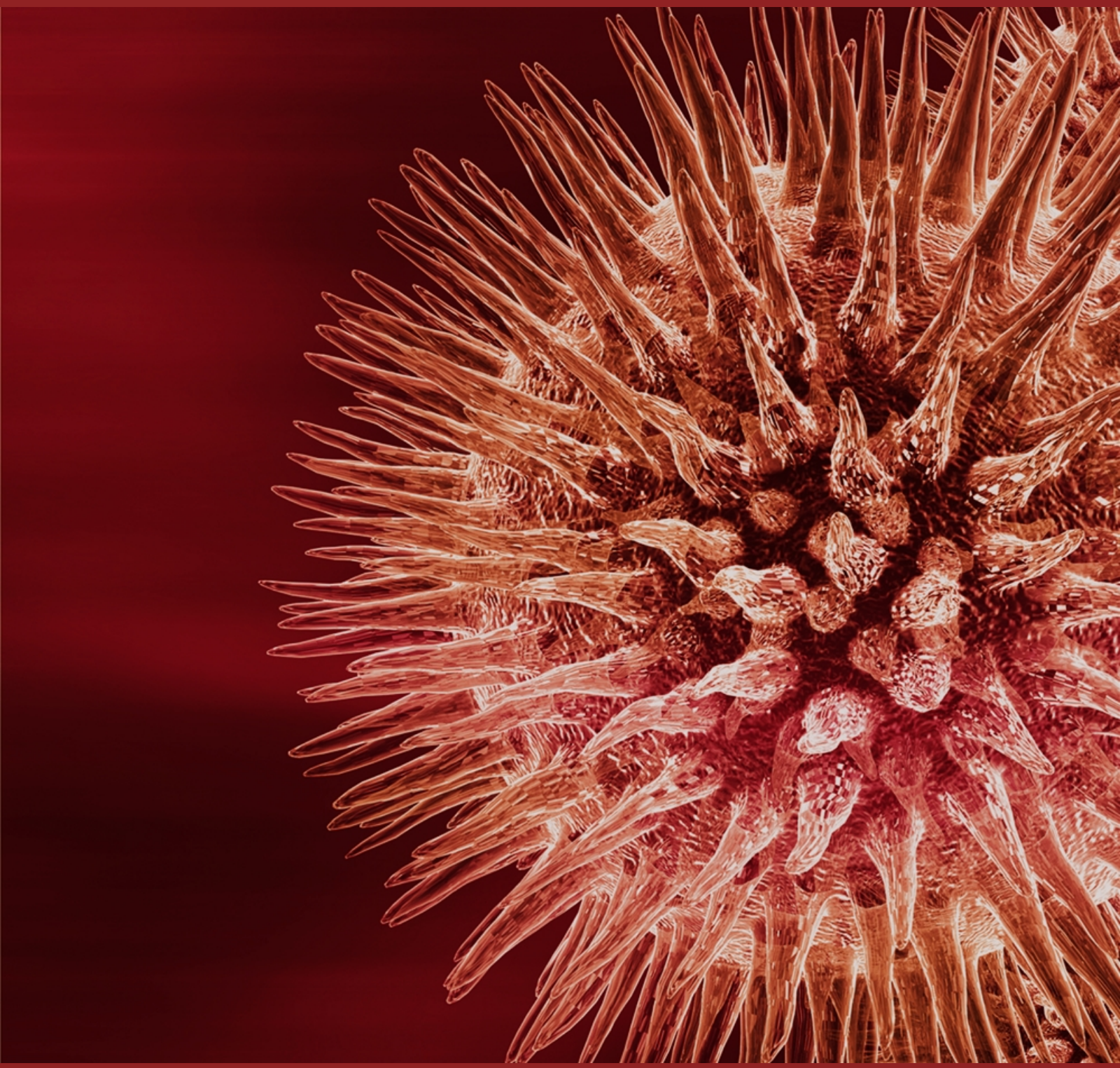
Journal of Biomedicine and Biotechnology

Gene Therapy—Part II

Guest Editors: Nicol Keith and Claude Bagnis



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REVIEW ARTICLE

Baculoviruses as Vectors for Gene Therapy against Human Prostate Cancer

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Current curative strategies for prostate cancer are restricted to the primary tumour, and the effect of treatments to control metastatic disease is not sustained. Therefore, the application of gene therapy to prostate cancer is an attractive alternative. Baculoviruses are highly restricted insect viruses, which can enter, but not replicate in mammalian cells. Baculoviruses can incorporate large amounts of extra genetic material, and will express transgenes in mammalian cells when under the control of a mammalian or strong viral promoter. Successful gene delivery has been achieved both in vitro and in vivo and into both dividing and nondividing cells, which is important since prostate cancers divide relatively slowly. In addition, the envelope protein gp64 is sufficiently mutable to allow targeted transduction of particular cell types. In this review, the advantages of using baculoviruses for prostate cancer gene therapy are explored, and the mechanisms of viral entry and transgene expression are described.

WHY GENE THERAPY FOR PROSTATE CANCER?

The case for new therapies in prostate cancer is particularly strong, given the frequency of the disease in a western population that is increasingly elderly (Dijkman and Debruyne [1]). Conventional therapies, such as surgery and radiotherapy can be effective if early stage disease is detected and targeted for therapy, a strategy employed on a wide scale in the USA (Bubolz et al [2]; Pirtskhalaishvili et al [3]). Even in the later stages of the disease, intervention to block the necessary supply of androgens is effective in the short term, although resistant tumours develop relatively rapidly, within 1–2 years (BJ Feldman and B Feldman [4]). Cytotoxic chemical therapies are rarely effective. Thus a therapeutic strategy, in which the genetic nature of the prostate tumour is turned against the cancer is very attractive. The mantra of successful gene therapy for prostate cancer has been repeated many times since the earliest reports of successful gene transfer were published (reviewed in Roth and Grammer [5]). In this respect, the prostate is both a good and bad target for specific therapy. On the credit side, the prostate itself, like most secretory organs, displays radically different patterns of gene expression from most other organs, and many of these "tissue-specific" products are retained in the tumours. Tissue specificity can be turned against the tumour, both at the cell surface level (attachment of therapeutic agents) and at the transcriptional level to direct expression of therapeutic genes. The range of candidates has been covered in an earlier review (Maitland [6]). In addition, there are a number of tumour associated antigens, whose expression is upregulated in prostate tumours. It is in this respect that prostate tumours remain a poor candidate for strictly gene-based therapy. Firstly, the range of tumour antigens is small, but increasing in view of recent stimulation of research in this area (Liu [7]; Luo et al [8]; Ornstein et al [9]). Secondly, the natural history of the disease is relatively poorly understood, in comparison with breast cancer, a disease of similar incidence and mortality. Prostate tumours display genetic and antigenic heterogeneity (Macintosh et al [10]), and the ability to accurately predict the course of the disease (and therefore to identify patients for gene therapy regimes) remains rather primitive relative to breast cancers (Van't Veer and De Jong [11]), despite the application of gene array technology (Dhanasekaran et al [12]). Finally, prostate tumours display an ability to shift phenotype, probably by selectively activating or inactivating gene expression, for example, in the development of androgen-independent disease (Karan et al [13]; Tso et al [14]) and the inactivation at the transcriptional level of genes encoding carcinogen-inactivating enzymes (Lee et al [15]). This would seem to be the ideal mechanism to inactivate the expression of exogenous therapeutic genes.

It is also likely, given the genetic and clinical heterogeneity of prostate cancers, that a range or even a combination of gene therapy strategies with conventional treatments will be needed to achieve a substantial effect. This is particularly true with viral vectors, where an existing immune memory against human viruses (eg, adenoviruses)

could preclude their use in certain cases. Indeed, to optimise the therapeutic effects, simultaneous infection with a "cocktail" of therapeutic viruses (to overcome the initial tumour heterogeneity) or sequential inoculation with different virus types (to escape either preexisting or therapy-induced antiviral immunity) may be necessary. However, neither strategy will be clinically feasible unless all unacceptable risks of side effects can be eliminated. Lastly, and perhaps most importantly, the method of inoculation and dosage has to be optimised. For example, should the primary target for gene therapy be organ confined disease, where the conventional therapeutic strategies are moderately effective, or against metastatic disease?

So can baculoviruses provide an alternative means of delivering therapeutic genes into organ confined and metastatic prostate cancer?

WHAT ARE BACULOVIRUSES?

It is perhaps surprising that there are more than 500 different types of baculoviruses (Martignoni and Iwai [16]). They are widespread pathogens of insects and invertebrates, ranging from shrimps to moths and butterflies. However, the most studied types are those which cause disease in common insect pests. Research was driven initially by the intention to use them as a biological insecticide (Ignoffo [17, 18]; Martignoni [19]). The individual baculoviral strains have a limited host range, which is usually restricted to one species [20, 21]. Pioneering studies at Texas A&M University, where the effects of the *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) on the fall army worm were first studied in molecular detail. MNPV's are a subgroup of baculoviruses that produce large polyhedral occlusion bodies as part of the viral life cycle [21]. Polyhedrin is a 246-amino acid (29 kd) protein, which forms a hard polyhedral protein matrix of between 0.15 to 15 μm in which Bv nucleocapsids, surrounded by a single unit membrane, are embedded. This provides the ultimate protection in the wild. These polyhedra, are however susceptible to the alkaline environment in the midgut of the host insect, releasing infectious virus, into susceptible host cells (reviewed in Harrap and Longworth [22]). Polyhedra can be visualised as refractive crystals in the nucleus of infected insect cells under light microscopy. The polyhedrin embedded occluded virus (OV) is formed very late in the baculoviral life cycle, in contrast to the budded virus (BuV) formed earlier in the infectious life cycle [20]. Both OV and BuV are rod shaped with a supercoiled dsDNA genome of 80–200 kilobasepairs (approximately 134 kbp in AcMNPV), which is condensed into a nucleoprotein core with proteins p39 and p87 [20, 23]. As a result of budding out through the cell plasma membrane, BuV acquire a loosely fitting viral envelope which has peplomers protruding around one end [20]. These peplomers are comprised of the major envelope glycoprotein gp64, which is responsible for cell to cell spread and secondary viral infection. In contrast, AcMNPVOV consists of multiple nucleocapsids surrounded by a *de novo* synthesised viral envelope which does not display gp64 peplomers [20]. This led to the development of the virus as a potential gene cloning vector, exploiting the readily available cell lines from the midgut of the moth, and the ability to recombine into the viral genome by cotransfection of intact viral DNA with a segment of the viral genome containing an exogenous gene, normally under the control of one of the very strong late promoters such as that for the polyhedrin gene. A diagrammatic version of the baculoviral infectious process is shown in Figure 1.

Many of the 151 recognised open reading frames (Genbank number NC_001623) encode proteins of as yet unknown function. However, like many larger DNA viruses, the Bv genome does contain a large number of proteins whose main function is to subvert both host cell and host organism defences. In many cases these are homologues of host cell proteins such as ubiquitin, PCNA, and viral DNA polymerase/RNA polymerase components (reviewed in Ayres et al [24]; Kool and Vlak [25]). There are also homologues of growth factors, intracellular signalling molecules, and perhaps most notably, a unique apoptosis suppressor p35, which is functional in both insect and human cells (Hsu et al [26]; Resnicoff et al [27]; Robertson et al [28]). The genomic location of some of these open reading frames is indicated in Figure 2. Clearly, the expression of some or all of these Bv functions, which are sufficiently closely related to human homologues, would be undesirable in a human gene therapy vector, particularly one designed to kill cancer cells.

GENERATION OF RECOMBINANT BACULOVIRUSES

When the first baculoviral protein expression vectors were generated, the selection system was based on the loss of the polyhedrin gene, by recombination from a transfer plasmid, which disrupted the PH coding sequence. This resulted in infected cells lacking the characteristic occlusion bodies, composed of enveloped nucleocapsids embedded in a polyhedrin matrix. In practice it took some time to become experienced in identifying nonoccluded viral plaques, and the recombinants were frequently contaminated by nonrecombinant wild-type virus, which in a large scale culture could outgrow the recombinants, particularly when the recombinant protein expressed had cytopathic properties.

More recent developments have used cotransfection of multiply-deleted viral genomes (eg, the commercially available BacVector 1000 series) together with the transfer vector (generated in a bacterial plasmid) which contains recombination sequences, normally from the nonessential polyhedrin gene. Capacity for recombinant inserts is at least 40 kbp in this rod form virus, although in practice this could be difficult to maintain in the transfer vector.

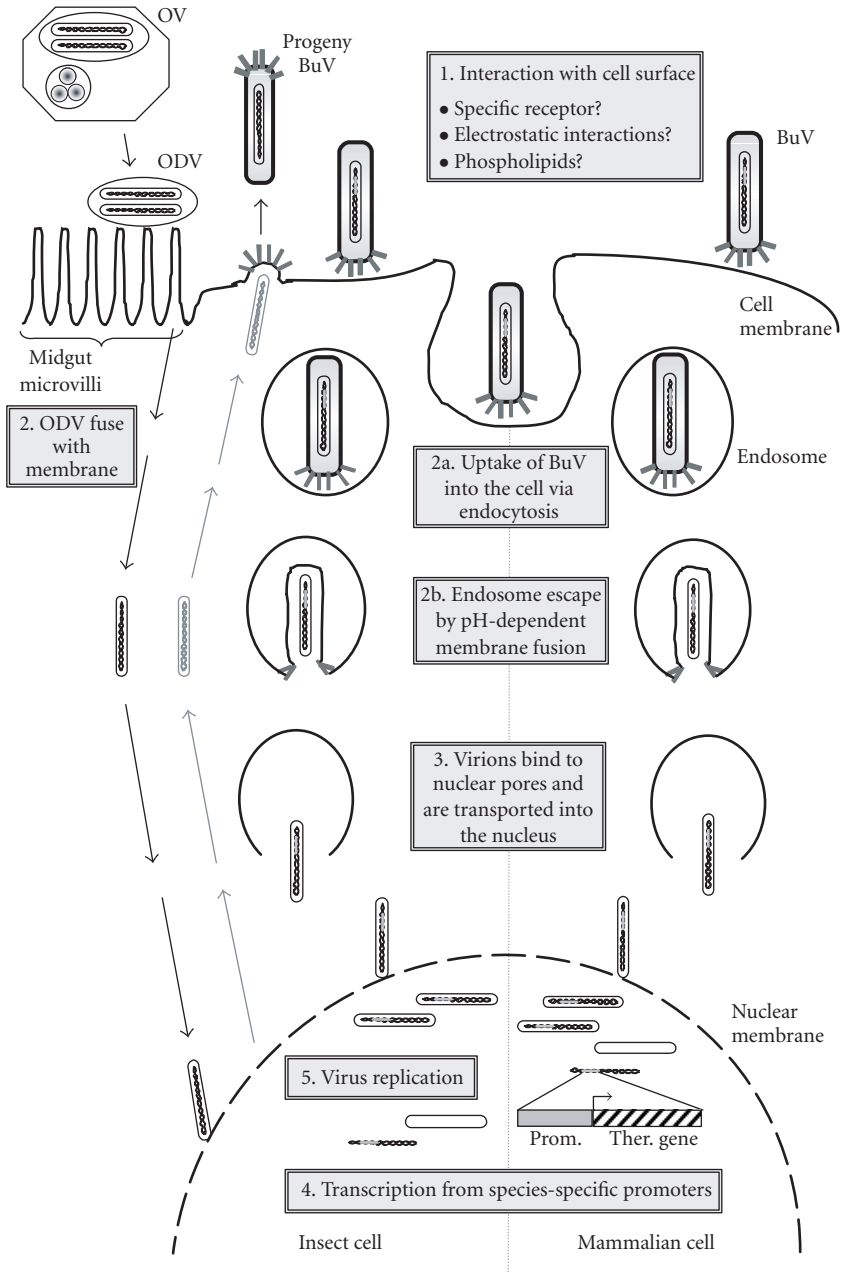


Figure 1. Baculovirus infection of insect and mammalian cells. (1) Budded virus (BuV) particles interact with the mammalian cell surface via the surface gp64 protein in both insect and mammalian cells (described in the text). The occluded virus (OV) polyhedrin matrix is digested in the insect midgut lumen releasing occlusion derived virions (ODV), which consist of multiple nucleocapsids surrounded by a membrane. (2) ODV particles fuse with the microvilli membranes of midgut epithelial cells, releasing nucleocapsids into the cytoplasm. (2a) BuV particles are taken up into endosomes from which they escape by endosome acidification and membrane fusion (2b). (3) Virus particles are transported to the nucleus where they bind the nuclear pores and whole virions containing the genome are actively transported through the pores into the nucleus. Viral DNA is released from the nucleocapsid. (4) In insect cells, the baculoviral genes are transcribed whereas in mammalian cells, only genes under the control of a mammalian promoter are transcribed. (5) In insect cells, viral DNA is replicated and packaged into nucleocapsids. During the late phase of infection, nucleocapsids exit the nucleus and bud from the cell membrane to form BuV. During the very late stage of infection, nucleocapsids remain in the nucleus where they are enveloped and embedded in a polyhedrin matrix to produce OV. In mammalian cells, virus replication does not take place and no progeny virus is produced.

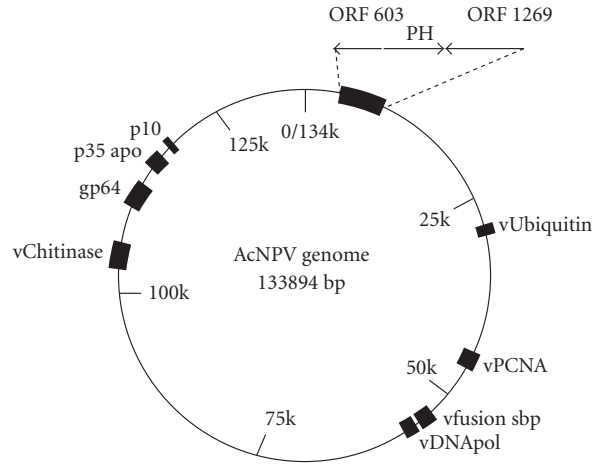


Figure 2. Genomic map of Bv (AcNPV) genome. Some significant open reading frames are indicated. Detail is the polyhedrin (PH) cloning sites for recombinants.

Recombinant viral yields from the BacVector (Novagen Inc, Madison, Wis, USA) system are high and wild-type viral yields are virtually eliminated, by use of the "triple cut" system, which uses Bv DNA in which the PH gene has been disrupted by insertion of the β gal gene, leading to blue nonrecombinant Bv plaques in the presence of Xgal indicator. The second and more important step is the introduction of a site for the rare cutting restriction endonuclease Bsu36I within the essential (for replication) Bv gene ORF1629. Further Bsu36I sites are located in the βgal and in the other Bv ORF flanking PH ORF: ORF603. The complementing ORF1629 protein is supplied by the recombination transfer vector, and the triple cut Bv DNA cannot produce viable virus without addition of the ORF1629 (see Figure 2). Thus the production of wild-type virus and isolation of recombinants is not a serious problem in this system.

The recent development of the BAC to BAC vector system (Invitrogen), in which the entire Bv genome has been cloned into a bacterial artificial chromosome, enables the recombination to be carried out with higher efficiency and control in bacteria. This results in higher yields and more rapid recombinant generation (Luckow et al [29]) as with similar adenoviral systems (eg, AdEasy from Q-Biogene, Illkirch, France).

ADVANTAGES OF BACULOVIRUSES AS A GENE THERAPY VECTOR

Firstly, Bv is a rod form, and limitations to the amount of extra genetic information inserted into the recombinants such as those imposed by the defined adenovirus capsid are not appropriate. It has been estimated that standard Bv, without further deletions, can accommodate more than 38 kilobases of extra genetic information (Cheshenko et al [30]).

Baculovirus does not express its own genes or replicate in human cells. As long ago as 1983, Tjia et al showed that there was an absence of Bv gene transcription in infected HeLa cells (Tjia et al [31]). In another study, no detectable expression from the polyhedrin promoter was demonstrated in Huh7 cells (Hofmann et al [32]). In a recent study in our laboratory (A. Jones et al, manuscript in preparation), using the most sensitive methods currently available (ie, RT-PCR) the expression of a number of the potentially pathogenic Bv genes was assessed after successful infection of human and insect cells. Whereas the appropriate PCR products were detected in the infected insect cells, they were absent in Bv infected human cells, which did however express a marker gene under human promoter control. Even extending PCR cycles up to 40 did not produce a Bv gene product in the human cells. These results are not entirely unexpected, particularly as the late and very late Bv genes are transcribed by an alpha-amanatin resistant RNA polymerase (BvAARP) (Huh and Weaver [33]), which is at least partly encoded within the Bv genome (Passarelli et al [34]). In addition, the absolute requirement for BvAARP mediated expression of the late structural proteins in the assembly and production of progeny virus provides another "fire wall" to prevent the generation of replicating virus in human cells and tissues.

Mammalian viruses, and in particular those currently employed in gene therapy trials are critically dependent on host cell functions to complete their life cycle. The viral proteins can interact with host cell proteins and nucleic acids, often perturbing the host cell cycle and viability. As the Bv genes, which perform these functions in insect cells are not expressed in mammalian cells, Bv infection is unlikely to affect the target cells. This is particularly important for gene therapy protocols involving correction of a cell gene defect rather than killing of the target cells, as proposed for cancer gene therapy. Encouragingly, Bv infection of primary pancreatic islet β-cells did not affect normal cellular calcium responses to glucose, which has important implications for gene therapy of diabetes (Ma et al [35]).

Baculovirus does not recombine with preexisting genetic material: a potential drawback of the mammalian viruses, where endogenous virus is widespread in the human population, and the potential for interspecific recombination could produce new replication-competent viruses with a new pathogenicity, or cell trophism. Baculovirus also cannot "help" replication of endogenous viruses in humans, such as adenovirus and adeno-associated viruses.

The BV gp64 envelope protein is sufficiently mutable to allow the rapid insertion of new and more specific attachment sequences, much more readily than those described recently for AdV fibre protein (Krasnykh et al [36]), without perturbing its function as the principal attachment protein. This technique has already been exploited to produce antigen display in the membrane of Bv (Ernst et al [37]). However, to be bifunctional in human

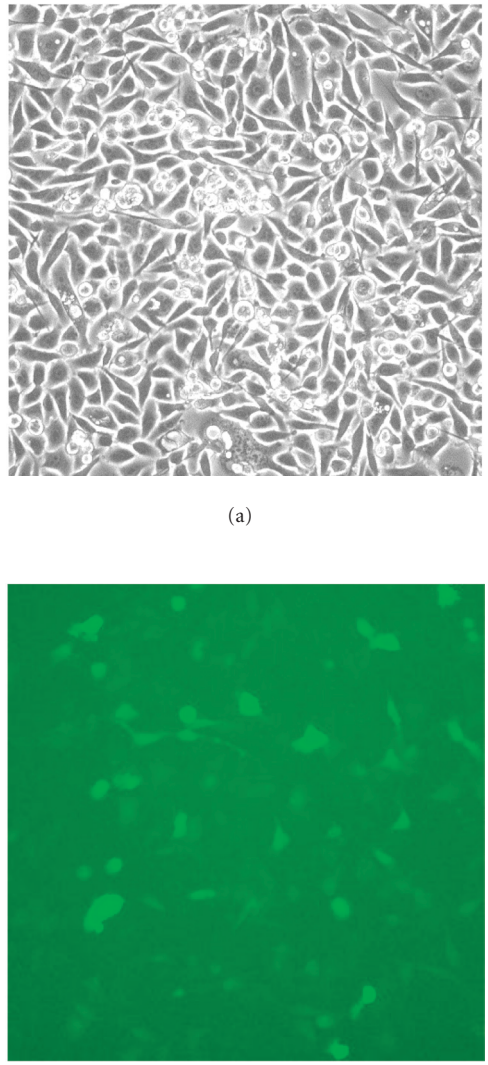
and insect cells, a mosaic envelope with wild-type and recombinant gp64 is required, which cuts down on infection efficiency in both cell types. However, effective retargeting has already been demonstrated.

In contrast to many of the other therapeutic viruses, Bv can be grown in serum-free culture media and in large quantities. In our hands, the viruses are completely stable and their production can be readily scaled up to industrial levels (currently 2–3 litres can be cultured without loss of viral viability or selection of mutant Bv). The industrial scale culture of Bv is also possible in serum-free culture conditions, which removes the potential hazard of serum contamination of the therapeutic agent with viral and prion agents from the donating animal. There is however a tendency in high level production for the virus particles (like many recombinant enveloped viruses) to aggregate, which could limit the dosages applied clinically.

Lastly, for in vivo use, there is no preexisting immune response against Bv in humans (a common problem associated with all human viral vectors, including adenoviruses). However a complement response has been demonstrated, although a number of investigations have produced inhibition strategies in animal models which prevent rapid elimination of intravenous injected Bv. This is dealt with later in more detail.

IN VITRO TRANSDUCTION OF MAMMALIAN CELL LINES WITH BACULOVIRAL VECTORS

Expression of a transgene under the control of a mammalian promoter in human cells following transduction with a recombinant baculovirus was first shown by Hofmann et al in 1995 (Hofmann et al [32]) and Boyce and Bucher in 1996 (Boyce and Bucher [38]). These studies and others have reported that hepatic cells, such as the human liver tumour cell lines HepG2 and Huh7, are generally the most susceptible mammalian cell type to infection by baculoviruses in vitro (Sandig et al [39]). Following infection with a recombinant baculovirus at an MOI of 100 pfu per cell, approximately 25–50% of HepG2 cells were shown to be positive for *LacZ* transgene expression whereas transduction of COS-7 (monkey kidney), A549 (human lung), and 293 (human kidney) cells was 10 to 100-fold less efficient (Boyce and Bucher [38]). However, there remains some controversy about the best target for Bv transduction in comparative tests of Cos-1 (SV40-transformed green monkey kidney epithelial cells), T47-D (mammary ductal carcinoma), A549, CHO (Chinese hamster ovary), HeLa (cervical carcinoma), HaCaT (keratinocyte), NIH 3T3 (fibroblasts), and COS-7 (Hofmann et al [32]; Sarkis et al [40]). The human osteogenic sarcoma cell line SAOS-2 cells expressed a baculoviral-mediated *LacZ* transgene at levels almost 20-times greater than in HepG2 cells (Song and Boyce [41]). Our own studies show that both 293 (human embryonic kidney) and PC3 (prostate cancer) cells can be transduced with approximately equal efficiency (25–50%) when infected with a CAG-EGFP baculovirus at an MOI of >100 (A. Jones, unpublished results, 2000). A typical result is shown in Figure 3.



(a)

(b)

Figure 3. Infection of PC3 prostate cancer cells with a humanised Bv expressing EGFP from a hybrid CAG promoter. Phase image of infected cells (a) after 24 hours indicates confluency of cultures while fluorescent image (b) confirms high infectivity (at > 100 particles/cell) of the tumour cells. After a further 24–48 hours more than 90% of the tumour cells express EGFP.

INFECTION OF PRIMARY CELL CULTURES WITH BACULOVIRUSES

Keratinocytes and bone marrow fibroblasts are among the primary human cell types to be successfully transduced with a baculoviral vector (Condreay et al [42]). Approximately 70% of primary hepatocytes were shown to express β-galactosidase following transduction with

an RSV-*LacZ* baculovirus at an MOI of 430 (Boyce and Bucher [38]). A much lower MOI of 25 was reportedly sufficient to transduce approximately 30% of undifferentiated primary human neural progenitor cells and approximately 55% of differentiated primary human neural cells with a CMV-EGFP baculovirus (Sarkis et al [40]).

BACULOVIRUS INFECTION OF EPITHELIAL CELLS IS INDEPENDENT OF THE CELL CYCLE

One unusual feature of prostate cancers is that the tumour cells multiply at an apparently slower rate than other cancers. Recombinant baculoviruses are however able to transduce both nondividing and actively dividing cells. For example, a G1/S arrested epithelial pig kidney cell line was infected as efficiently as dividing cells of the same type (van Loo et al [43]).

EFFICIENCY OF BACULOVIRAL-MEDIATED GENE TRANSFER IN VITRO

At a MOI of 10, comparing both levels of transgene expression and percentage of cells expressing the transgene, baculoviruses are comparable to lipofectamine and calcium phosphate precipitation as gene delivery vehicles for Huh7 cells (Hofmann et al [32]). At an MOI of 100, baculoviruses are much more efficient than both nonviral methods. A comparison with adenoviruses shows that at an MOI of both 10 and 100, transduction by a baculovirus vector results in higher β-galactosidase activity, than when the same expression cassette was transduced by an adenovirus. However, the overall percentage of cells expressing the *LacZ* gene following transduction with the adenoviral vector was approximately twice that obtained when transduced with the baculovirus vector (Hofmann et al [32]). The number of primary pancreatic islet β-cells expressing GFP following transduction with a CMV-EGFP baculovirus were comparable to that reported for lentiviral and adenoviral vectors (Ma et al [35]).

IN VIVO TRANSDUCTION OF MAMMALIAN CELLS WITH A BACULOVIRAL VECTOR

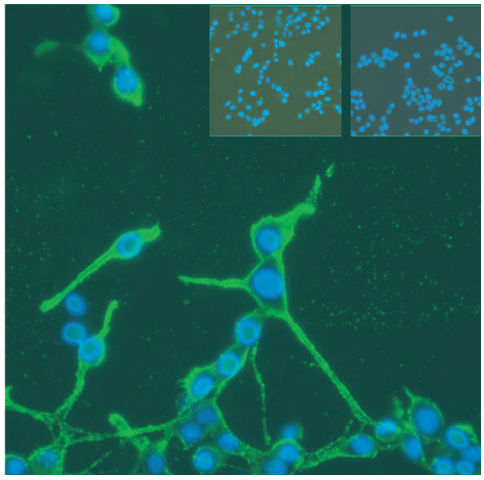
Initial attempts to use baculovirus vectors as gene delivery vehicles in vivo failed because the virus particles were inactivated by the complement immune response (Sandig et al [39]). However, in vivo gene delivery protocols that bypass the complement system have shown encouraging results. A CMV-*LacZ* baculovirus was administered to rabbit carotid arteries in vivo via a silastic collar fitted directly onto the artery (to sequester the Bv from exposure to blood). This resulted in expression of β-galactosidase in a comparable number of cells as achieved by administration with a CMV-*LacZ* adenovirus (Airenne et al [44]). Direct injection of a CMV-EGFP baculovirus into rat and mouse brain striatum resulted in transduction of neural cells in vivo, within a millimetre of the injection site (Sarkis et al [40]). The brain may represent a privileged site for Bv infection as the complement response did not abrogate transduction. In vivo gene transfer by direct injection into mouse skeletal muscle was achieved in the presence of complement with a baculoviral vector pseudotyped with VSVG, which has been shown to protect viral vectors from complement (Pieroni et al [45]).

MECHANISM OF MAMMALIAN CELL TRANSDUCTION BY BACULOVIRAL VECTORS

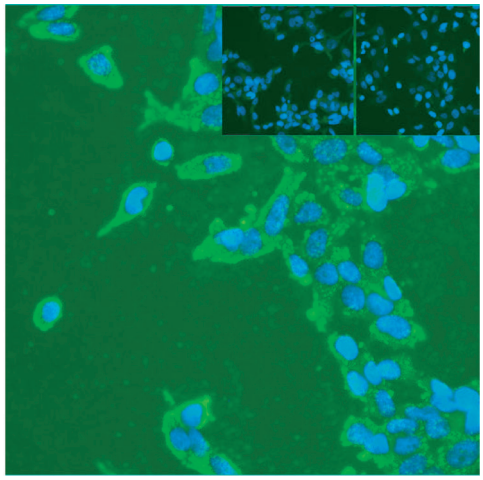
For infection of both insect and mammalian cells, baculoviruses are required to interact with the cell surface via its surface gp64 protein (Hefferon et al [46]; Tani et al [47]). It has been suggested that the cell binding and uptake mechanism may be via a specific receptor since permissiveness varies widely between different cell types. For example, the rat hepatoma cell line H35, may be nonpermissive (van Loo et al [43]). This could reflect differences in receptor expression levels but to date, no such receptor has been identified. The asialoglycoprotein receptor was initially suggested as a candidate but it was subsequently shown that a cell line expressing the cloned receptor did not show significant viral uptake (Hofmann et al [32]) and van Loo et al demonstrated efficient transduction of an epithelial pig kidney cell line (Pk1) that did not express this receptor (van Loo et al [43]). Electrostatic interactions between the viral and cellular membranes have been shown to be critical for baculovirus transduction, probably via negatively charged cell surface epitopes such as heparan sulphate (Duisit et al [48]).

At high MOI, Bv particles can be seen to completely coat mammalian cells. For example, in Figure 4a, a 3T3 cell has been infected at 4°C with an excess of Bv, and the extracellular gp64 stained with a monoclonal antibody. The fluorescent ring follows the murine cell membrane, indicating high efficiency attachment. A similar result is obtained with prostate epithelial cells (PNT1A), which contract after 4°C exposure.

Evidence that baculoviruses are taken up by endocytosis has been provided by (i) electron microscopy of CHO cells (Condreay et al [42]), and (ii) the observed reduction of virus transduction in the presence of chloroquine (Boyce and Bucher [38]; Hofmann et al [32]). As with other viruses (eg, influenza and adenoviruses), endosome acidification is required for release of the baculoviruses from the endocytotic pathway into the cytoplasm and subsequent transport, probably involving actin filaments, to the nucleus (Blissard and Wenz [49]; Boyce and Bucher [38]; van Loo et al [43]). Unlike many other viruses, both nucleocapsids and viral genomes can be detected inside the nucleus of infected cells (van Loo et al [43]). Electron microscopy images indicate that the baculoviral nucleocapsids dock onto nuclear pores in infected cells, before being transported through into the nucleus.



(a)



(b)

FIGURE 4. Specific attachment of recombinant Bv to mammalian cells. 3T3 cells (a) and prostate epithelial cells PNT1A (b) were infected with > 100 particles/cell with a humanised Bv for 1 hour at 4°C to permit attachment but not penetration. The cells were then fixed and stained with an antibody against the Bv gp64 protein. Controls are shown in the inserts (no virus infection and no primary antibody). Note the even coating of the 3T3 cells and the patchy but strong staining of the PNT1A cells.

This is observed in mitotic and nonmitotic cells. Therefore, it is likely that the capsids are transported through nuclear pores rather than taken up during mitosis (van Loo et al [43]).

The lack of transgene expression in cell lines less permissive to baculoviral transduction is more likely to be due to a block at the level of viral uncoating or transcription rather than virus entry, since viral DNA can be detected at approximately equal amounts in highly permissive (HepG2) and less permissive (Sk-Hep-1) cells 24 hours postinfection (Boyce and Bucher [38]). Also, RNA transcribed from a mammalian promoter-gene cassette can only be detected in transduced HepG2 cells and not in Sk-Hep-1 cells (Boyce and Bucher [38]). Barsoum et al (Barsoum et al [50]) demonstrated that in the highly permissive HepG2 cells, baculovirus DNA was present in the nucleus 24 hours after infection and that the DNA was packaged into chromatin as determined by digestion with staphylococcal nuclease. Conversely, in HeLa cells, DNA was not detected in the nucleus, and EM analysis supports the theory that much of the virus infected into HeLa cells is trapped inside intracellular vesicles. Effective escape from endosomes is thus a critical step in baculoviral transduction of mammalian cells.

