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Parvovirus vectors: use and optimisation in cancer gene therapy

Boris Blechacz and Stephen J. Russell

With the increasing incidence and mortality of cancer worldwide, there is an urgent need for new therapeutic approaches. Gene therapy is one such approach and preliminary data are promising. Viral and nonviral vector systems for gene delivery are available, but most of the current systems suffer from disadvantages such as low transfection efficiencies, in vivo instability, targeting problems, mutagenic potential and immunogenicity. Viruses of the Parvoviridae family, which are characterised by their oncotropism, oncosuppression, long-term gene expression and human apathogenicity, potentially offer advantages as viral vectors. This article evaluates their usefulness in gene therapy strategies for cancer.

In 2000, 12% of nearly 56 million deaths worldwide were caused by malignant diseases (Ref. 1). By 2020, it is expected that cancer rates might increase by 50%, with up to 15 million deaths worldwide (Ref. 1). Thus, despite all the developments in modern medicine, cancer is still an enormous health problem. Current therapy for malignant disease is mostly based on surgery, chemotherapy and radiotherapy, and there is an urgent need for new therapeutic approaches.

Gene therapy is one of the most promising approaches for cancer treatment because it has the potential to provide tumour cell selectivity and/or protection of untransformed cells of the body. There are four main strategies in cancer gene therapy: (1) immunogene therapy to produce an antitumour vaccine effect or to enhance T-cell antitumour capability; (2) anti-angiogenic gene therapy; (3) cytoreductive gene therapy; and (4) transduction of haematopoietic stem cells (HSCs) with drug-resistance genes to enhance their resistance to cytotoxic drugs. In order to transduce the gene of interest, either nonviral vectors or viral vectors are used. Nonviral vector strategies include naked plasmid DNA, liposome–DNA complexes, peptide-bound DNA and electroporation (Refs 2, 3, 4, 5, 6). The most widely used viral vectors are retroviruses, adenoviruses, adeno-associated viruses (AAVs; members of the Parvoviridae family and

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discussed further below) and herpesviruses, and there is a great diversity of new vector systems being developed (Refs 7, 8, 9, 10). Although the preliminary data are promising, most vectors suffer from disadvantages that limit their usefulness for gene therapy. Nonviral vector systems have low transfection efficiencies, and most of the viral systems have the problems of poor tumour targeting, immunogenicity and low transduction efficiencies.

Certain viruses of the Parvovirinae subfamily of the Parvoviridae family are characterised by their oncotropism, oncosuppression and ability to mediate long-term gene expression. Together with their human apathogenicity, these characteristics make Parvoviridae very interesting vector systems for cancer gene therapy. This article gives a brief overview of the Parvoviridae family, reviews the current status of utilising these viruses as gene therapy vectors for cancer and evaluates their usefulness with regard to the various gene therapy strategies.

Parvoviridae

The family Parvoviridae is subdivided into the subfamily Parvovirinae (which use vertebrate hosts) and the subfamily Densovirinae (arthropod hosts). The subfamily Parvovirinae, which is the main focus of this review article, includes three genera: *Parvovirus*, *Erythrovirus* and *Dependovirus* (Table 1). The main difference between parvoviruses and dependoviruses is the dependence of the latter on co-infection with an unrelated helper virus for productive infection. For ease of discussion, this article will refer to autonomously replicating parvovirus (ARP) when discussing the genus *Parvovirus* and to AAV when discussing the genus *Dependovirus*.

Table 1. The Parvovirinae subfamily[a]

Genus	Virus[b]
Parvovirus	**H1** **LuIII** **MVM** (minute virus of mice) CPV (canine parvovirus) FPV (feline parvovirus)
Erythrovirus	**B19**
Dependovirus	**AAV** (adeno-associated virus) serotypes 1–8

[a] For other genera of the Parvoviridae family (which are not discussed in this article), see Ref. 11.
[b] Viruses of the Parvovirinae subfamily that are commonly used for gene therapy are shown in bold.

Structure

Parvoviridae are icosahedral, nonenveloped viruses and, with a diameter of 18–26 nm and a molecular mass of $5.5–6.2 \times 10^6$ Da, they are among the smallest known viruses (Ref. 11). Differences between AAV and ARPs result from structural differences of the loops between the β-strands of the capsid proteins, a region that is responsible for interaction with antibodies and receptors on cells (Refs 12, 13, 14). The capsid is formed by the three capsid proteins VP1, VP2 and VP3 – 90% of the capsid is formed by VP3, 5% by VP1 and 5% by VP2. Half of the mass of the virus is DNA, and half is protein.

Genome and proteins

ARPs

ARPs encapsidate a genome of ~5.1 kb of single-stranded linear DNA of negative polarity, with palindromic sequences at the termini that serve as self-priming origins of replication (Fig. 1a) (Refs 15, 16). The inverted repeats at the left and right end are unique and form Y-shaped and T-shaped hairpin loops, respectively. Within the genome there are two open reading frames (ORFs) coding for the nonstructural and structural viral proteins, driven by promoters P4 and P38 (Refs 17, 18, 19, 20, 21). Expression of the nonstructural proteins (NS1 and 2) is driven by the P4 promoter, whose activation is induced at the G1–S-phase transition and stimulated by oncoproteins (Ref. 22). NS1 is an 83 kDa phosphoprotein that has helicase, ATPase, DNA-nicking and sequence-specific DNA-binding activities. It is therefore a multifunctional protein essential for viral replication and promoter transactivation (Refs 23, 24, 25, 26, 27, 28, 29, 30, 31). It is also considered the major mediator of cytotoxicity (Refs 32, 33). NS2 is a 22 kDa protein that is essential for replication, virus production, nuclear egress of progeny virions and host-specific infection (Refs 34, 35, 36, 37, 38, 39). In addition, NS2 seems to enhance NS1-mediated cytotoxicity (Refs 40, 41). The P38 promoter is trans-activated by the NS1 protein and regulates transcription of the capsid-coding genes (VP1 and VP2) (Refs 42, 43, 44, 45).

