which are significantly overexpressed in melanoma and breast cancer cells. A single intravenous injection of CVA21 produced significant regression of pre-established breast tumors *in vivo*, as well as targeting and elimination of metastases in the orthotopic model [127]. Another recent study examined the efficacy of oncolytic rat parvovirus H-1PV on treatment of human lymphoma [128].

Noticeably, microRNA (miRNA) technology has emerged as a powerful tool for selective cancer targeting. For example, by taking advantage of the fact that mir-143 and mir-145 are highly expressed in normal tissues but are significantly down-regulated in prostate cancer cells, an oncolytic HSV-1, which incorporated multiple copies of miRNA complementary target sequences for mir-143 or mir-145 into the 3′-untranslated region (3′-UTR) of an HSV-1 essential viral gene, *ICP4*, was generated. A recent study demonstrated that these miRNA-regulated oncolytic viruses have a selective killing to prostate cancer cells both *in vitro* and *in vivo* while retaining significantly attenuated virulence to normal tissues [129]. Furthermore, the combination of replication-competent virus and GDEPT strategy [124,130-133], or tumor-specific replication-competent with another therapeutic gene (so-called 'double targeting virus–dual gene therapy') [134-136], demonstrated great potential for the optimal systemic therapy targeting for cancer. The latter strategy uses two genes with compensative or synergistic effect in one oncolytic virus to maximize the antitumor effect. Examples include using oncolytic virus to deliver apoptosis-inducing Trail gene and short hairpin RNA to knock down apoptosis-inhibiting protein XIAP in the hepatocellular carcinoma [136].

7. Expert opinion

With increased human life expectancy resulting from decreased causation of other major disease deaths, such as cardiovascular diseases, cancer incidence will continue to increase and remain as one of the most devastating killers worldwide. One of the major causes of failure in the treatment of cancer is the occurrence of metastasis. The major challenge in viral-based targeted cancer therapy is therefore the systemic delivery and targeting of metastatic cancer cells. The current gene therapy strategies are not effective in eradicating distant cancer cells. For example, in prostate cancer, most current *in vivo* gene therapy uses intraprostatic injection of viral vectors because it is the most efficient delivery route at present to target primary prostate cancer. However, this approach is unable to target the distant cancer cells. The dilemma is that we cannot deliver the viral vectors systemically without strong consideration for the safety of the patient, because nonspecific viral promoter may cause tissue damage in undesired organs and tissues. Therefore, the best strategy to eliminate distant metastatic cancer is the combination of tumor (or cell type)-specific targeting virus and a systemic delivery manner.

Targeting can be achieved at the level of viral capsid binding or at the transcriptional regulation by the use of tissue-specific promoters. Still, targeting at the level of binding is preferred because: i) therapeutic viral vectors will not be diluted during delivery; ii) the interaction between non-targeted cells (especially those involved in the hepatic trapping and clearance path) and viral capsids will be avoided or reduced, which may lead to toxic effects; and iii) there will be less viral vector uptake by the reticuloendothelial system. The specificity of virus to target the specific organ or cancer cells can be achieved by tissue/cell-specific targeting at the delivery level. By modifying the adenoviral fiber protein and adding cell-specific ligand, the adenoviral vectors are able to change its cellular/tissue tropism and commit desired cell-specific gene delivery [137,138]. Other developed approaches to the targeted delivery include genetic or chemical modification of the adenovirus capsid and use of molecular adaptors to target/re-target specific cells or tissues [11,139-141]. Viral vectors targeting cancer cell-specific surface components may hold promise for targeting metastatic cancer cells. Although many obstacles that limit the effectiveness of the systemic delivery approach remain to be solved, it is conceivable that a new generation of tumor-selective oncolytic virus or cancer-targeted GDEPT virus, either by targeting cancer cells via a tissue- or tumor-specific receptor or cell-surface antigen [142] or under transcriptional control by a tissue- or tumor-specific promoter, will emerge in the future and lead to effective targeting of distant metastatic cancer cells by means of systemic delivery. Notably, with the recent understanding of hypoxic and necrotic regions within solid tumors and rapid development of recombinant DNA technology, anaerobic bacteria are being used as new gene delivery systems for targeted cancer therapy. These bacterial vectors have unique advantages over other existing delivery systems and may become a major vector of choice for future cancer gene therapy [143]. On the flip side, the safety concern of systemic delivery of replication-competent virus requires no or minimal toxicity to vulnerable organs/tissues (such as liver). A recent study using a modified wild-type adenovirus, which contained binding sites for hepatocyte-selective microRNA mir-122 within the 3′ UTR of the E1a transcription cassette, showed that the inclusion of mir-122 binding sites led to 80-fold decreased hepatic expression of E1a following intravenous viral delivery to mice and a 50-fold decreased viral replication and virtually no liver toxicity, whereas the virus retained full oncolytic activity within cancer cells [144].

An important and yet unresolved issue in viral-based gene therapy is the host immune response against the viral-mediated gene transfer, especially when adenoviral vectors are used. Although the repeated administration of viral vectors is required in most cases in order to achieve a successful therapeutic effect, the host immune response can significantly hamper the repeated viral-mediated gene transfer. Several strategies have been attempted to overcome this problem, including: i) modification of the virus (such as deleting some viral genetic components that are most antigenic) to decrease its immunogenicity [145]; and ii) suppression of the host immune response by coadministering an immune-suppressing agent to induce tolerance to viral antigens [146] or abrogation of the T-cell function with antibodies [147]. Owing to the scope/focus and space limitations, clinical trials are not provided in this review. Details of current active cancer clinical trials can be accessed at www.clinicaltrials.gov.

In summary, the success of targeted cancer therapy demands a maximum therapeutic efficacy at the target site with no or minimal toxicity to normal tissues. The lack of an ideal gene delivery system at present remains a major obstacle for the successful translation of gene therapy to clinical application. Progress in this field relies on advances in the vector system and delivery strategy, exploration of new cancer-specific targets and therapeutic genes, better understanding of biology of cancer, and the invention of more clinically relevant animal models for human cancers. With the nature of cancer cells that are metastatic and develop resistance to chemotherapy and radiotherapy, it is conceivable that targeted cancer therapy will be best executed through a combination of multiple therapeutic modalities, including surgery, hormone therapy, chemotherapy, radiotherapy and gene therapy, to ensure a complete eradication of local and distant tumors.

Acknowledgments

The authors thank Andrew Lu for reviewing and editing this manuscript.

Declaration of interest

The authors state no conflict of interest and have received no payment in the preparation of this manuscript.

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