BACULOVIRUS-MEDIATED TRANSGENE EXPRESSION IN MAMMALIAN CELLS

The onset of transgene expression has been shown as early as 6 hours posttransduction with a recombinant baculovirus and can reach peak expression levels after 12 to 24 hours (Boyce and Bucher [38]). Expression has consistently been shown to persist at approximately peak levels for at least a week both in vitro (Hofmann et al [32]; Ma et al [35]) and in vivo (Airenne et al [44]; Haeseleer et al [51]; Sarkis et al [40]). In the absence of complement, transgene expression has been detected for up to 178 days in vivo (Pieroni et al [45]).

It is possible to generate stable cell lines from cultured cells by selection of baculovirus transduced cells with G418 when a neomycin resistance cassette is included in the baculoviral transfer vector (Condreay et al [42]). Following infection of CHO cells at an MOI of 1 pfu per cell, approximately 1–2% of cells that had been transduced by the virus went on to form G418 resistant colonies (Condreay et al [42]). This is relatively inefficient and is even less likely to occur in vivo since there will be no selective pressure for integration to become a selective advantage. Further analysis revealed that fragments of the baculoviral genome ranging in size from 5 to 18 kb had integrated into the CHO cell genome (Merrihew et al [52]). The breakpoints in the virus genome were randomly located and with little homology between baculovirus and CHO cell DNA at recombination sites, suggests a mechanism of illegitimate recombination (Merrihew et al [52]). If stable integration is required for a particular gene therapy protocol, site specific integration should be safer since the risk of insertional inactivation is much lower. This has been approached by creation of a hybrid baculovirus-AAV (adeno-associated virus) vector resulting in Chromosome 19-specific integration in mammalian cells (Palombo et al [53]).

HDAC inhibitors such as trichostatin A (TCA) and butyrate have been shown to increase expression levels of a baculovirus encoded mammalian transgene in a wide variety of cultured cells including HeLa, Huh7, CHO, COS7, and 293 in addition to primary cultures of human keratinocytes, bone marrow fibroblasts, and neural

cells (Airenne et al [44]; Condreay et al [42]; Sarkis et al [40]). Although probably not feasible to use for in vivo gene therapy protocols, this could be applied to *ex vivo* gene transfer systems.

ENGINEERING PROSTATE SPECIFICITY FOR THERAPEUTIC GENE EXPRESSION IN Bv VECTORS

Although most studies of baculoviral-mediated gene transfer to date have employed the use of strong, virus derived promoters such as the CMV immediate early promoter or RSV, expression from a tissue specific promoter has also been demonstrated. The α-fetoprotein (AFP) promoter was successfully used to direct expression of a transgene specifically in AFP-expressing hepatic cells in vitro (Park et al [54]). This has important implications for gene therapy in vivo, demonstrating that transcriptional targeting is a possibility for baculoviral gene therapy.

There are now numerous reports of successful use of prostate-specific gene promoters incorporated into other gene therapy vectors that have been tested in vivo, including a PSA promoter-based lentivirus (Yu et al [55]), a PSA promoter-based adenovirus (Li et al [56]), an osteocalcin promoter-based adenovirus (Matsubara et al [57]), a probasin promoter-based adenovirus (Lowe et al [58]; Martiniello-Wilks et al [59]) (Xie et al [60]), and a human kallikrein 2 promoter-based adenovirus (Xie et al [61]).

In addition, a number of "prostate-specific" promoters have been tested for specificity after transfection into cultured cells. Amongst the most promising are PSMA (O'Keefe et al [62]), DD3 (Verhaegh et al [63]), PART-1 (Lin et al [64]), prostate transglutaminase (Dubbink et al [65]), prostatic acid phosphatase (Zelivianski et al [66]), and NKX3.1 (Prescott et al [67]; Xu et al [68]). There are no good reasons to suspect that their enhanced activity in prostate cells will be compromised in any way by insertion into Bv vectors.

Also, prostate tumour cells in vitro are highly susceptible to Bv infection, as demonstrated not only by the attachment results shown in Figure 4, but also confirmed by the result shown in Figure 3, where the strong hybrid CAG promoter has been used to drive EGFP expression in PC3 prostatic carcinoma cells, after transduction by a recombinant Bv.

The additional genetic capacity of the recombinant Bv should also allow coexpression of transcriptional modulatory genes. The best example of this might be androgen receptor, whose activity is frequently depressed in androgen insensitive tumours. In addition, many of the prostate-specific gene promoters are positively regulated by male sex hormnes, but could be inactive (mutated) or transcriptionally inactivated in hormone insensitive metastatic tumours. Therefore, coexpression of an intact or partial androgen receptor to stimulate expression from the androgen responsive promoters (Suzuki et al [69]) should be possible.

BACULOVIRUS VECTOR MODIFICATIONS FOR GENE THERAPY

Attachment targeting of baculoviruses to specific receptors on the surface of mammalian cells can be achieved by inserting attachment modifying sequences into the gp64 membrane protein, for example, insertion of a functional single chain antibody fragment specific for carcinoembryonic antigen (CEA) or two copies of a synthetic IgG binding domain of protein A (Ojala et al [70]). According to one study, addition of a modified gp64 coding region into the baculoviral genome resulted in an expression ratio of approximately 1 : 1 between wild-type and modified gp64 protein (Hüser et al [71]).

ELISA analysis indicated that the gp64 fusion proteins were capable of binding to their specific ligands and that the inserted coding region was located in an accessible part of the gp64 protein loop (Ojala et al [70]). The CEA fusion was incubated with PC-3 cells (previously shown to express CEA) and the IgG binding domain expressing baculovirus was bound to cells by preincubation of BHK cells with an anti-$\alpha 5\beta 1$ integrin polyclonal antibody and subsequent addition of the virus. Both methods result in detection of greater numbers of baculovirus particles bound to the cell surface as detected using an antibody against gp64. However, this increase in binding did not appear to enhance transduction of the cells as assessed by EGFP transgene expression, by both fluorescence microscopy and FACS (Ojala et al [70]). Thus the modification of the gp64 protein could be compromising normal functions such as endosomal escape. Thus if the rate-determining step of transduction is endosomal escape, increasing the number of bound viral particles would only have a limited effect on transduction efficiency. However, this result is still very important for gene therapy, since viral targeting could be utilised to reduce the number of viral particles required for a gene therapy regime in vivo; if the viruses can be engineered to bind more efficiently to a specific cell type (eg, prostate cancer cells) than to other cell types.

This is particularly relevant for liver, to where most intravenously injected virus will locate, as judged by studies with adenoviruses (Mizuguchi and Hayakawa [72]). By achieving retargeting, and by eliminating the liver cell trophism, the number of particles required to be administered systemically would be reduced to the benefit of the patient (and also co-incidentally increasing cost effectiveness). To date, there are no publications that assess whether a low MOI of targeted virus can achieve the same level of transduction as a nontargeted virus at a high MOI.

If the rate-limiting step of transduction is endosome escape, then modification of the baculoviral vector to mediate endosome lysis should further increase transduction efficiency. To overcome this block, the vesicular stomatitis virus G protein (VSVG) has been used. For example, efficiency of transduction and expression of the *LacZ*

transgene from a recombinant baculovirus in mammalian cells is increased by up to 200 fold by incorporation of VSVG on the virus surface (Barsoum et al [50]; Pieroni et al [45]). VSVG mediates escape from endosomes by membrane fusion (Eidelman et al [73]) but may also play a role in binding and entry of the baculovirus into mammalian cells (Tani et al [47]). Cell lines that are less permissive for baculovirus transduction, such as HeLa, A549, CHO, and NIH 3T3 cells, show the greatest difference in transgene expression between the nonpseudotyped and VSVG-expressing viruses, but even susceptible HepG2 cells show a 10-fold-increase in transgene expression (Barsoum et al [50]). Importantly, baculovirus-mediated transgene expression in HeLa cells, which are not very susceptible to baculovirus transduction, can be seen at an MOI of 1 with the VSVG-pseudotyped virus compared to an MOI of 100 with the nonpseudotyped virus (Barsoum et al [50]).

In vivo, VSVG pseudotyping may confer protection from the complement response since the transduction efficiency of mouse skeletal muscle cells was 5–10 times greater than transduction with nonpseudotyped virus. This improvement cannot be fully attributed to the enhanced transport into the cells, as the VSVG-pseudotyped virus only transduced twice as efficiently as a nonpseudotyped virus in complement deficient animals (Pieroni et al [45]). Furthermore, VSVG-pseudotyped retroviruses have been shown to be more resistant to complement than nonpseudotyped retroviruses (Ory et al [74]).

However, VSVG pseudotyping could compromise targeting strategies, since VSVG has also previously been shown to complement several functions of gp64 in a gp64 null baculovirus (Mangor et al [75]). In addition to mediating endosome escape, VSVG-enhances entry into mammalian cells, since competition with an anti-gp64 antibody did not completely inhibit cell transduction with a VSVG-pseudotyped virus. A non-VSVG-pseudotyped virus was inhibited to the normal extent (Tani et al [47]).

IMMUNE RESPONSES TO BACULOVIRUS VECTORS IN VIVO

The earliest attempts to achieve baculoviral-mediated gene transfer in vivo failed because of vector inactivation by serum components, most probably those involved in the complement response (Sandig et al [39]). The complement response is however also activated by other agents used for gene delivery such as liposomes (Szebeni [76]) and synthetic DNA complexes (Plank et al [77]). Although there is no preexisting humoral or cell mediated memory against Bv in humans and other mammals, repeated administration does give rise to neutralising antibodies. However, transgene expression has been shown to persist in the absence of complement (Pieroni et al [45]).

COMPLEMENT MANIPULATION TO POTENTIATE Bv TRANSDUCTION IN VIVO

Activation of the complement response following baculoviral infection has been investigated in more detail and it appears that complement is being activated via the classical pathway since serum depletion of C1q, unique to the classical pathway, allowed complete survival of the baculoviral vectors in vitro (Hofmann and Strauss [78]). Hofmann and Strauss (Hofmann and Strauss [78]) explored various strategies for complement inhibition to promote baculoviral vector survival in vivo. Incubation of human serum with an antibody against complement component C5, involved in both classical and alternative pathways, promoted vector survival in a dose dependent manner in vitro (Hofmann and Strauss [78]). In addition, treatment of human blood and plasma with cobra venom factor (CVF), an inhibitor of the complement component C3, also resulted in almost complete survival of baculovirus vectors as opposed to the 1% that survived in the absence of this factor (Hofmann and Strauss [78]). CVF has successfully been used to deplete the complement response in mammals in vivo, including monkeys (Chen et al [79]).

A recombinant soluble complement receptor type 1 (sCR1) lacking transmembrane and cytoplasmic domains has been shown to inhibit both classical and alternative pathways of complement activation. Presence of this factor promoted baculoviral vector survival following incubation with human serum and subsequent transduction of Huh7 cells was approximately 5 times more efficient than with baculoviruses incubated with serum in the absence of sCR1 (Hofmann et al [80]).

A further strategy designed to inhibit complement activation by baculoviruses has been to engineer expression of decay accelerating factor (DAF), a naturally occurring negative regulator of both classical and alternative complement pathways, on the virus surface as a fusion protein with gp64 (Hüser et al [71]). Following incubation with human serum, the DAF modified baculoviruses were shown to be able to transduce Huh7 cells in the presence of complement at a much higher frequency than unmodified viruses. This was shown to be due to increased survival of the vectors in the presence of complement (Hüser et al [71]). The same vectors were injected into rat livers in vivo and transgene expression, measured after 3 days, was five times higher in the livers treated with DAF-modified baculoviruses than the unmodified form (Hüser et al [71]).

Thus for cancer gene therapy, the complement response against Bv can be overcome by short term biochemical manipulation during viral inoculation. The optimal conditions have not been determined however, and a number of alternative inhibition systems, to those employed above can still be developed, particularly for prostate cancer patients. In the brain however, the complement response has no effect on Bv gene transduction, and it has been proposed as a safe and effective

agent (Sarkis et al [40]) for treatment of neural disorders.

CONCLUSIONS

Thus baculoviruses are a novel and sophisticated vector to carry therapeutic genes into human prostate cancers. They can be readily manipulated, using established and now commercially available technology, and perhaps most importantly, they have a vast capacity for exogenous DNA. This will allow larger control sequences, and even genes for transcriptional control proteins, which will offer greater independence from intracellular factors (which could simply be turned off, for example, by genome CpG methylation, as a defence mechanism) and provide greater control and specificity. The ability to target specific cell subtypes has been adequately demonstrated, although definitive data for prostate remains to be confirmed. The absence of preexisting humoral and cell-mediated immune memory against nonhuman viruses is well established, and if the complement inactivation can be overcome, their efficiency should exceed that of human viruses.

They are not without other serious problems, as industrial scale culture can be compounded by aggregation, although in our experience when employed for protein production, good virological practice prevents the build up of defective interfering particles. There is also the relatively high affinity of Bv for liver cells, which could produce undesirable hepatic side effects, unless other forms of targeting (eg, transcriptional/therapeutic gene targeting) have been included in the final vector construct. Repeated Bv inoculation will undoubtedly elicit a potent immune response, but use of Bv in combination with other viral (or nonviral) agents, could conceivably keep therapy ahead of the defence mechanisms of both the patient and his tumour, which have rendered prostate tumours so recalcitrant to normal anticancer therapy.

REFERENCES

[1] Dijkman GA, Debruyne FMJ. Epidemiology of prostate cancer. *Eur Urol*. 1996;30(3):281–295.

[2] Bubolz T, Wasson JH, Lu-Yao G, Barry MJ. Treatments for prostate cancer in older men: 1984–1997. *Urology*. 2001;58(6):977–982.

[3] Pirtskhalaishvili G, Hrebinko RL, Nelson JB. The treatment of prostate cancer: an overview of current options. *Cancer Pract*. 2001;9(6):295–306.

[4] Feldman BJ, Feldman D. The development of androgen-independent prostate cancer. *Nat Rev Cancer*. 2001;1(1):34–45.

[5] Roth JA, Grammer SF. Gene therapy—tumour-suppressor gene replacement/oncogene suppression. In: Malcolm RA, ed. *The Cancer Handbook*. Basingstoke: Macmillan Online Publishing, Macmillan Publishers Ltd; 2001.

[6] Maitland NJ. Targeting therapeutic gene expression to human prostate cancers. *Curr Opin Mol Ther*. 2000;2(4):389–399.

[7] Liu AY. Differential expression of cell surface molecules in prostate cancer cells. *Cancer Res*. 2000; 60(13):3429–3434.

[8] Luo J, Duggan DJ, Chen Y, et al. Human prostate cancer and benign prostatic hyperplasia: molecular dissection by gene expression profiling. *Cancer Res*. 2001;61(12):4683–4688.

[9] Ornstein DK, Gillespie JW, Paweletz CP, et al. Proteomic analysis of laser capture microdissected human prostate cancer and *in vitro* prostate cell lines. *Electrophoresis*. 2000;21(11):2235–2242.

[10] Macintosh CA, Stower M, Reid N, Maitland NJ. Precise microdissection of human prostate cancers reveals genotypic heterogeneity. *Cancer Res*. 1998;58(1):23–28.

[11] Van't Veer LJ, De Jong D. The microarray way to tailored cancer treatment. *Nat Med*. 2002;8(1):13–14.

[12] Dhanasekaran SM, Barrette TR, Ghosh D, et al. Delineation of prognostic biomarkers in prostate cancer. *Nature*. 2001;412(6849):822–826.

[13] Karan D, Kelly DL, Rizzino A, Lin MF, Batra SK. Expression profile of differentially-regulated genes during progression of androgen-independent growth in human prostate cancer cells. *Carcinogenesis*. 2002;23(6):967–975.

[14] Tso CL, McBride WH, Sun J, et al. Androgen deprivation induces selective outgrowth of aggressive hormone-refractory prostate cancer clones expressing distinct cellular and molecular properties not present in parental androgen-dependent cancer cells. *Cancer J*. 2000;6(4):220–233.

[15] Lee WH, Morton RA, Epstein JI, et al. Cytidine methylation of regulatory sequences near the π-class glutathione S-transferase gene accompanies human prostatic carcinogenesis. *Proc Natl Acad Sci USA*. 1994;91(24):11733–11737.

[16] Martignoni ME, Iwai PJ. *A Catalog of Viral Diseases of Insects, Mites, and Ticks*. Portland, Ore, U.S. Department of Agriculture, Forest Service, Pacific Northwest Research Station; 1986:51. General Technical Report PNW; No. 195.

[17] Ignoffo CM. Development of a viral insecticide: concept to commercialization. *Exp Parasitol*. 1973;33(2): 380–406.

[18] Ignoffo CM. Living microbial insecticides. In: Norris JR, Richmond MH, eds. *Essays in Applied Microbiology*. Chichester: John Wiley & Sons; 1981:1–31.

[19] Martignoni ME. Baculovirus: an attractive biological alternative. In: Garner WY, Harvey J Jr, eds. *Chemical and Biological Controls in Forestry*. Washington DC: American Chemical Society; 1984:55–67.

[20] Rohrmann GF. Baculovirus structural proteins. *J Gen Virol*. 1992;73(4):749–761.

[21] Moscardi F. Assessment of the application of baculoviruses for control of lepidoptera. *Annu Rev Entomol*. 1999;44:257–289.

[22] Harrap KA, Longworth JF. An evaluation of purification methods for baculoviruses. *J Invertebr Pathol*. 1974;24(1):55–62.

[23] O'Reilly DR, Miller LK, Luckow VA. *Baculovirus expression vectors: A laboratory manual*. New York, NY: WH Freeman and Company, 1992.

[24] Ayres MD, Howard SC, Kuzio J, Lopez-Ferber M, Possee RD. The complete DNA sequence of *Autographa californica* nuclear polyhedrosis virus. *Virology*. 1994;202(2):586–605.

[25] Kool M, Vlak JM. The structural and functional organization of the Autographa californica nuclear polyhedrosis virus genome. *Arch Virol*. 1993;130(1-2):1–16.

[26] Hsu SY, Kaipia A, Zhu L, Hsueh AJ. Interference of BAD (Bcl-xL/Bcl-2-associated death promoter)-induced apoptosis in mammalian cells by 14-3-3 isoforms and P11. *Mol Endocrinol*. 1997;11(12):1858–1867.

[27] Resnicoff M, Valentinis B, Herbert D, et al. The baculovirus anti-apoptotic p35 protein promotes transformation of mouse embryo fibroblasts. *J Biol Chem*. 1998;273(17):10376–10380.

[28] Robertson NM, Zangrilli J, Fernandes-Alnemri T, Friesen PD, Litwack G, Alnemri ES. Baculovirus p35 inhibits the glucocorticoid-mediated pathway of cell death. *Cancer Res*. 1997;57(1):43–47.

[29] Luckow VA, Lee SC, Barry GF, Olins PO. Efficient generation of infectious recombinant baculoviruses by site-specific transposon-mediated insertion of foreign genes into a baculovirus genome propagated in Escherichia coli. *J Virol*. 1993;67(8):4566–4579.

[30] Cheshenko N, Krougliak N, Eisensmith RC, Krougliak VA. A novel system for the production of fully deleted adenovirus vectors that does not require helper adenovirus. *Gene Ther*. 2001;8(11):846–854.

[31] Tjia ST, zu Altenschildesche GM, Doerfler W. Autographa californica nuclear polyhedrosis virus (AcNPV) DNA does not persist in mass cultures of mammalian cells. *Virology*. 1983;125(1):107–117.

[32] Hofmann C, Sandig V, Jennings G, Rudolph M, Schlag P, Strauss M. Efficient gene transfer into human hepatocytes by baculovirus vectors. *Proc Natl Acad Sci USA*. 1995;92(22):10099–10103.

[33] Huh NE, Weaver RF. Identifying the RNA polymerases that synthesize specific transcripts of the *Autographa californica* nuclear polyhedrosis virus. *J Gen Virol*. 1990;71(pt 1):195–201.

[34] Passarelli AL, Todd JW, Miller LK. A baculovirus gene involved in late gene expression predicts a large polypeptide with a conserved motif of RNA polymerases. *J Virol*. 1994;68(7):4673–4678.

[35] Ma L, Tamarina N, Wang Y, et al. Baculovirus-mediated gene transfer into pancreatic islet cells. *Diabetes*. 2000;49(12):1986–1991.

[36] Krasnykh V, Belousova N, Korokhov N, Mikheeva G, Curiel DT. Genetic targeting of an adenovirus vector via replacement of the fiber protein with the phage T4 fibritin. *J Virol*. 2001;75(9):4176–4183.

[37] Ernst WJ, Spenger A, Toellner L, Katinger H, Grabherr RM. Expanding baculovirus surface display. Modification of the native coat protein gp64 of *Autographa californica* NPV. *Eur J Biochem*. 2000;267(13):4033–4039.

[38] Boyce FM, Bucher NLR. Baculovirus-mediated gene transfer into mammalian cells. *Proc Natl Acad Sci USA*. 1996;93(6):2348–2352.

[39] Sandig V, Hofmann C, Steinert S, Jennings G, Schlag P, Strauss M. Gene transfer into hepatocytes and human liver tissue by baculovirus vectors. *Hum Gene Ther*. 1996;7(16):1937–1945.

[40] Sarkis C, Serguera C, Petres S, et al. Efficient transduction of neural cells in vitro and in vivo by a baculovirus-derived vector. *Proc Natl Acad Sci USA*. 2000;97(26):14638–14643.

[41] Song SU, Boyce FM. Combination treatment for osteosarcoma with baculoviral vector mediated gene therapy (p53) and chemotherapy (adriamycin). *Exp Mol Med*. 2001;33(1):46–53.

[42] Condreay JP, Witherspoon SM, Clay WC, Kost TA. Transient and stable gene expression in mammalian cells transduced with a recombinant baculovirus vector. *Proc Natl Acad Sci USA*. 1999;96(1):127–132.

[43] van Loo ND, Fortunati E, Ehlert E, Rabelink M, Grosveld F, Scholte BJ. Baculovirus infection of nondividing mammalian cells: mechanisms of entry and nuclear transport of capsids. *J Virol*. 2001;75(2):961–970.

[44] Airenne KJ, Hiltunen MO, Turunen MP, et al. Baculovirus-mediated periadventitial gene transfer to rabbit carotid artery. *Gene Ther*. 2000;7(17):1499–1504.

[45] Pieroni L, Maione D, La Monica N. *In vivo* gene transfer in mouse skeletal muscle mediated by baculovirus vectors. *Hum Gene Ther*. 2001;12(8):871–881.

[46] Hefferon KL, Oomens AG, Monsma SA, Finnerty CM, Blissard GW. Host cell receptor binding by baculovirus GP64 and kinetics of virion entry. *Virology*. 1999;258(2)455–468.

[47] Tani H, Nishijima M, Ushijima H, Miyamura T, Matsuura Y. Characterization of cell-surface determinants important for baculovirus infection. *Virology*. 2001;279(1):343–353.

[48] Duisit G, Saleun S, Douthe S, Barsoum J, Chadeuf G, Moullier P. Baculovirus vector requires electrostatic interactions including heparan sulfate for efficient gene transfer in mammalian cells. *J Gene Med*. 1999;1(2)93–102.

[49] Blissard GW, Wenz JR. Baculovirus gp64 envelope glycoprotein is sufficient to mediate pH-dependent membrane fusion. *J Virol*. 1992;66(11):6829–6835.

[50] Barsoum J, Brown R, McKee M, Boyce FM. Efficient transduction of mammalian cells by a recombinant baculovirus having the vesicular stomatitis virus

G glycoprotein. *Hum Gene Ther*. 1997;8(17):2011–2018.

[51] Haeseleer F, Imanishi Y, Saperstein DA, Palczewski K. Gene transfer mediated by recombinant baculovirus into mouse eye. *Invest Ophthalmol Vis Sci*. 2001;42(13):3294–3300.

[52] Merrihew RV, Clay WC, Condreay JP, Witherspoon SM, Dallas WS, Kost TA. Chromosomal integration of transduced recombinant baculovirus DNA in mammalian cells. *J Virol*. 2001;75(2):903–909.

[53] Palombo F, Monciotti A, Recchia A, Cortese R, Ciliberto G, La Monica N. Site-specific integration in mammalian cells mediated by a new hybrid baculovirus-adeno-associated virus vector. *J Virol*. 1998;72(6):5025–5034.

[54] Park SW, Lee HK, Kim TG, Yoon SK, Paik SY. Hepatocyte-specific gene expression by baculovirus pseudotyped with vesicular stomatitis virus envelope glycoprotein. *Biochem Biophys Res Commun*. 2001;289(2):444–450.

[55] Yu D, Chen D, Chiu C, Razmazma B, Chow YH, Pang S. "Prostate-specific targeting using PSA promoter-based lentiviral vectors. *Cancer Gene Ther*. 2001;8(9):628–635.

[56] Li Y, McCadden J, Ferrer F, et al. Prostate-specific expression of the diphtheria toxin A chain (DT-A): studies of inducibility and specificity of expression of prostate-specific antigen promoter-driven DT-A adenoviral-mediated gene transfer. *Cancer Res*. 2002;62(9):2576–2582.

[57] Matsubara S, Wada Y, Gardner TA, et al. A conditional replication-competent adenoviral vector, Ad-OC-E1a, to cotarget prostate cancer and bone stroma in an experimental model of androgen-independent prostate cancer bone metastasis. *Cancer Res*. 2001;61(16):6012–6019.

[58] Lowe SL, Rubinchik S, Honda T, McDonnell TJ, Dong JY, Norris JS. Prostate-specific expression of Bax delivered by an adenoviral vector induces apoptosis in LNCaP prostate cancer cells. *Gene Ther*. 2001;8(18):1363–1371.

[59] Martiniello-Wilks R, Tsatralis T, Russell P, et al. Transcription-targeted gene therapy for androgen-independent prostate cancer. *Cancer Gene Ther*. 2002;9(5):443–452.

[60] Xie X, Zhao X, Liu Y, et al. Adenovirus-mediated tissue-targeted expression of a caspase-9-based artificial death switch for the treatment of prostate cancer. *Cancer Res*. 2001;61(18):6795–6804.

[61] Xie X, Zhao X, Liu Y, et al. Robust prostate-specific expression for targeted gene therapy based on the human kallikrein 2 promoter. *Hum Gene Ther*. 2001;12(5):549–561.

[62] O'Keefe DS, Uchida A, Bacich DJ, et al. Prostate-specific suicide gene therapy using the prostate-specific membrane antigen promoter and enhancer. *Prostate*. 2000;45(2):149–157.

[63] Verhaegh GW, van Bokhoven A, Smit F, Schalken JA, Bussemakers MJG. Isolation and characterization of the promoter of the human prostate cancer-specific *DD3* gene. *J Biol Chem*. 2000;275(48):37496–37503.

[64] Lin B, White JT, Ferguson C, et al. *PART-1*: a novel human prostate-specific, androgen-regulated gene that maps to chromosome 5q12. *Cancer Res*. 2000;60(4):858–863.

[65] Dubbink HJ, de Waal L, van Haperen R, Verkaik NS, Trapman J, Romijn JC. The human prostate-specific transglutaminase gene (TGM4): genomic organization, tissue-specific expression, and promoter characterization. *Genomics*. 1998;51(3):434–444.

[66] Zelivianski S, Comeau D, Lin MF. Cloning and analysis of the promoter activity of the human prostatic acid phosphatase gene. *Biochem Biophys Res Commun*. 1998;245(1):108–112.

[67] Prescott JL, Blok L, Tindall DJ. Isolation and androgen regulation of the human homeobox cDNA, *NKX3.1*. *Prostate*. 1998;35(1):71–80.

[68] Xu LL, Srikantan V, Sesterhenn IA, et al. Expression profile of an androgen regulated prostate specific homeobox gene *NKX3.1* in primary prostate cancer. *J Urol*. 2000;163(3):972–979.

[69] Suzuki S, Tadakuma T, Asano T, Hayakawa M. Coexpression of the partial androgen receptor enhances the efficacy of prostate-specific antigen promoter-driven suicide gene therapy for prostate cancer cells at low testosterone concentrations. *Cancer Res*. 2001;61(4):1276–1279.

[70] Ojala K, Mottershead DG, Suokko A, Oker-Blom C. Specific binding of baculoviruses displaying gp64 fusion proteins to mammalian cells. *Biochem Biophys Res Commun*. 2001;284(3):777–784.

[71] Hüser A, Rudolph M, Hofmann C. Incorporation of decay-accelerating factor into the baculovirus envelope generates complement-resistant gene transfer vectors. *Nat Biotechnol*. 2001;19(5):451–455.

[72] Mizuguchi H, Hayakawa T. Enhanced antitumor effect and reduced vector dissemination with fiber-modified adenovirus vectors expressing herpes simplex virus thymidine kinase. *Cancer Gene Ther*. 2002;9(3):236–242.

[73] Eidelman O, Schlegel R, Tralka TS, Blumenthal R. pH-dependent fusion induced by vesicular stomatitis virus glycoprotein reconstituted into phospholipid vesicles. *J Biol Chem*. 1984;259(7):4622–4628.

[74] Ory DS, Neugeboren BA, Mulligan RC. A stable human-derived packaging cell line for production of high titer retrovirus/vesicular stomatitis virus G pseudotypes. *Proc Natl Acad Sci USA*. 1996;93(21):11400–11406.

[75] Mangor JT, Monsma SA, Johnson MC, Blissard GW. A GP64-null baculovirus pseudotyped with vesicular stomatitis virus G protein. *J Virol*. 2001;75(6):2544–2556.

[76] Szebeni J. The interaction of liposomes with the complement system. *Crit Rev Ther Drug Carrier Syst*. 1998;15(1):57–88.

[77] Plank C, Mechtler K, Szoka FC Jr, Wagner E. Activation of the complement system by synthetic DNA complexes: a potential barrier for intravenous gene delivery. *Hum Gene Ther*. 1996;7(12):1437–1446.
[78] Hofmann C, Strauss M. Baculovirus-mediated gene transfer in the presence of human serum or blood facilitated by inhibition of the complement system. *Gene Ther*. 1998;5(4):531–536.
[79] Chen G, Wang XM, Sun QY, et al. Prevention of hyperacute rejection of pig-to-monkey cardiac xenografts by Chinese cobra venom factor. *Transplant Proc*. 2001;33(7-8):3857–3858.
[80] Hofmann C, Hüser A, Lehnert W, Strauss M. Protection of baculovirus-vectors against complement-mediated inactivation by recombinant soluble complement receptor type 1. *Biol Chem*. 1999; 380(3):393–395.

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REVIEW ARTICLE

In Vivo Noninvasive Imaging for Gene Therapy

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Gene therapy is reaching a stage where some clinical benefits have been demonstrated on patients involved in phase I/II clinical trials. However, in many cases, the clinical benefit is hardly measurable and progress in the improvement of gene therapy formulations is hampered by the lack of objective clinical endpoints to measure transgene delivery and to quantitate transgene expression. However, these endpoints rely almost exclusively on the analysis of biopsies by molecular and histopathological methods. These methods provide only a limited picture of the situation. Therefore, there is a need for a technology that would allow precise, spacio-temporal measurement of gene expression on a whole body scale upon administration of the gene delivery vector. In the field of gene therapy, a considerable effort is being invested in the development of noninvasive imaging of gene expression and this review presents the various strategies currently being developed.

INTRODUCTION

Gene therapy can be defined as the introduction of genetic material into cells for a therapeutic purpose. The field itself can be divided into *ex vivo* gene therapy, where the target cells are taken from a patient, genetically engineered and reinfused into the patient and in vivo gene therapy, where the gene medicine formulation is directly injected into the patient. There are now some reports where clinical efficacy [1, 2, 3, 4, 5] or even complete cure [6] have been demonstrated. However, these successes remain exceptional and the large majority of phase I/II trials have failed to demonstrate any objective clinical efficacy. Therefore, the challenges of the field are to understand the reasons for the inefficacy of the current formulations in order to develop new and more efficient gene delivery vectors. For this purpose, the definition of relevant clinical endpoints is crucial. Currently, these endpoints rely on analysis of biopsies. From these patient samples, the presence and expression of the transgene can be detected by PCR, RT-PCR, or histopathological methods and in some cases enzymatic activity of the transgene can be measured. However, the information that can be gathered from this type of approach is restricted to a few cubic millimeters of biopsy material and, therefore, is only a partial reflection of the real situation in vivo.

Ideally, the monitoring of transgene delivery and expression should cover the whole body, should be noninvasive and could be repeated over time in the same patient to provide information on the location, magnitude, and kinetics of gene expression. The availability of such noninvasive techniques could be pivotal in the rational development of new formulations designed to selectively target particular tissues, organs, or disease sites and, therefore, a significant effort is currently being invested by the gene therapy community to develop in vivo, noninvasive molecular imaging technologies.

METHODS TO DETECT GENE EXPRESSION IN VIVO IN PRECLINICAL MODELS

As in the case of in vitro transfection experiments, detection of gene expression in vivo requires a reporter gene and a technology capable of detecting its activity or presence in a particular tissue and in a noninvasive way. Two types of methodologies that are already used in medicine for other purposes are currently being adapted to usage in gene therapy: nuclear medical methods and magnetic resonance tomography. More recently, a technology exploiting the fact that bioluminescence can travel through tissues and be detected by very sensitive cameras has been developed.

The general principle is that upon expression of the reporter gene the biodistribution of a tracer molecule is altered, leading to its local concentration at the site of reporter gene expression. Three types of reporter genes are currently being considered and developed: enzymes, receptors, and transport proteins (Figure 1). A general overview of the different reporter genes (proven or potential) is presented in Table 1.

Enzymes

The reporter gene can be an enzyme expressed inside the cell that alters a labelled compound. The most common approach consists of the phosphorylation of a

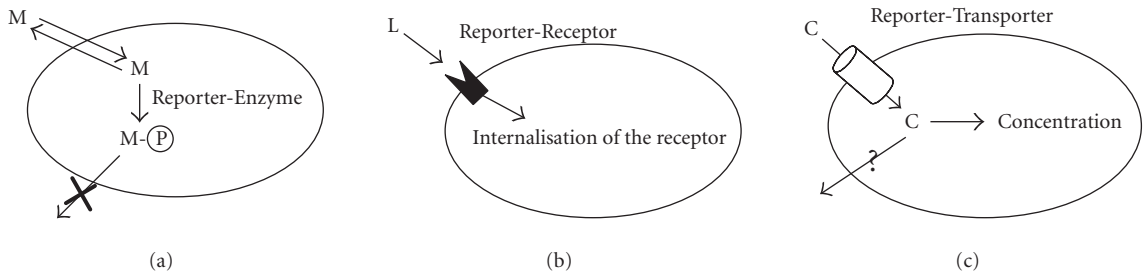

Figure 1. General principles of biological imaging. (a) Enzymes as reporter genes; upon expression of the reporter gene, the imaging marker (M) that can freely cross the plasmic membrane is metabolised within the cell (the most commonly used enzymatic reaction is a phosphorylation). As a result, the phosphorylated marker becomes incapable of recrossing the plasmic membrane and is trapped within the cell. (b) Receptors as reporter genes; upon expression of the reporter gene, the imaging ligand (L) binds to its receptor, resulting in the accumulation of the detectable ligand in the transduced tissue. This signal can be amplified when the receptor gets internalised. (c) Transporter as reporter genes; upon expression of the transporter, the imaging compound (C) is selectively transported into the cell where it concentrates. Depending on its nature, the imaging tracer is either trapped into the cell, or released when the extracellular concentration decreases (question mark), leading to a transient signal.