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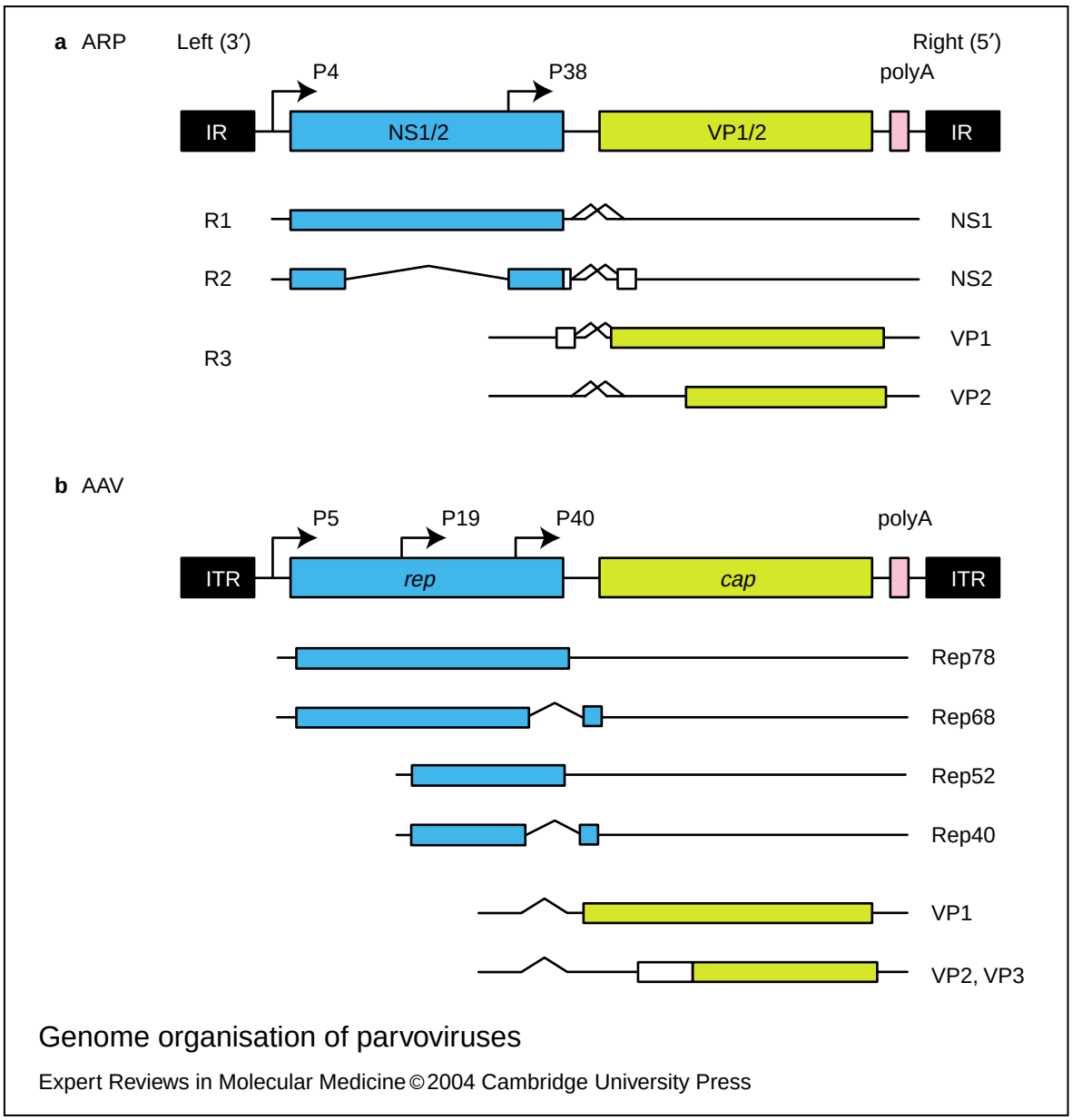

Figure 1. Genome organisation of parvoviruses. (a) Autonomously replicating parvovirus (ARP). The 5.1 kb single-stranded linear DNA genome has inverted repeat (IR) sequences at the termini that serve as self-priming origins of replication. There are two open-reading frames, coding for the structural (VP1 and VP2) and nonstructural (NS1 and NS2) proteins. Transcription is regulated by the P4 and P38 promoters. The transcripts R1–3 and the corresponding parvoviral proteins that they encode are indicated below the schematic of the genome. (b) Adeno-associated virus (AAV). The 4.7 kb single-stranded linear DNA genome is packaged as plus and minus strands in separate virus particles. The termini consist of short inverted terminal repeats (ITRs) that, in contrast to the ARP palindromes, are identical in their sequence. Three different promoters drive transcription: P5, P19 and P40. The transcripts that encode the nonstructural Rep proteins and the structural capsid proteins (VPs) are shown below the schematic of the genome.

AAVs

AAVs encapsidate a genome of ~4.7 kb of single-stranded linear DNA, and plus and minus strands are packaged in separate virus particles (Fig. 1b) (Ref. 11). The termini consist of short inverted terminal repeats (ITRs) that, in contrast to the ARP

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palindromes, are identical in their sequence and form T-shaped hairpin structures. Three different promoters drive transcription: P5, P19 and P40. The P5 promoter drives the expression of the nonstructural proteins Rep78 and 68, which are involved in replication, autoregulation of the P19 and P40 promoters, and site-directed integration of the AAV genome into human chromosome 19 at a site called AAVS1 (Refs 46, 47, 48). The P19 promoter drives expression of the nonstructural proteins Rep40 and 52, which are involved in the production of single-stranded vector genomes. The P40 promoter drives transcription of the three capsid proteins VP1–3, which are translated from different initiation codons.

Life cycle

The life cycle of the Parvoviridae starts with receptor-mediated binding of the virus to the cell surface (Fig. 2). Although not yet completely characterised, the receptor for binding of MVM (minute virus of mice) and H1 is known to contain *N*-acetylneuraminic acid (sialyl)-containing glycoproteins (Ref. 16). For the erythrovirus B19, erythrocyte P antigen has been identified as a binding receptor and α5β1 integrin as a cellular coreceptor (Refs 49, 50, 51). Recently, the differential use of the transferrin receptor (TfR) as a binding receptor for canine parvovirus (CPV) and feline parvovirus (FPV) has been shown (Refs 52, 53, 54, 55). AAV-2 binding is mediated by the heparin sulphate proteoglycan (HSPG) receptor, and virus entry through fibroblast growth factor receptor 1 (FGFR1) and αvβ5 integrin acting as coreceptors (Refs 56, 57). Internalisation of the bound virus occurs by receptor-mediated endocytosis of clathrin-coated pits, as has been demonstrated for AAV-2 and CPV (Refs 58, 59).

The processes of intracellular trafficking and endosomal release are still not completely understood but seem to be in part responsible for differences in transduction efficiencies between different cell lines (Ref. 60). In the nucleus, the single-stranded DNA is converted to its duplex replicative form. A key difference between ARPs and AAVs is that, for ARP, the process of DNA replication is S-phase dependent but is independent from helper virus proteins whereas AAV rely on helper virus proteins derived from adenovirus or herpesvirus (Ref. 61) (Fig. 2). In the absence of a helper virus, AAV is able to integrate into the host chromosome. In humans, integration occurs on chromosome 19, at the AAVS1 site. By superinfection of a latently AAV-infected cell with a helper virus, the AAV genome can be excised and productive infection initiated (Refs 46, 47, 48). By contrast, ARP does not integrate into the host genome (Ref. 62). Replication of parvoviral DNA follows the rolling hairpin model, during which continuous elongation of the viral DNA results in the formation of double-stranded mono- and multimeric intermediates and finally the single-stranded parvoviral genome, which is packaged in progeny virions (Ref. 11).