Table 1. Reporter genes and corresponding probes for in vivo imaging.

Reporter genes	Mechanism	Imaging agents	Imaging	References
Cytosine deaminase	Deamination	5-[^{19}F]fluorocytosine	MRS	[50]
HSV1-*tk*	Phosphorylation	[^{131}I]FIAU, [^{131}I]FIAU	SPECT, gamma camera	[51, 52]
		[^{124}I]FIAU	PET	[53]
		[$^{123/125}$I]FIAU	Gamma camera	[54]
		[^{14}C]GCV, [^{3}H]GCV	Autoradiography	[55, 56]
		[^{18}F]GCV	PET	[57, 58]
		[^{18}F]PCV	PET	[14]
		[^{18}F]FHPG	PET	[59, 60]
		[^{18}F]FHBG	PET	[12]
HSV1-sr39*tk*	Phosphorylation	[^{18}F]PCV, [^{18}F]FHBG	PET	[10, 12, 61, 62]
D2R	Receptor-ligand	[^{18}F]FESP	PET	[13]
Mutant D2R	Receptor-ligand	[^{18}F]FESP	PET	[11]
Somatostatin receptor	Affinity binding	[^{111}In]DTPA-D-Phe^{1}-octreotide	Gamma camera	[63]
		[^{64}Cu]-TETA-octreotide	Tissue dose counting	[64]
		[^{188}Re]-somatostatin analogue, ^{99m}Tc somatostatin analogue	Gamma camera	[65, 66, 67]
Na/I symporter	Active transport	[^{131}I], [^{123}I]	Gamma camera	[24, 30, 68]
		PET		[69]
Luciferase	Luciferin reaction	Bioluminescence	CCD camera	[41, 70]
Cathepsin D	Quenched fluorochromes	Fluorescence activation	CCD camera	[71]
Metalloproteinase	Quenched fluorochromes	Fluorescence activation	CCD camera	[72]
β-galactosidase	Hydrolysis of β-glycoside bond	EgadMe	MRI	[73]
Mutated transferrin receptor	Receptor-ligand	Tf-MION	MRI	[40, 74]
Creatine kinase	Dephosphorylation	Phosphocreatine	MRS	[75]
Arginine kinase	Dephosphorylation	Phosphoarginine	MRS	[76]

substrate that can cross the plasma membrane of mammalian cells. The phosphorylated product becomes incapable of traversing the cell membrane and gets trapped inside the cell.

The first tracers were developed for cytosine deaminase [7, 8] but cellular uptake proved slow and this system was superseded by an evolution of tracers that have been created for the prodrug-activating enzyme herpes simplex virus-thymidine kinase (HSV-1-Tk, [9]). In the search for medications against herpes simplex virus, compounds have been found that are preferential substrates for the HSV-1-*tk* rather than for cellular thymidine kinase. Radiolabelled derivatives have been produced that can be used for imaging. Currently, FPCV (8-[^{18}F]fluoropenciclovir) is the tracer compound that enables the highest sensitivity even with weak expression of HSV-1-*tk*. A mutated HSV-1-*tk* (HSV1-sr39*tk*) featuring a higher specificity to acycloguanosines such as FPCV provides a further increase in sensitivity [10]. Adenoviral delivery of CMV-driven HSV1-sr39tk probed with FHPG (9-[(3-[^{18}F]fluoro-1-hydroxy-2-propoxy) methyl] guanine) induced uptake of 8.5% ID/g in the liver of nude mice if 2×10^9 plaque forming units (pfu) were injected intravenously [11]. Following intratumoural injection of a similar adenovirus at a dose of 1×10^9 pfu an FHPG concentration of 6.3% ID/g has been described [12]. Images can be obtained about one hour after tracer injection [11].

Receptor binding

Much experience exists in the imaging community with tracers that bind to surface receptors. These receptors can be expressed as transgenes and can serve as reporter genes together with their specific labelled ligand.

Dopamine receptor

The dopamine D2 receptor can be expressed as a transgene in the cell membrane to induce binding of the ligand FESP (3-(2′-[^{18}F]fluoroethyl)spiperone) which can be imaged by positron emission tomography (PET) [13]. Because ectopic expression of the D2 receptor sensitises cells to circulating adrenergic signals, a mutated receptor (D80RA) has been reported uncouples ligand binding from intracellular signal transduction [11]. When nude mice were intravenously injected with 2×10^9 pfu, adenovirus in which CMV drives either D2R or D2R80A, binding of 17.5% ID/g liver of FESP was observed [11]. FESP requires about 3-hours binding time before obtaining the image [11]. In a direct comparison of the D2 receptor/FESP combination and the HSV-1-*tk*/FPCV system, similar results were obtained [14].

Somatostatin receptor type 2 (SSTR2)

Radiolabelled somatostatin analogues, such as [^{111}In]octreotide, are routinely used in the clinic for the detection of rare neuroendocrine tumours expressing the SSTR2. Expression of the receptor in tissues by gene delivery has been shown to lead to uptake of the ligand. When subcutaneous tumours in nude mice were injected with 1×10^9 pfu adenovirus carrying the sstr2 gene driven by a CMV promoter, 8% ID/g located to the tumour if probed with [^{99m}Tc]P2045, another somatostatin analogue. The time delay between injection and imaging was 5 hours [15]. In another study, intraperitoneal injection of 1×10^9 pfu of the same virus led to uptake of 2.2% ID/g [^{99m}Tc]P2045 in an intraperitoneal nude mice model for ovarian cancer [16]. The native SSTR2 activates intracellular signalling pathways resulting in cell cycle arrest [17]. The engineering of a mutated variant has been recommended [11] although the potential growth arrest properties of the SSTR2 receptor may be beneficial for application in cancer gene therapy [18, 19].

Transporter proteins: the Na/I symporter (NIS)

Transport proteins have high specificity for certain compounds and can be expressed in the cell membrane as reporter genes. They use active transport to concentrate the labelled compound in a defined compartment such as the cell cytosol.

Figure 1 illustrates the function of NIS in the thyroid gland. Driven by the sodium gradient across the basal membrane it transports iodide into the cytoplasm and concentrates it twenty to forty fold [20]. Iodide then leaves the cytoplasm entering the thyroid follicle through the apical membrane by facilitated transport, a process which involves at least one (pendrin) [21]. NADPH oxidase on the luminal side of the apical membrane generates H_2O_2 which oxydizes iodide to iodine through the action of thyroperoxidase (TPO). TPO allows binding of iodide to the tyrosine residues in the thyroglobulin present in the thyroid follicle. Iodine is therefore trapped in the thyroid follicle and is organified.

Soon after the cloning of the rat NIS gene in 1996 [22], imaging of the transgene in nonthyroid cells was demonstrated by gamma camera [23]. Intratumoural injection of 2×10^9 adenovirus, in which NIS expression is controlled by the immediate-early CMV promoter control, was shown to redirect 11% ID/g of the injected radioiodine to the tumour [24].

Imaging using NIS offers several advantages. Iodide is a tracer without requirement for radiochemistry and this has significant logistic and cost advantages. Decaying isotope does not produce cold tracer but disappears from the system. Iodide has several isotopes with different nuclear physical properties that are widely used in different imaging protocols. [^{99m}Tc]pertechnetate can be used in place of iodide and is the tracer of choice for thyroid scintigraphy. [^{188}Re]pertechnetate, a powerful beta emitter, is transported by NIS in a similar way to [^{99m}Tc]pertechnetate and has been suggested for targeted radiotherapy delivering higher tissue doses than can be achieved with ^{131}I [25]. These two isotopes can easily be obtained from generators. However, one potential limitation of the system is that NIS alone is incapable of the organification of iodide. Therefore, the accumulation of iodide can be predicted to be a dynamic phenomenon, largely dependent on the clearance of the tracer and for

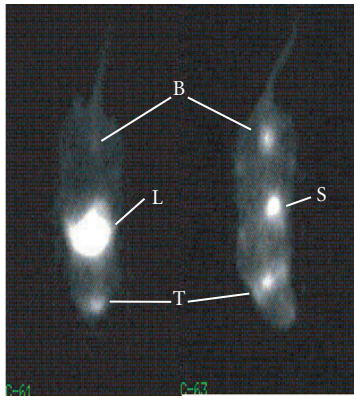

Figure 2. Imaging of hNIS expression by PET. Individual coronal slices from PET images of two nude mice intravenously treated with 5×10^7 GFU adenovirus in which a CMV promoter drives hNIS expression (left) and a PBS-treated control (right) followed, 72 hours later, by injection of [^{124}I] iodide as a tracer. In both pictures, the chosen slice lies at the level of the thyroid region (T). Tracer is seen in the stomach (S) and in the bladder (B) of the control animal. Tracer uptake in the treated animal is in the upper abdomen consistent with adenoviral homing to the liver (for detailed experimental conditions, see [69]).

which this clearance effect will vary between organs. A PET image is presented in Figure 2.

Transgenes with combined therapeutic and in vivo imaging potential

By definition, reporter genes should demonstrate a total lack of toxicity. However, an attractive alternative would be able to monitor the expression of a therapeutic transgene. This would combine monitoring of gene expression with monitoring of the efficacy of the treatment which has implications in reducing the cost of these experiments. Such an approach has been developed in cancer gene therapy where a large number of studies have aimed to develop tracer compounds that could be used to detect the activity of prodrug-converting enzymes (HSV-1-*tk*, cytosine deaminase).

In this context, the potential of the human NIS gene (hNIS) transfer for targeted radiotherapy has to be recognised. Concentration of radioiodine for the treatment of differentiated metastatic thyroid cancer has been successfully utilized since 1943 [26]. Several authors have reported the effect of high doses of radioiodine on experimental tumours following expression of NIS [27, 28]. In some cases, tumour reduction was demonstrated in permanently NIS-expressing cell lines [29]. The biological half-life of radioiodine in these studies is usually short [24, 30] and the benefit of a therapeutic dose has been shown in only one in vivo model [27]. From clinical reports it is known that failures of radioiodine therapy are linked to a short biological half-life of activity in the tumour [31] and pharmacological agents such as lithium have been described to prolong the biological half-life of iodide thyroid tissue [32]. Differentiated thyroid tissue generally expresses NIS and TPO and maintains the capability of retaining activity by organification. Coexpression of thyroid peroxidase has recently been reported in vitro but the effect on iodide retention is limited [33, 34].

Finally, the somatostatin receptor SSTR2 has been described to induce cell cycle arrest [17] and reports have demonstrated that this effect can provide therapeutic benefits in pancreatic carcinoma models [18, 19].

NON-NUCLEAR-MEDICINE METHODS

Nuclear medical methods currently offer the greatest potential to be translated into clinical applications. They are highly sensitive, provide good time resolution, and acceptable spatial resolution. But alternative methods are currently in development.

Magnetic resonance imaging (MRI)

MRI techniques have recently shown some remarkable images at very high anatomical resolution in small animals [35], during development [36, 37] and in clinical practice. However, compared to nuclear techniques, the temporal resolution remains limited and the detection of the probes by MRI is several orders of magnitude lower. In an attempt to improve the sensitivity of MR for gene therapy applications, an engineered transferrin receptor has recently been used to shuttle and accumulate supramagnetic nanoparticles into the cells [38]. The MR tracer consists of 3 nm monocristalline iron oxide nanoparticles (MION), sterically protected by a layer of dextran [39]. These MION can be covalently conjugated to the human holotransferrin (Tf-MION). The use of Tf-MION allows an amplification of the signal due to the fact that an average of 2064 Fe molecules are taken up through the transferrin receptor, as opposed to two molecules of Fe in holotransferrin. Upon binding of the Tf-MION to the transferrin receptor, the complex is internalised and MION are accumulated into the endosome. The proof of principle of noninvasive in vivo imaging of gene expression has been demonstrated [40] but it is likely that the development of complementary strategies will be necessary before MRI imaging can be used as a gene therapy tool in the clinic or even in preclinical models.

Detection of biolumunescence

Very recently, some highly sensitive devices capable of detecting and quantifying bioluminescent light have been designed. These devices are capable of detecting photons that are transmitted through mammalian tissues from internal sources [41]. This imaging of very weak visible light is rendered possible by the use of charged coupled device (CCD) cameras that include microchannel plate intensifiers and liquid nitrogen-cooled detectors. This technology aims at enhancing signal-to-noise ratio by decreasing the background (cooling) or amplifying the signal (intensifiers). These instruments are now commercially available.

Table 2. Comparison between SPECT and PET.

Method	Advantages	Disadvantages	In vivo animal use	Clinical use
SPECT	- resolution limited by technology only (submillimeter) - low sensitivities - can differentiate between isotopes with different radiation energies	- 2D (planar images) and reconstructed 3D - semiquantitative data only	- converted clinical cameras (pinhole collimator) - dedicated cameras evolving	- readily available and in widespread use - wide range of clinically tested tracers
PET	- high sensitivity - 3D acquisition - good resolution, but with a physical limit - quantification possible	- short-lived isotopes - isotopes produced in cyclotrons - expensive tracer production - expensive equipment - higher tissue doses, but balanced by higher sensitivity	currently evolving: - HIDAC - microPET	- [^{18}F]FDG becoming routine in oncology - special applications in neurology and cardiology

The gene therapy applications of this technology use luciferase genes as reporter genes. Upon addition of luciferin, the product of the luciferase gene produces luninescence. In addition, luciferin has shown a remarkably good and rapid biodistribution that allows detection of gene expression in vivo feasible [42]. Most commonly, the firefly luciferase gene is employed but other luciferases emit light at different wavelengths. This allows the monitoring of different transgenes, simultaneously in the same animal [43]. More recently, this technology has been used successfully in preclinical models of diseases [44, 45]. However, if this technology appears to be very effective in small animals (mice, rats), there is no evidence that bioluminescence imaging will be adaptable to larger animals, where the distances between organs and the skin are greater and, therefore, the photonic signal is scattered and attenuated. In addition, the signal is bidimentional and tomographic images cannot be obtained by this method. Therefore, bioluminescence detection currently appears to be limited to the laboratory. An exception to this could be clinical gene therapy for skin or occular diseases in which the target tissue is directly accessible to the detector. However, this assumption is highly hypothetical and, to our knowledge, no clinical protocol for such human studies has been submitted to the regulatory bodies.

NUCLEAR MEDICAL DETECTION METHODS

The benefits and shortcoming of two main nuclear imaging technologies and their potential for application in the laboratory animal and in the clinic are outlined in Table 2. Nuclear medical methods of gene expression imaging offer sufficient sensitivity and currently hold the best potential to be scaled up for use in patients. The principle methods are gamma cameras, single photon emission-computed tomography (SPECT), and positron emission tomography (PET).

Single photon emission-computed tomography (SPECT)

SPECT uses arrays of detectors to identify individual photons emitted by the isotope independent of their direction. A directed view is obtained by fitting metal collimators. Acting like blinkers, they filter out all photons not travelling in a certain direction (eg, either right angle to detectors or through a pinhole) relative to the detector panel. However, the greatest problem with SPECT in respect to gene therapy applications is its lack of potential for quantitation.

Positron-emitting tomography (PET)

Because of its potential in terms of quantitation as well as its high sensitivity, PET appears to be the technique of choice to collect information on the location, magnitude, and kinetics of gene expression upon delivery of genetic material.

Detection in a PET scanner requires tracers that incorporate positron-emitting isotopes. These isotopes decay by a number of different decay mechanisms. In a certain percentage of these decays, positrons are released. The positron will travel for some distance, defined by its energy and by the surrounding matter, before being annihilated when hitting an electron. Two photons of 511 MeV are created by the annihilation event and these travel in opposite directions at an angle of almost exactly 180°. PET scanner detectors register all events in a ring of detectors around the radioactivity source but process only events that occur simultaneously within a certain time window (Figure 3c). The annihilation event must have taken place on a line between these two detectors recording the event. Reconstruction from the raw data is performed by different mathematical methods that can be either back-projections or iterative reconstruction methods. They differ in their resolution,

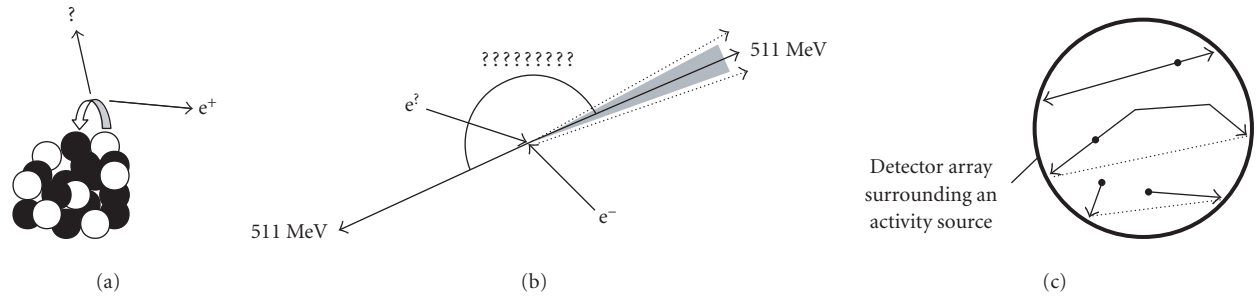

Figure 3. (a) A positron and a neutrino are released when a proton becomes a neutron. (b) Two annihilation photons travel away from each other at 180 ± 0.25°. (c) The scanner records simultaneous events within a 10–80 ns time window. These are from true coincidences, scattered coincidences, and random coincidences from independent annihilation events. Single events are not processed further (see [77]).

Table 3. Energy and half-life characteristics of selected positron-emitting isotopes.

Isotope	Half-life	Maximum positron energy	Maximum range	Spatial resolution (FWHM)	Radiation dose with internal intake{890}
^{18}F	109 min	0.63 MeV	2.6 mm	0.22 mm	0.049 mSv/MBq
^{11}C	20 min	0.96 MeV	4.2 mm	0.28 mm	0.024 mSv/MBq
^{13}N	9.9 min	1.2 MeV	5.4 mm	0.35 mm	n/a
^{15}O	122 s	1.74 MeV	8.4 mm	1.22 mm	n/a
^{82}Rb	1.3 min	3.15 MeV	17.1 mm	2.6 mm	n/a
^{124}I	4.2 d	2.14 MeV		~ 1.5 mm	13 mSv/MBq
^{64}Cu	12.7 h	3.3 MeV			0.12 mSv/MBq
^{120}I	81 min	5.6 MeV			n/a

resolution-noise ratio, contrast, and required processing time [46].

Spatial resolution in PET imaging has a physical limit (a) because of the distance the photon travels between the nuclear decay and the annihilation event and (b) because the angle between the two annihilation photons deviates slightly around 180° (Figure 3b). The former is influenced by the choice of isotope (Table 3), the latter is improved with smaller dimensions of the scanner. Spatial resolution is also reduced by scattering of the photons in tissue altering the angle between them ("Scattered," in Figure 3c). The statistical quality of the image reconstruction is reduced by photons that are simultaneously registered but originate from different events ("Random," in Figure 3c) and by multiple simultaneous registrations. Both (a) and (b) increase when the count rate approaches the saturation rate of the detection system.

Another artifact arises from attenuation of photons when travelling through the tissue. Signals from near the body surface and in the lungs are registered with relatively higher intensity. This is of less importance for smaller animals but for patient imaging attenuation correction can be performed during image reconstruction.

Positron-emitting isotopes are generally short-lived (Table 3). They are produced in cyclotrons that should be near the laboratory or hospital where tracer synthesis, transport, and application take place. Small variations in timing have important consequences. The tissue dose from positron-emitting isotopes is relatively high because the energy of the positron is completely absorbed in the tissue. This is balanced by a short half-life but is more significant for ^{124}I with a relatively long duration in the tissue.

HUMAN STUDY

The development of these new technologies is just reaching the clinic and, to our knowledge, only one report has been published on the use of in vivo molecular imaging in clinical gene therapy [47]. This study was preceded by the establishment of the pharmacodynamic and biodistribution parameters of the radioactive tracer [^{124}I] FIAU (to monitor HSV-1-*tk* expression) in humans [48], with a particular emphasis on the head region. This radioactive tracer was shown not to be able to cross the blood-brain barrier in normal brain but showed rapid and nonspecific accumulation within recurrent glioma [48], demonstrating that [^{124}I] FIAU was a useful tracer when the blood-brain barrier was disrupted. In a follow-up study, this tracer was used in a small clinical trial testing liposomal intratumoural delivery of the HSV-1-*tk* gene to patients with recurrent glioma [47]. The authors demonstrated accumulation of [^{124}I] FIAU, indicative of HSV-1-*tk* expression, in the immediate periphery of the needle tract in one

out of five patients. In this patient, the overall therapeutic effect was limited to a portion of the tumour. However, in the four remaining patients, histology sections of the tumour showed a significant decrease in the number of proliferating cells. This observation tends to indicate that a critical threshold of gene expression has to be reached before HSV-1-*tk*-associated FIAU could be detected by PET. The authors concluded that the extent of HSV-1-tk expression appeared to predict the therapeutic response. However, the overall [^{124}I] FIAU accumulation, as well as the therapeutic response were limited [47].

CONCLUDING REMARK

There is little doubt that using whole body, noninvasive imaging technology will help to design gene therapy formulations tailored to target diseases. But the need for clinicians to monitor gene expression, and more generally to monitor biodistribution and pharmacodynamics of gene therapy formulations in a more precise and quantitative way has been sadly highlighted by the death of Jesse Gelsinger, who became the first person to die from experimental techniques of gene therapy. Following his death, one of the recommendations of the Recombinant DNA Advisory Committee (RAC) of the National Institute of Health in the USA was to develop better ways for measuring transgene expression in cells and tissues in order to improve the safety of these interventions [49].

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REFERENCES

[1] Khuri FR, Nemunaitis J, Ganly I, et al. A controlled trial of intratumoral ONYX-015, a selectively-replicating adenovirus, in combination with cisplatin and 5-fluorouracil in patients with recurrent head and neck cancer. *Nat Med.* 2000;6(8):879–885.

[2] Sandmair AM, Loimas S, Puranen P, et al. Thymidine kinase gene therapy for human malignant glioma, using replication-deficient retroviruses or adenoviruses. *Hum Gene Ther*. 2000;11(16):2197–2205.

[3] Nemunaitis J, Swisher SG, Timmons T, et al. Adenovirus-mediated p53 gene transfer in sequence with cisplatin to tumors of patients with non-small-cell lung cancer. *J Clin Oncol*. 2000;18(3):609–622.

[4] Isner JM, Asahara T. Angiogenesis and vasculogenesis as therapeutic strategies for postnatal neovascularization. *J Clin Invest*. 1999;103(9):1231–1236.

[5] Kay MA, Manno CS, Ragni MV, et al. Evidence for gene transfer and expression of factor IX in haemophilia B patients treated with an AAV vector. *Nat Genet*. 2000;24(3):257–261.

[6] Cavazzana-Calvo M, Hacein-Bey S, de Saint Basile G, et al. Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. *Science*. 2000;288(5466):669–672.

[7] Gambhir SS, Barrio JR, Herschman HR, Phelps ME. Assays for noninvasive imaging of reporter gene expression. *Nucl Med Biol*. 1999;26(5):481–490.

[8] Brix G, Bellemann ME, Haberkorn U, Gerlach L, Lorenz WJ. Assessment of the biodistribution and metabolism of 5-fluorouracil as monitored by 18F PET and 19F MRI: a comparative animal study. *Nucl Med Biol*. 1996;23(7):897–906.

[9] Blasberg RG, Tjuvajev JG. Herpes simplex virus thymidine kinase as a marker/reporter gene for PET imaging of gene therapy. *Q J Nucl Med*. 1999; 43(2):163–169.

[10] Gambhir SS, Bauer E, Black ME, et al. A mutant herpes simplex virus type 1 thymidine kinase reporter gene shows improved sensitivity for imaging reporter gene expression with positron emission tomography. *Proc Natl Acad Sci USA*. 2000; 97(6):2785–2790.

[11] Liang Q, Satyamurthy N, Barrio JR, et al. Noninvasive, quantitative imaging in living animals of a mutant dopamine D2 receptor reporter gene in which ligand binding is uncoupled from signal transduction. *Gene Ther*. 2001;8(19):1490–1498.

[12] Yaghoubi SS, Wu L, Liang Q, et al. Direct correlation between positron emission tomographic images of two reporter genes delivered by two distinct adenoviral vectors. *Gene Ther*. 2001;8(14):1072–1080.

[13] MacLaren DC, Gambhir SS, Satyamurthy N, et al. Repetitive, non-invasive imaging of the dopamine D2 receptor as a reporter gene in living animals. *Gene Ther*. 1999;6(5):785–791.

[14] Iyer M, Barrio JR, Namavari M, et al. 8-[^{18}F]Fluoropenciclovir: an improved reporter probe for imaging HSV1-tk reporter gene expression in vivo using PET. *J Nucl Med*. 2001;42(1):96–105.

[15] Zinn KR, Chaudhuri TR. The type 2 human somatostatin receptor as a platform for reporter gene imaging. *Eur J Nucl Med Mol Imaging*. 2002;29(3):388–399.

[16] Chaudhuri TR, Rogers BE, Buchsbaum DJ, Mountz JM, Zinn KR. A noninvasive reporter system to image adenoviral-mediated gene transfer to ovarian cancer xenografts. *Gynecol Oncol*. 2001;83(2):432–438.

[17] Pages P, Benali N, Saint-Laurent N, et al. sst2 somatostatin receptor mediates cell cycle arrest and induction of p27(Kip1). Evidence for the role of SHP-1. *J Biol Chem*. 1999;274(21):15186–15193.

[18] Rochaix P, Delesque N, Esteve JP, et al. Gene therapy for pancreatic carcinoma: local and distant antitumor effects after somatostatin receptor

sst2 gene transfer. *Hum Gene Ther*. 1999;10(6):995–1008.

[19] Benali N, Cordelier P, Calise D, et al. Inhibition of growth and metastatic progression of pancreatic carcinoma in hamster after somatostatin receptor subtype 2 (sst2) gene expression and administration of cytotoxic somatostatin analog AN-238. *Proc Natl Acad Sci USA*. 2000;97(16):9180–9185.

[20] Filetti S, Bidart JM, Arturi F, Caillou B, Russo D, Schlumberger M. Sodium/iodide symporter: a key transport system in thyroid cancer cell metabolism. *Eur J Endocrinol*. 1999;141(5):443–457.

[21] Royaux IE, Suzuki K, Mori A, et al. Pendrin, the protein encoded by the Pendred syndrome gene (PDS), is an apical porter of iodide in the thyroid and is regulated by thyroglobulin in FRTL-5 cells. *Endocrinology*. 2000;141(2):839–845.

[22] Dai G, Levy O, Carrasco N. Cloning and characterization of the thyroid iodide transporter. *Nature*. 1996;379(6564):458–460.

[23] Shimura H, Haraguchi K, Miyazaki A, Endo T, Onaya T. Iodide uptake and experimental ^{131}I therapy in transplanted undifferentiated thyroid cancer cells expressing the Na^+/I^- symporter gene. *Endocrinology*. 1997;138(10):4493–4496.

[24] Boland A, Ricard M, Opolon P, et al. Adenovirus-mediated transfer of the thyroid sodium/iodide symporter gene into tumors for a targeted radiotherapy. *Cancer Res*. 2000;60(13):3484–3492.

[25] Dadachova E, Bouzahzah B, Zuckier LS, Pestell RG. Rhenium-188 as an alternative to Iodine-131 for treatment of breast tumors expressing the sodium/iodide symporter (NIS). *Nucl Med Biol*. 2002;29(1):13–18.

[26] Siegel E. The beginnings of radioiodine therapy of metastatic thyroid carcinoma: a memoir of Samuel M. Seidlin, M. D. (1895–1955) and his celebrated patient. *Cancer Biother Radiopharm*. 1999;14(2):71–79.

[27] Spitzweg C, O'Connor MK, Bergert ER, Tindall DJ, Young CY, Morris JC. Treatment of prostate cancer by radioiodine therapy after tissue-specific expression of the sodium iodide symporter. *Cancer Res*. 2000;60(22):6526–6530.

[28] Smit JW, Schroder-van der Elst JP, Karperien M, et al. Iodide kinetics and experimental ^{131}I therapy in a xenotransplanted human sodium-iodide symporter-transfected human follicular thyroid carcinoma cell line. *J Clin Endocrinol Metab*. 2002; 87(3):1247–1253.

[29] Spitzweg C, Harrington KJ, Pinke LA, Vile RG, Morris JC. Clinical review 132: The sodium iodide symporter and its potential role in cancer therapy. *J Clin Endocrinol Metab*. 2001;86(7):3327–3335.

[30] La Perle KMD, Shen D, Buckwalter TLF, et al. In vivo expression and function of the sodium iodide symporter following gene transfer in the MATLyLu rat model of metastatic prostate cancer. *Prostate*. 2002;50(3):170–178.

[31] Maxon HR, Thomas SR, Hertzberg VS, et al. Relation between effective radiation dose and outcome of radioiodine therapy for thyroid cancer. *N Engl J Med*. 1983;309(16):937–941.

[32] Koong SS, Reynolds JC, Movius EG, et al. Lithium as a potential adjuvant to ^{131}I therapy of metastatic, well differentiated thyroid carcinoma. *J Clin Endocrinol Metab*. 1999;84(3):912–916.

[33] Huang M, Batra RK, Kogai T, et al. Ectopic expression of the thyroperoxidase gene augments radioiodide uptake and retention mediated by the sodium iodide symporter in non-small cell lung cancer. *Cancer Gene Ther*. 2001;8(8):612–618.

[34] Boland A, Magnon C, Filetti S, et al. Transposition of the thyroid iodide uptake and organification system in nonthyroid tumor cells by adenoviral vector-mediated gene transfers. *Thyroid*. 2002;12(1):19–26.

[35] Johnson GA, Benveniste H, Black RD, Hedlund LW, Maronpot RR, Smith BR. Histology by magnetic resonance microscopy. *Magn Reson Q*. 1993;9(1):1–30.

[36] Smith BR, Johnson GA, Groman EV, Linney E. Magnetic resonance microscopy of mouse embryos. *Proc Natl Acad Sci USA*. 1994;91(9):3530–3533.

[37] Jacobs RE, Fraser SE. Magnetic resonance microscopy of embryonic cell lineages and movements. *Science*. 1994;263(5147):681–684.

[38] Moore A, Basilion JP, Chiocca EA, Weissleder R. Measuring transferrin receptor gene expression by NMR imaging. *Biochim Biophys Acta*. 1998;1402(3): 239–249.

[39] Shen T, Weissleder R, Papisov M, Bogdanov A Jr, Brady TJ. Monocrystalline iron oxide nanocompounds (MION): physicochemical properties. *Magn Reson Med*. 1993;29(5):599–604.

[40] Weissleder R, Moore A, Mahmood U, et al. In vivo magnetic resonance imaging of transgene expression. *Nat Med*. 2000;6(3):351–355.

[41] Contag PR, Olomu IN, Stevenson DK, Contag CH. Bioluminescent indicators in living mammals. *Nat Med*. 1998;4(2):245–247.

[42] Sweeney TJ, Mailander V, Tucker AA, et al. Visualizing the kinetics of tumor-cell clearance in living animals. *Proc Natl Acad Sci USA*. 1999;96(21):12044–12049.

[43] Bhaumik S, Gambhir SS. Optical imaging of *Renilla* luciferase reporter gene expression in living mice. *Proc Natl Acad Sci USA*. 2002;99(1):377–382.

[44] Nakajima A, Seroogy CM, Sandora MR, et al. Antigen-specific T cell-mediated gene therapy in collagen-induced arthritis. *J Clin Invest*. 2001; 107(10):1293–1301.

[45] Lipshutz GS, Gruber CA, Cao Y, Hardy J, Contag CH, Gaensler KM. In utero delivery of adeno-associated viral vectors: intraperitoneal gene transfer produces long-term expression. *Mol Ther*. 2001;3(3):284–292.

[46] Walledge RJ, Manavaki R, Reader AJ, et al. Comparison of four images reconstruction techniques for

high resolution imaging. IEEE Nuclear Science Symposium and Medical Conference papers. Submitted to *IEEE T Nucl Sci*. 2002.

[47] Jacobs A, Voges J, Reszka R, et al. Positron-emission tomography of vector-mediated gene expression in gene therapy for gliomas. *Lancet*. 2001;358(9283):727–729.

[48] Jacobs A, Braunlich I, Graf R, et al. Quantitative kinetics of [^{124}I]FIAU in cat and man. *J Nucl Med*. 2001;42(3):467–475.

[49] Hollon T. Researchers and regulators reflect on first gene therapy death. *Nat Med*. 2000;6(1):6.

[50] Stegman LD, Rehemtulla A, Beattie B, et al. Noninvasive quantitation of cytosine deaminase transgene expression in human tumor xenografts with in vivo magnetic resonance spectroscopy. *Proc Natl Acad Sci USA*. 1999;96(17):9821–9826.

[51] Tjuvajev JG, Finn R, Watanabe K, et al. Noninvasive imaging of herpes virus thymidine kinase gene transfer and expression: a potential method for monitoring clinical gene therapy. *Cancer Res*. 1996;56(18):4087–4095.

[52] Tjuvajev JG, Chen SH, Joshi A, et al. Imaging adenoviral-mediated herpes virus thymidine kinase gene transfer and expression in vivo. *Cancer Res*. 1999;59(20):5186–5193.

[53] Tjuvajev JG, Avril N, Oku T, et al. Imaging herpes virus thymidine kinase gene transfer and expression by positron emission tomography. *Cancer Res*. 1998;58(19):4333–4341.

[54] Haubner R, Avril N, Hantzopoulos PA, Gansbacher B, Schwaiger M. In vivo imaging of herpes simplex virus type 1 thymidine kinase gene expression: early kinetics of radiolabelled FIAU. *Eur J Nucl Med*. 2000;27(3):283–291.

[55] Haberkorn U, Altmann A, Morr I, et al. Monitoring gene therapy with herpes simplex virus thymidine kinase in hepatoma cells: uptake of specific substrates. *J Nucl Med*. 1997;38(2):287–294.

[56] Haberkorn U, Khazaie K, Morr I, Altmann A, Muller M, van Kaick G. Ganciclovir uptake in human mammary carcinoma cells expressing herpes simplex virus thymidine kinase. *Nucl Med Biol*. 1998;25(4):367–373.

[57] Gambhir SS, Barrio JR, Wu L, et al. Imaging of adenoviral-directed herpes simplex virus type 1 thymidine kinase reporter gene expression in mice with radiolabeled ganciclovir. *J Nucl Med*. 1998;39(11):2003–2011.

[58] Gambhir SS, Barrio JR, Phelps ME, et al. Imaging adenoviral-directed reporter gene expression in living animals with positron emission tomography. *Proc Natl Acad Sci USA*. 1999;96(5):2333–2338.

[59] Alauddin MM, Conti PS, Mazza SM, Hamzeh FM, Lever JR. 9-[(3-[^{18}F]-fluoro-1-hydroxy-2-propoxy)methyl]guanine ([^{18}F]-FHPG): a potential imaging agent of viral infection and gene therapy using PET. *Nucl Med Biol*. 1996;23(6):787–792.

[60] Alauddin MM, Shahinian A, Kundu RK, Gordon EM, Conti PS. Evaluation of 9-[(3-^{18}F-fluoro-1-hydroxy-2-propoxy)methyl]guanine ([18F]-FHPG) in vitro and in vivo as a probe for PET imaging of gene incorporation and expression in tumors. *Nucl Med Biol*. 1999;26(4):371–376.

[61] Yu Y, Annala AJ, Barrio JR, et al. Quantification of target gene expression by imaging reporter gene expression in living animals. *Nat Med*. 2000;6(8):933–937.

[62] Sun X, Annala AJ, Yaghoubi SS, et al. Quantitative imaging of gene induction in living animals. *Gene Ther*. 2001;8(20):1572–1579.

[63] Rogers BE, McLean SF, Kirkman RL, et al. In vivo localization of [(111)In]-DTPA-D-Phe1-octreotide to human ovarian tumor xenografts induced to express the somatostatin receptor subtype 2 using an adenoviral vector. *Clin Cancer Res*. 1999;5(2):383–393.

[64] Buchsbaum DJ, Rogers BE, Khazaeli MB, et al. Targeting strategies for cancer radiotherapy. *Clin Cancer Res*. 1999;5(Suppl 10):3048s–3055s.

[65] Rogers BE, Zinn KR, Buchsbaum DJ. Gene transfer strategies for improving radiolabeled peptide imaging and therapy. *Q J Nucl Med*. 2000;44(3):208–223.

[66] Zinn KR, Buchsbaum DJ, Chaudhuri TR, Mountz JM, Grizzle WE, Rogers BE. Noninvasive monitoring of gene transfer using a reporter receptor imaged with a high-affinity peptide radiolabeled with ^{99m}Tc or ^{188}Re. *J Nucl Med*. 2000;41(5):887–895.

[67] Hemminki A, Zinn KR, Liu B, et al. In vivo molecular chemotherapy and noninvasive imaging with an infectivity-enhanced adenovirus. *J Natl Cancer Inst*. 2002;94(10):741–749.

[68] Haberkorn U, Henze M, Altmann A, et al. Transfer of the human NaI symporter gene enhances iodide uptake in hepatoma cells. *J Nucl Med*. 2001;42(2):317–325.

[69] Groot-Wassink T, Aboagye EO, Glaser M, Lemoine NR, Vassaux G. Adenovirus biodistribution and noninvasive imaging of gene expression in vivo by positron emission tomography using the sodium/iodide symporter as a reporter gene. *Hum Gene Ther*. 2002;13(14):1723–1735.

[70] Edinger M, Sweeney TJ, Tucker AA, Olomu AB, Negrin RS, Contag CH. Noninvasive assessment of tumor cell proliferation in animal models. *Neoplasia*. 1999;1(4):303–310.

[71] Tung CH, Mahmood U, Bredow S, Weissleder R. In vivo imaging of proteolytic enzyme activity using a novel molecular reporter. *Cancer Res*. 2000;60(17):4953–4958.

[72] Bremer C, Bredow S, Mahmood U, Weissleder R, Tung CH. Optical imaging of matrix metalloproteinase-2 activity in tumors: feasibility study in a mouse model. *Radiology*. 2001;221(2):523–529.

[73] Louie AY, Huber MM, Ahrens ET, et al. In vivo visualization of gene expression using magnetic resonance imaging. *Nat Biotechnol*. 2000;18(3):321–325.

[74] Moore A, Josephson L, Bhorade RM, Basilion JP, Weissleder R. Human transferrin receptor gene as a marker gene for MR imaging. *Radiology*. 2001;221(1):244–250.
[75] Auricchio A, Zhou R, Wilson JM, Glickson JD. In vivo detection of gene expression in liver by 31P nuclear magnetic resonance spectroscopy employing creatine kinase as a marker gene. *Proc Natl Acad Sci USA*. 2001;98(9):5205–5210.
[76] Walter G, Barton ER, Sweeney HL. Noninvasive measurement of gene expression in skeletal muscle. *Proc Natl Acad Sci USA*. 2000;97(10):5151–5155.
[77] Budinger TF. PET instrumentation: what are the limits? *Semin Nucl Med*. 1998;28(3):247–267.

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REVIEW ARTICLE

Targeting Radiotherapy to Cancer by Gene Transfer

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Targeted radionuclide therapy is an alternative method of radiation treatment which uses a tumor-seeking agent carrying a radioactive atom to deposits of tumor, wherever in the body they may be located. Recent experimental data signifies promise for the amalgamation of gene transfer with radionuclide targeting. This review encompasses aspects of the integration of gene manipulation and targeted radiotherapy, highlighting the possibilities of gene transfer to assist the targeting of cancer with low molecular weight radiopharmaceuticals.

INTRODUCTION

While tumors which are confined to their site of origin may often be cured by local treatment such as surgery or conventional external beam irradiation, cancer which has spread to locations distant from the primary tumor requires a treatment which is applied to the whole body of the patient. Total body irradiation is effective in the management of leukemia but normal tissue intolerance restricts the radiation dose which can be given, so that it cannot be used against less radiosensitive neoplasia. Biologically targeted radionuclide therapy is an alternative method of systemic irradiation treatment which circumvents the two problems of widespread distribution of disease and the intolerance of normal tissues.

Targeted radiotherapy uses a molecular vehicle which either localises on the surface of malignant cells or is preferentially accumulated within them. For many tumours, monoclonal antibodies or their fragments represent the only targeting agents. With the notable exception of B-cell lymphoma [1, 2], clinical applications of these radiolabelled macromolecules have generally been unsatisfactory due to low tumor specificity of targeted epitopes, limited penetration into tumors, and the provocation of anti-mouse immunoglobulin responses. These considerations favour the use of nonimmunogenic small molecules with higher uptake in tumours. These criteria are fulfilled by peptides, meta-iodobenzylguanidine (MIBG), and sodium iodide (NaI), which are readily available in radioiodinated form. MIBG and NaI have been used extensively for the treatment of neural crest-derived tumors (neuroblastoma and phaeochromocytoma) and thyroid carcinoma, respectively.

The new challenge is to enhance targeted radiotherapy by combining it with the transfer into tumour cells of genes encoding specific transporters. The success of this approach has been demonstrated in model systems. Efforts are now underway to optimise tumour to normal tissue uptake ratios; to limit the expression of transporter genes to malignant sites; and to compare the therapeutic potential of α- and β-emitting radionuclides conjugated to tumour-seeking agents. These investigations will determine optimal promoter/transgene/radionuclide combinations for effective human anticancer gene therapy.

TRANSDUCTION OF ANTIGEN ENCODING GENES FOR ANTIBODY TARGETING

The efficacy of radiolabelled antibodies is not due to radiation kill alone [3] but results also from the cytotoxic effects of unlabelled antibody, namely apoptosis, complement-mediated cytolysis and antibody-dependent cellular cytotoxicity. This type of treatment is proving to be useful in the management of B-cell lymphomas [4] which are more radiosensitive than solid tumours. They have a propensity to apoptose after radiation insult and have limited capacity for the repair of sublethal damage. However, radioimmunotherapy of other tumour types has been less impressive. An average value, computed from a wide range of articles, for the accumulation of radiolabelled antibodies—0.005% per gram of the administered activity—is insufficient for tumour sterilisation [5]. An impediment to radioimmunotherapy is the sparsity of antigen presentation on the membranes of tumour cells.

This problem has been addressed by transfection of genes to induce tumour cells to express high concentrations of membrane-associated epitopes which have affinity for monoclonal antibodies. Radiolabelled antibodies directed against carcinoembryonic antigen (CEA) have

been used extensively in experimental and clinical detection and treatment of various tumours (reviewed by Buchsbaum and Curiel [6]). Raben et al [7] have demonstrated that adenovirus-mediated gene transfer resulted in high levels of CEA expression in vitro in tumour cells that did not constitutively express CEA, rendering those cells capable of binding radiolabelled COL-1 anti-CEA antibody. They have further demonstrated the applicability of this approach in vivo in nude mice resulting in enhanced, radiolabelled, antibody localisation to tumours transduced by intratumoural injection of the recombinant virus.

After extensive preclinical testing, the most exciting clinical data has come from the use of radioimmunotherapy in the treatment of lymphoma and haematological malignancies and it would now appear highly likely that radioimmunotherapy in combination with gene transfer will play a major role in treatment strategies for these diseases. Results in solid tumours are improving. However, much progress remains to be made before RIT becomes a component of standard practice for common malignancies in the clinic.

FACILITATION OF PEPTIDE TARGETING BY RECEPTOR GENE EXPRESSION

Tumour-targeting peptides offer several advantages over antibodies such as improved penetration characteristics, ease of labelling, and lack of immunogenicity. Several studies show the feasibility of inducing the expression of receptors by tumour cells to improve the accumulation of toxic radionuclides transported by a variety of peptide ligands. For example, virus-assisted gene transfer stimulated the expression of the receptor for thyrotropin-releasing hormone (TRH) with subsequent binding of radiolabelled TRH [8]. Unfortunately, this tripeptide has a short biological half-life in serum and loses receptor affinity after conjugation to radionuclides. Using in vitro and in vivo models, Rogers et al [9] have demonstrated the potential of a seven-peptide analogue of bombesin to target tumour cells transfected with the gene encoding gastrin releasing peptide receptor. This ligand is attractive because it is readily bound to several different radionuclides, maintains receptor binding affinity and exhibits in vivo stability. Recently, Stackhouse et al [10] introduced a greater level of sophistication to this scheme by placing the expression of the GPR transgene under the control of promoter elements of one of two different genes—*erbB-2* and *MUC1*—which are overexpressed in a number of tumours. This strategy was successful in restricting GPR expression to human breast and cholangiocarcinoma cell lines thereby demonstrating the potential for tumour-specific targeting with radiolabelled peptide. An exciting application of the control of expression by the MUC1 carcinoma-promoter has been described by Chen et al [11]. They eliminated six logs of contaminating cancer cells from haemopoietic stem cell preparations by ganciclovir treatment after adenoviral-mediated transduction of *HSV-tk* gene under control of the *MUC1* promoter. This study illustrates the potential of the gene transfer approach to the purging of marrow or peripheral blood stem cells.

An attractive means of improving the specificity of targeting is to express a nonhuman receptor on tumour cells and target these with a xenogeneic molecule. For example, the murine glycoprotein, interleukin-4 (IL-4), does not bind to the human IL-4 receptor nor does the human IL-4 have affinity for the murine receptor. The mouse receptor cDNA has been expressed in heterologous cells, resulting in a five-fold increase in binding of ligand to transfectants [12]. It is hoped that this promising strategy for the transport of therapeutic radionuclides can be developed using xenogeneic systems which involve smaller targeting agents such as peptides or steroids [13].

Somatostatin receptors, which are expressed on many tumours of neuroendocrine origin, constitute another peptide target which may be exploitable for radionuclide therapy. Octreotide is an octapeptide analogue of somatostatin which has greater stability in plasma than the natural ligand [14]. Rogers et al [15] recently employed recombinant adenoviral vectors to induce somatostatin receptors on human nonsmall cell lung cancer cells which were grown as xenografts. Tumour localisation was demonstrated using [^{111}In]-labelled octreotide and therapeutic efficacy was obtained with [^{90}Y] octreotide. This is the first illustration in vivo of the effectiveness of a radiolabeled peptide targeted to a receptor expressed on the surface of tumor cells following gene transfer. It is expected that these studies will form the basis of future therapeutic investigations using gene transfer to enhance tumour targeting by radiolabelled octreotide.

RADIOHALIDE CONCENTRATION VIA THE SODIUM IODIDE SYMPORTER

Unlike the above schemes for radionuclide targeting, sodium radioiodide ($Na^{131}I$) therapy requires no radiochemical synthetic procedure. Most well-differentiated thyroid tumours retain iodide-concentrating capacity—mediated by the sodium (Na) iodide (I) symporter (NIS). Therefore, $Na^{131}I$ is used to ablate postsurgical remnants and to treat recurrent and metastatic disease. The overall prognosis following radioiodine therapy is good for differentiated thyroid cancer. This is the most basic, yet most efficacious form of radionuclide therapy to date.

Cloning and characterisation of the sodium iodide symporter (NIS) gene has stimulated cytoreductive gene therapy based on NIS gene transfer followed by the administration of radioiodide. Transfer of the NIS gene into a variety of nonthyroidal cancer cell lines has induced iodide uptake activity and cytotoxicity [16, 17, 18]. Following PSA promoter-mediated NIS gene delivery, prostate-specific iodide accumulation was achieved in prostate cancer cells that was sufficient to attain a therapeutic

response of ^{131}I in vitro and in vivo [19, 20]. These observations indicate that, following thyroid blockade, it may be possible to deliver Na^{131}I to transfected, NIS-expressing, tumour cells with minimal uptake of radioactivity in normal tissues.

However, rapid efflux of radioiodide from NIS gene transfectants imposes a severe limitation on tumor cell killing. Means of circumventing this problem are under investigation. Unlike NIS-transfected tumor cells, thyrocytes exhibit, in addition, peroxidase activity which is responsible for the oxidation of accumulated iodide, leading to its conjugation to tyrosyl residues of thyroglobulin and prolonged entrapment within the cell. Huang et al [21] reported an increase in radioiodide uptake and retention and enhanced apoptosis in nonsmall cell lung cancer cells transfected with both NIS and thyroperoxidase genes.

As well as iodide, several other monovalent anions have the affinity for NIS [22]. Those with ionic radii similar to iodide are more readily transported [23]. Of special interest are [^{211}At$^-$] astatide for therapy and pertechnetate ($^{99\,m}$TcO$_4^-$) for imaging.

The choice of therapeutic radionuclide (Table 1) is influenced by the expected intracellular residence time of the radioactivity and the range of cross fire radiation needed to sterilise clumps of tumor cells of different sizes. Ideally, the physical half-life of the radionuclide should closely match its intracellular half-time of retention. Therefore, an alternative tactic to overcome the rapid efflux of iodide from NIS transfectants is to employ radiohalides with short $t_{1/2}$ values. Experimental evidence indicates that the highly radiotoxic α-emitter astatide (Na^{211}At) has an affinity for NIS-expressing cells [24] and could be an alternative radiohalide to [^{131}I]-iodide for NIS-based endoradiotherapy.

The demonstration that NIS was expressed not only in the lactating mammary gland but also in more than 80% of breast cancer [25] indicated that radioiodide may be an option for the diagnosis and treatment of mammary gland malignancies. To assess the feasibility of imaging NIS-expressing tumors, we compared the rate of loss of actively transported ^{131}I and $^{99\,m}$TcO$_4^-$ in transduced UVW human glioma cells (Figure 1). The enhanced retention of $^{99\,m}$TcO$_4^-$ suggested its utility for scintigraphic localisation of tumors after NIS-transfection. Recently, Moon et al [26] established a correlation between the expression of NIS mRNA and the uptake of $^{99\,m}$TcO$_4^-$ in 25 breast tumors. Because of its chemical similarity to pertechnetate, the perrhenate anion (^{188}ReO$_4^-$) is also concentrated by NIS-positive cells. ^{188}ReO$_4^-$ could be a potent therapeutic agent because it is able to deliver a dose 4.5 times higher than ^{131}I [27].

Exploitation of the NIS has potentially important applications in the management of breast cancer and other tumor types. Future directions in NIS-targeting will include the enhancement of radiohalide retention in tumors, circumvention of normal tissue uptake by differential regulation of NIS expression, and the investigation of tumor-imaging agents.

Table 1. Radiohalogens for targeted radiotherapy.

Radionuclide	Half-life	Emitted particles	Particle range
^{131}I	8 d	β	0.8 mm
^{125}I	60 d	Auger electrons	~ 10 nm
^{123}I	13 h	Auger electrons	~ 10 nm
^{211}At	7 h	α	0.05 mm

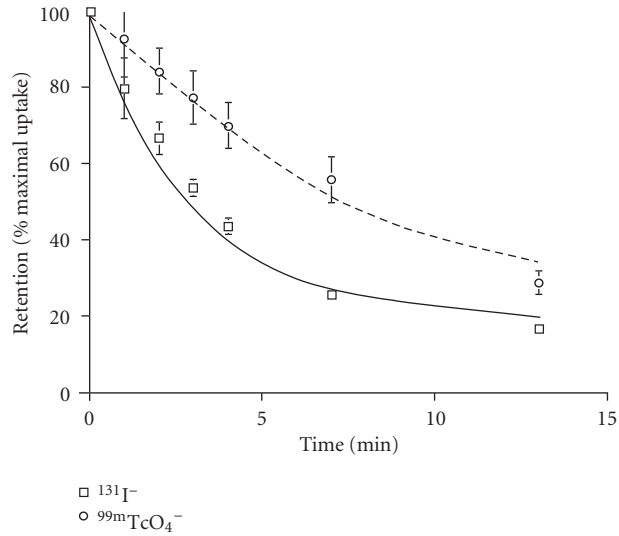

Figure 1. Rate of iodide efflux from UVW-hNIS cells. 10^6 cells were incubated with 7 kBq/ml Na^{131}I or Na$^{99\,m}$TcO$_4^-$ for 1 hour. The radioactive solution was replaced with fresh medium which was removed at various intervals for measurement of activity by gamma counting. Each point represents the mean and S.D. of three experiments performed in triplicate. Intracellular half-times of retention: ^{131}I = 3 min; $^{99\,m}$TcO$_4^-$ = 8 min.

TRANSFECTION OF THE NORADRENALINE TRANSPORTER GENE FOR MIBG-TARGETED RADIOTHERAPY

One impediment to the wider application of radioiodide therapy is the localisation of radioactivity in nontarget organs. This has prompted the search for tumor-affinic radiolabelled compounds which have a low tendency to deiodinate in vivo. One such agent is [^{131}I]-labelled meta-iodobenzylguanidine ([^{131}I]MIBG). The structure of MIBG is based on those of the adrenergic neurone blockers guanethidine and bretylium [28] (Figure 2). Because MIBG has high affinity for the noradrenaline transporter (NAT) [29], it is used in the imaging and treatment of tumors derived from the neural crest, such as neuroblastoma and phaeochromocytoma. NAT expression is predictive for MIBG uptake capacity [30] and quantification of NAT mRNA could be used for the selection of patients for MIBG therapy [31].

Approximately 15% of neuroblastoma patients are MIBG negative by scintigraphy and therefore do not progress to [^{131}I]MIBG therapy. Moreover, [^{131}I]MIBG

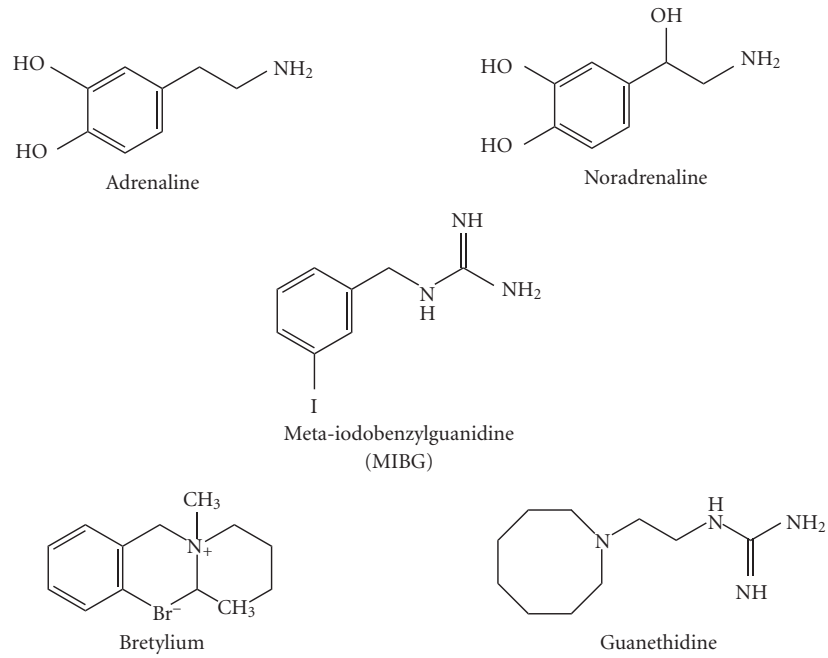

Figure 2. Catecholamines, adrenergic neurone blockers, and MIBG.

uptake in malignant sites is heterogeneous [32], suggesting that this therapy alone is unlikely to cure advanced stage disease. Our efforts to increase MIBG accumulation in malignant cells have considered means of enhancing NAT gene transcription. Recently, we have transfected the NAT gene into neuroblatoma cells, resulting in stimulated MIBG uptake. Transfectants more readily succumbed in a dose-dependent manner to treatment with [^{131}I]MIBG [33]. While presently, there is only an in vitro demonstration of the effect, these results are promising and indicate that it may be possible to elevate the intracellular levels of the radiopharmaceutical so that cure, rather than temporary control of tumour growth, could be the outcome. Given an appropriate delivery vehicle and tumour-specific control of expression, the introduction of exogenous NAT may make more neuroblastoma tumours amenable to MIBG therapy. In collaboration with the Gaslini Institute, Genoa, we are currently developing immunoliposomal systems for the delivery to neuroblastoma of NAT cDNA and MIBG. [^{131}I]MIBG is one of the best agents for targeted radiotherapy but its utility is restricted to a small number of NAT-expressing tumour types. We observed that after transfection of the NAT gene into a human glioma cell line, it actively concentrated [^{131}I]MIBG resulting in substantial toxicity, demonstrating the potential of gene therapy-assisted MIBG-targeted radiotherapy for the treatment of nonneuroectodermal tumors [34]. These findings are extremely encouraging for the development of NAT gene transfer-mediated [^{131}I]MIBG therapy. We expect the first clinical applications to be in the treatment of glioma or prostate carcinoma.

Targeting gene expression specifically to tumour cells, is one of the most important goals of research in cancer gene therapy. Appropriate control elements for the expression of transgenes that facilitate targeted radiotherapy are radiation-inducible gene promoters. Aspects of this type of gene regulation are reviewed by Robson in this issue.

Telomerase is the most common general marker of cancer cells and its activity has been detected in every major category of human malignancy, whereas normal somatic tissues have negligible activity [35, 36, 37, 38]. Therefore, an alternative method of specifying gene expression to malignant cells is provided by the telomerase promoters. Unfortunately, mammalian promoter elements are generally inefficient transcriptional activators [39]. However, we observed 17-fold enhancement of [^{131}I]MIBG uptake by UVW glioma cells transfected with the NAT gene whose expression was driven by the human telomerase RNA (hTR) promoter. This level of induction was 70% of the uptake achieved by a strong viral promoter and was sufficient to sterilise all clonogens in multicellular spheroids, suggesting that hTR-regulated expression of NAT may be an effective therapeutic strategy [40].

EVALUATION OF RADIOLOGICAL BYSTANDER EFFECTS

It is unlikely that gene therapy technology will achieve 100% transfection of cells in a clinical tumour. Bystander effects are therefore an important requirement for effective gene therapy. Current gene therapy is mostly based

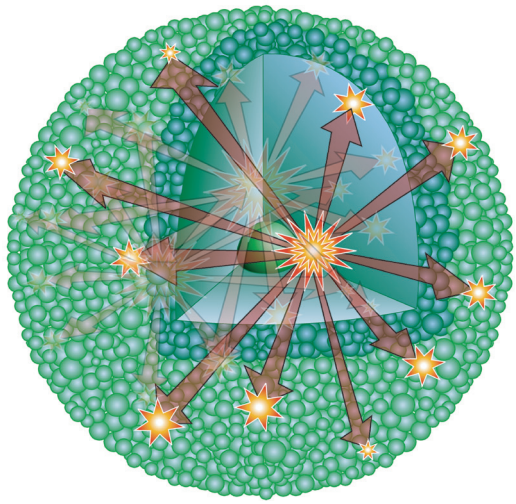

FIGURE 3. Radiation cross fire. An advantage of the concentration in tumor cells of radionuclides with long-range emissions (eg, ^{131}I) is the presence of a radiological bystander effect. That is the bombardment of untargeted cells by beta decay particles emanating from neighbouring, successfully targeted cells which have actively accumulated ^{131}I-labelled drug.

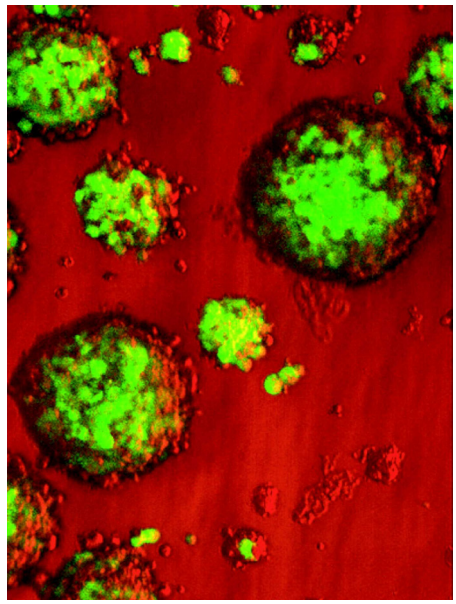

FIGURE 4. Transfectant mosaic spheroids. These multicellular spheroids were prepared by mixing human glioma cells (UVW) with UVW cells transfected with the gene encoding the jellyfish green fluorescent protein (GFP). The GFP can be used as a marker for the transfer of therapeutically useful genes such as those encoding receptors for targeting agents. Spheroids composed of a range of proportions of transfected to untransfected cells allow the determination of the influence of gene transfer efficiency upon the efficacy of targeted radiotherapy.

on prodrug activation, with bystander effects dependent on gap junctions which often diminish with tumour progression [41]. Novel systems providing a freely diffusable product are under investigation [42].

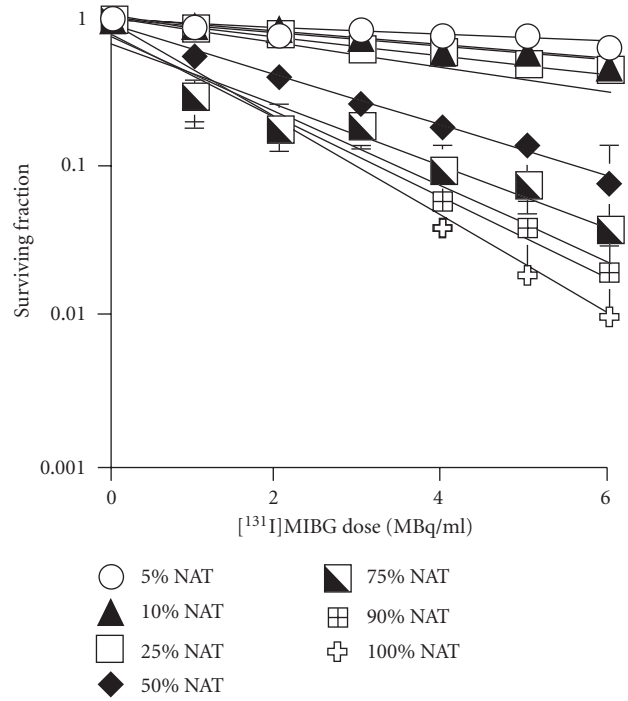

FIGURE 5. The effect on clonogenic survival of TMS treated with [^{131}I]MIBG. TMS composed of various proportions of NAT-expressing cells were assessed for clonogenic potential after administration of [^{131}I]MIBG. Spheroids were treated with 0 to 6 MBq/ml of [^{131}I]MIBG before washing and leaving intact for 24 hours to allow radiation cross fire to occur. The various spheroid groups were then disaggregated to single cells and seeded for clonogenic survival. The data is mean values of three experiments with error bars showing standard deviations.

Multicellular spheroids are substantially more susceptible than monolayer cultures to the lethal effects of ^{131}I β-radiation [43]. The increased effectiveness of radionuclide treatment of cellular aggregates is due to the enhanced efficiency of absorption of decay energy in three-dimensional cultures [34, 44]. This cross fire irradiation phenomenon (Figure 3) is an especially attractive feature of gene therapy schemes involving cellular concentration of radionuclides since gene transfer is notoriously inefficient, necessitating a mechanism which achieves bystander cell kill. The radiological bystander effect resulting from radiation cross fire provides exciting new possibilities, especially when transfection rates are low. This and other bystander effects require further investigation, for which a suitable experimental model would be very valuable.

Very recently, we have developed a modified spheroid system—transfectant mosaic spheroids (TMS) [45]—which is well suited to the experimental investigation of bystander effects (Figure 4). TMS are hybrids formed by coculturing gene-transfected and nontransfected (but otherwise identical) cells in controlled proportions. This allows the experimental simulation of small tumours in which a range of proportions of cells have been

transfected. The mosaic spheroid model is used in our investigation of the radiological bystander effect to quantify the effects of radiation cross fire as a function of the proportion of the cells transfected.

To examine the utility of this system, we prepared TMS from glioma cells transfected with the NAT gene or the GFP gene. Cell killing by [^{131}I]MIBG was proportional to the percentage of MIBG-uptaking cells (Figure 5). We conclude that TMS provide a useful model for assessment of the effectiveness of targeted radiotherapy in combination with gene therapy when less than 100% of the target cell population is expressing the NAT transgene [46].

CONCLUSION

Despite the attractiveness of the concept of sterilising cancer cells by means of the specific delivery of radionuclides, targeted radiotherapy has been applied only to a few malignant diseases with suitable biochemical features. The advent of gene transfer technology, promises to widen the scope of targeted radiotherapy by enabling the expression by tumour cells of membrane-bound proteins which actively accumulate radiopharmaceuticals.

Recent research findings augur well for the development of gene transfer to enable the targeting of a variety of tumour types with low molecular weight radiopharmaceuticals. It is envisaged that this approach combined with conventional therapeutic modalities, will improve cancer treatment in the new century. The immediate goals are to achieve efficient, tumour-specific, gene expression and efficacy in animal models before proceeding to clinical evaluation.

We envisage that the first clinical usage of this combined approach will be for the treatment of regionally confined malignancies such as glioma and bladder carcinoma or for the purging of bone marrow or peripheral blood stem cells prior to autologous rescue. To turn these dreams into reality, we must now develop efficient and specific vectors for the delivery of therapeutic transgenes.

REFERENCES

[1] Kaminski MS, Estes J, Zasadny KR, et al. Radioimmunotherapy with iodine (131)I tositumomab for relapsed or refractory B-cell non-Hodgkin lymphoma: updated results and long- term follow-up of the University of Michigan experience. *Blood*. 2000; 96(4):1259–1266.

[2] Kaminski MS, Zelenetz AD, Press OW, et al. Pivotal study of iodine I 131 tositumomab for chemotherapy-refractory low-grade or transformed low-grade B-cell non-Hodgkin's lymphomas. *J Clin Oncol*. 2001;19(19):3918–3928.

[3] Illidge T, Honeychurch J, Vandersteen A, Cragg M. Radioimmunotherapy in the pi-BCL1 B cell lymphoma model: efficacy depends on more than targeted irradiation alone. *Cancer Biother Radiopharm*. 2000;15(6):581–591.

[4] Press OW, Rasey J. Principles of radioimmunotherapy for hematologists and oncologists. *Semin Oncol*. 2000;27(6 Suppl 12):62–73.

[5] Vaughan AT, Anderson P, Dykes PW, Chapman CE, Bradwell AR. Limitations to the killing of tumours using radiolabelled antibodies. *Br J Radiol*. 1987; 60(714):567–572.

[6] Buchsbaum DJ, Curiel DT. Gene therapy for the treatment of cancer. *Cancer Biother Radiopharm*. 2001;16(4):275–288.

[7] Raben D, Buchsbaum DJ, Khazaeli MB, et al. Enhancement of radiolabeled antibody binding and tumor localization through adenoviral transduction of the human carcinoembryonic antigen gene. *Gene Ther*. 1996;3(7):567–580.

[8] Buchsbaum DJ, Raben D, Stackhouse MA, et al. Approaches to enhance cancer radiotherapy employing gene transfer methods. *Gene Ther*. 1996;3(12):1042–1068.

[9] Rogers BE, Rosenfeld ME, Khazaeli MB, et al. Localization of iodine-125-mIP-Des-Met14-bombesin (7-13)NH2 in ovarian carcinoma induced to express the gastrin releasing peptide receptor by adenoviral vector-mediated gene transfer. *J Nucl Med*. 1997; 38(8):1221–1229.

[10] Stackhouse MA, Buchsbaum DJ, Kancharla SR, et al. Specific membrane receptor gene expression targeted with radiolabeled peptide employing the erbB-2 and DF3 promoter elements in adenoviral vectors. *Cancer Gene Ther*. 1999;6(3):209–219.

[11] Chen L, Pulsipher M, Chen D, et al. Selective transgene expression for detection and elimination of contaminating carcinoma cells in hematopoietic stem cell sources. *J Clin Invest*. 1996;98(11):2539–2548.

[12] Curiel DT. High-efficiency gene transfer employing adenovirus-polylysine-DNA complexes. *Nat Immun*. 1994;13(2-3):141–164.

[13] Romano G, Pacilio C, Giordano A. Gene transfer technology in therapy: current applications and future goals. *Oncologist*. 1998;3(4):225–236.

[14] Katzenellenbogen JA, Coleman RE, Hawkins RA, et al. Tumor receptor imaging: proceedings of the National Cancer Institute workshop, review of current work, and prospective for further investigations. *Clin Cancer Res*. 1995;1(8):921–932.

[15] Rogers BE, Zinn KR, Lin CY, Chaudhuri TR, Buchsbaum DJ. Targeted radiotherapy with [(90)Y]-SMT 487 in mice bearing human nonsmall cell lung tumor xenografts induced to express human somatostatin receptor subtype 2 with an adenoviral vector. *Cancer*. 2002;94(suppl 4):1298–1305.

[16] Mandell RB, Mandell LZ, Link CJ Jr. Radioisotope concentrator gene therapy using the sodium/iodide symporter gene. *Cancer Res*. 1999;59(3):661–668.