Infectivity, pathogenicity and oncosuppression

Viruses of the Parvovirinae subfamily have the ability to infect a variety of different vertebrates. Although the natural hosts of parvovirus H1, MVM and LuIII are rodents, these parvoviruses can also infect human cells (Refs 11, 63). AAV serotypes 2, 3 and 5 are endemic in humans, AAV-4 infects mainly nonhuman primates and the host for serotypes 1, 6, 7 and 8 is unclear (Refs 64). Despite this, neither ARPs nor AAVs are pathogenic in humans. Although viraemia after human exposure to AAV and H1 has been described, B19 (of the *Erythrovirus* genus) is the only virus of the Parvovirinae subfamily known to cause human diseases, which include erythema infectiosum, hydrops fetalis, transient aplastic anaemia, myocarditis, hepatitis, arthritis, vasculitis and neurological disorders (Ref. 65).

In the context of cancer, ARPs have special characteristics. They were first isolated from human tumour tissue and for that reason were believed to be oncogenic (Ref. 66). It was then observed that ARPs possess an oncosuppressive potential, inhibiting the formation of spontaneous and chemically or virally induced tumours in vivo and in vitro (Refs 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76). Oncogenic transformation of several human and rodent cells resulted in an enhanced capacity for parvoviral DNA amplification and gene expression and correlated with significantly increased susceptibility towards the parvoviral cytotoxicity (Refs 77, 78, 79). It has been shown that the oncogenic transformation of cells resulted in P4 promoter activation (Refs 22, 80, 81). A precondition for parvoviral cytotoxicity is cell proliferation. Parvovirus P4 promoter activation and replication is S-phase dependent (Refs 82, 83, 84). Several oncoproteins have been shown

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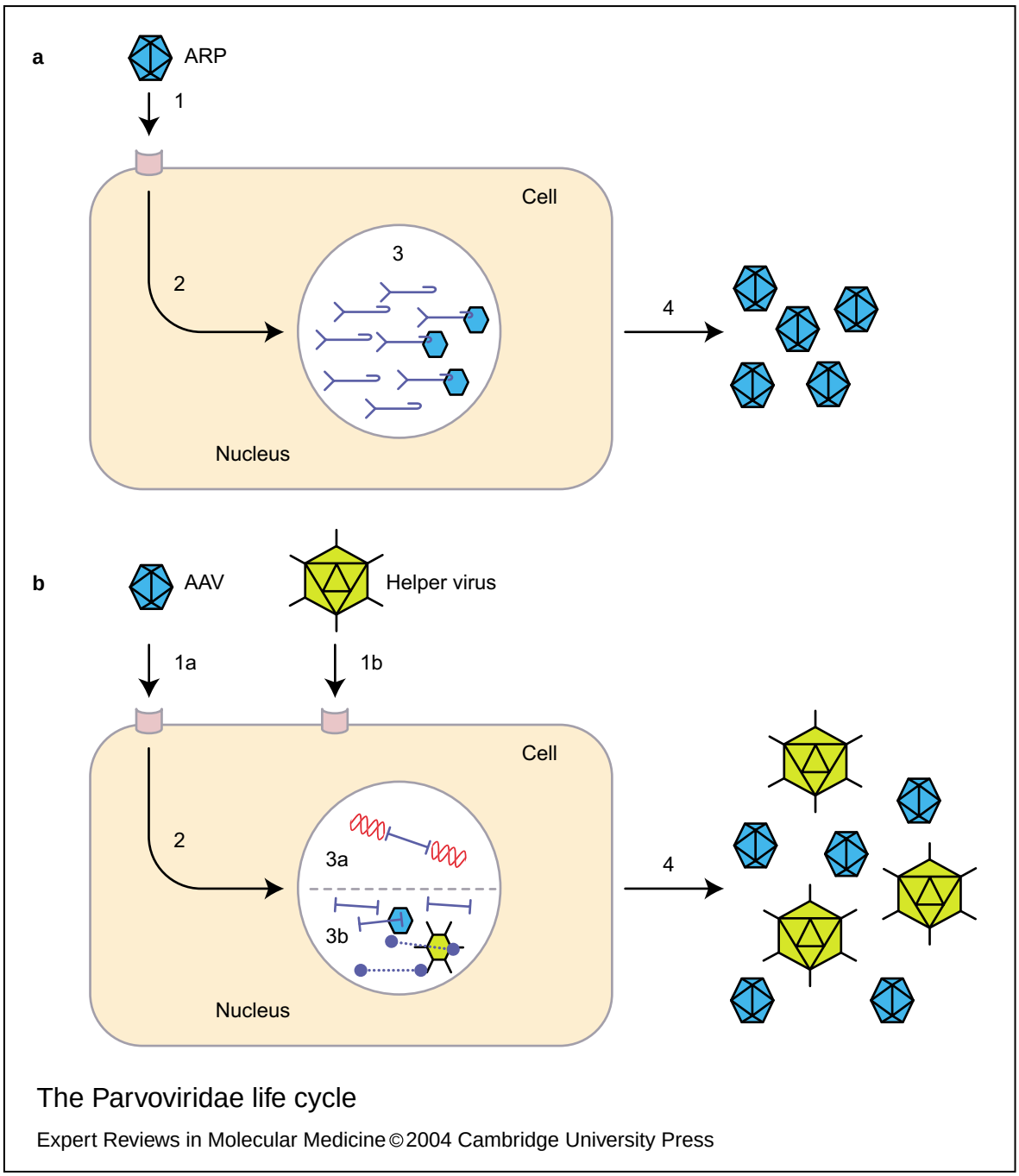

Figure 2. The Parvoviridae life cycle. Life cycle of (a) autonomously replicating parvovirus (ARP) and (b) adeno-associated virus (AAV). Stages shown are: (1) virus binding and entry into the cell; (2) intracellular trafficking; (3) intracellular replication and virus production; and (4) release of intracellular viral particles. In the case of ARP, the virus replicates autonomously in the host cell nucleus. In the case of AAV, stages 1 and 3 include the absence (1a, 3a) or presence (1b, 3b) of helper virus; in the absence of helper virus, the AAV genome integrates into the host cell DNA in order to replicate (Ref. 11).

to contribute to the activation of the P4 promoter during its stimulation at the G1–S-phase transition, resulting in NS1 production at a level high enough to initiate viral replication (Refs 22, 80, 81, 82, 83, 84). The exact mechanism of parvoviral tumour suppression is not fully

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understood but is thought to involve several factors, with the NS1 protein as the major mediator of parvoviral cytotoxicity (Refs 85, 86, 87, 88). Depending on the cell line, the mechanism of parvovirus-mediated cytotoxicity is either apoptosis or necrosis (Refs 89, 90, 91).