[17] Boland A, Ricard M, Opolon P, et al. Adenovirus-mediated transfer of the thyroid sodium/iodide

symporter gene into tumors for a targeted radiotherapy. *Cancer Res*. 2000;60(13):3484–3492.
[18] Carlin S, Cunningham SH, Boyd M, McCluskey AG, Mairs RJ. Experimental targeted radioiodide therapy following transfection of the sodium iodide symporter gene: effect on clonogenicity in both two-and three-dimensional models. *Cancer Gene Ther*. 2000; 7(12):1529–1536.
[19] Spitzweg C, O'Connor MK, Bergert ER, Tindall DJ, Young CY, Morris JC. Treatment of prostate cancer by radioiodine therapy after tissue-specific expression of the sodium iodide symporter. *Cancer Res*. 2000;60(22):6526–6530.
[20] Spitzweg C, Dietz AB, O'Connor MK, et al. In vivo sodium iodide symporter gene therapy of prostate cancer. *Gene Ther*. 2001;8(20):1524–1531.
[21] Huang M, Batra RK, Kogai T, et al. Ectopic expression of the thyroperoxidase gene augments radioiodide uptake and retention mediated by the sodium iodide symporter in non-small cell lung cancer. *Cancer Gene Ther*. 2001;8(8):612–618.
[22] Eskandari S, Loo DD, Dai G, Levy O, Wright EM, Carrasco N. Thyroid Na + /I– symporter. Mechanism, stoichiometry, and specificity. *J Biol Chem*. 1997;272(43):27230–27238.
[23] Halmi NS. Thyroidal iodide transport. *Vitam Horm*. 1961;19:133–163.
[24] Carlin S, Mairs RJ, Welsh P, Zalutsky MR. Sodium-iodide symporter (NIS)-mediated accumulation of [^{211}At]astatide in NIS-transfected human cancer cells. *Nucl Med Biol*. 2002;29(7):729–739.
[25] Tazebay UH, Wapnir IL, Levy O, et al. The mammary gland iodide transporter is expressed during lactation and in breast cancer. *Nat Med*. 2000;6(8):871–878.
[26] Moon DH, Lee SJ, Park KY, et al. Correlation between 99mTc-pertechnetate uptakes and expressions of human sodium iodide symporter gene in breast tumor tissues. *Nucl Med Biol*. 2001;28(7):829–834.
[27] Dadachova E, Bouzahzah B, Zuckier LS, Pestell RG. Rhenium-188 as an alternative to Iodine-131 for treatment of breast tumors expressing the sodium/iodide symporter (NIS). *Nucl Med Biol*. 2002;29(1):13–18.
[28] Wieland DM, Wu J, Brown LE, Mangner TJ, Swanson DP, Beierwaltes WH. Radiolabeled adrenergi neuron-blocking agents: adrenomedullary imaging with [^{131}I]iodobenzylguanidine. *J Nucl Med*. 1980; 21(4):349–353.
[29] Jaques S Jr, Tobes MC, Sisson JC, Baker JA, Wieland DM. Comparison of the sodium dependency of uptake of meta-lodobenzylguanidine and norepinephrine into cultured bovine adrenomedullary cells. *Mol Pharmacol*. 1984;26(3):539–546.
[30] Mairs RJ, Livingstone A, Gaze MN, Wheldon TE, Barrett A. Prediction of accumulation of ^{131}I-labelled meta-iodobenzylguanidine in neuroblastoma cell lines by means of reverse transcription and polymerase chain reaction. *Br J Cancer*. 1994;70(1): 97–101.
[31] Mairs RJ. Neuroblastoma therapy using radiolabelled [^{131}I]meta-iodobenzylguanidine ([^{131}I]MIBG) in combination with other agents. *Eur J Cancer*. 1999;35(8):1171–1173.
[32] Moyes JSE, Babich JW, Carter R, Meller ST, Agrawal M, McElwain TJ. Quantitative study of radioiodinated metaiodobenzylguanidine uptake in children with neuroblastoma: correlation with tumor histopathology. *J Nucl Med*. 1989;30(4):474–480.
[33] Cunningham SH, Boyd M, Brown MM, et al. A gene therapy approach to enhance the targeted radiotherapy of neuroblastoma. *Med Pediatr Oncol*. 2000; 35(6):708–711.
[34] Boyd M, Cunningham SH, Brown MM, Mairs RJ, Wheldon TE. Noradrenaline transporter gene transfer for radiation cell kill by [^{131}I]meta-iodobenzylguanidine. *Gene Ther*. 1999;6(6):1147–1152.
[35] Kim NW, Piatyszek MA, Prowse KR, et al. Specific association of human telomerase activity with immortal cells and cancer. *Science*. 1994; 266(5193):2011–2015.
[36] Broccoli D, Young JW, de Lange T. Telomerase activity in normal and malignant hematopoietic cells. *Proc Natl Acad Sci USA*. 1995;92(20):9082–9086.
[37] Harle-Bachor C, Boukamp P. Telomerase activity in the regenerative basal layer of the epidermis inhuman skin and in immortal and carcinoma-derived skin keratinocytes. *Proc Natl Acad Sci USA*. 1996; 93(13):6476–6481.
[38] Tahara H, Yasui W, Tahara E, et al. Immunohistochemical detection of human telomerase catalytic component, hTERT, in human colorectal tumor and non-tumor tissue sections. *Oncogene*. 1999; 18(8):1561–1567.
[39] Chen J, Bezdek T, Chang J, et al. A glial-specific, repressible, adenovirus vector for brain tumor gene therapy. *Cancer Res*. 1998;58(16):3504–3507.
[40] Boyd M, Mairs RJ, Mairs SC, et al. Expression in UVW glioma cells of the noradrenaline transporter gene, driven by the telomerase RNA promoter, induces active uptake of [^{131}I]MIBG and clonogenic cell kill. *Oncogene*. 2001;20(53):7804–7808.
[41] Denning C, Pitts JD. Bystander effects of different enzyme-prodrug systems for cancer gene therapy depend on different pathways for intercellular transfer of toxic metabolites, a factor that will govern clinical choice of appropriate regimes. *Hum Gene Ther*. 1997;8(15):1825–1835.
[42] Bridgewater JA, Knox RJ, Pitts JD, Collins MK, Springer CJ. The bystander effect of the nitroreductase/CB1954 enzyme/prodrug system is due to a cell-permeable metabolite. *Hum Gene Ther*. 1997; 8(6):709–717.

[43] Cunningham SH, Mairs RJ, Wheldon TE, Welsh PC, Vaidyanathan G, Zalutsky MR. Toxicity to neuroblastoma cells and spheroids of benzylguanidine conjugated to radionuclides with short-range emissions. *Br J Cancer*. 1998;77(12):2061–2068.

[44] Neshasteh-Riz A, Mairs RJ, Angerson WJ, et al. Differential cytotoxicity of [123I]IUdR, [125I]IUdR and [131I]IUdR to human glioma cells in monolayer or spheroid culture: effect of proliferative heterogeneity and radiation cross-fire. *Br J Cancer*. 1998;77(3):385–390.

[45] Boyd, M, Wheldon, TE. Tumour Spheroids in the Culture of Animal Cells. In: Freshney RI, ed. 4th ed. New York, NY: Alan R. Liss; 1999.

[46] Boyd M, Mairs SC, Stevenson K, et al. Transfectant mosaic spheroids: a new model for evaluation of tumour cell killing in targeted radiotherapy and experimental gene therapy. *J Gene Med*. 2002;4(5):567–576.

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REVIEW ARTICLE

Transcriptional Targeting in Cancer Gene Therapy

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Cancer gene therapy has been one of the most exciting areas of therapeutic research in the past decade. In this review, we discuss strategies to restrict transcription of transgenes to tumour cells. A range of promoters which are tissue-specific, tumour-specific, or inducible by exogenous agents are presented. Transcriptional targeting should prevent normal tissue toxicities associated with other cancer treatments, such as radiation and chemotherapy. In addition, the specificity of these strategies should provide improved targeting of metastatic tumours following systemic gene delivery. Rapid progress in the ability to specifically control transgenes will allow systemic gene delivery for cancer therapy to become a real possibility in the near future.

INTRODUCTION

Gene therapy is the transfer of exogenous genes, called transgenes, into the somatic cells of a patient to obtain a therapeutic effect. Initially, gene therapy was viewed as an approach for treating hereditary diseases, but its potential role in the treatment of acquired diseases such as cancer is now widely recognised. In theory, gene therapy offers the potential for enormous improvements in the targeting of cancer therapy, but it is clear that this has not yet been achieved, although early trials are promising.

One of the main goals for all cancer therapies is the selective targeting and killing of tumour cells, thereby increasing the therapeutic ratio. Both chemotherapy and radiotherapy induce dose limiting normal tissue toxicities, which reduce their clinical effectiveness. Cancer gene therapy has the advantage that normal tissue toxicity might be avoided if suitable strategies can be employed to target the therapeutic transgene directly to tumour cells; an outcome that conventional therapeutic approaches have failed to achieve.

To date, clinical trials have focused on the delivery of genes directly to the tumour site by intratumoural injection using both viral and nonviral delivery agents, thereby largely avoiding normal tissues. However, the goal of most cancer gene therapy is to be able to administer a suitably packaged transgene systemically and achieve a high level of tumour targeting. This will be important for targeting the majority of tumours, that are not accessible for direct injection, and to ensure adequate distribution of the transgene throughout the tumour mass. The blood supply still offers the best opportunity to do this. Systemic delivery of transgenes would also allow targeting of both the primary tumour and metastatic deposits, which must be controlled if therapy is to be successful. A number of strategies are now being developed to target both viral and nonviral delivery agents to tumour cells. These include exploitation of natural viral tropisms, such as those exhibited by adenoviruses to target lung epithelium; retargeting viruses using a bispecific molecule to simultaneously block native receptor binding, redirecting virus to a tissue-specific receptor; genetically modifying the virus to ablate native receptor interactions and incorporating a novel ligand into one of the virus' coat proteins; using tissue-specific ligands or monoclonal antibodies incorporated onto the surface of liposomes to direct them to target cells. A detailed description of these targeted delivery strategies, lies beyond the scope of this review (for reviews covering this area see references [1, 2, 3, 4]).

As well as controlling the delivery of the therapeutic gene to the tumour tissue, controlled regulation of transgene expression is now playing a major role in targeted cancer gene therapy strategies. Indeed, by combining targeted delivery with tumour-specific expression, the level of transgene product in nontarget normal tissues, compared with that in tumours, can be greatly reduced. The purpose of this review is to focus on transcriptional targeting of transgenes, which will undoubtedly be an essential component of systemic cancer gene therapy.

TRANSCRIPTIONAL CONTROL OF EXPRESSION

In the last decade, it has become increasingly clear that gene expression is regulated by a complex interplay of factors that function in a cell-type-specific manner to produce diverse effects. These subtleties arise from the balance of tissue-specific transcriptional control elements present in the various cell types, for example, hepatic, melanocyte, neuronal, and erythroid or tumour-specific promoters/enhancers that are activated

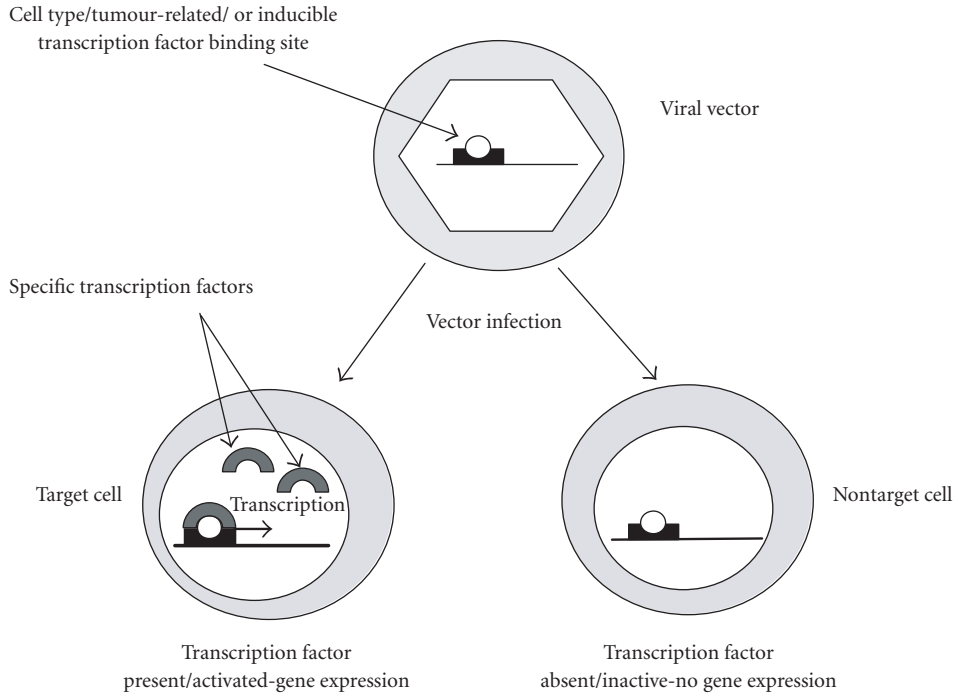

Figure 1. The use of specific tissue/tumour/inducible promoters to allow targeted transcription in tumour cells. Tissue-specific, tumour-specific, or inducible (eg, by radiation, drugs, etc) promoters can limit gene expression to target cells which express a specific transcription factor, or in which the transcription factors are activated exogenously.

in diseased states, or as a result of being exposed to an unfavourable tumour-associated microenvironment, for example, hypoxia. These cis-acting elements can be harnessed to drive the transcription of a therapeutic gene in a tissue- or tumour-specific manner (Figure 1). The use of tissue-specific cellular promoters to restrict transgene expression usually results in constitutive expression in the target tissue. However, for some therapeutic strategies, it will be preferable to regulate the duration and level of expression exogenously. This may be achieved by the use of cellular promoters that are preferentially activated under certain conditions to drive transgene expression in the target cell population. Research has focused on promoters induced by ionising radiation, heat, and small molecules. The use of tissue- and tumour-specific and exogenously controlled inducible promoters will now be discussed, as well as novel molecular adaptations of these promoters to strengthen gene expression. However, because the number of promoters that have been used for transcriptional targeting of transgenes for cancer gene therapy is very large, it is not feasible to discuss all of these in detail. The available data to date will therefore be presented in a series of tables.

TISSUE-SPECIFIC PROMOTERS

Transcriptional targeting utilising tissue-specific promoters exploits genes that are switched on only in certain tissues. A list of tissue-specific promoters currently being used in cancer gene therapy is given in Table 1. One of the main limitations of this type of promoter is that transgene expression may lead to cytotoxic effects in normal as well as tumour tissue derived from that same cell type. Therefore, the use of such promoters must be restricted to tissues in which damage is not critical for the survival of the host, for example, prostate, melanocytes, or thyroid. If the tissue/organ is critical, then the transgenes must be delivered directly to the tumour site to prevent normal tissue toxicity or be delivered with retroviruses which are less efficient at infecting nondividing slowly proliferating normal tissues, such as the liver.

Melanocyte-restricted expression

The use of tissue-specific promoters was pioneered by Vile and Hart in 1993, with the use of the tyrosinase promoter to target melanocytes/melanomas. Melanin biosynthesis is restricted to melanocytes, partly, as a consequence of transcriptional regulation of gene products, such as tyrosinase or tyrosinase-related protein 1, involved in this biochemical pathway. Vile and Hart demonstrated that small elements of the 5′ flanking regions of these two genes could drive reporter gene expression in murine and human melanoma cell lines in vitro and in vivo, but they could detect only low level expression in other cell types in vitro and no expression in Colo 26 tumours grown in vivo following direct injection of the transgenes [5]. They went on to demonstrate efficacy in vivo using the herpes simplex thymidine kinase (HSV-

Table 1. Tissue-specific promoters used in cancer gene therapy.

Promoter	Target tissue/tumour	Reference
Tyrosinase	Melanocytes/melanoma	[5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18]
Prostate-specific antigen (PSA)	Prostate	[19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33]
Prostate-specific membrane antigen (PSMA)	Prostate/also targets vascular endothelium of other tumours	[31, 34, 35, 36]
Probasin	Prostate	[28, 37, 38, 39, 40, 41]
Human glandular kallikrein (hK2)	Prostate	[25, 37, 38, 42]
Glial fibrillary acidic protein (GFAP)	Glial/glioma	[43, 44, 45, 46, 47, 48, 49, 50, 51]
Myelin basic protein (MBP)	Glial and astocytes/glioma	[43, 44, 52, 53, 54, 55]
Myelin proteolipid protein	Glial/glioma	[43, 44]
Neural specific enolase	Neuronal/SCLC	[48, 56]
Neuronal specific synapsin 1	Neuronal	[57, 58]
Ncx/Hox11L.1	Neural crest derived cells/neurobalstoma	[59]
Albumin	Liver/hepatocellular carcinoma	[60, 61, 62, 63, 64, 65, 66]
Surfactant protein B	Type II alveolar and bronchial cells/lung cancer	[67, 68]
Thyroglobulin	Thyroid/thyroid carcinomas	[69, 70, 71, 72, 73]
Ovarian-specific promoter	Ovarian	[74, 75]

tk)/gancyclovir (GCV) combination, which gave a significant tumour growth delay following intratumoural injection of the tyrosinase-driven constructs and decreased metastatic potential in mice injected with melanoma cells expressing these constructs following treatment with GCV [6]. In a separate study, tissue-specific expression of IL-2, IL-4, and GM-CSF was also seen in vitro and in vivo [7]. As an extension to this work, retroviruses were developed containing the murine tyrosinase promoter to achieve transcriptionally targeted expression of HSV-*tk* or IL-2 genes [8, 9]. Antitumour efficacy and tissue specificity were observed after intratumoural and IV delivery using localised and metastatic melanoma models [7]. Other studies have demonstrated antitumour efficacy using both retrovirus [10] and cationic liposomes [11] to achieve tyrosinase driven expression of cytosine deaminase (CD), which in turn catalyses the conversion of the nontoxic prodrug 5-fluorocytosine (5-FC) to 5-fluorouracil (5-FU). Adenoviral delivery of the murine tyrosinase promoter, introduced into the E1 region, has been used to drive melanoma-specific expression of the reporter gene β-galactosidase [12]. Both studies showed effective transcriptional targeting. More recently, tissue-specific elements of the tyrosinase promoter have been incorporated into more elaborate DNA constructs, that incorporate either a second tumour-specific promoter, cyclin A [13] for added specificity, or elements to help increase tyrosinase promoter expression [14]. As an extension to this, both targeted delivery and transcriptional targeting of melanoma cells via the tyrosinase promoter were combined in one study. Anionic liposomes carrying a peptide ligand for targeting α-v-β3-integrin receptors, commonly upregulated on malignant melanoma cells, were used in this instance for the transductional targeting [15]. These more elaborate combined approaches will be discussed in detail later as future perspectives.

Prostate-specific expression

Another well-characterised tissue-specific promoter is that of prostate-specific antigen (PSA). Prostate-specific antigen is expressed predominantly in the prostate and is transcriptionally upregulated by androgens. Pang et al [19] demonstrated that elements from the 5′ region of this gene can drive reporter gene expression in the PSA-producing cell line, LNCaP, but not in the nonproducing tumour cell lines, DU145 and PC-3, nor in renal or breast cancer cell lines. Tumour cell growth was inhibited when the PSA promoter was used to drive expression of antisense constructs targeting DNA polymerase α and topoisomerase α in prostate tumour cell lines, but not in cell lines of any other tumour [20]. In addition, a replication-competent adenovirus, CN706, has been developed with a selective toxicity for PSA-positive prostate cancer cells, using minimal enhancer/promoter constructs derived from the 5′ flank of the PSA gene, to drive the E1A gene [21]. A single intratumoural injection of the virus destroyed large LNCaP tumours and abolished PSA production in mice. Gotoh et al [22] have developed an attenuated adenovirus which expresses HSV-*tk* from a 5837-bp fragment of the PSA promoter. This

promoter was active in both androgen-dependent and independent PSA-producing prostate cancer cells in vitro, and in prostate tumours in castrated hosts. Chimeric constructs which have also been engineered improved the activity and specificity of the PSA enhancer. Importantly, intravenous delivery of adenoviral constructs, with either duplication of the enhancer core or insertion of tandem copies of the proximal androgen response elements, not only showed enhanced activity and inducibility but also retained tissue discrimination for human LAPC-9 tumour xenografts [23]. Constructs utilising PSA promoter elements have also been developed to drive the expression of the sodium iodide transporter to concentrate radioiodine in the prostate [24]. They have also been used to increase the expression of nitroreductase or CD allowing sensitisation of prostate cells to the prodrugs, CB1954 [25] or 5-FC, respectively [76]. PSA promoter driven transgenes have also been delivered using an HIV-1-based lentiviral vector [26] and liposomes [27].

Promoters of prostate-specific genes other than PSA are now being utilised. Prostate-specific membrane antigen (PSMA) is a type-2 membrane protein expressed in virtually all prostate cancers and their metastases. Moreover, unlike PSA, PSMA expression is upregulated by androgen deprivation. These advantages make PSMA a useful target for advanced prostate cancer therapy, especially in combination with conventional hormonal treatment. O'Keefe et al [34] have determined the most active regions of the PSMA enhancer and revealed a 1.6-kbp region which was active in driving prostate-specific CD expression, sensitising cells in vitro to 5-FC more than 50-fold compared to control cells. Furthermore, C4-2 prostate tumours grown in nude mice were eliminated by 5-FC when CD expression was induced by the PSMA promoter/enhancer [35]. This type of promoter will be particularly useful for patients who have undergone androgen ablation therapy and are suffering from a relapse of the disease.

Human glandular kallikrein (hK2) and PSA are related members of the human kallikrein gene family, however, unlike PSA whose expression displays an inverse correlation with prostate cancer stage and grade, hK2 is upregulated in higher grade and stage of the disease. Yu et al [37] identified an androgen-dependent and prostate-specific enhancer located between −3.4 and −5.2 kbp upstream of the hK2 transcription start site. A replication-competent adenovirus was constructed, CV763, in which the E1A gene was driven by the hK2 enhancer/promoter. Specificity for prostate tumours in vivo was impressive. More recently, a recombinant adenovirus expressing EGFP under the control of a triplicate hK2 enhancer/promoter led to robust tumour-restricted EGFP expression [38].

Probasin is another prostate-specific promoter, which has been used by many groups for transcriptional targeting of therapeutic transgenes. Steiner et al [28] used probasin to drive the expression of the *LacZ* reporter gene in a canine model following intraprostatic injection. They were able to demonstrate that, although adenovirus was detected in other tissue, lacZ mRNA could be detected by RT-PCR only in the prostate and not in other tissues. A small potent composite rat probasin promoter has been developed, ARR(2)PB, which is both androgen- and glucocorticoid-inducible [39]. When used to drive cell cycle-independent caspases, in an adenoviral system, it can lead to apoptosis in prostate cancer xenografts, but not in xenografts of other tumours, when the dimeric ligand AP20187 is delivered to trigger caspase activation [77]. More importantly, systemic delivery of a similar adenovirus, utilising the ARR(2)PB composite promoter to drive *BAX* expression, revealed no toxicity in the liver, lung, kidney, or spleen, but resulted in highly specific cytotoxicity in the prostatic cancer cell line LNCaP [40]. Lastly, a tumour-specific rather than prostate-specific promoter from the osteocalcin gene has shown a considerable promise in targeting prostate cancer bone metastases. This will be discussed in the next section.

Glial-restricted expression

A number of tissue-specific promoters have now been tested for targeting of gliomas. In 1993, Miyao et al [43], described 2.5 kbp, 1.3 kbp, and 1.5 kbp 5′ flanking regions of the mouse glial fibrillary acidic protein (GFAP) gene, myelin basic protein (MBP) gene, and myelin proteolipid protein (PLP) gene, respectively, which conferred specificity for glial cells. Following retroviral delivery, transcription was initiated more effectively in glioma cells via the MBP promoter where the HSV-*tk* transgene sensitised cells to gancyclovir in both murine and human glioma cells [44]. GFAP promoter driven expression of HSV-*tk* and therapeutic efficacy for gliomas was demonstrated by other groups [45, 46]. The RL1 variant herpes simplex virus 1 (HSV-1) has also been engineered to target intracerebral tumours of glial and astrocyte origin by using the GFAP promoter. These viruses, 1774 and 1775, showed high levels of β-galactosidase expression specifically in astrocytes following intracerebral inoculation [47]. Replication-defective adenoviral vectors have also been engineered to contain either the GFAP promoter or the neuronal specific enolase promoter [48, 49]. Importantly, in the Morelli study, Fas ligand (FasL) transgene expression and induced-toxicity following IV delivery were restricted to glial tissue, unlike the noncell-type-specific CMV-driven viruses that induced acute liver haemorrhage with hepatocyte apoptosis. Tight control of transgene expression was therefore achieved following systemic delivery. In the Chen study, a repressible adenovirus was developed by driving expression of a tetracycline-controlled transactivator. This was the first glial-specific gene delivery system that allowed for repression of ectopic gene expression. More recently, an elegant liposomal delivery system has been developed; it incorporates both transport across the blood brain barrier following IV administration of a targeting ligand such as the peptidomimetic rat 8D3 mAb to the mouse transferrin

receptor, and restricted expression to glial cells by the use of the GFAP promoter. This particular study demonstrates that tissue-specific gene expression in brain is possible after IV administration of a nonviral vector combined with gene targeting technology and glial-specific promoters [50]. Shinoura et al [52, 53, 54] have used the MBP promoter to drive expression of two apoptosis-related genes, *BAX* and caspase 8, selectively, in glioma cells both in vitro, and in vivo using adenoviruses. Massive apoptosis was seen in glioma cells only, demonstrating effective tissue specificity and good antitumour efficacy was observed in vivo.

As well as promoters that can target gliomas, a promoter that could be useful in targeting neuroblastomas has also been described. The human NCX gene, is a homologue of the murine neural crest homeobox (Ncx/Hox11L.1) gene, whose expression is restricted to a subset of neural crest-derived tissues. A 1.7-kbp fragment upstream showed preferential promoter activity in neuroblastoma cells and when linked to the HSV-*tk* gene caused increased sensitivity to gancyclovir [59]. The regulatory region of this gene may therefore be useful for neuroblastoma-specific gene therapy.

Liver-specific expression

The enhancer/promoter of the albumin gene is the only tissue-specific promoter that has been shown to preferentially target liver cells. The first demonstration of efficacy using this promoter was by Kuriyama et al [60]. When this promoter was delivered using a retrovirus, which is efficient only in dividing cells, β-galactosidase expression was restricted to hepatoma cell lines in vitro. When this retrovirus was injected via the spleen or directly into the liver, gene expression was observed only in dividing heptocytes in partially hepatectomised mice, but not in nondividing hepatocytes in normal mice. These authors went on to show that the susceptibility of murine and rat hepatocellular carcinoma (HCC) cells, infected with retroviruses expressing HSV-*tk* under the control of the albumin promoter, were 100-fold more sensitive to gancyclovir than non-HCC cells. Systemic gancyclovir administration resulted in complete regression of retroviral-infected HCC cells and significant inhibition of tumour growth even when only 5% of the cells were infected with retrovirus [61]. The albumin promoter has also been used to develop a retrovirus expressing the TNF-α, IL-2, and IL-3 genes [62, 63], an adenovirus expressing HSV-*tk* [64, 65] and a replication-competent herpes simplex virus, HSV G92A [66].

Thyroid-specific promoters

Thyroid cancer is a disease with a relatively good prognosis, but about 30% of the tumours dedifferentiate and may finally develop into highly malignant anaplastic thyroid carcinomas which are inaccessible to standard therapeutic procedures such as radioiodine therapy and thyroxine-mediated thyrotrophin suppression. Several gene therapy strategies are now available to target therapeutic genes to thyroid tissues using tissue-specific promoters. Zeiger first described the use of the thyroglobulin (TG) gene promoter for the selective targeting of thyroid cell lines. Nearly, 100% of thyroid carcinoma cells treated with an adenovirus expressing HSV-*tk* under the control of a TG promoter were killed by GCV compared to only 5% of control cells [69]. A retrovirus carrying HSV-*tk* under the control of the TG promoter also showed preferential GCV toxicity for malignant rat thyroid carcinoma cells compared to a normal rat thyroid cell line in vitro and in vivo [70]. Furthermore, systemic delivery of an adenovirus expressing thyroglobulin-driven HSV-*tk* did not induce luciferase activity in the liver or spleen, indicating that TG does not induce luciferase expression in non-TG producing tissues [71]. In addition, transfer of the HSV-*tk* gene driven by the TG promoter following administration of GCV is specifically cytotoxic to thyroid cells [71]. In order to address the issue that most poorly differentiated and anaplastic thyroid carcinomas have lost the ability to express the TG gene through loss of the thyroid transcription factor-1 (TTF-1), TTF-2, or Pax 8, Shimura et al infected thyroid cells with both adenoviruses expressing TTF-1 and adenoviruses carrying HSV-*tk* driven by the thyroglobulin promoter [72]. Cotransduction permitted 90% cytotoxicity in thyroid carcinoma cells lacking this transcription factor and normally unable to express the TG gene. A putative enhancer element for the TG gene has also been recently identified which appears to increase the TG promoter activity and specificity for thyroid cells. Interestingly, the TG promoter is also being used to target feline thyroid disease and neoplasia by selectively increasing expression of the nitroreductase gene allowing subsequent activation of a prodrug, CB1954 [73].

Several other novel tissue-specific genes have been used to target tumours in a tissue-specific manner, although only one or two reports are available. These include an ovarian-specific promoter, OSP-1, a 462-bp sequence that is a part of a retrovirus-like element specifically expressed in rat ovary [74]. The expression of HSV-*tk* under the control of this promoter significantly sensitised ovarian cancer xenografts (OVCAR) to GCV following delivery with a cationic lipid [75]. In addition, the promoter for surfactant protein B (SPB), whose activity is restricted to adult type II alveolar epithelial cells and bronchial epithelial cells, has been used to target both replication-deficient [67] and -competent adenoviruses by replacement of the E4 promoter [68]. This promoter showed good specificity for bronchial cancer cells in vivo.

TUMOUR-SPECIFIC PROMOTERS

Ideally, tumour-specific promoters should be highly active in tumour cells and have little or no activity in normal cells. Harrington et al [78] elegantly split this vast group of promoters into subgroups depending on

their discrete characteristics and for the purpose of this review this convention will be applied. Tumour-specific promoters can be described as (1) cancer-specific promoters, that is, specific for the malignant process, but showing no tissue specificity (2) tumour-type-specific promoters, that is, oncofoetal related with tissue specificity (3) tumour microenvironment-related promoters, that is, promoters which respond to the tumour microenvironment and physiology, for example, hypoxia and glucose regulated (4) tumour vasculature-related promoters, that is, promoters which are more active in the tumour vasculature rather than normal vasculature.

Cancer-specific promoters

One of the main obstacles to current cancer therapies is the lack of tumour specificity. Targeting gene expression specifically to tumour cells is therefore one of the most important goals of cancer gene therapy strategies. A common problem with the use of the tissue-specific promoters which were described in the previous section, is that transgene expression can also occur in the normal tissue and whilst some tissue damage may be acceptable in certain organs, in other organs such as brain, liver, and so forth, it could be catastrophic. There is probably only one gene, telomerase, that can be genuinely classified as cancer specific and whose promoter is being used to drive the expression of transgenes selectively in a wide variety of tumour cells.

Telomerase activation is considered to be a critical step in cancer progression because of its role in cellular immortilisation. Approximately 90% of human cancers possess active telomerase, whereas normal somatic tissues have either much lower or undetectable activity [79, 80, 81]. Human telomerase activity depends on the presence of both the RNA subunit (hTR) and the catalytic protein component (hTERT) [82]. For both genes there is a clear differential expression between normal and malignant cells. A number of studies have used these two promoters to drive transgene expression for therapeutic benefit. Pan and Koeneman [83] first described the use of the hTERT promoter in a retroviral system and combined it with a Cre/*loxP* site-specific recombination system to allow the killing of p53-negative tumour cells while sparing normal wild-type cells. Since then, a host of papers have reported the use of both promoters in several different tumour types in combination with several different transgenes. Bladder and liver tumour cell lines were targeted using both hTR and hTERT to drive expression of the diphtheria toxin A chain gene [84]. The glioma cell line, UVW, was targeted using the hTR promoter to drive expression of the noradrenaline transporter gene (NAT) where a 17-fold enhancement of the radionuclide [131]meta-iodobenzylguanidine uptake was reported resulting in complete sterilisation of tumour spheroids [85]. Gliomas were also the target for hTERT-driven expression of Fas-associated protein with death domain (FADD) or rev-caspase-6. FADD is a protein which induces apoptosis in cells regardless of Fas expression on the cell surface and rev-caspase 6 induces apoptosis independent of initiator caspases. Subcutaneously implanted human gliomas treated with this construct were significantly reduced in volume compared to control tumours [86, 87, 88]. More recently, the apoptotic pathway was successfully targeted both in human tumour xenograft and syngeneic mouse UV-2237m fibrosarcomas, using Bax as a transgene, driven by the hTERT promoter [89, 90]. In a suicide gene therapy approach, hTERT was also used to drive expression of HSV-*tk* in an osteosarcoma xenograft model following intratumoural injection. Targeted delivery using this approach avoided the liver toxicity observed using a CMV driven construct following GCV administration. Finally, Keith's group [91] tested hTR and hTERT in a panel of 10 cell lines consisting of lung, colon, ovarian, bladder, and cervical origin, demonstrating the truly "cancer specific" nature of these promoters. The hTR promoter appeared to have the superior activity in the telomerase-positive cell lines and when used to drive the nitroreductase gene to increase the activation of the prodrug, CB1954, in two xenograft models, it showed even better antitumour effects (up to 97% reduction in tumour volume) than a CMV-driven construct [91]. These promoters clearly have a real potential in targeting a wide range of different tumour types.

Tumour-type-specific promoters

Certain types of tumour often overexpress genes of oncofoetal origin that are silent in normal tissue. The most well-characterised promoters of these tumour-specific genes are the carcinoembryonic antigen (CEA) and α fetoprotein (AFP), which are expressed in adenocarcinomas and hepatocellular carcinomas, respectively. There are also many other genes that are overexpressed in certain tumour types and, as such, their promoters are being utilised for transcriptional regulation of therapeutic genes.