Tissue targeting with Parvovirinae vectors

In order to prevent damage to healthy tissue, gene therapy strategies for tumour cells have to have high specificity, particularly when the vector is to be used systemically. Unfortunately, the therapeutic index of most existing vectors is low (Ref. 92), and the undesired transduction of noncancerous tissue can result in damage and cell death. In general, there are two different ways to achieve specificity of a gene therapy vector: transductional targeting and transcriptional targeting (Refs 92, 93).

Transductional targeting describes the selective uptake of the vector into the cells of interest, where the transgene is transcribed. Depending on the gene therapy strategy, this can be the tumour cells, the endothelial cells of the tumour vasculature or other target cells. Selective uptake can be achieved by various strategies, such as modification of the viral capsid or pseudotyping of viruses. In transcriptional targeting, although the transgene might be taken up by many different cells, it is transcribed only in the target cells. In this approach, selective expression of the transgene is achieved by replacing the natural promoter or by modifying the transcription-factor-binding sites within a promoter. There are promoters based on aberrant tumour biology (e.g. promoter induction by telomerase), tissue-specific expression (e.g. promoter induction by tyrosinase for cell type-specific expression in melanomas) and externally inducible promoters (e.g. heat shock protein 70).

Transductional targeting

AAVs have serotype-specific tissue tropism; thus, one approach to achieving tissue-specific transduction with a therapeutic gene is the use of different AAV serotypes (Refs 94, 95, 96, 97, 98, 99, 100). For example, AAV-2 preferentially transduces the liver, AAV-1 transduces the muscle and AAV-5 transduces airway epithelium (Refs 98, 99). In vitro studies with primary human cell lines showed that AAV-2 is also a promising vector for the transduction of human skeletal and smooth muscle cells in terms of transduction efficiency and duration of transgene expression (Ref. 101). Another approach is to cross-package the genome of one AAV serotype into virions of different AAV serotypes. Cross-packaging broadens the host range of a recombinant (r) AAV and might also be advantageous in avoiding elimination of the vector by preformed antibodies: up to 96% of the population have antibodies against AAV-2, which is the most commonly used serotype (Refs 102, 103).

The basis for serotype-specific tissue tropism is the expression of cytoplasmic determinants as well as distinct receptors by the target cells (Refs 104, 105). As mentioned above, AAV-2 uses HSPG as its primary attachment receptor and $\alpha v\beta 5$ integrin and fibroblast growth factor receptor 1 as coreceptors (Refs 56, 57, 106). AAV-3 uses HSPG as its binding receptor, AAV-4 binds to $\alpha 2$-3 O-linked sialic acid and AAV-5 to 2,3-linked sialic acid (Refs 107, 108). AAV-6, which is thought to be a hybrid of AAV-1 and AAV-2, also binds to sialic acid (Ref. 99).

However, serotype-specific tissue tropism enhances transgene expression in certain tissues but does not provide absolute specificity of transgene expression in other tissues. In cancer gene therapy, restriction of transgene expression to one specific target tissue is especially important. To increase the efficiency and safety of AAV vectors, different re-targeting strategies have been attempted, in particular: (1) direct re-targeting by manipulation of the viral capsid using the optimal insertion site that guarantees display of a targeting peptide on the viral surface yet does not interfere with packaging (Refs 109, 110, 111, 112, 113, 114, 115, 116, 117, 118); and (2) indirect re-targeting using a molecule bound to the viral surface that binds specifically and stably to the target cell (e.g. glycoside molecules and bispecific antibodies) (Refs 119, 120) (Fig. 3).

Transductional re-targeting of ARPs has been reported for FPV, a parvovirus that normally infects feline cells, with the aim of modifying it to target human tumour cells for cancer gene therapy. Although modification of the FPV capsid to bind αv integrins enabled transduction of a human rhabdomyosarcoma cell line, other human tumour cell lines expressing αv integrins were not transduced (Ref. 121). Thus, other factors are likely to be required.

Transcriptional targeting

Transcriptional targeting of AAV is mostly used to enhance transgene expression in a

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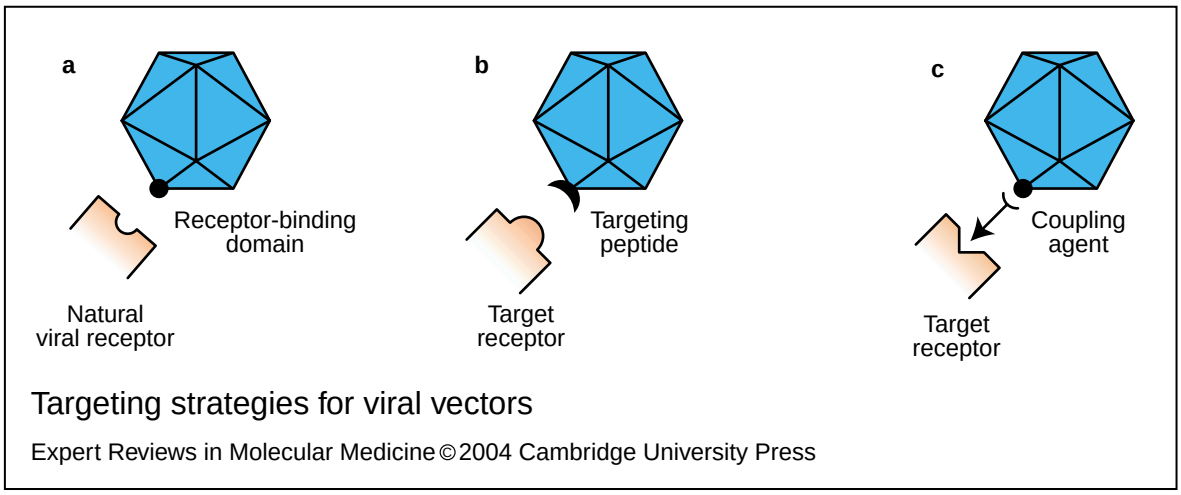

Figure 3. Targeting strategies for viral vectors. To increase the efficiency and safety of viral vectors, strategies to restrict transgene expression to one specific target tissue have been developed (b, c). (a) Interaction between the natural viral receptor and its corresponding binding domain of the capsid protein. (b) Direct re-targeting: the natural binding domain of the viral capsid has been modified so that it is targeted to the specific receptor of the target cell. Examples for this strategy are the insertion of the endothelial cell targeting peptide SIGYPLP or the integrin-binding motif RGD (Refs 114, 115). (c) Indirect re-targeting: a molecule bound to the viral surface interacts specifically and stably with the target cell. Examples of molecules successfully used for this strategy are bispecific antibodies or high-affinity avidin–biotin molecular bridges (Refs 119, 120).

tissue-specific manner rather than to restrict its expression to certain tissues. A variety of promoters have been used successfully, including an albumin gene promoter and a retroviral long terminal repeat promoter to express human α_1-antitrypsin in hepatocytes (Ref. 122), a myelin basic protein (MBP) gene promoter to direct MBP expression specifically to oligodendrocytes (Refs 123, 124) and regulatory elements of the F4/80-gene promoter for specific expression in primary microglia (Ref. 125).

Transcriptional targeting of ARPs has the advantage that the vectors are already oncoselective, as explained above. Transcriptional targeting of ARPs has been used to achieve cell-type specific transgene expression of the parvovirus LuIII: rLuIII vectors expressing the luciferase marker gene under the control of a chimaeric promoter containing a liver-specific enhancer directed the preferential expression of the luciferase marker in transduced human hepatoma cells (Refs 126, 127). Another approach targeted colon carcinoma by using hybrid H-1–MVM parvovirus vectors carrying binding sites for the heterodimeric β-catenin/Tcf transcription factor in the P4 promoter; this transcription factor functions in the wnt signalling pathway, which is constitutively activated in colon carcinoma. NS1 expression and viral burst size were similar to wild-type levels in colon carcinoma cell lines, but wnt-inactive cell lines showed a 1000-fold reduction of viral burst size (Ref. 128).

Parvovirinae in cancer gene therapy

The aim of cancer gene therapy is the transduction of a specific cell population with a specific gene in order to destroy the tumour, or to protect noncancerous cells and thereby enable high-dose chemotherapy. The cell populations of interest to achieve these goals are the tumour cells themselves, cells of the immune system or HSCs. Gene therapy strategies for cancer can be grouped as follows: (1) immunogene therapy with the aim of achieving either an antitumour vaccine effect or enhancing T-cell antitumour effector capability; (2) anti-angiogenic gene therapy to reduce the supply of oxygen and nutrients to the tumour; (3) cytoreductive gene therapy by gene transfer to a large number of tumour cells in situ to achieve nonimmune tumour reduction by direct cytotoxicity or by an indirect bystander effect; and (4) transduction of HSCs with drug-resistance genes to enhance their resistance to cytotoxic drugs.

Depending on the desired gene therapy approach, there are different requirements

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the vector must fulfil with regard to safety and efficiency of the vector, specific targeting of gene transduction, expression level of the transduced gene and ease of manufacture (Ref. 129). The specific requirements for vectors in the various gene therapy strategies are discussed in the following sections and are summarised in Table 2.

Immunogene therapy

In oncological diseases, neoplastic cells have escaped the immune surveillance that normally prevents tumour formation. Mechanisms by which tumours escape an immune response include development of tolerance towards tumour-associated antigens (TAAs), development in an immunoprivileged site, and suppression of cytotoxic T lymphocytes (CTLs) by immunosuppressive factors expressed on the tumour cell surface (e.g. apoptotic ligands) or secreted by the tumour cells [e.g. interleukin 10 (IL-10) and transforming growth factor β (TGF-β)] (Ref. 130). The rationale underlying immunogene therapy is to enhance either tumour immunogenicity or the antitumour effector capability of T cells to overcome the tolerance of the immune system.

The first approach can be achieved by transduction of tumour cells or antigen-presenting cells (APCs) such as dendritic cells with cytokine genes, costimulatory molecules, strong immunogenic tumour rejection antigens or foreign major histocompatibility complex (MHC) molecules. This can be accomplished either by ex vivo transduction of explanted tumour cells for use as cell-based cancer vaccines or by in vivo transduction of tumour cells or APCs to achieve a vaccine effect. In the second approach, immune effector cells can be re-targeted by transduction of T cells with chimaeric antigen receptors (CARs) or human leukocyte antigen (HLA)-restricted αβ heterodimeric T-cell receptors (TCRs). Both strategies result in immune-mediated destruction of the tumour (Refs 130, 131, 132, 133, 134).

Immunogene therapy to achieve an antitumour vaccine effect

Unmodified Parvoviridae (MVM, H-1, AAV-2 or -5) are not able to induce significant cytokine production [interferon (IFN), tumour necrosis factor α (TNF-α) and IL-6] either in rodent or human cells (Ref. 135). However, induction has been reported of a cytokine-like factor called AAV-induced factor (AIF), which can indirectly affect distant AAV-free target cells, and AIF was shown to have a growth-inhibiting effect on human hepatoma and melanoma cells and a growth-stimulating effect on fibroblasts in vitro (Ref. 136). Also, induction of heat shock protein release after H1 infection has been reported (Ref. 137). Heat

Table 2. Requirements of a gene delivery system with respect to different target cells[a]

	Tumour cells ex vivo	Tumour cells in vivo	VECs	T cells or HSCs ex vivo	T cells or HSCs in vivo
Transfer to progeny	–	–	–	+	+
Expression in nondividing cells	+	+	+	–	+
In vivo stability	–	+	+	–	+
Transcriptional targeting	–	+	+	–	+
Targeted attachment	–	+	+	–	+

[a] The table shows the requirements that vector systems have to fulfil in the different cancer gene therapy strategies. Further details and explanations are described in the text of the corresponding sections. Reproduced, with permission, from Ref. 129 (Copyright © 1996, Kluwer Academic Publishers).
Abbreviations: +, required; –, not required; VEC, vascular endothelial cell; HSC, haematopoietic stem cell.

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shock proteins are intracellular molecules that are released in necrotic but not apoptotic cell death; their antigenic peptides are chaperoned into APCs and induce an immune response after presentation to CTLs (Ref. 138). Enhancement of the immune-mediated parvoviral antitumour effect can be achieved by transduction of tumour cells or APCs with specific transgenes. Both ex vivo and in vivo transduction approaches have been used.

Ex vivo transduction

In ex vivo transduction, tumour cells are explanted, transduced with the therapeutic gene and readministered to provoke a host immune response. The vector must be capable of mediating relatively short-term gene expression in the explanted tumour cell. The helper-dependent as well as the ARPs fulfil that requirement. rAAV viral vectors or rAAV-based plasmids have been used most frequently for this application.