CEA

The CEA promoter is active in a proportion of breast, lung, colorectal, and pancreatic cancers. The 5′ promoter sequences have been analysed and identified by reporter gene assays and have lead to high-level selective expression of transgenes in CEA-positive cell lines [92]. The CEA promoter has been used to drive CD expression [92, 93, 94], HSV-*tk* [95, 96, 97, 98, 99], *BAX* [100], and mammalian degenerin, a sodium ion channel gene, overexpression of which leads to sodium influx and cell bursting [101]. Importantly, CEA appears to be truly selective for tumour tissues in animal models as several reports have not detected transgene expression or related toxicities following systemic delivery of these constructs via tail vein injection [98] or intraperitoneally [94]. Although a significant number of studies have shown that the CEA promoter is effective for selectively targeting tumour cells, there have been few studies which have demonstrated

truly significant in vivo antitumour effects possibly because of the low activity of the promoter. More recently, several studies have made use of a Cre recombinase/*lox P* system [102, 103, 104] or a GAL4 regulatory system [100], both of which significantly increased transgene expression by up to 100 fold, while maintaining tumour specificity. These strategies will be discussed in more detail later. Finally, Qiao et al [105] constructed an adenovirus containing a binary promoter system with a CEA promoter driving a transcription activator, which then activates a minimal promoter to express HSV-*tk*. Importantly, in vivo dose-escalation studies demonstrated significantly reduced toxicity following intravenous administration of this construct and provide a proof of principle for the powerful enhancement of a weak promoter for effective tumour-restricted gene expression.

AFP

The α-fetoprotein gene is normally expressed in foetal liver and is transcriptionally silent in adult liver. It is also expressed in around 70% of the hepatocellular carcinomas and its promoter has been used extensively to drive transgene expression for targeting these tumours. Studies have clearly shown that transgene expression is restricted to tumours that overexpress AFP and that transgene expression is less efficient in tumours with lower AFP levels. A variant 0.3-kbp promoter has also been developed with a G to A substitution at nucleotide −119; a point mutation responsible for hereditary persistence of human AFP. This promoter has proved particularly useful for transgene expression in low AFP producing hepatoma cells [106]. Ishikawa et al have also shown that by placing the AFP promoter/enhancer in the reverse orientation to the long terminal repeats with a retroviral construct dramatically increases both HSV-*tk* transgene expression and GCV-mediated cytotoxicity in a HepG2 tumour in vivo [107]. The AFP promoter has also been used to drive CD [108], IL-2 [109, 110], diphtheria toxin A [111], and interestingly, a replication-competent adenovirus has been constructed using the AFP promoter to drive an attenuated E1B gene. Systemic delivery of this virus resulted in tumour-specific regression [112]. The AFP promoter activity can be weak, but it can be enhanced using Cre/*loxP* technology to increase transgene expression [113], a similar strategy to that applied to CEA.

ErbB2

ErbB2/HER2 is an oncogene that codes for a receptor tyrosine kinase that is overexpressed by transcriptional upregulation in approximately one third of breast and pancreatic tumours, and in a small proportion of other tumours. This promoter has been used to drive the expression of CD and when delivered using a retrovirus, CD expression and significant 5-FC-mediated cell death was observed only in ErbB2-positive cell lines [114, 115]. ErbB2-driven expression of a membrane receptor, murine gastrin releasing peptide, has also been used effectively to target breast cancer cell lines with a radiolabelled peptide [116]. More recently, studies to identify a minimal ErbB2 promoter were successful. Maeda et al [117] identified a 251 bp fragment (−213/+38) that could direct transcription of a luciferase reporter gene better than the SV40 immediate early promoter in breast cancer cells. When linked to the HSV-*tk* gene, increased sensitivity to GCV was observed in breast but not nonbreast cancer cell lines, and suppressed the growth of these lines in nude mice.

MUC1/DF3

The MUC1 gene encodes a high molecular weight mucin-like glycoprotein and is overexpressed at the transcriptional level in breast and cholangiocarcinomas. A 114 bp enhancer region has been identified that can modulate transcription from heterologous promoters [118]. DF3-positive breast cancer cell lines were more sensitive to GCV-mediated cell killing when the HSV-*tk* gene was delivered and driven by this enhancer. A replication-defective adenovirus containing this construct was subsequently developed and inhibition of tumour growth was observed in an intraperitoneal breast cancer metastases model [119]. This strategy could transfect and kill carcinoma cells within haematopoetic stem cell populations without causing associated normal tissue toxicity [120]. An adenovirus expressing *BAX* under the control of this promoter has also been constructed for targeting ovarian cancer cell lines expressing DF3. Transgene expression was restricted to tumour tissue in a nude mouse model and administration of the virus 2–3 days after tumour inoculation eradicated > 99% of tumour implants [121].

Osteocalcin

Osteocalcin (OC) is a noncollagenous bone matrix protein expressed at high levels in the neonate. It functions primarily in osteoblasts found in growing bone and is highly expressed in osteogenic sarcomas. Koeneman et al [122] have also provided evidence of expression in ovarian, lung, brain, and prostate tumours (especially those showing androgen-independence). A phase I trial is now underway to target androgen-independent prostate cancer using an adenoviral construct expressing HSV-*tk* under the control of this promoter. Of all the transcriptional targeting strategies discussed so far, the use of the osteocalcin promoter has been tested thoroughly using systemic delivery. Matsubara et al [123] have successfully constructed a replication-competent adenovirus using this promoter to drive E1a expression. They demonstrated that 100% of mice carrying prostate xenografts responded to systemic adenoviral delivery and more significantly 40% of mice were cured. Phase I/II trials are now being carried out using the same virus to target pulmonary metastases of osteosarcomas, following systemic delivery of this virus, which must pass through the lung before being sequestered in the liver [124].

Shirakawa et al [125] also used an adenovirus to target osteosarcoma pulmonary metastases with an osteocalcin driven HSV-*tk*. In an established animal model, systemic delivery of these constructs via tail vein injection and subsequent intraperitoneal acyclovir treatment significantly decreased the number of tumour nodules in the lung, while significantly increasing the survival of these animals. Interestingly, because the osteocalcin promoter can also target bone stromal cells, which are important for supporting the growth of prostate tumour metastases, an adenovirus expressing HSV-*tk* under the control of this promoter was effective at destroying prostate cancer xenografts in both subcutaneous and bone sites [122]. It is quite clear that this promoter will be important for the treatment of both prostate and osteosarcoma, but it will be some time before we learn about its true specificity and any associated toxicities. Certainly, because this promoter is effective in androgen-independent prostate tumours, it will ultimately be more effective than the tissue-specific PSA promoter discussed in the last section. A clearer understanding of this promoter may help to increase specificity even further and Yeung et al have already identified elements important for prostate specificity, in particular, the elements important for androgen-independent specificity [126].

L-plastin

L-plastin is constitutively expressed at high levels in malignant epithelial cells, but is not expressed in normal tissue, except at low levels in haemopoetic cells. A replication-incompetent adenovirus has been constructed to express either LacZ or CD under the control of the human L-plastin promoter. High levels of expression of β-galactosidase was seen in breast, ovarian, and fibrosarcoma cell lines [127] and in explants of ovarian cancer cells from patients compared to normal mesothelial cells from surgical specimens [128]. A greater reduction in tumour size was seen when the adenovirus expressing CD under the control of the L-plastin promoter was injected into human ovarian tumour xenografts than when the CD was controlled by the CMV promoter. L-plastin may therefore be an effective promoter for targeting ovarian cancer.

Midkine

Midkine (MK) is a heparin-binding growth factor that is transiently expressed in the early stages of retinoic acid-induced differentiation of embryonal carcinoma cells. Many malignant tumours express high levels of MK mRNA or protein with no expression in human liver. This is important as adenovirus' have a natural tropism for liver and such promoters could be useful for preventing transgene expression and associated hepatotoxicity in the liver. Miyauchi et al [129] examined the expression of MK in human oesophageal cancer cells. Positive staining with anti-MK antibody was found in 8 out of 14 tumour specimens, while the surrounding normal oesophageal tissues in these specimens were not stained. The 5′ flanking 2.3-kbp region of the MK gene was then used to drive the expression of HSV-*tk*, where expression conferred the sensitivity to GCV. Since then, an adenoviral vector has been constructed to express HSV-*tk* under the control of the MK promoter to target Wilm's tumour and neuroblastoma cell lines [130]. Importantly, systemic delivery of these constructs into mice resulted in much lower hepatotoxicites; by day 8 of systemic delivery, four out of five mice treated with an adenovirus expressing HSV-*tk* via a CMV promoter had died, whereas all the mice treated with the midkine adenovirus survived at least 10 days [131]. The midkine promoter has also proved useful for targeting ovarian cancer cells and pancreatic cells, as has the cyclooxygensae-2 gene promoter [132, 133].

Other tumour-type-specific promoters

Many other tumour-specific promoters have been utilised to target particular types and are listed in Table 2. Particularly interesting is the secretory leukoprotease inhibitor gene (SPL1) promoter for targeting cervical carcinoma cells [157], lung, breast oropharyngeal, bladder, endometrial, and colorectal carcinomas [158]. Yamamura et al [170] have used the promoter from the human calponin gene, normally expressed in maturated smooth muscle cells and overexpressed in human soft tissue and bone tumour cells, to drive expression of ICP4, a major transacting factor for viral genes to produce a novel conditionally replicating herpes simples virus. The viral treatment showed stable and significant inhibition of tumourigenicity with cures in four out of five mice harbouring synovial sarcoma, leimyosarcoma, and osteosarcoma cells, while sparing normal vascular smooth muscle cells. Chen and McCormick [172] report on a novel transcriptional targeting strategy for colon cancer. Since many colon cancer cells show mutations in either the adenomatous polyposis coli or β-catenin genes that lead to stabilisation of β-catenin and activation of downstream T-cell factor (Tcf) target genes, tcf promoter elements were used to control expression of an apoptosis gene, FADD, which appears to effectively and selectively kill colon cancer cells. The regulatory regions of the H19 gene, is differentially regulated in normal and cancer cells and has been linked to diphtheria toxin A or HSV-*tk* to successfully target syngeneic bladder tumours in vivo with no obvious systemic toxicity towards the host animals [171]. A 2.2-kbp 5′ flanking region of the human calretinin gene has been used to drive the expression of HSV-*tk* selectively in mesothelioma cell lines [174] and the human calcitonin promoter has been used to drive expression of HSV-*tk* in medullary thyroid carcinomas following systemic delivery by adenovirus without any evident toxicity [175, 176, 177]. The gastrin-releasing peptide gene promoter has been used to selectively control HSV-*tk* expression in small-cell lung cancer cells [167]; injection of an adenovirus containing this construct resulted in complete regression of lung

Table 2. Tumour-specific promoters.

Promoter	Tumour target	Reference
Telomerase	Lung, colon, ovarian, bladder, cervical, liver, glioma	[83, 84, 85, 86, 87, 91, 134, 135, 136, 137]
CEA	Colorectal, pancreatic, cholangiocarcinoma, breast, lung	[92, 93, 94, 95, 96, 97, 98, 100, 138, 139, 140, 141, 142, 143, 144]
Alpha feto protein (AFP)	Hepatoma	[64, 106, 107, 108, 145, 146, 147, 148, 149]
Erb B2	Breast, pancreatic, ovarian	[114, 115, 116, 117, 150, 151, 152]
DF3/MUC1	Breast, choloangiocarcinoma	[116, 118, 119, 120, 121, 153, 154]
Osteocalcin	Prostate, ovary, lung, brain, osteoblasts	[122, 123, 124, 125, 126, 127, 155, 156]
L-plastin	Ovarian, breast, fibrosarcoma	[127, 128]
Midkine	Embryonal carcinoma; Wilm's tumours, neuroblastoma, pancreatic, oespohageal	[129, 130, 131, 132, 133]
Secretory leukoprotease inhibitor (SLP1)	Lung, breast, oropharyngeal, bladder, endometrial, ovarian, colorectal, cervival	[157, 158]
Alpha lactalbumin	Breast	[159]
Myc-max	Breast, lung	[140, 160, 161]
Somatostatin	Malignant melanoma of soft parts	[162]
Cox2	Ovarian, pancreatic, gastroin-testinal	[132, 133, 163]
Ornithine decarboxylase	Colon and neuroblastoma	[164]
Epithelial glyocoprotein 2 (EPG2)	Carcinomas	[165]
c-Myb-responsive promoters	Hematopoietic tumours	[166]
Gastrin-releasing peptide	Lung	[167, 168]
Metallothionein	Ovarian	[169]
Calponin	Soft tissue and bone tumours	[170]
H19	Bladder	[171]
Tcf	Colon	[172, 173]
Calretinin	Mesothelioma	[174]
Calcitonin/calcitonin gene-related peptide	Thyroid/thyroid medullary cancer	[175, 176, 177]
Cell cycle-related		
Cdc25C	Melanoma	[178]
CyclinA	Melanoma	[13, 178, 179]
Endoglin	Endothelial cells	[179, 180]
IGF-1-R	Tumours mutant for p53, cMyb or EWS/WT1	[181]
E2F-1	Glioma	[182]

tumour xenografts following IP administration of GCV [168]. Lastly, Vandier et al [169] have been successful in targeting cisplatin-resistant ovarian carcinoma cells which overexpress the metallothionein gene, a metal binding protein. The human metallothionein promoter was therefore used to drive expression of HSV-*tk*, resulting in marked sensitisation to GCV in the cisplatin-resistant cell lines compared to the cisplatin sensitive cells.

Cell cycle-related promoters

The expression of proliferation-associated genes is a hallmark of both cancer cells and tumour endothelial cells. The retinoblastoma tumour suppressor gene (RB), is a cell cycle-associated gene, which has the ability to repress E2F-responsive promoters, such as E2F-1. E2F-responsive promoters should therefore be more active in tumour cells relative to normal cells because of an excess of free E2F and loss of RB/E2F repressor complexes. Parr et al [182] have shown that adenoviral vectors, containing transgenes driven by an E2F-1 promoter, can mediate tumour-selective gene expression in vivo, allowing for eradication of established tumours. Significantly, less toxicity was seen with this adenovirus than standard constitutively regulated adenoviral constructs.

Table 3. Tumour environment-specific promoters.

Promoter	Tumour target	Reference
Endothelial cell-specific promoters		
KDR/Flk-1	Sarcoma, ovarian, endothelioma	[184, 185, 186, 187]
Flt-1	Breast, gynaecological, teratocarcinoma	[188]
E-selectin	Endothelioma	[184, 186, 189]
von Willebrand factor (vWF)	Endothelial cells	[190]
Preproendothelin-1	Endothelial cells	[191, 192, 193]
VCAM-1	Endothelial cells	[186]
Hypoxia-responsive promoters		
VEGF (HRE elements)	Hepatoma	[145, 194, 195, 196, 197]
Erythropoetin (HRE elements)	Brain	[198]
Phosphoglycerate kinase 1 (HRE elements)	Fibrosarcoma	[33, 199]
Glucose-responsive promoters		
GRP78	Breast	[198, 200, 201, 202, 203, 204, 205]
Hexokinase II	Lung	[206]

Muller's group have published several papers where the cell cycle-related promoter, cyclin A or cdc25c, has been used in combination with a tissue-specific tyrosinase promoter to selectively target proliferating melanoma cells [13, 178]. These dual specificity promoters showed > 20 fold and > 50 fold increase in cell cycle-related and cell-type-specific regulation, respectively, and will be discussed in more detail later.

Finally, the insulin-like growth factor-1 (IGF-1) has a central role in normal cellular proliferation. Transcription of this gene is controlled by a number of tumour suppressors. On the other hand, a number of oncogenes, including mutant *p53* and *c-Myb* and the fusion protein EWS-WT1 significantly stimulate promoter activity. This promoter may therefore be a good candidate for use in transcriptional targeting in cancer gene therapy protocols [181].

Promoters that respond to the tumour microenvironment and physiology

There are many aspects of the tumour microenvironment that are unique, and can be targeted with strategies that take advantage of genes upregulated in response to these environments. For tumours to grow, they must stimulate angiogenesis; this requires proliferation of endothelial cells, a process specific (in normal adults) to the tumour microenvironment. An additional bonus of this strategy is that vectors delivered systemically will have direct access to the proliferating endothelial cells of the tumour vasculature. Furthermore, specific genes are upregulated in proliferating endothelial cells whose promoters are attractive for targeting transgenes to the tumour vasculature. Secondly, because the tumour vasculature is often disorganised and inadequate, areas of low oxygen tension (hypoxia) are prevalent in malignant tumours [183]. Many genes transcriptionally upregulated in response to hypoxia are mediated by the inducible transcription complex, hypoxia-inducible factor-1 (HIF-1). HIF-1 binds to DNA motifs known as hypoxia response elements (HREs) within these genes. HREs can therefore be used to drive transgene expression specifically within areas of tumour hypoxia. It is extremely important to target this population of cells since they are highly resistant to other forms of treatment, such as radio and chemotherapy. Lastly, as well as oxygen starvation, tumours can also be deprived of glucose leading to the increased expression of genes involved in glucose metabolism. The promoters of these genes are therefore also being used to drive transgene expression specifically within a tumour. A list of tumour microenvironment and physiology-related promoters can be found in Table 3.

Endothelial cell-specific promoters

Over the past 10 years a number of genes have been reported to be upregulated in proliferating endothelium of tumour blood vessels. The endothelial-specific kinase insert domain receptor (KDR/flk-1) and E-selectin have been shown to be upregulated in tumour endothelium, and defined regions of these promoters have been used to drive TNF-α carried by a retroviral vector. A 10–11-fold increase in TNF-α expression within sEND endothelioma cells was observed compared to NIH-3T3 fibroblasts [184]. More recently, it was demonstrated that the KDR/flk-1 promoter, is not only endothelial cell-specific, but also active in two human ovarian cancer cell lines where CD expression sensitised these cells to 5-FC both in vitro and in vivo [185]. E-selectin expression is very low in normal adult capillaries, but is significantly elevated in newly formed tumour vessels. It has been shown that the E-selectin promoter is also activated by TNF-α, generating a 30-fold increase in transgene expression compared

to untreated cells. Furthermore, endothelial cells exposed to tumour-conditioned medium as an in vitro simulation of the tumour microenvironment resulted in even higher induction of transgene expression [189].

Flt-1, a receptor for vascular endothelial growth factor (VEGF), is known to display dysregulated expression in both tumour vasculature and tumour cells per se, and adenoviral vectors containing transgenes under the control of the flt-1 promoter achieve very low levels of toxicity in normal liver. This is important, as this is the major organ responsible for blood clearance of adenoviruses and most at risk from inadvertent transgene-related toxicity. Bauerschmitz et al [188] have shown that the flt-1 promoter is useful for the transcriptional targeting of adenoviruses to teratocarcinomas.

The promoter of human preproendothelin-1 has been used to generate a retrovirus that demonstrates endothelial-specific expression of the β-galactosidase gene [191, 192]. An adenovirus containing the preproendothelin-1 promoter driving luciferase expression has also been constructed. Following systemic injection, higher transgene expression was seen using this promoter rather than a CMV promoter, resulting in a high level of activity specifically in the new vasculature of primary tumours and lung metastases, with the highest level in the angiogenic endothelial cells of the metastasis [193]. However, an impediment to the use of the preproendothelin-1 promoter may be that it is also active in some epithelial cells [207], compromising its vascular targeting specificity.

The promoter for human von Willebrand factor (vWF) has also been used to drive transgene expression selectively in proliferating endothelial cells [190, 208]. Both reports emphasise that although this promoter is very weak, it appears to be highly selective for endothelial cells and is effective at suppressing endothelial cell growth when driving the HSV-*tk* gene in the presence of GCV. Furthermore, a novel strategy has been developed to enhance this promoter > 14–100 fold while maintaining > 30–100 fold specificity and will be discussed in more detail later [208]. Nettelbeck also tested the PECAM-1, endoglin, P-selectin, and KDR promoters, but vWF promoter was clearly the most specific for endothelial cells.

Hypoxia-responsive promoters

Hypoxia response elements (HREs) found within the hypoxia-responsive genes of vascular endothelial growth factor (VEGF), erythropoietin (Epo), and phosphoglycerate kinase-1 (PGK-1), have been tested for their use in cancer gene therapy. Shibata et al examined the HREs of human VEGF and Epo and found high inducibility of both in mammalian cells, while only the HREs from the VEGF promoter were active in murine cells, and then to a lesser extent than in human cells. In addition, the introduction of multiple copies of the HRE and an E1B minimal promoter improved the hypoxia responsiveness [194]. Shibata then tested other HRE promoter combinations and found that 5HREs and a CMV minimal promoter exhibited the most effective hypoxia response (over 500 fold) when compared to an intact CMV promoter [195]. In addition, significant antitumour effects were achieved with intraperitoneal injections of the prodrug, CB1954, in tumours that expressed bacterial nitroreductase (NTR) constitutively or via this hypoxia-inducible promoter. Furthermore, respiration of 10% O_2 increased tumour hypoxia in vivo and enhanced the antitumour effects [196]. The HREs of the mouse VEGF gene have also been used to construct a retroviral vector expressing HSV-*tk* and tumours expressing this construct regressed following administration of GCV [197]. Dachs et al [199] have successfully utilised HREs from the PGK-1 gene and have shown that it is active in hypoxic tumour cells in vivo and Ruan et al [198] have constructed an adeno-associated virus carrying HREs from the Epo gene that can increase gene expression in brain tumour cell lines, under anoxia, by 79–110 fold. Combinations of both hypoxia and tumour/tissue-specific elements are now being developed to increase specificity for hypoxic tumour cells [145, 150, 186, 209]. These will be discussed in more detail later.

Glucose-responsive promoters

The glucose-regulated protein GRP78 functions as a molecular chaperone and can bind to malfolded proteins and unassembled complexes. They are induced in response to low glucose, hypoxia, acidic pH, or oxidative stress, which are hallmark features of solid tumours. The use of these promoters for cancer gene therapy was first proposed by Lee's group [200]. This group has fully characterised the use of this promoter in several tumour systems. A truncated rat GRP78 promoter with most of its distal basal elements removed drove high level expression in a murine fibrosarcoma model [201] and when GRP78 was used in a retroviral system to drive HSV-*tk*, HSV-*tk* expression levels were much higher [202] in tumours in vivo than cells in vitro, resulting in complete tumour eradication with no recurrence of tumour growth. Similarly, impressive results were obtained in a murine mammary adenocarcinoma cell line (TSA) in syngeneic, immune-competent hosts [203]; in addition, immune memory was induced in these hosts with this system. Finally, Luna et al [204] have used photodynamic therapy (PDT) to activate the GRP78 promoter, since this modality generates cytotoxic singlet oxygen species, which are potent activators of this promoter. Inducible expression of HSV-*tk* was observed after PDT in stably retroviral-transduced mouse mammary carcinoma cells (TSA), and enhanced tumouricidal activity was seen. The GRP78 promoter is, therefore, also inducible and can function as a molecular switch following exposure to PDT.

Hexokinase II is also overexpressed in solid tumours, partly due to elevated glucose catabolism in rapidly proliferating cells. Hexokinase II catalyses the first committed step of glycolysis and is overexpressed in some tumours since it is no longer responsive to the physiological

Table 4. Exogenously controlled inducible promoters.

Promoter	Tumour target	Reference
Radiation-inducible promoters		
Egr-1 and CArG elements	Glioma, hepatocellular carcinoma, soft tissue sarcomas	[209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221]
Waf-1	Murine fibrosarcoma	[222, 223]
RecA	Clostridia	[224, 225]
c-IAP2	Colon	[226]
Heat-inducible promoters		
HSP70B	Breast, melanoma, and prostate	[227, 228, 229, 230, 231, 232, 233, 234]
Gadd 153		[235]
Drug-inducible promoters		
MDR-1	Breast, colon	[236, 237, 238]
Tetracycline inducible	Breast, melanoma, brain, glioma, prostate	[49, 146, 239, 240, 241, 242, 243, 244, 245, 246, 247]
Rapamycin inducible	Fibrosarcomas	[248]
Tamoxifen-inducible estrogen response elements	Breast	[150, 249, 250]

inhibitor, glucagon. Adenovirus carrying *LacZ* under the control of the hexokinase promoter showed preferential β-galactosidase expression in nonsmall cell lung carcinomas compared to primary normal human bronchial epithelial cells. Cells infected with an adenovirus expressing HSV-*tk* under the control of this promoter, demonstrated selective toxicity, with a 10 to 100-fold increase in IC50 between the lung cancer cell lines and the normal bronchial epithelial cells and a 100-fold increase in the IC50 when compared to MCF-7 breast carcinoma cells [206].

EXOGENOUSLY CONTROLLED INDUCIBLE PROMOTERS

The use of tissue/tumour-specific cellular promoters to restrict transgene expression usually results in constitutive expression in the target tissue. However, in many situations it might be preferable to exogenously regulate the duration and level of expression. A list of exogenously controlled inducible promoters currently being used for cancer gene therapy purposes can be found in Table 4.

Radiation-inducible promoters

Radiation therapy is one of the main strategies used to treat solid tumours and although successful in some cases, there is the potential for enhancing radiation effects by combining with other agents such as gene therapy. Radiation-mediated gene therapy exploits the fact that as well as the cell killing effect, radiation will also activate transgenes which are driven by a radiation-inducible promoter. Confinement of transcription to the radiation field should be achievable by either using conformal, interstitial, or brachytherapy and by targeted delivery of radioisotopes. The cellular response to ionising radiation includes the transcriptional activation of a variety of genes, for example, *c-jun, NFB, EGR1, and p21*$^{WAF1/CIP1}$, to name but a few. However, although numerous radiation-inducible genes and proteins have been identified, relatively few radiation-inducible promoters/enhancers have been fully characterised.

Datta et al [251] identified a sequence of the EGR-1 gene that confers radiation inducibility. This sequence, CC(A+T rich)GG, is known as the radiation-responsive CArG element. In vitro studies, using the CArG, elements of the EGR1 promoter have demonstrated a dose-dependent expression of reporter gene activity of up to 28 fold following radiation exposure (0–20 Gy) [210]. However, at therapeutically relevant doses of radiation (2 Gy) only a 3-fold increase in reporter gene activity was achievable [211]. The thymidine analogue 5-iodo-2′-deoxyuridine radiolabelled with the Auger electron-emitter iodine-125 also activated the promoter and resulted in increased β-galactosidase activity in the 9L gliosarcoma tumour model in vivo. A number of studies have used CArG elements to drive expression of TNF using liposomes [212], a haemopoetic cell carrier [213] and adenovirus [214, 215, 216]. These studies have led to clinical trials where an adenovirus carrying TNF driven by CArG elements of the Egr-1 promoter are injected intratumourally into patients with soft-tissue sarcomas, followed by irradiation [217]. Enhanced antitumour effects were also observed when the Egr-1 promoter was combined with HSV-*tk* [210, 252]. Tumours regressed significantly after GCV and radiation in comparison to GCV alone. Furthermore, tumour regrowth did not occur when GCV treatment was withdrawn. As well as being radiation-inducible the Egr-1 promoter was tumour-specific for hepatocellular carcinomas whereas its expression was barely detectable within normal liver [210]. It

was evident from some of these papers that reporter gene expression was detectable in nonirradiated cells, which could limit its use in combination with cytotoxic agents. Weichselbaum et al [218] are also developing a herpes simplex virus (HSV-1) that is induced to proliferate within the tumour volume following ionising radiation exposure. Finally, Marples and Scott are developing a novel molecular switch using CArG elements derived from this promoter, in combination with a Cre/*loxP* site-specific recombination system of the P1 bacteriophage, to generate high level and constitutive expression of transgenes following activation by therapeutically relevant radiation doses [209, 219, 253]. This system will be discussed in more detail later.

The promoter of the $P21^{WAF1/CIP1}$ gene is also radiation-sensitive and its activation is also increased in tumour cells [254, 255, 256, 257, 258, 259, 260, 261]. Interestingly, expression appears to be independent of p53 status in a variety of tumour types [257, 262, 263]. Furthermore, we have evidence that this promoter is also highly active following exposure of cell in vitro to hypoxia (unpublished data, 2002). This promoter therefore, may have several layers of specificity, that is, tumour-, radiation-, and hypoxia-specific. Research in our laboratory has shown that WAF-1 can successfully drive expression of inducible nitric oxide synthase which leads to the generation of the vasoactive, cytotoxic, and radiosensitising product, nitric oxide (NO), close to therapeutically relevant doses. As well as causing significant vasorelaxation in isolated arteries [222], it also caused abundant apoptosis in a murine fibrosarcoma model and a highly significant tumour growth delay in vivo when used in combination with 20 Gy radiation [223]. This promoter is also being used to drive expression of cytochrome P450 enzymes to increase the activation of the bioreductive drug, AQ4N [264, 265].

Radiation-inducible bacterial promoters have also been used such as RecA, delivered using the anaerobic apathogenic bacterium, clostridia, as a gene transfer system to target anoxic areas within tumours. Significant increases in β-galactosidase activity and TNF-α secretion was seen following 2-Gy irradiation [224, 225, 266]; fractionation of the radiation dose lead to repeated gene induction.

Lastly, a retrovirus gene trap screening identified c-IAP2 as an X-ray-inducible gene with radiation-sensitive NFkB binding sites. Construction of a plasmid with four tandem repeats of the NFkB binding site driving the expression of the apoptotic suicide gene *BAX*, resulted in significant cell kill following a therapeutically relevant dose of 2-Gy X-rays [226].

Heat-inducible promoters

Hyperthermia is known to improve the response of tumours to radiation and chemotherapeutic treatment [267]. The cellular response to hyperthermia is associated with the synthesis of heat shock proteins (HSP). Strategies are now being developed to combine hyperthermia with gene therapy by using the promoter of the HSP70B gene to selectively activate therapeutic transgenes following hyperthermia treatment, thereby controlling the location, duration, and level of expression of the therapeutic transgene. MRI-guided ultrasound is also being used to accurately focus gene activation [227]. The HSP70B promoter has been used to drive expression of TGF-β in an adenoviral construct [268]; HSV-*tk*, where sensitivity to GCV in a mouse mammary cancer cell line was increased more than 50,000 times [228]; HSV-*tk*/cytosine deaminase fusion gene and a replication-competent E1B-attenuated adenoviral vector [229]; and IL-12 in combination with radiation [230, 231]. The HSP70B promoter appears to be highly active and a 200–950-fold increase in reporter gene expression was observed when additional heat shock elements (HSE) were introduced to the HSP70 promoter with a 1–2°C decrease in the activation temperature [232]. Two very elegant constructs have now been developed which incorporate elements from the HSP70 promoter. Emiliusen et al and Lipinski et al have developed constructs which incorporate effective feedback loops which can enhance transgene expression and immune response [14] or inhibit transgene expression in normal cells expressing wild-type p53 [173]. These will be discussed in more detail later. Interestingly, electromagnetic fields have been reported to activate the HSP70 promoter and could possibly be used as switches to regulate transgenes for cancer gene therapy purposes [269]. This has already been achieved with another stress-inducible promoter, Gadd153. The promoter from this gene was placed upstream of the TNF-α gene and delivered using magnetite cationic liposomes (MCLs). In nude mice, these constructs induced cell death throughout much of the tumour area on heating under an alternating magnetic field as a result of a 3-fold increase in TNF-α production compared with that of a nonheated tumour [235].

Drug-inducible promoters

As already mentioned many tissue/tumour-specific promoters are often weak and are therefore less effective than constitutive promoters. Much work has therefore focused on developing promoters which are both strong and regulatable exogenously. The use of tetracycline (tc)-responsive promoters is probably best researched. Two systems exist; the allosteric-off system or the allosteric-on system which have been described in detail by Harrington et al [78]. A tc inducible promoter has been used to drive expression of HSV-*tk* in breast carcinoma cells and increased the IC50 for GCV by 50 fold, although bystander effects were not enhanced [239]. Both IL-2 and IL-1β converting enzyme (ICE) have been shown to be tightly regulated, respectively, in human melanoma cells [240] and in a rat brain tumour model [241] in vivo using a similar approach. However, the real beauty of this system has been to selectively regulate transgene expression in

certain tissues by combining with tissue/tumour-specific promoters. A replication-incompetent adenovirus, which has the glial fibrillary acidic protein coupled to a minimal CMV promoter, has been constructed to drive expression of a tc-controlled transactivator. High levels of expression were reported in cells of glial origin and expression was tightly controlled by the addition of tc. This system is both glial-specific and allows for repression of ectopic gene expression [49]. Both a parvovirus and an adenovirus have been constructed to combine cell-type specificity with high-level regulated expression for prostate tissues using the ARR2BP promoter [57] or for hepatoma cells using a liver-specific promoter [146]. In a similar study, Rubinchik et al [242] have placed the tc transactivator gene under the control of the ARR2PB promoter and an FASL fusion gene under the control of a tc responsive promoter, within a single construct. Not only is the expression of this apoptotic related gene FASL restricted to prostate cancer cells and is regulatable by the tc analogue, doxycycline, but also, in vivo studies confirmed that systemic delivery of this vector was well tolerated at doses that were lethal for the FASL controlled by a CMV promoter. Overall, tc promoters show high-level inducibility compared to other inducible promoters and when combined with other tissue- and tumour-specific elements, they can be highly inducible and specific.

Dimerisation switch-on systems are also being developed, which make use of the immunosuppressive agent, rapamycin, and again this area has been reviewed by Harrington et al [78]. Rapamycin has been used to control regulation of adenoviral vectors where inhibition of tumour growth was seen following subcutaneous injection into the human fibrosarcoma, HT1080 [248].

As well as tc, there is now much interest in the use of estrogen response elements (EREs) which are responsive to estrogens and can be controlled by antiestrogens, such as tamoxifen. EREs have been used to drive expression of the E1a and E4 units in response to estrogens-generating conditionally replicative adenoviruses, selective for ER-positive breast cancer cells, which can also be modulated by the addition of tamoxifen [249]. When these EREs were combined with hypoxia response elements, these viruses can kill ER-positive breast cancer cells or cells growing under hypoxic conditions [150].

Lastly, a series of papers have described the use of the multidrug resistance (mdr1) gene promoter which harbours drug-inducible response elements to drive expression of a reporter gene [270] and TNF-α within a retroviral construct both in vitro in mammary and colon carcinoma cell lines [271] and in vivo in MCF-7 mammary xenografts [236]. Chemotherapy regulation by doxorubicin or vincristine showed a 25-fold increase in TNF-α secretion within the xenografts and was more effective in inhibiting tumour growth than constitutively TNF-α-expressing vectors. More recently, Walther et al [237] have shown that the mdr1 promoter also harbours heat-inducible elements.

STRATEGIES FOR ENHANCING THE EFFICIENCY OF WEAK TISSUE/TUMOUR-INDUCIBLE PROMOTERS

The transcriptional activity of the tissue-specific elements used as promoters for gene therapy is often too weak to generate adequate concentrations of transgene product in target cells. The dilemma is how to achieve high levels of transgene expression without compromising specificity. As mentioned previously, several elegant strategies have been developed which combine both inducible and tissue/tumour-specific elements, thereby increasing transcriptional activation while allowing tissue specificity to be retained. Furthermore, other systems have been developed which are tissue-specific but also highly active by encouraging positive feedback loops incorporating strong transcriptional activators.