Cytokines such as IL-2, IL-12 and granulocyte–macrophage colony-stimulating factor (GM-CSF) have shown their antitumour potential in different models (Refs 139, 140, 141, 142, 143, 144, 145, 146, 147). IL-2 and IL-12 mediate their antineoplastic potential mainly by activation and expansion of tumour-specific T cells and the activation of the cytotoxic activity of natural killer (NK) cells. In addition, they are able to stimulate IFN-γ production, induce the differentiation of T helper type 1 (Th1) cells and activate lymphokine-activated killer cells (LAKs) (Refs 142, 147). GM-CSF stimulates $CD11c^+$ dendritic cells type 1 (DC1), resulting in an enhancement of antigen processing and presentation (Ref. 130). AAV-based vectors have been used for transduction of a great variety of different tumour types with cytokines (Refs 148, 149, 150, 151). Initial studies used mostly AAV-based plasmids/liposome complexes for in vitro transfection either in in vitro transformed cell lines or in primary tumour cell lines. The results of these experiments revealed high-level IL-2 expression that was reported to exceed expression levels achieved by other non-AAV plasmids or by retroviral transduction (Refs 148, 149, 150, 151, 152). The use of ARP vectors (MVM and H-1) resulted in even higher, although not stable, IL-2 levels after transduction of transformed cells (Refs 153 , 154), and in vivo experiments showed up to 90% tumour reduction of human cervical carcinoma (HeLa) xenografts (Ref. 154).

Another widely used family of proteins for cancer gene therapy are the monocyte chemotactic proteins (MCP), which belong to the chemokine family. MCP-1 mostly recruits monocyte/macrophages; MCP-3 exerts its effect on a broader variety of cells including monocytes, T cells, basophils, eosinophils, neutrophils, granulocytes, NK cells and DCs (Refs 155, 156, 157, 158, 159, 160, 161, 162). Transduction of tumour cells with MCPs has been shown to have a tumour-suppressive effect. The observation that endogenous MCP-1 is absent in human cervical carcinoma cell lines made it a therapeutic transgene of interest for cancer gene therapy (Refs 163, 164). Infection of HeLa cells with rAAV or hH1 carrying a gene encoding MCP-1 or MCP-3, respectively, followed by transplantation of the infected cells into mouse models, resulted in a significant reduction in tumour size and growth, although a complete tumour suppression could not be achieved (Refs 165, 166).

Another popular approach to enhance tumour immunogenicity is transduction with costimulatory molecules, resulting in an induction of an antitumour CTL response. CD80 (B7.1) and CD86 (B7.2) stimulate CD28, which is expressed on the T-cell surface; the costimulation of CD28 and the TCR leads to expansion of $CD8^+$ CTLs. Expression of B7 proteins in tumours has been shown to increase antitumour immune responses, resulting in rejection and even protection against tumours not expressing B7 proteins (Refs 167, 168, 169, 170, 171). However, there are certain factors that influence the antitumour efficiency such as quantity of the B7 protein expressed by the tumour cell (Ref. 172). For the achievement of high transduction as well as high expression levels, Parvovirinae have proved to be promising vectors. For instance, AAV-mediated transduction of multiple myeloma cell lines with B7 genes has led to transduction efficiencies of up to 80% and high-level B7 expression, with a resulting increase in T-cell proliferation (Ref. 173).

An alternative to transduction of tumour cells is the transduction of APCs such as DCs with TAAs or tumour-specific antigens (TSAs) to induce a tumour-specific immune response (Ref. 174). AAVs are favourable for this strategy as one of their advantages is their ability to transduce proliferating as well as quiescent cells, which permits transduction of DCs from activation through maturation. In addition, they provide stable expression of the antigen and do not express

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viral proteins, thereby minimising competition between viral petides and the transgene for MHC presentation. Cervical cancer is an appealing target for this approach because 90% of the cancer cells contain human papillomavirus (HPV) DNA and express the oncogenes E6 and E7, which serve as TAAs. The transduction of DCs with the E6 and the E7 genes using AAV induced a strong CTL response, resulting in killing of up 86% human cervical carcinoma cells in vitro (Refs 175, 176, 177). Also, a strong induction of CTLs was achieved when AAV was used to transduce DCs with the gene coding for the fusion region of the Bcr–Abl fusion oncoprotein, which is expressed in chronic myeloid leukaemia (Ref. 178).

In vivo transduction

The requirements a vector must fulfil for in vivo transduction are more demanding compared with the ex vivo approach. The vector should be transductionally or transcriptionally targeted to the tumour. In addition, it has to be sufficiently stable in vivo to tolerate systemic administration, although direct intratumoural delivery is also possible.

An MCP-1-expressing rAAV-2 has been used for in vivo transduction of a human cervical carcinoma cell line, resulting in a reduction of tumour-growth rate; unfortunately, complete regression could not be achieved in this system (Ref. 165). These results confirm observations made in the ex vivo models described above regarding MCP-1.

More effective has been the transduction of glioma cells in vivo with a bicistronic gene coding for IL-2 and herpes simplex virus thymidine kinase (HSV-TK) using AAV as the gene transfer vector. TK converts the drug ganciclovir into ganciclovir-monophosphate, which is then converted to the di- and triphosphate forms by endogenous kinases (Ref. 179). Integration of the triphosphate form into nascent DNA causes premature chain termination, resulting in apoptosis of the host cell. HSV-TK is also known to elicit a strong bystander effect. Examination 17 days after tumour implantation into mice showed that rAAV and ganciclovir treatment resulted in a 35-fold size reduction of the tumours without damaging normal brain tissue (Ref. 180). However, complete remissions could not be achieved and residual tumours proliferated rapidly after ganciclovir treatment was terminated, leading to death after 28.8 days in the treated group in comparison with the untreated group in which the animals died after 20.6 days on average.

MVMp has been used for in vivo transduction of metastatic haemangiosarcoma with interferon-inducible protein 10 (IP-10), with very promising results; a significant slowing of recurring haemangioma growth and metastasis suppression was observed in immunocompetent mice, as well as survival up to 6 months in a third of the animals (Ref. 181). IP-10 is a chemokine with previously demonstrated antitumour potential, which is thought to be mediated by anti-angiogenic factors as well as immune stimulation (Refs 182, 183).

These in vivo data are promising and show the usefulness of parvoviruses for this cancer gene therapy strategy. However, further evaluation and optimisation will be necessary to increase the tumour-suppressive effects.

Immunogene therapy to enhance T-cell antitumour effector capability

T cells are essential in the immune response against tumours. Enhancement of their antitumour capability might be achieved by transduction of autologous T cells with CARs or HLA-restricted TCRs or by transduction of tumour-infiltrating lymphocytes with T-cell-stimulatory cytokines. T cells might be isolated, expanded and transduced ex vivo before being readministered, or in vivo transduction of T cells might be accomplished by transductional or transcriptional targeting of the vector (Ref. 129).