In one approach, a weak tissue-specific promoter (vWF or gastrointestinal-specific sucrose-maltase) initiates transcription of both the transgene and a gene encoding a VP16-LexA fusion protein (Figure 2). This protein, in turn, interacts with LexA binding sites inserted upstream of the tissue-specific promoter, leading to transactivation and enhanced transcription. Repeated cycles of this amplification lead to very high levels (14–100 fold) of tissue-specific expression [208]. An alternative strategy using a GAL4/VP16 fusion protein to enhance the weak tumour-specific CEA promoter achieved similar amplification of the reporter genes *LacZ* or *GV16* [100]. This concept has been further developed by using a tissue-specific promoter (PSA) to drive expression of GAL4 derivatives fused with up-to-4 VP16 activation domains. By incorporating up-to-5 GAL4 binding sites upstream of the reporter gene (firefly luciferase) 800-fold amplification was achieved [272].

The Cre/*loxP* system offers a different approach to the enhancement of tissue-specific promoter activity (Figure 3). Target cells are transfected with a plasmid incorporating the therapeutic transgene separated from a strong constitutive promoter by a "stop" cassette enclosed by two *loxP* sites. Cotransfection with a site-specific Cre gene driven by the tissue-specific promoter generates Cre, which excises the stop cassette, bringing the therapeutic transgene under the control of the constitutive promoter [113]. This system has been used to achieve therapeutic levels of transgenes in mouse tumours in vivo with a high degree of tissue specificity. CEA-driven expression of Cre together with expression of lox-HSV-*tk* caused significant suppression of tumours in mice treated with GCV, without significant side effects [99, 104]. CEA-driven expression of Cre also achieved cytotoxicity in tumours in mice transfected with cytosine deaminase and dosed with the 5-FU precursor, 5-FC, and this was reflected in enhanced overall survival. Diphtheria toxin (DT) has also been used therapeutically in growth hormone (GH)-producing pituitary tumours; the DT gene was activated via the Cre/*loxP* system driven by the GH promoter [273]. Cre/*loxP* has also been used to increase HSV-*tk* transgene

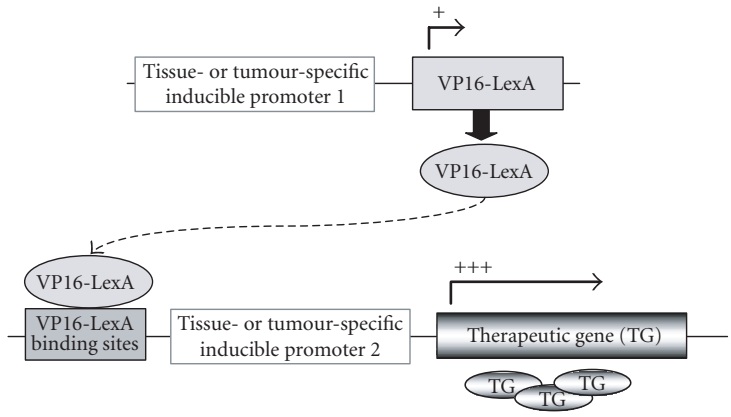

Figure 2. Schematic outline of a positive feedback loop utilising VP16/LexA to enhance weak promoters. Transcription is initiated by the cell-type-specific promoter in target cells which leads to expression of the VP16/LexA fusion protein. The VP16/LexA protein then interacts with the LexA binding sites upstream of a possible second cell-type-specific promoter leading to transactivation and thus enhancement of transcription. Alternatively, the VP16/LexA fusion protein could be expressed along with the transgene expressed via an IRES. Adapted from Nettelbeck et al [179].

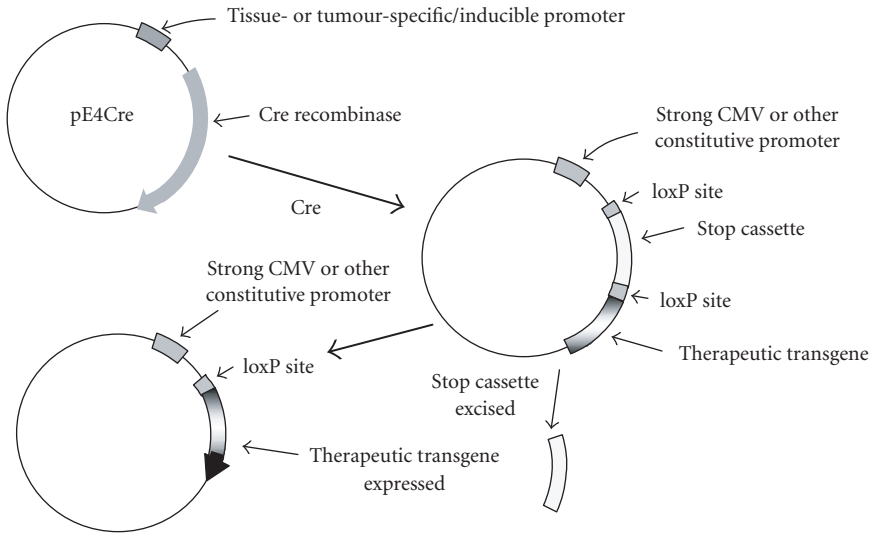

Figure 3. The Cre recombinase/loxP system for enhancing weak tissue or tumour-specific promoters. Cre, induced by the specific promoter excises the transcriptional stop cassette via the loxP sites. This brings the therapeutic transgene under the control of a strong constitutive promoter (eg, CMV).

expression following expression of Cre via the radiation-inducible CArG elements [209, 219].

Another approach that has a potential for tumour-specific activation incorporates specificity for mutated p53 [173]. In this study, a double recombinant adenovirus consisting of two independent expression cassettes was constructed; one controlled expression of the *E. coli* nitroreductase gene via an hsp70 promoter containing Lac1 binding sites, which was capable of binding a p53-inducible lac repressor, incorporated into the second expression cassette. This resulted in growth inhibition of human tumour xenografts with mutated p53 following exposure to the prodrug, CB1954, but not in tumours with wild-type p53 status.

Lastly, high-level tissue-specific expression of a directly cytotoxic protein, gibbon ape leukemia virus hyperfusogenic envelope protein (GALV-FMG), has been achieved in melanoma cells by elegantly driving both GALV and the gene for heat shock factor 1 (HSF-1) from a human tyrosinase promoter, preferentially activated in melanoma cells [14]. Promoter activity initiates low-level expression of the toxic transgene, but also HSF-1, which binds to a heat shock binding element upstream of the tyrosinase promoter. This enhances promoter

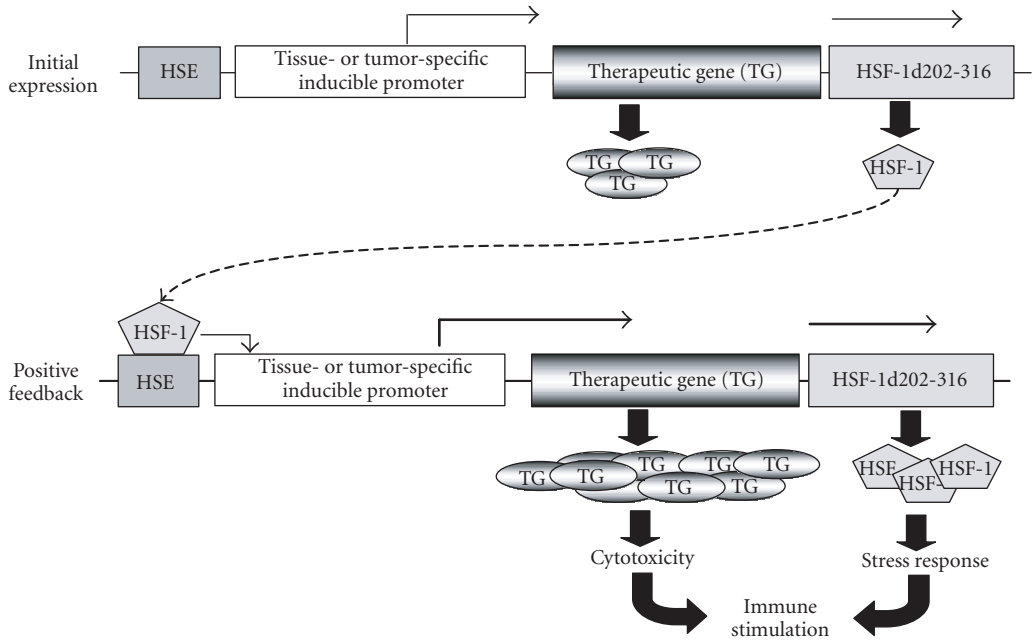

FIGURE 4. A transcriptional feedback loop for tissue-specific expression of highly cytotoxic genes which incorporate an immunostimulatory component. A weak tissue- or tumour-specific promoter will initially express the therapeutic transgene (TG) at low levels, however coexpression of the heat shock transcription factor (HSF-1) will activate the heat shock element (HSE) causing progressively increased TG and HSF-1 expression via a positive feedback loop. In addition, the expression of HSF-1 causes an immune response. Adapted from Emiliusen et al [14].

activation and completes the transcriptional feedback loop (Figure 4). This strategy has achieved long-term eradication of melanomas in mice [14].

CONCLUSIONS AND FUTURE PERSPECTIVES

The ultimate goal of all currently used cancer therapy is to target damaging events to tumour cells while sparing normal tissues sufficiently that they can recover to an adequate functional level; this applies equally to gene therapy. However, while conventional therapies rely on one, or at the most two, targeting characteristics such as spatial deposition, biochemical pathway specificity, or cellular proliferation, there is no theoretical limit to the number of elements that can be incorporated into a gene therapy strategy to confer tumour specificity. There is also the potential for controlling temporal expression of transgene products in tumours much more precisely than the concentration of conventional anticancer agents.

The first opportunity for targeting specificity is at the level of vector delivery. There is currently an immense amount of research activity in this area (beyond the scope of this review) reflecting the recognition that advances in this process are critical to unleashing the potential of all the recent developments in transcriptional control. Direct injection systems have been important in providing the proof of principle for several gene therapy strategies, but however sophisticated these may become, the number of accessible sites will always be limited and perhaps more importantly the crucial problem of disseminated disease cannot be addressed.

As detailed in this review, the possibilities for targeting via transcriptional control are endless. It is unlikely for most cancers that any one promoter will offer the ideal combination of interrelated characteristics: high level specificity to endogenous or exogenous factors, silence in the absence of these factors and sufficient strength to induce the therapeutic transgene many fold. In any event, the use of a single promoter/transgene combination fails to exploit one of the main advantages of gene therapy: the ability to custom design, even within a single plasmid, a combination of diverse elements that confer exquisite spatial and temporal specificity for an individual tumour type. It may even be possible using gene/protein array technology to tailor this specificity to an individual patient's tumour. It could be envisaged that promoters specific to the tumour tissue type could be combined with factors inducible by the tumour microenvironment and/or exogenous control elements (eg, radiation). This has already been successfully achieved in vivo by combining cell cycle and tyrosinase promoters [179], HREs and KDR/E- selectin promoters [186], and HREs and the AFP promoter [145]. Furthermore, these could be combined with elements incorporating strong promoters/enhancers in positive feedback loops giving progressively stronger and more specific transgene expression [14, 179] or by using a Cre/*loxP* system [274]. The packaging of such complex constructs, ideally within a single construct, will

require a more thorough understanding of the control domains from individual promoters such that multiple copies of the elements that confer tumour specificity can be incorporated while eliminating those sequences that permit expression in nontarget tissues.

A vital issue when considering the clinical application of any gene therapy protocol is the basal expression of the transgene. Many of the papers discussed in this review have failed to adequately assess basal-level expression in other tissues following systemic delivery in an appropriate in vivo model. Highly sensitive systems now exist for assessing promoter leakiness in vitro [42] and recent developments in imaging technology have permitted whole animal detection of fluorescent reporter gene products in small rodents [272, 275]. Although this technology is theoretically applicable in man, considerable caution must be exercised in extrapolating from animal data. It is likely in early trials that transgene activation by a given promoter combination will need to be assessed using a nontoxic reporter gene, particularly where tissue-specific promoters are to be used. This use of a surrogate marker is an additional and important advantage of gene therapy over conventional chemotherapy that can be applied even at the level of the individual patient.

Finally, the therapeutic transgene product will usually be an enzyme, so the opportunity arises to achieve additional specificity at the level of enzyme biochemistry or enzyme product characteristics. For example, reductive enzymes can metabolise bioreductive prodrugs, such as AQ4N [264, 265] or tirapazamine [276], specifically in a hypoxic environment thereby adding an additional layer of tumour specificity even after transductional and transcriptional targeting. A similarly enhanced specificity can be achieved, where the final product of the enzyme specifically sensitises hypoxic tumour cells [222, 223]. Alternatively, a powerful approach for tight regulation has been to make the activity of the apoptotic target gene, E2F1, tamoxifen-dependent by fusing it to the ligand-binding domain of the estrogen receptor; massive apoptosis was seen upon activation by tamoxifen [277].

The rate of development of gene therapy over the past decade has been dramatic and if this continues, it is reasonable to speculate that the next decade will see widespread introduction of this new technology to clinical practice. We can anticipate a level of anticancer efficacy combined with normal tissue sparing that few small molecules have yet achieved and with the current advances in systemic gene delivery this is a very real possibility. By combining the very best of cancer gene therapy strategies with current chemotherapy and radiotherapy treatment regimens, real patient benefit with reduced toxicities should be achievable.

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REFERENCES

[1] Maron DJ, Choi EA, Spitz FR. Gene therapy of metastatic disease: progress and prospects. *Surg Oncol Clin N Am*. 2001;10(2):449–460.

[2] Brown MD, Schatzlein AG, Uchegbu IF. Gene delivery with synthetic (non viral) carriers. *Int J Pharm*. 2001;229(1-2):1–21.

[3] McCormick F. Cancer gene therapy: fringe or cutting edge? *Nat Rev Cancer*. 2001;1(2):130–141.

[4] Barnett BG, Crews CJ, Douglas JT. Targeted adenoviral vectors. *Biochim Biophys Acta*. 2002;1575(1–3):1–14.

[5] Vile RG, Hart IR. In vitro and in vivo targeting of gene expression to melanoma cells. *Cancer Res*. 1993;53(5):962–967.

[6] Vile RG, Hart IR. Use of tissue-specific expression of the herpes simplex virus thymidine kinase gene to inhibit growth of established murine melanomas following direct intratumoral injection of DNA. *Cancer Res*. 1993;53(17):3860–3864.

[7] Vile RG, Nelson JA, Castleden S, Chong H, Hart IR. Systemic gene therapy of murine melanoma using tissue specific expression of the HSVtk gene involves an immune component. *Cancer Res*. 1994; 54(23):6228–6234.

[8] Vile R, Miller N, Chernajovsky Y, Hart I. A comparison of the properties of different retroviral vectors containing the murine tyrosinase promoter to achieve transcriptionally targeted expression of the HSVtk or IL-2 genes. *Gene Ther*. 1994;1(5):307–316.

[9] Diaz RM, Eisen T, Hart IR, Vile RG. Exchange of viral promoter/enhancer elements with heterologous regulatory sequences generates targeted hybrid long terminal repeat vectors for gene therapy of melanoma. *J Virol*. 1998;72(1):789–795.

[10] Cao G, Zhang X, He X, Chen Q, Qi Z. A safe, effective in vivo gene therapy for melanoma using tyrosinase promoter-driven cytosine deaminase gene. *In Vivo*. 1999;13(2):181–187.

[11] Szala S, Missol E, Sochanik A, Strozyk M. The use of cationic liposomes DC-CHOL/DOPE and DDAB/DOPE for direct transfer of Escherichia coli cytosine deaminase gene into growing melanoma tumors. *Gene Ther*. 1996;3(11):1026–1031.

[12] Siders WM, Halloran PJ, Fenton RG. Transcriptional targeting of recombinant adenoviruses to human and murine melanoma cells. *Cancer Res*. 1996;56(24):5638–5646.

[13] Jerome V, Muller R. Tissue-specific, cell cycle-regulated chimeric transcription factors for the targeting of gene expression to tumor cells. *Hum Gene Ther*. 1998;9(18):2653–2659.

[14] Emiliusen L, Gough M, Bateman A, et al. A transcriptional feedback loop for tissue-specific expression of highly cytotoxic genes which incorporates

an immunostimulatory component. *Gene Ther*. 2001;8(13):987–998.

[15] Nahde T, Muller K, Fahr A, Muller R, Brusselbach S. Combined transductional and transcriptional targeting of melanoma cells by artificial virus-like particles. *J Gene Med*. 2001;3(4):353–361.

[16] Vile RG, Hart IR. Targeting of cytokine gene expression to malignant melanoma cells using tissue specific promoter sequences. *Ann Oncol*. 1994;5 (suppl 4):59–65.

[17] Hart IR. Tissue specific promoters in targeting systemically delivered gene therapy. *Semin Oncol*. 1996;23(1):154–158.

[18] Bazzoni F, Regalia E. Triggering of antitumor activity through melanoma-specific transduction of a constitutively active tumor necrosis factor (TNF) R1 chimeric receptor in the absence of TNF-alpha. *Cancer Res*. 2001;61(3):1050–1057.

[19] Pang S, Taneja S, Dardashti K, et al. Prostate tissue specificity of the prostate-specific antigen promoter isolated from a patient with prostate cancer. *Hum Gene Ther*. 1995;6(11):1417–1426.

[20] Lee CH, Liu M, Sie KL, Lee MS. Prostate-specific antigen promoter driven gene therapy targeting DNA polymerase-alpha and topoisomerase II alpha in prostate cancer. *Anticancer Res*. 1996;16(4A): 1805–1811.

[21] Rodriguez R, Schuur ER, Lim HY, Henderson GA, Simons JW, Henderson DR. Prostate attenuated replication competent adenovirus (ARCA) CN706: a selective cytotoxic for prostate-specific antigen-positive prostate cancer cells. *Cancer Res*. 1997; 57(13):2559–2563.

[22] Gotoh A, Ko SC, Shirakawa T, et al. Development of prostate-specific antigen promoter-based gene therapy for androgen-independent human prostate cancer. *J Urol*. 1998;160(1):220–229.

[23] Wu L, Matherly J, Smallwood A, et al. Chimeric PSA enhancers exhibit augmented activity in prostate cancer gene therapy vectors. *Gene Ther*. 2001;8(18):1416–1426.

[24] Spitzweg C, Zhang S, Bergert ER, et al. Prostate-specific antigen (PSA) promoter-driven androgen-inducible expression of sodium iodide symporter in prostate cancer cell lines. *Cancer Res*. 1999; 59(9):2136–2141.

[25] Latham JP, Searle PF, Mautner V, James ND. Prostate-specific antigen promoter/enhancer driven gene therapy for prostate cancer: construction and testing of a tissue-specific adenovirus vector. *Cancer Res*. 2000;60(2):334–341.

[26] Yu D, Chen D, Chiu C, Razmazma B, Chow YH, Pang S. Prostate-specific targeting using PSA promoter-based lentiviral vectors. *Cancer Gene Ther*. 2001;8(9):628–635.

[27] Suzuki S, Tadakuma T, Kunitomi M, et al. Liposome-mediated gene therapy using HSV-TK/ganciclovir under the control of human PSA promoter in prostate cancer cells. *Urol Int*. 2001; 67(3):216–223.

[28] Steiner MS, Zhang Y, Carraher J, Lu Y. In vivo expression of prostate-specific adenoviral vectors in a canine model. *Cancer Gene Ther*. 1999;6(5):456–464.

[29] Culig Z, Hobisch A, Cronauer MV, et al. Androgen receptor activation in prostatic tumor cell lines by insulin-like growth factor-I, keratinocyte growth factor, and epidermal growth factor. *Cancer Res*. 1994;54(20):5474–5478.

[30] Spitzweg C, O'Connor MK, Bergert ER, Tindall DJ, Young CY, Morris JC. Treatment of prostate cancer by radioiodine therapy after tissue-specific expression of the sodium iodide symporter. *Cancer Res*. 2000;60(22):6526–6530.

[31] Gong MC, Chang SS, Watt F, et al. Overview of evolving strategies incorporating prostate-specific membrane antigen as target for therapy. *Mol Urol*. 2000;4(3):217–222; discussion 223.

[32] Suzuki S, Tadakuma T, Asano T, Hayakawa M. Co-expression of the partial androgen receptor enhances the efficacy of prostate-specific antigen promoter-driven suicide gene therapy for prostate cancer cells at low testosterone concentrations. *Cancer Res*. 2001;61(4):1276–1279.

[33] Zhang S, Gu J, Yang NS, et al. Relative promoter strengths in four human prostate cancer cell lines evaluated by particle bombardment-mediated gene transfer. *Prostate*. 2002;51(4):286–292.

[34] O'Keefe DS, Uchida A, Bacich DJ, et al. Prostate-specific suicide gene therapy using the prostate-specific membrane antigen promoter and enhancer. *Prostate*. 2000;45(2):149–157.

[35] Uchida A, O'Keefe DS, Bacich DJ, Molloy PL, Heston WD. In vivo suicide gene therapy model using a newly discovered prostate-specific membrane antigen promoter/enhancer: a potential alternative approach to androgen deprivation therapy. *Urology*. 2001;58(2)suppl 1:132–139.

[36] Watt F, Martorana A, Brookes DE, et al. A tissue-specific enhancer of the prostate-specific membrane antigen gene, FOLH1. *Genomics*. 2001;73(3): 243–254.

[37] Yu DC, Sakamoto GT, Henderson DR. Identification of the transcriptional regulatory sequences of human kallikrein 2 and their use in the construction of calydon virus 764, an attenuated replication competent adenovirus for prostate cancer therapy. *Cancer Res*. 1999;59(7):1498–1504.

[38] Xie X, Zhao X, Liu Y, et al. Robust prostate-specific expression for targeted gene therapy based on the human kallikrein 2 promoter. *Hum Gene Ther*. 2001;12(5):549–561.

[39] Zhang J, Thomas TZ, Kasper S, Matusik RJ. A small composite probasin promoter confers high levels of prostate-specific gene expression through regulation by androgens and glucocorticoids in vitro and in vivo. *Endocrinology*. 2000;141(12):4698–4710.

[40] Lowe SL, Rubinchik S, Honda T, McDonnell TJ, Dong JY, Norris JS. Prostate-specific expression of Bax delivered by an adenoviral vector induces apoptosis in LNCaP prostate cancer cells. *Gene Ther*. 2001;8(18):1363–1371.

[41] Martiniello-Wilks R, Tsatralis T, Russell P, et al. Transcription-targeted gene therapy for androgen-independent prostate cancer. *Cancer Gene Ther*. 2002;9(5):443–452.

[42] van der Poel HG, McCadden J, Verhaegh GW, et al. A novel method for the determination of basal gene expression of tissue-specific promoters: an analysis of prostate-specific promoters. *Cancer Gene Ther*. 2001;8(12):927–935.

[43] Miyao Y, Shimizu K, Moriuchi S, et al. Selective expression of foreign genes in glioma cells: use of the mouse myelin basic protein gene promoter to direct toxic gene expression. *J Neurosci Res*. 1993;36(4):472–479.

[44] Miyao Y, Shimizu K, Tamura M, et al. Usefulness of a mouse myelin basic protein promoter for gene therapy of malignant glioma: myelin basic protein promoter is strongly active in human malignant glioma cells. *Jpn J Cancer Res*. 1997;88(7):678–686.

[45] Vandier D, Rixe O, Brenner M, Gouyette A, Besnard F. Selective killing of glioma cell lines using an astrocyte-specific expression of the herpes simplex virus-thymidine kinase gene. *Cancer Res*. 1998;58(20):4577–4580.

[46] Vandier D, Rixe O, Besnard F, et al. Inhibition of glioma cells in vitro and in vivo using a recombinant adenoviral vector containing an astrocyte-specific promoter. *Cancer Gene Ther*. 2000;7(8):1120–1126.

[47] McKie EA, Graham DI, Brown SM. Selective astrocytic transgene expression in vitro and in vivo from the GFAP promoter in a HSV RL1 null mutant vector—potential glioblastoma targeting. *Gene Ther*. 1998;5(4):440–450.

[48] Morelli AE, Larregina AT, Smith-Arica J, et al. Neuronal and glial cell type-specific promoters within adenovirus recombinants restrict the expression of the apoptosis-inducing molecule Fas ligand to predetermined brain cell types, and abolish peripheral liver toxicity. *J Gen Virol*. 1999;80(pt 3):571–583.

[49] Chen J, Bezdek T, Chang J, et al. A glial-specific, repressible, adenovirus vector for brain tumor gene therapy. *Cancer Res*. 1998;58(16):3504–3507.

[50] Shi N, Zhang Y, Zhu C, Boado RJ, Pardridge WM. Brain-specific expression of an exogenous gene after i.v. administration. *Proc Natl Acad Sci USA*. 2001;98(22):12754–12759.

[51] Glenn GM, Chatterjee S. Generation of adenoviruses encoding the herpes simplex virus vhs gene: a novel strategy to generate adenoviruses expressing genes toxic to producer cells. *Cancer Gene Ther*. 2001;8(8):566–572.

[52] Shinoura N, Ohashi M, Yoshida Y, et al. Adenovirus-mediated overexpression of Fas induces apoptosis of gliomas. *Cancer Gene Ther*. 2000;7(2):224–232.

[53] Shinoura N, Saito K, Yoshida Y, et al. Adenovirus-mediated transfer of bax with caspase-8 controlled by myelin basic protein promoter exerts an enhanced cytotoxic effect in gliomas. *Cancer Gene Ther*. 2000;7(5):739–748.

[54] Shinoura N, Koike H, Furitu T, et al. Adenovirus-mediated transfer of caspase-8 augments cell death in gliomas: implication for gene therapy. *Hum Gene Ther*. 2000;11(8):1123–1137.

[55] Martin V, Cortes ML, de Felipe P, Farsetti A, Calcaterra NB, Izquierdo M. Cancer gene therapy by thyroid hormone-mediated expression of toxin genes. *Cancer Res*. 2000;60(12):3218–3224.

[56] Tanaka M, Inase N, Miyake S, Yoshizawa Y. Neuron specific enolase promoter for suicide gene therapy in small cell lung carcinoma. *Anticancer Res*. 2001;21(1A):291–294.

[57] Ralph GS, Bienemann A, Harding TC, Hopton M, Henley J, Uney JB. Targeting of tetracycline-regulatable transgene expression specifically to neuronal and glial cell populations using adenoviral vectors. *Neuroreport*. 2000;11(9):2051–2055.

[58] Glover CP, Bienemann AS, Heywood DJ, Cosgrave AS, Uney JB. Adenoviral-mediated, high-level, cell-specific transgene expression: a SYN1-WPRE cassette mediates increased transgene expression with no loss of neuron specificity. *Mol Ther*. 2002;5(pt 1):509–516.

[59] Narita M, Bahar R, Hatano M, et al. Tissue-specific expression of a suicide gene for selective killing of neuroblastoma cells using a promoter region of the NCX gene. *Cancer Gene Ther*. 2001;8(12):997–1002.

[60] Kuriyama S, Yoshikawa M, Ishizaka S, et al. A potential approach for gene therapy targeting hepatoma using a liver-specific promoter on a retroviral vector. *Cell Struct Funct*. 1991;16(6):503–510.

[61] Kuriyama S, Sakamoto T, Masui K, et al. Tissue-specific expression of HSV-tk gene can induce efficient antitumor effect and protective immunity to wild-type hepatocellular carcinoma. *Int J Cancer*. 1997;71(3):470–475.

[62] Cao G, Kuriyama S, Du P, et al. Construction of retroviral vectors to induce strong hepatoma cell-specific expression of cytokine genes. *J Gastroenterol Hepatol*. 1996;11(11):1053–1061.

[63] Cao G, Kuriyama S, Du P, et al. Complete regression of established murine hepatocellular carcinoma by in vivo tumor necrosis factor alpha gene transfer. *Gastroenterology*. 1997;112(2):501–510.

[64] Su H, Chang JC, Xu SM, Kan YW. Selective killing of AFP-positive hepatocellular carcinoma cells by adeno-associated virus transfer of the herpes

simplex virus thymidine kinase gene. *Hum Gene Ther*. 1996;7(4):463–470.

[65] Su H, Lu R, Chang JC, Kan YW. Tissue-specific expression of herpes simplex virus thymidine kinase gene delivered by adeno-associated virus inhibits the growth of human hepatocellular carcinoma in athymic mice. *Proc Natl Acad Sci USA*. 1997;94(25):13891–13896.

[66] Miyatake SI, Tani S, Feigenbaum F, et al. Hepatoma-specific antitumor activity of an albumin enhancer/promoter regulated herpes simplex virus in vivo. *Gene Ther*. 1999;6(4):564–572.

[67] Strayer MS, Guttentag SH, Ballard PL. Targeting type II and Clara cells for adenovirus-mediated gene transfer using the surfactant protein B promoter. *Am J Respir Cell Mol Biol*. 1998;18(1):1–11.

[68] Doronin K, Kuppuswamy M, Toth K, et al. Tissue-specific, tumor-selective, replication-competent adenovirus vector for cancer gene therapy. *J Virol*. 2001;75(7):3314–3324.

[69] Zeiger MA, Takiyama Y, Bishop JO, Ellison AR, Saji M, Levine MA. Adenoviral infection of thyroid cells: a rationale for gene therapy for metastatic thyroid carcinoma. *Surgery*. 1996;120(6):921–925.

[70] Braiden V, Nagayama Y, Iitaka M, Namba H, Niwa M, Yamashita S. Retrovirus-mediated suicide gene/prodrug therapy targeting thyroid carcinoma using a thyroid-specific promoter. *Endocrinology*. 1998;139(9):3996–3999.

[71] Zhang R, Straus FH, DeGroot LJ. Adenoviral-mediated gene therapy for thyroid carcinoma using thymidine kinase controlled by thyroglobulin promoter demonstrates high specificity and low toxicity. *Thyroid*. 2001;11(2):115–123.

[72] Shimura H, Suzuki H, Miyazaki A, et al. Transcriptional activation of the thyroglobulin promoter directing suicide gene expression by thyroid transcription factor-1 in thyroid cancer cells. *Cancer Res*. 2001;61(9):3640–3646.

[73] Blackwood L, O'Shaughnessy PJ, Reid SW, Argyle DJ. E. coli nitroreductase/CB1954: in vitro studies into a potential system for feline cancer gene therapy. *Vet J*. 2001;161(3):269–279.

[74] Selvakumaran M, Bao R, Crijns AP, Connolly DC, Weinstein JK, Hamilton TC. Ovarian epithelial cell lineage-specific gene expression using the promoter of a retrovirus-like element. *Cancer Res*. 2001;61(4):1291–1295.

[75] Bao R, Selvakumaran M, Hamilton TC. Targeted gene therapy of ovarian cancer using an ovarian-specific promoter. *Gynecol Oncol*. 2002;84(2):228–234.

[76] Yoshimura I, Suzuki S, Tadakuma T, Hayakawa M. Suicide gene therapy on LNCaP human prostate cancer cells. *Int J Urol*. 2001;8(7):S5–S8.

[77] Xie X, Zhao X, Liu Y, et al. Adenovirus-mediated tissue-targeted expression of a caspase-9-based artificial death switch for the treatment of prostate cancer. *Cancer Res*. 2001;61(18):6795–6804.

[78] Harrington KJ, Linardakis E, Vile RG. Transcriptional control: an essential component of cancer gene therapy strategies? *Adv Drug Deliv Rev*. 2000;44(2-3):167–184.

[79] Kim NW, Piatyszek MA, Prowse KR, et al. Specific association of human telomerase activity with immortal cells and cancer. *Science*. 1994; 266(5193):2011–2015.

[80] Broccoli D, Young JW, de Lange T. Telomerase activity in normal and malignant hematopoietic cells. *Proc Natl Acad Sci USA*. 1995;92(20):9082–9086.

[81] Tahara H, Yasui W, Tahara E, et al. Immunohistochemical detection of human telomerase catalytic component, hTERT, in human colorectal tumor and non-tumor tissue sections. *Oncogene*. 1999;18(8):1561–1567.

[82] Shay JW, Wright WE. Ageing and cancer: the telomere and telomerase connection. *Novartis Found Symp*. 2001;235:116–125.

[83] Pan CX, Koeneman KS. A novel tumor-specific gene therapy for bladder cancer. *Med Hypotheses*. 1999;53(2):130–135.

[84] Abdul-Ghani R, Ohana P, Matouk I, et al. Use of transcriptional regulatory sequences of telomerase (hTER and hTERT) for selective killing of cancer cells. *Mol Ther*. 2000;2(6):539–544.

[85] Boyd M, Mairs RJ, Mairs SC, et al. Expression in UVW glioma cells of the noradrenaline transporter gene, driven by the telomerase RNA promoter, induces active uptake of [131I]MIBG and clonogenic cell kill. *Oncogene*. 2001;20(53):7804–7808.

[86] Komata T, Koga S, Hirohata S, et al. A novel treatment of human malignant gliomas in vitro and in vivo: FADD gene transfer under the control of the human telomerase reverse transcriptase gene promoter. *Int J Oncol*. 2001;19(5):1015–1020.

[87] Koga S, Hirohata S, Kondo Y, et al. FADD gene therapy using the human telomerase catalytic subunit (hTERT) gene promoter to restrict induction of apoptosis to tumors in vitro and in vivo. *Anticancer Res*. 2001;21(3B):1937–1943.

[88] Komata T, Kondo Y, Kanzawa T, et al. Treatment of malignant glioma cells with the transfer of constitutively active caspase-6 using the human telomerase catalytic subunit (human telomerase reverse transcriptase) gene promoter. *Cancer Res*. 2001;61(15):5796–5802.

[89] Gu J, Andreeff M, Roth JA, Fang B. hTERT promoter induces tumor-specific Bax gene expression and cell killing in syngenic mouse tumor model and prevents systemic toxicity. *Gene Ther*. 2002;9(1):30–37.

[90] Gu J, Kagawa S, Takakura M, et al. Tumor-specific transgene expression from the human telomerase reverse transcriptase promoter enables targeting of the therapeutic effects of the Bax gene to cancers. *Cancer Res*. 2000;60(19):5359–5364.

[91] Plumb JA, Bilsland A, Kakani R, et al. Telomerase-specific suicide gene therapy vectors expressing bacterial nitroreductase sensitize human cancer cells to the pro-drug CB1954. *Oncogene*. 2001; 20(53):7797–7803.

[92] Richards CA, Austin EA, Huber BE. Transcriptional regulatory sequences of carcinoembryonic antigen: identification and use with cytosine deaminase for tumor-specific gene therapy. *Hum Gene Ther*. 1995;6(7):881–893.

[93] Cao G, Kuriyama S, Gao J, et al. In vivo gene transfer of a suicide gene under the transcriptional control of the carcinoembryonic antigen promoter results in bone marrow transduction but can avoid bone marrow suppression. *Int J Oncol*. 1999;15(1):107–112.

[94] Cao G, Kuriyama S, Gao J, et al. Effective and safe gene therapy for colorectal carcinoma using the cytosine deaminase gene directed by the carcinoembryonic antigen promoter. *Gene Ther*. 1999;6(1):83–90.

[95] Osaki T, Tanio Y, Tachibana I, et al. Gene therapy for carcinoembryonic antigen-producing human lung cancer cells by cell type-specific expression of herpes simplex virus thymidine kinase gene. *Cancer Res*. 1994;54(20):5258–5261.