In order to achieve immunogene therapy to enhance T-cell antitumour effector capability, the vector in question must enable stable transduction and expression of the transgene in the progeny of the initially transduced T cell. AAV is known to integrate site-specifically in the host cell genome and has shown stable expression of different transgenes in different cell models (Refs 46, 47, 184, 185, 186, 187). However, the Parvoviridae are not optimal vectors for gene transfer to T cells. Low transduction efficiencies as well as loss of transferred DNA would be significant disadvantages minimising the therapeutic effect of this approach (Refs 188, 189).

Anti-angiogenic cancer gene therapy

Tumour growth is dependent on angiogenesis because it provides the tumour with oxygen and nutrients. The degree of vascularisation of a

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tumour has been associated with its aggressiveness (Refs 190, 191, 192). Tumour angiogenesis is driven by the proliferation and migration of vascular endothelial cells (VECs), which are therefore important targets in cancer therapy. These endothelial cells are stimulated by pro-angiogenic proteins such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF). These proteins are synthesised by tumour cells, endothelial cells or both. VEGF in particular is highly expressed in many different tumour cells and has been correlated with tumour growth, tumour invasion and metastasis (Refs 193, 194).

In cancer therapy, there are three different strategies to disrupt tumour vessel growth. In the first strategy, endothelial cells are directly targeted to block proliferation or migration or to induce apoptosis. The second strategy is to attack tumour-associated endothelial cells indirectly by blocking the expression or activity of tumour proteins that stimulate angiogenesis. This can be achieved by neutralising pro-angiogenic proteins in the microcirculation, by preventing their binding to their cognate receptors or by blocking signal transduction by targeting the intracellular domains of receptor tyrosine kinases. For example, the inhibition of epidermal growth factor receptor (EGFR) blocks the synthesis of VEGF, bFGF and TGF-α. A third strategy is the inhibition of multiple signal transduction pathways on both the tumour-associated endothelial cells and the tumour cells. These various anti-angiogenic approaches can be used in combination (Ref. 195).

The requirements a vector must fulfil for an anti-angiogenic gene therapy approach are demanding. Since the route of administration has to be systemic, the vector has to be stable in vivo. Also, since long-term tumours can reoccur as soon as the inhibition of the tumour angiogenesis is stopped, the vector has to provide stable expression of the transgene. Although no toxic side effects have been observed up to now, there are still concerns about the effect of angiogenesis inhibition on physiological angiogenesis.

Within the family Parvoviridae, AAV fulfils the requirements for anti-angiogenic gene therapy: the virus is able to introduce transgenes in a great variety of mature and differentiated cells with high efficiency and long-term stability (Ref. 129). Several studies have shown that selective expression in tumour VECs can be achieved by transductional or transcriptional targeting of AAV. An rAAV vector encoding a soluble truncated form of VEGF receptor 2 (Flk-1/KDR), which inhibits VEGF activity, was used for transduction of the liver in a mouse model of Wilms' tumour, and resulted in 90% tumour growth inhibition with a 48% reduction in mean intratumoural endothelial density. In another experiment under the same conditions, treatment resulted in prevention of tumour development in 67% of cases and a reduction of tumour burden of 83–87% up to 60 days after tumour cell injection (Ref. 196).

Another angiogenesis-inhibiting protein is endostatin, a 20 kDa C-terminal proteolytic fragment of collagen XVIII. It inhibits endothelial cell proliferation and migration, and induces endothelial cell apoptosis. Its systemic administration led to suppression of primary tumours as well as metastasis in different tumour models (Refs 197, 198, 199). A single intramuscular injection into mice of human endostatin as a transgene cloned into an AAV vector resulted in a constant expression of biologically active human endostatin starting 4 weeks after injection, reaching a plateau at 6–8 weeks and persisting for up to 4 months. The vector also showed anti-angiogenic and tumour-suppressive potential in a human colon carcinoma xenograft model in nude mice (Ref. 200). Using a bicistronic AAV vector coding for endostatin and angiostatin, complete tumour protection has been reported in a human ovarian carcinoma xenograft model in mice (Ref. 185). It should be mentioned that the optimal choice of anti-angiogenic genes might differ for different tumours: endostatin did not have an oncosuppressive effect in a xenotransplant model of human acute lymphocytic leukaemia (Ref. 201).

In summary, helper-dependent parvoviruses, especially AAV, could be advantageous for anti-angiogenic cancer gene therapy. In comparison with other gene therapy vectors, they have the advantage of being less immunogenic and providing long-term transgene expression in different dividing and nondividing tissues. Since it was shown that a maximum anticancer effect occurs with constant low-level expression of anti-angiogenic protein, and not with a one-time peak expression with subsequent fall-off as provided by adenovirus vectors, AAV is optimal for anti-angiogenic cancer gene therapy. In addition, it was

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reported that AAV vectors seem to have a direct cytotoxic effect on tumour cells besides their anti-angiogenic properties (Ref. 196).

Cytoreductive cancer gene therapy

Cytoreductive cancer gene therapy is used to reduce the number of tumour cells by transducing them with a therapeutic gene encoding a cytotoxic protein or a prodrug-sensitising protein (Ref. 129). To achieve this, a vector requires in vivo stability, high transduction efficiency and the ability to target, either by transcriptional control using a tumour-specific promoter or transductional targeting, to avoid damage to normal tissue (Refs 126, 202).

As discussed earlier, ARPs are characterised by their oncotropism and oncosuppressive potential and provide high-level transgene expression. These characteristics make them promising vectors for cytoreductive cancer gene therapy. Furthermore, AAVs have been shown to cause a decreased risk for high-grade cervical neoplasms and to enhance the cytotoxic effect of chemotherapeutics (Refs 203, 204). However, in general, the parvoviral cytotoxicity is not sufficiently potent to achieve 100% tumour regression. The resistance of some tumour cells to parvoviral cytotoxicity is thought to be in part correlated to the p53 status of the cell, as wild-type $p53^{+}$ cells are less susceptible to parvoviral cytotoxicity (Refs 91, 205). One approach to overcome this problem of tumour-cell resistance towards parvoviral cytotoxicity is through the use of toxic transgenes. This can be achieved either by replacing the capsid-coding region with a therapeutic transgene or by replacing the complete parvoviral coding region with a different expression cassette flanked by parvoviral palindromic sequences. Great efforts have been made to modulate the parvoviral genome to enhance its cytotoxicity, and two transgenes that have been used are apoptin and HSV-TK.

Apoptin is a 13.8 kDa protein encoded by the chicken anaemia virus (Ref. 206). It induces apoptosis in tumour cells in a p53-independent manner. Its apoptosis induction is mediated by the caspase signalling pathway. Interestingly, apoptin does not harm untransformed cells, most probably owing to a cytosolic localisation in contrast to its nuclear localisation in transformed cells. Its antitumoural potential has been shown in different tumour models (Refs 207, 208, 209, 210, 211). The susceptibility of H1-resistant tumour cell lines could be increased by infection with the rhH1–apoptin: a cytotoxic effect was achieved that was up to threefold higher than that achieved by parvovirus hH1–GFP (green fluorescent protein) infection. It remains to be determined if this effect can also be achieved in vivo (Ref. 212).

In in vitro tumour models of melanoma, breast cancer and glioma, HSV-TK was tested as a therapeutic transgene cloned in MVMp (Ref. 213). Infection of these tumour cell lines with the rMVMp–HSV-TK followed by the addition of ganciclovir resulted in up to 95% cell killing dependent on the tumour type. Ganciclovir-independent MVM-mediated cytotoxicity was 30–65%. AAV has also been used for transduction of the gene encoding HSV-TK. Using rAAV–HSV-TK and ganciclovir in an in vitro model of human soft tissue sarcoma, transduction efficiencies of more than 96% were achieved, and tumour cells were completely eliminated. When rAAV/HSV-TK-infected and ganciclovir-treated sarcoma cells were transplanted in severe combined immunodeficient (SCID) mice, survival times of more than 5 months were observed in comparison with a 1 month survival time in the mock-treated group (Ref. 214). In a glioma model, AAV containing a bicistronic gene coding for HSV-TK and human IL-2 was tested. In vitro as well as in vivo tumour cell reduction was observed after ganciclovir administration following AAV-mediated gene transduction. In vivo, a 35-fold reduction of mean tumour volume was achieved without damage to normal tissue (Ref. 180).

HSC transduction with drug-resistance genes

One of the limiting factors in chemotherapy of malignancy is bone marrow (myelo) suppression (Ref. 215). Autologous stem cell transplantation is therefore a widely used method to facilitate high-dose chemotherapies. However, as the transplant is harvested before the therapy, the possibility of contamination of the transplant with tumour cells remains. As an alternative to this procedure, transduction of HSCs with a drug-resistance gene makes these stem cells, as well as their offspring, more resistant to the cytotoxic effect of the chemotherapeutic agent, allowing the administration of higher doses of chemotherapy, which in turn increases the likelihood of a curative therapy of the malignancy (Ref. 129). The most widely used multidrug-resistance gene is the gene encoding MDR1.

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Other examples of genes having a protective potential against chemotherapeutic drugs are those encoding dihydrofolate reductase (DHFR), O^6-methylguanine-DNA methyltransferase (MGMT), aldehyde dehydrogenase (ALDH), glutathione-*S*-transferase (GST), cytidine deaminase (CDD) and dihydropyrimidine dehydrogenase (DPD) (Ref. 216). The major requirement for the transduction of drug-resistance genes is the restriction of their expression to HSCs, because transduction of even a small number of tumour cells with drug-resistance genes would render them resistant to chemotherapy. HSCs can be collected from bone marrow, peripheral blood or umbilical cord blood, and selective transduction can be achieved ex vivo by transduction of the HSCs after $CD34^+$ selection and stimulation with growth factors (Refs 129, 217, 218). For direct in vivo gene transfer, the vectors would have to be targeted against proteins such as CD34 and c-kit, both of which are human haematopoietic progenitor cell-surface markers (Refs 219, 220, 221).

Adenovirus vectors do not integrate or self-replicate (Ref. 11) so they are not suitable for HSC transduction. Retroviruses and lentiviruses are well suited for this approach but have the disadvantage of having low transduction efficiencies (Ref. 216). Of the Parvoviridae, the AAVs have been assessed for this cancer gene therapy approach. They efficiently transduce proliferating and nonproliferating cells and lead to stable transgene expression (Ref. 222). Their ability to transduce human haematopoietic progenitor cells has been shown; however, transduction efficiencies were low and varied from donor to donor, which would be a considerable disadvantage in the treatment of human patients (Refs 223, 224). To date, the transduction of a drug-resistance gene using a parvovirus has been reported only in NIH-3T3 cells using an AAV–plasmid/liposome complex and in $CD34^+$ human bone marrow using AAV (Refs 225, 226). Unfortunately, the variability of transduction, the low transduction efficiencies and the observed loss of episomal forms of AAV over time indicated that AAV is not the optimal vector for this gene therapy approach.

Conclusions and summary

Parvoviridae are very promising vectors for gene therapy of cancer. Depending on the specific cancer gene therapy approach, each Parvoviridae genus has its advantages and disadvantages. AAVs are able to transduce a variety of nondividing and terminally differentiated cells, provide long-term expression, induce a weaker CTL response than other viral vectors and are human apathogenic. Depending on the target cell, they have the potential of high transduction efficiencies and low immunogenic potential. In addition, owing to the various tissue tropisms of different serotypes and the recent progress in terms of retargeting AAV, they offer the possibility of specific transductional targeting (Refs 61, 102, 113, 227). AAV vectors are well suited for certain cancer gene therapy approaches, such as immunogene therapy targeting APCs and anti-angiogenic gene therapy, where long-term gene expression is desired. ARPs are appealing vectors for cytoreductive gene therapy as well as immunogene therapy approaches to target tumour cells. They have the advantage of being oncotropic, oncosuppressive, human apathogenic and providing high-level transgene expression.

A disadvantage of ARPs is the limitation of transgene size owing to inefficient encapsidation beyond a size limit of 106% of the wild-type size (Refs 228, 229). The original packaging capacity of <5 kb for rAAV vectors has meanwhile been expanded to 10 kb using strategies based on heterodimerisation of separate AAV vectors in head-to-tail orientation during concatemer formation (Refs 230, 231, 232). Another serious problem concerns the production of vector. Although there has been much progress in the production of rARPs and rAAVs, there is still the need for optimisation. Higher vector yields must be achieved and contamination with wild-type and helper viruses must be completely eliminated if the vectors are to be used in human clinical trials.

In summary, Parvoviridae have proved in recent years to be a vector system that is in many aspects equal if not superior to other viral and nonviral gene-delivery systems. These promising findings indicate that they should be further evaluated in vivo.

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Website

A helpful overview of the Parvovirinae can be found at 'All the Virology on the WWW':

http://www.tulane.edu/~dmsander/WWW/335/Parvoviruses.html

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Figures

Figure 1. Genome organisation of parvoviruses.
Figure 2. The Parvoviridae life cycle.
Figure 3. Targeting strategies for viral vectors.

Table

Table 1. The Parvovirinae subfamily.
Table 2. Requirements of a gene delivery system with respect to different target cells.

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