[96] DiMaio JM, Clary BM, Via DF, Coveney E, Pappas TN, Lyerly HK. Directed enzyme pro-drug gene therapy for pancreatic cancer in vivo. *Surgery*. 1994;116(2):205–213.

[97] Tanaka T, Kanai F, Okabe S, et al. Adenovirus-mediated prodrug gene therapy for carcinoembryonic antigen-producing human gastric carcinoma cells in vitro. *Cancer Res*. 1996;56(6):1341–1345.

[98] Brand K, Loser P, Arnold W, Bartels T, Strauss M. Tumor cell-specific transgene expression prevents liver toxicity of the adeno-HSVtk/GCV approach. *Gene Ther*. 1998;5(10):1363–1371.

[99] Goto H, Osaki T, Kijima T, et al. Gene therapy utilizing the Cre/loxP system selectively suppresses tumor growth of disseminated carcinoembryonic antigen-producing cancer cells. *Int J Cancer*. 2001;94(3):414–419.

[100] Koch PE, Guo ZS, Kagawa S, Gu J, Roth JA, Fang B. Augmenting transgene expression from carcinoembryonic antigen (CEA) promoter via a GAL4 gene regulatory system. *Mol Ther*. 2001;3(3):278–283.

[101] Horimoto M, Sasaki Y, Ugawa S, et al. A novel strategy for cancer therapy by mutated mammalian degenerin gene transfer. *Cancer Gene Ther*. 2000;7(10):1341–1347.

[102] Ueda K, Iwahashi M, Nakamori M, Nakamura M, Yamaue H, Tanimura H. Enhanced selective gene expression by adenovirus vector using Cre/loxP regulation system for human carcinoembryonic antigen-producing carcinoma *Oncology*. 2000;59(3):255–265.

[103] Ueda K, Iwahashi M, Nakamori M, et al. Carcinoembryonic antigen-specific suicide gene therapy of cytosine deaminase/5-fluorocytosine enhanced by the cre/loxP system in the orthotopic gastric carcinoma model. *Cancer Res*. 2001;61(16):6158–6162.

[104] Kijima T, Osaki T, Nishino K, et al. Application of the Cre recombinase/loxP system further enhances antitumor effects in cell type-specific gene therapy against carcinoembryonic antigen-producing cancer. *Cancer Res*. 1999;59(19):4906–4911.

[105] Qiao J, Doubrovin M, Sauter BV, et al. Tumor-specific transcriptional targeting of suicide gene therapy. *Gene Ther*. 2002;9(3):168–175.

[106] Ishikawa H, Nakata K, Mawatari F, et al. Utilization of variant-type of human alpha-fetoprotein promoter in gene therapy targeting for hepatocellular carcinoma. *Gene Ther*. 1999;6(4):465–470.

[107] Ishikawa H, Nakata K, Mawatari F, et al. Retrovirus-mediated gene therapy for hepatocellular carcinoma with reversely oriented therapeutic gene expression regulated by alpha-fetoprotein enhancer/promoter. *Biochem Biophys Res Commun*. 2001;287(4):1034–1040.

[108] Kanai F, Lan KH, Shiratori Y, et al. In vivo gene therapy for alpha-fetoprotein-producing hepatocellular carcinoma by adenovirus-mediated transfer of cytosine deaminase gene. *Cancer Res*. 1997; 57(3):461–465.

[109] Bui LA, Butterfield LH, Kim JY, et al. In vivo therapy of hepatocellular carcinoma with a tumor-specific adenoviral vector expressing interleukin-2. *Hum Gene Ther*. 1997;8(18):2173–2182.

[110] Cao G, Kuriyama S, Tsujinoue H, Chen Q, Mitoro A, Qi Z. A novel approach for inducing enhanced and selective transgene expression in hepatocellular-carcinoma cells. *Int J Cancer*. 2000; 87(2):247–252.

[111] Murayama Y, Tadakuma T, Kunitomi M, et al. Cell-specific expression of the diphtheria toxin A-chain coding sequence under the control of the upstream region of the human alpha-fetoprotein gene. *J Surg Oncol*. 1999;70(3):145–149.

[112] Ohashi M, Kanai F, Tateishi K, et al. Target gene therapy for alpha-fetoprotein-producing hepatocellular carcinoma by E1B55k-attenuated adenovirus. *Biochem Biophys Res Commun*. 2001;282(2): 529–535.

[113] Sato Y, Tanaka K, Lee G, et al. Enhanced and specific gene expression via tissue-specific production of Cre recombinase using adenovirus vector. *Biochem Biophys Res Commun*. 1998;244(2):455–462.

[114] Harris JD, Gutierrez AA, Hurst HC, Sikora K, Lemoine NR. Gene therapy for cancer using tumour-specific prodrug activation. *Gene Ther*. 1994;1(3):170–175.

[115] Sikora K, Harris J, Hurst H, Lemoine N. Therapeutic strategies using c-erbB-2 promoter-controlled

drug activation. *Ann N Y Acad Sci*. 1994;716:115–124; discussion 124–125, 140–143.

[116] Stackhouse MA, Buchsbaum DJ, Kancharla SR, et al. Specific membrane receptor gene expression targeted with radiolabeled peptide employing the erbB-2 and DF3 promoter elements in adenoviral vectors. *Cancer Gene Ther*. 1999;6(3):209–219.

[117] Maeda T, O-Wang J, Matsubara H, et al. A minimum c-erbB-2 promoter-mediated expression of herpes simplex virus thymidine kinase gene confers selective cytotoxicity of human breast cancer cells to ganciclovir. *Cancer Gene Ther*. 2001;8(11):890–896.

[118] Manome Y, Abe M, Hagen MF, Fine HA, Kufe DW. Enhancer sequences of the DF3 gene regulate expression of the herpes simplex virus thymidine kinase gene and confer sensitivity of human breast cancer cells to ganciclovir. *Cancer Res*. 1994;54(20):5408–5413.

[119] Chen L, Chen D, Manome Y, Dong Y, Fine HA, Kufe DW. Breast cancer selective gene expression and therapy mediated by recombinant adenoviruses containing the DF3/MUC1 promoter. *J Clin Invest*. 1995;96(6):2775–2782.

[120] Chen L, Pulsipher M, Chen D, et al. Selective transgene expression for detection and elimination of contaminating carcinoma cells in hematopoietic stem cell sources. *J Clin Invest*. 1996;98(11):2539–2548.

[121] Tai YT, Strobel T, Kufe D, Cannistra SA. In vivo cytotoxicity of ovarian cancer cells through tumor-selective expression of the BAX gene. *Cancer Res*. 1999;59(9):2121–2126.

[122] Koeneman KS, Kao C, Ko SC, et al. Osteocalcin-directed gene therapy for prostate-cancer bone metastasis. *World J Urol*. 2000;18(2):102–110.

[123] Matsubara S, Wada Y, Gardner TA, et al. A conditional replication-competent adenoviral vector, Ad-OC-E1a, to cotarget prostate cancer and bone stroma in an experimental model of androgen-independent prostate cancer bone metastasis. *Cancer Res*. 2001;61(16):6012–6019.

[124] Benjamin R, Helman L, Meyers P, Reaman G. A phase I/II dose escalation and activity study of intravenous injections of OCaP1 for subjects with refractory osteosarcoma metastatic to lung. *Hum Gene Ther*. 2001;12(12):1591–1593.

[125] Shirakawa T, Ko SC, Gardner TA, et al. In vivo suppression of osteosarcoma pulmonary metastasis with intravenous osteocalcin promoter-based toxic gene therapy. *Cancer Gene Ther*. 1998;5(5):274–280 .

[126] Yeung F, Law WK, Yeh CH, et al. Regulation of human osteocalcin promoter in hormone-independent human prostate cancer cells. *J Biol Chem*. 2002;277(4):2468–2476.

[127] Chung I, Schwartz PE, Crystal RG, Pizzorno G, Leavitt J, Deisseroth AB. Use of L-plastin promoter to develop an adenoviral system that confers transgene expression in ovarian cancer cells but not in normal mesothelial cells. *Cancer Gene Ther*. 1999;6(2):99–106.

[128] Peng XY, Won JH, Rutherford T, et al. The use of the L-plastin promoter for adenoviral-mediated, tumor-specific gene expression in ovarian and bladder cancer cell lines. *Cancer Res*. 2001; 61(11):4405–4413.

[129] Miyauchi M, Shimada H, Kadomatsu K, et al. Frequent expression of midkine gene in esophageal cancer suggests a potential usage of its promoter for suicide gene therapy. *Jpn J Cancer Res*. 1999;90(4):469–475.

[130] Adachi Y, Reynolds PN, Yamamoto M, et al. Midkine promoter-based adenoviral vector gene delivery for pediatric solid tumors. *Cancer Res*. 2000;60(16):4305–4310.

[131] Adachi Y, Matsubara S, Muramatsu T, Curiel DT, Reynolds PN. Midkine promoter-based adenoviral suicide gene therapy to midkine-positive pediatric tumor. *J Pediatr Surg*. 2002;37(4):588–592.

[132] Casado E, Gomez-Navarro J, Yamamoto M, et al. Strategies to accomplish targeted expression of transgenes in ovarian cancer for molecular therapeutic applications. *Clin Cancer Res*. 2001;7(8):2496–2504.

[133] Wesseling JG, Yamamoto M, Adachi Y, et al. Midkine and cyclooxygenase-2 promoters are promising for adenoviral vector gene delivery of pancreatic carcinoma. *Cancer Gene Ther*. 2001;8(12):990–996.

[134] Majumdar AS, Hughes DE, Lichtsteiner SP, Wang Z, Lebkowski JS, Vasserot AP. The telomerase reverse transcriptase promoter drives efficacious tumor suicide gene therapy while preventing hepatotoxicity encountered with constitutive promoters. *Gene Ther*. 2001;8(7):568–578.

[135] Gu J, Andreeff M, Roth JA, Fang B. hTERT promoter induces tumor-specific Bax gene expression and cell killing in syngenic mouse tumor model and prevents systemic toxicity. *Gene Ther*. 2002;9(1):30–37.

[136] Komata T, Kanzawa T, Kondo Y, Kondo S. Telomerase as a therapeutic target for malignant gliomas. *Oncogene*. 2002;21(4):656–663.

[137] Kyo S, Takakura M, Inoue M. Telomerase activity in cancer as a diagnostic and therapeutic target. *Histol Histopathol*. 2000;15(3):813–824.

[138] Lan KH, Kanai F, Shiratori Y, et al. In vivo selective gene expression and therapy mediated by adenoviral vectors for human carcinoembryonic antigen-producing gastric carcinoma. *Cancer Res*. 1997;57(19):4279–4284.

[139] Cao GW, Qi ZT, Pan X, et al. Gene therapy for human colorectal carcinoma using human CEA promoter contro led bacterial ADP-ribosylating toxin genes human CEA: PEA & DTA gene transfer. *World J Gastroenterol*. 1998;4(5):388–391.

[140] Nishino K, Osaki T, Kumagai T, et al. Adenovirus-mediated gene therapy specific for small cell lung cancer cells using a Myc-Max binding motif. *Int J Cancer*. 2001;91(6):851–856.

[141] Humphreys MJ, Ghaneh P, Greenhalf W, et al. Hepatic intra-arterial delivery of a retroviral vector expressing the cytosine deaminase gene, controlled by the CEA promoter and intraperitoneal treatment with 5-fluorocytosine suppresses growth of colorectal liver metastases. *Gene Ther*. 2001;8(16):1241–1247.

[142] Tanaka S, Iwai M, Harada Y, et al. Targeted killing of carcinoembryonic antigen (CEA)-producing cholangiocarcinoma cells by polyamidoamine dendrimer-mediated transfer of an Epstein-Barr virus (EBV)-based plasmid vector carrying the CEA promoter. *Cancer Gene Ther*. 2000;7(9):1241–1250.

[143] Nyati MK, Sreekumar A, Li S, et al. High and selective expression of yeast cytosine deaminase under a carcinoembryonic antigen promoter-enhancer. *Cancer Res*. 2002;62(8):2337–2342.

[144] Tanaka T, Kanai F, Lan KH, et al. Adenovirus-mediated gene therapy of gastric carcinoma using cancer-specific gene expression in vivo. *Biochem Biophys Res Commun*. 1997;231(3):775–779.

[145] Ido A, Uto H, Moriuchi A, et al. Gene therapy targeting for hepatocellular carcinoma: selective and enhanced suicide gene expression regulated by a hypoxia-inducible enhancer linked to a human alpha-fetoprotein promoter. *Cancer Res*. 2001;61(7):3016–3021.

[146] Maxwell IH, Spitzer AL, Long CJ, Maxwell F. Autonomous parvovirus transduction of a gene under control of tissue-specific or inducible promoters. *Gene Ther*. 1996;3(1):28–36.

[147] Wills KN, Huang WM, Harris MP, Machemer T, Maneval DC, Gregory RJ. Gene therapy for hepatocellular carcinoma: chemosensitivity conferred by adenovirus-mediated transfer of the HSV-1 thymidine kinase gene. *Cancer Gene Ther*. 1995;2(3):191–197.

[148] Kaneko S, Hallenbeck P, Kotani T, et al. Adenovirus-mediated gene therapy of hepatocellular carcinoma using cancer-specific gene expression. *Cancer Res*. 1995;55(22):5283–5287.

[149] Ido A, Nakata K, Kato Y, et al. Gene therapy for hepatoma cells using a retrovirus vector carrying herpes simplex virus thymidine kinase gene under the control of human alpha-fetoprotein gene promoter. *Cancer Res*. 1995;55(14):3105–3109.

[150] Hernandez-Alcoceba R, Pihalja M, Nunez G, Clarke MF. Evaluation of a new dual-specificity promoter for selective induction of apoptosis in breast cancer cells. *Cancer Gene Ther*. 2001;8(4):298–307.

[151] Takakuwa K, Fujita K, Kikuchi A, et al. Direct intratumoral gene transfer of the herpes simplex virus thymidine kinase gene with DNA-liposome complexes: growth inhibition of tumors and lack of localization in normal tissues. *Jpn J Cancer Res*. 1997;88(2):166–175.

[152] Zheng S, Wang S, Ma L, Sun K. HER2 promoter controlled specific expression of the reporter gene in ovarian cancer cell line [in Chinese]. *Zhonghua Yi Xue Yi Chuan Xue Za Zhi*. 2000;17(5):313–315.

[153] Patterson A, Harris AL. Molecular chemotherapy for breast cancer. *Drugs Aging*. 1999;14(2):75–90.

[154] Zaretsky JZ, Sarid R, Aylon Y, Mittelman LA, Wreschner DH, Keydar I. Analysis of the promoter of the MUC1 gene overexpressed in breast cancer. *FEBS Lett*. 1999;461(3):189–195.

[155] Ko SC, Cheon J, Kao C, et al. Osteocalcin promoter-based toxic gene therapy for the treatment of osteosarcoma in experimental models. *Cancer Res*. 1996;56(20):4614–4619.

[156] Cheon J, Ko SC, Gardner TA, et al. Chemogene therapy: osteocalcin promoter-based suicide gene therapy in combination with methotrexate in a murine osteosarcoma model. *Cancer Gene Ther*. 1997;4(6):359–365.

[157] Robertson MW 3rd, Wang M, Siegal GP, et al. Use of a tissue-specific promoter for targeted expression of the herpes simplex virus thymidine kinase gene in cervical carcinoma cells. *Cancer Gene Ther*. 1998;5(5):331–336.

[158] Garver RI Jr, Goldsmith KT, Rodu B, Hu PC, Sorscher EJ, Curiel DT. Strategy for achieving selective killing of carcinomas. *Gene Ther*. 1994;1(1):46–50.

[159] Anderson LM, Krotz S, Weitzman SA, Thimmapaya B. Breast cancer-specific expression of the Candida albicans cytosine deaminase gene using a transcriptional targeting approach. *Cancer Gene Ther*. 2000;7(6):845–852.

[160] Kumagai T, Tanio Y, Osaki T, et al. Eradication of Myc-overexpressing small cell lung cancer cells transfected with herpes simplex virus thymidine kinase gene containing Myc-Max response elements. *Cancer Res*. 1996;56(2):354–358.

[161] Sugaya S, Fujita K, Kikuchi A, et al. Inhibition of tumor growth by direct intratumoral gene transfer of herpes simplex virus thymidine kinase gene with DNA-liposome complexes. *Hum Gene Ther*. 1996;7(2):223–230.

[162] Lung RW, Lee KA. The cellular oncogene EWS/activating transcription factor 1 is unable to activate adenovirus-borne promoters: implications for cytotoxic prodrug therapy of malignant melanoma of soft parts. *Cancer Gene Ther*. 2000;7(3):396–406.

[163] Yamamoto M, Alemany R, Adachi Y, Grizzle WE, Curiel DT. Characterization of the cyclooxygenase-2 promoter in an adenoviral vector and its application for the mitigation of toxicity in suicide gene therapy of gastrointestinal cancers. *Mol Ther*. 2001;3(3):385–394.

[164] Iyengar RV, Pawlik CA, Krull EJ, et al. Use of a modified ornithine decarboxylase promoter to achieve efficient c-MYC- or N-MYC-regulated protein expression. *Cancer Res*. 2001;61(7):3045–3052.

[165] McLaughlin PM, Harmsen MC, Dokter WH, et al. The epithelial glycoprotein 2 (EGP-2) promoter-driven epithelial-specific expression of EGP-2 in transgenic mice: a new model to study carcinoma-directed immunotherapy. *Cancer Res*. 2001;61(10):4105–4111.

[166] Nawrath M, Pavlovic J, Moelling K. Inhibition of human hematopoietic tumor formation by targeting a repressor Myb-KRAB to DNA. *Cancer Gene Ther*. 2000;7(6):963–972.

[167] Inase N, Horita K, Tanaka M, Miyake S, Ichioka M, Yoshizawa Y. Use of gastrin-releasing peptide promoter for specific expression of thymidine kinase gene in small-cell lung carcinoma cells. *Int J Cancer*. 2000;85(5):716–719.

[168] Morimoto E, Inase N, Mlyake S, Yoshizawa Y. Adenovirus-mediated suicide gene transfer to small cell lung carcinoma using a tumor-specific promoter. *Anticancer Res*. 2001;21(1A):329–331.

[169] Vandier D, Calvez V, Massade L, et al. Transactivation of the metallothionein promoter in cisplatin-resistant cancer cells: a specific gene therapy strategy. *J Natl Cancer Inst*. 2000;92(8):642–647.

[170] Yamamura H, Hashio M, Noguchi M, et al. Identification of the transcriptional regulatory sequences of human calponin promoter and their use in targeting a conditionally replicating herpes vector to malignant human soft tissue and bone tumors. *Cancer Res*. 2001;61(10):3969–3977.

[171] Ohana P, Bibi O, Matouk I, et al. Use of H19 regulatory sequences for targeted gene therapy in cancer. *Int J Cancer*. 2002;98(5):645–650.

[172] Chen RH, McCormick F. Selective targeting to the hyperactive beta-catenin/T-cell factor pathway in colon cancer cells. *Cancer Res*. 2001;61(11):4445–4449.

[173] Lipinski KS, Djeha AH, Krausz E, et al. Tumour-specific therapeutic adenovirus vectors: repression of transgene expression in healthy cells by endogenous p53. *Gene Ther*. 2001;8(4):274–281.

[174] Inase N, Miyake S, Yoshizawa Y. Calretinin promoter for suicide gene expression in malignant mesothelioma. *Anticancer Res*. 2001;21(2A):1111–1114.

[175] Messina M, Yu DM, Learoyd DL, Both GW, Molloy PL, Robinson BG. High level, tissue-specific expression of a modified calcitonin/calcitonin gene-related peptide promoter in a human medullary thyroid carcinoma cell line. *Mol Cell Endocrinol*. 2000;164(1-2):219–224.

[176] Minemura K, Takeda T, Minemura K, et al. Cell-specific induction of sensitivity to ganciclovir in medullary thyroid carcinoma cells by adenovirus-mediated gene transfer of herpes simplex virus thymidine kinase. *Endocrinology*. 2000;141(5):1814–1822.

[177] Yamazaki M, Zhang R, Straus FH, et al. Effective gene therapy for medullary thyroid carcinoma using recombinant adenovirus inducing tumor-specific expression of interleukin-12. *Gene Ther*. 2002;9(1):64–74.

[178] Nettelbeck DM, Zwicker J, Lucibello FC, et al. Cell cycle regulated promoters for the targeting of tumor endothelium. *Adv Exp Med Biol*. 1998;451:437–440.

[179] Nettelbeck DM, Jerome V, Muller R. A dual specificity promoter system combining cell cycle-regulated and tissue-specific transcriptional control. *Gene Ther*. 1999;6(7):1276–1281.

[180] Velasco B, Ramirez JR, Relloso M, et al. Vascular gene transfer driven by endoglin and ICAM-2 endothelial-specific promoters. *Gene Ther*. 2001;8(12):897–904.

[181] Werner H, Shalita-Chesner M, Abramovitch S, Idelman G, Shaharabani-Gargir L, Glaser T. Regulation of the insulin-like growth factor-I receptor gene by oncogenes and antioncogenes: implications in human cancer. *Mol Genet Metab*. 2000;71(1-2):315–320.

[182] Parr MJ, Manome Y, Tanaka T, et al. Tumor-selective transgene expression in vivo mediated by an E2F-responsive adenoviral vector. *Nat Med*. 1997;3(10):1145–1149.

[183] Hockel M, Vaupel P. Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. *J Natl Cancer Inst*. 2001;93(4):266–276.

[184] Jaggar RT, Chan HY, Harris AL, Bicknell R. Endothelial cell-specific expression of tumor necrosis factor-alpha from the KDR or E-selectin promoters following retroviral delivery. *Hum Gene Ther*. 1997;8(18):2239–2247.

[185] Szary J, Kalita K, Przybyszewska M, et al. KDR promoter can transcriptionally target cytosine deaminase suicide gene to cancer cells of nonendothelial origin. *Anticancer Res*. 2001;21(5):3471–3475.

[186] Modlich U, Pugh CW, Bicknell R. Increasing endothelial cell specific expression by the use of heterologous hypoxic and cytokine-inducible enhancers. *Gene Ther*. 2000;7(10):896–902.

[187] Heidenreich R, Kappel A, Breier G. Tumor endothelium-specific transgene expression directed by vascular endothelial growth factor receptor-2 (Flk-1) promoter/enhancer sequences. *Cancer Res*. 2000;60(21):6142–6147.

[188] Bauerschmitz GJ, Nettelbeck DM, Kanerva A, et al. The flt-1 promoter for transcriptional targeting of teratocarcinoma. *Cancer Res*. 2002;62(5):1271–1274.

[189] Walton T, Wang JL, Ribas A, Barsky SH, Economou J, Nguyen M. Endothelium-specific expression of

an E-selectin promoter recombinant adenoviral vector. *Anticancer Res*. 1998;18(3A):1357–1360.

[190] Ozaki K, Yoshida T, Ide H, et al. Use of von Willebrand factor promoter to transduce suicidal gene to human endothelial cells, HUVEC. *Hum Gene Ther*. 1996;7(13):1483–1490.

[191] Jager U, Zhao Y, Porter CD. Endothelial cell-specific transcriptional targeting from a hybrid long terminal repeat retrovirus vector containing human prepro-endothelin-1 promoter sequences. *J Virol*. 1999;73(12):9702–9709.

[192] Mavria G, Jager U, Porter CD. Generation of a high titre retroviral vector for endothelial cell-specific gene expression in vivo. *Gene Ther*. 2000;7(5):368–376.

[193] Varda-Bloom N, Shaish A, Gonen A, et al. Tissue-specific gene therapy directed to tumor angiogenesis. *Gene Ther*. 2001;8(11):819–827.

[194] Shibata T, Akiyama N, Noda M, Sasai K, Hiraoka M. Enhancement of gene expression under hypoxic conditions using fragments of the human vascular endothelial growth factor and the erythropoietin genes. *Int J Radiat Oncol Biol Phys*. 1998;42(4):913–916.

[195] Shibata T, Giaccia AJ, Brown JM. Development of a hypoxia-responsive vector for tumor-specific gene therapy. *Gene Ther*. 2000;7(6):493–498.

[196] Shibata T, Giaccia AJ, Brown JM. Hypoxia-inducible regulation of a prodrug-activating enzyme for tumor-specific gene therapy. *Neoplasia*. 2002;4(1):40–48.

[197] Koshikawa N, Takenaga K, Tagawa M, Sakiyama S. Therapeutic efficacy of the suicide gene driven by the promoter of vascular endothelial growth factor gene against hypoxic tumor cells. *Cancer Res*. 2000;60(11):2936–2941.

[198] Ruan H, Su H, Hu L, Lamborn KR, Kan YW, Deen DF. A hypoxia-regulated adeno-associated virus vector for cancer-specific gene therapy. *Neoplasia*. 2001;3(3):255–263.

[199] Dachs GU, Patterson AV, Firth JD, et al. Targeting gene expression to hypoxic tumor cells. *Nat Med*. 1997;3(5):515–520.

[200] Little E, Ramakrishnan M, Roy B, Gazit G, Lee AS. The glucose-regulated proteins (GRP78 and GRP94): functions, gene regulation, and applications. *Crit Rev Eukaryot Gene Expr*. 1994;4(1):1–18.

[201] Gazit G, Kane SE, Nichols P, Lee AS. Use of the stress-inducible grp78/BiP promoter in targeting high level gene expression in fibrosarcoma in vivo. *Cancer Res*. 1995;55(8):1660–1663.

[202] Gazit G, Hung G, Chen X, Anderson WF, Lee AS. Use of the glucose starvation-inducible glucose-regulated protein 78 promoter in suicide gene therapy of murine fibrosarcoma. *Cancer Res*. 1999;59(13):3100–3106.

[203] Chen X, Zhang D, Dennert G, Hung G, Lee AS. Eradication of murine mammary adenocarcinoma through HSVtk expression directed by the glucose-starvation inducible grp78 promoter. *Breast Cancer Res Treat*. 2000;59(1):81–90.

[204] Luna MC, Chen X, Wong S, et al. Enhanced photodynamic therapy efficacy with inducible suicide gene therapy controlled by the grp promoter. *Cancer Res*. 2002;62(5):1458–1461.

[205] Lee AS. The glucose-regulated proteins: stress induction and clinical applications. *Trends Biochem Sci*. 2001;26(8):504–510.

[206] Katabi MM, Chan HL, Karp SE, Batist G. Hexokinase type II: a novel tumor-specific promoter for gene-targeted therapy differentially expressed and regulated in human cancer cells. *Hum Gene Ther*. 1999;10(2):155–164.

[207] Harats D, Kurihara H, Belloni P, et al. Targeting gene expression to the vascular wall in transgenic mice using the murine preproendothelin-1 promoter. *J Clin Invest*. 1995;95(3):1335–1344.

[208] Nettelbeck DM, Jerome V, Muller R. A strategy for enhancing the transcriptional activity of weak cell type-specific promoters. *Gene Ther*. 1998;5(12):1656–1664.

[209] Marples B, Greco O, Joiner MC, Scott SD. Molecular approaches to chemo-radiotherapy. *Eur J Cancer*. 2002;38(2):231–239.

[210] Kawashita Y, Ohtsuru A, Kaneda Y, et al. Regression of hepatocellular carcinoma in vitro and in vivo by radiosensitizing suicide gene therapy under the inducible and spatial control of radiation. *Hum Gene Ther*. 1999;10(9):1509–1519.

[211] Manome Y, Kunieda T, Wen PY, Koga T, Kufe DW, Ohno T. Transgene expression in malignant glioma using a replication-defective adenoviral vector containing the Egr-1 promoter: activation by ionizing radiation or uptake of radioactive iododeoxyuridine. *Hum Gene Ther*. 1998;9(10):1409–1417.

[212] Seung LP, Mauceri HJ, Beckett MA, Hallahan DE, Hellman S, Weichselbaum RR. Genetic radiotherapy overcomes tumor resistance to cytotoxic agents. *Cancer Res*. 1995;55(23):5561–5565.

[213] Weichselbaum RR, Hallahan DE, Beckett MA, et al. Gene therapy targeted by radiation preferentially radiosensitizes tumor cells. *Cancer Res*. 1994;54(16):4266–4269.

[214] Mauceri HJ, Hanna NN, Wayne JD, Hallahan DE, Hellman S, Weichselbaum RR. Tumor necrosis factor alpha (TNF-alpha) gene therapy targeted by ionizing radiation selectively damages tumor vasculature. *Cancer Res*. 1996;56(19):4311–4314.

[215] Mauceri HJ, Seung LP, Grdina WL, Swedberg KA, Weichselbaum RR. Increased injection number enhances adenoviral genetic radiotherapy. *Radiat Oncol Investig*. 1997;5(5):220–226.

[216] Hallahan DE, Mauceri HJ, Seung LP, et al. Spatial and temporal control of gene therapy using ionizing radiation. *Nat Med*. 1995;1(8):786–791.

[217] Hallahan DE, Qu S, Geng L, et al. Radiation-mediated control of drug delivery. *Am J Clin Oncol*. 2001;24(5):473–480.
[218] Weichselbaum RR, Kufe DW, Advani SJ, Roizman B. Molecular targeting of gene therapy and radiotherapy. *Acta Oncol*. 2001;40(6):735–738.
[219] Scott SD, Marples B, Hendry JH, et al. A radiation-controlled molecular switch for use in gene therapy of cancer. *Gene Ther*. 2000;7(13):1121–1125.
[220] Hanna NN, Hallahan DE, Wayne JD, Weischselbaum RR. Modification of the radiation response by the administration of exogenous genes. *Semin Radiat Oncol*. 1996;6(4):321–328.
[221] Kaminski JM, Kaminski RJ, Dicker AP, Urbain JL. Defining a future role for radiogenic therapy. *Cancer Treat Rev*. 2001;27(5):289–294.
[222] Worthington J, Robson T, Murray M, O'Rourke M, Keilty G, Hirst DG. Modification of vascular tone using iNOS under the control of a radiation-inducible promoter. *Gene Ther*. 2000;7(13):1126–1131.
[223] Worthington J, Robson T, O'Keeffe M, Hirst DG. Tumour cell radiosensitization using constitutive (CMV) and radiation inducible (WAF1) promoters to drive the iNOS gene: a novel suicide gene therapy. *Gene Ther*. 2002;9(4):263–269.
[224] Nuyts S, Van Mellaert L, Theys J, et al. Radio-responsive recA promoter significantly increases TNFalpha production in recombinant clostridia after 2 Gy irradiation. *Gene Ther*. 2001;8(15):1197–1201.
[225] Nuyts S, Van Mellaert L, Theys J, Landuyt W, Lambin P, Anne J. The use of radiation-induced bacterial promoters in anaerobic conditions: a means to control gene expression in clostridium-mediated therapy for cancer. *Radiat Res*. 2001;155(5):716–723.
[226] Ueda T, Akiyama N, Sai H, et al. c-IAP2 is induced by ionizing radiation through NF-kappaB binding sites. *FEBS Lett*. 2001;491(1-2):40–44.
[227] Madio DP, van Gelderen P, DesPres D, et al. On the feasibility of MRI-guided focused ultrasound for local induction of gene expression. *J Magn Reson Imaging*. 1998;8(1):101–104.
[228] Braiden V, Ohtsuru A, Kawashita Y, et al. Eradication of breast cancer xenografts by hyperthermic suicide gene therapy under the control of the heat shock protein promoter. *Hum Gene Ther*. 2000;11(18):2453–2463.
[229] Lee YJ, Galoforo SS, Battle P, Lee H, Corry PM, Jessup JM. Replicating adenoviral vector-mediated transfer of a heat-inducible double suicide gene for gene therapy. *Cancer Gene Ther*. 2001;8(6):397–404.
[230] Lohr F, Hu K, Huang Q, et al. Enhancement of radiotherapy by hyperthermia-regulated gene therapy. *Int J Radiat Oncol Biol Phys*. 2000;48(5):1513–1518.
[231] Lohr F, Huang Q, Hu K, Dewhirst MW, Li CY. Systemic vector leakage and transgene expression by intratumorally injected recombinant adenovirus vectors. *Clin Cancer Res*. 2001;7(11):3625–3628.
[232] Brade AM, Ngo D, Szmitko P, Li PX, Liu FF, Klamut HJ. Heat-directed gene targeting of adenoviral vectors to tumor cells. *Cancer Gene Ther*. 2000;7(12):1566–1574.
[233] Huang Q, Hu JK, Lohr F, et al. Heat-induced gene expression as a novel targeted cancer gene therapy strategy. *Cancer Res*. 2000;60(13):3435–3439.
[234] Vekris A, Maurange C, Moonen C, et al. Control of transgene expression using local hyperthermia in combination with a heat-sensitive promoter. *J Gene Med*. 2000;2(2):89–96.
[235] Ito A, Shinkai M, Honda H, Kobayashi T. Heat-inducible TNF-alpha gene therapy combined with hyperthermia using magnetic nanoparticles as a novel tumor-targeted therapy. *Cancer Gene Ther*. 2001;8(9):649–654.
[236] Walther W, Stein U, Fichtner I, Alexander M, Shoemaker RH, Schlag PM. Mdr1 promoter-driven tumor necrosis factor-alpha expression for a chemotherapy-controllable combined in vivo gene therapy and chemotherapy of tumors. *Cancer Gene Ther*. 2000;7(6):893–900.
[237] Walther W, Stein U, Schlag PM. Use of the human MDR1 promoter for heat-inducible expression of therapeutic genes. *Int J Cancer*. 2002;98(2):291–296.
[238] Walther W, Stein U, Fichtner I, et al. In vivo evaluation of a drug-inducible vector system for the combined gene- and chemotherapy of cancer. *Adv Exp Med Biol*. 1998;451:139–144.
[239] Patil R, Chavez JB, Yee D. Inducible expression of herpes simplex virus thymidine kinase increases sensitivity to ganciclovir but does not enhance bystander effect in breast cancer cells. *Breast Cancer Res Treat*. 2000;62(2):109–115.
[240] Pitzer C, Schindowski K, Pomer S, Wirth T, Zoller M. In vivo manipulation of interleukin-2 expression by a retroviral tetracycline (tet)-regulated system. *Cancer Gene Ther*. 1999;6(2):139–146.
[241] Yu JS, Sena-Esteves M, Paulus W, Breakefield XO, Reeves SA. Retroviral delivery and tetracycline-dependent expression of IL-1beta-converting enzyme (ICE) in a rat glioma model provides controlled induction of apoptotic death in tumor cells. *Cancer Res*. 1996;56(23):5423–5427.
[242] Rubinchik S, Wang D, Yu H, et al. A complex adenovirus vector that delivers FASL-GFP with combined prostate-specific and tetracycline-regulated expression. *Mol Ther*. 2001;4(5):416–426.
[243] Iida A, Chen ST, Friedmann T, Yee JK. Inducible gene expression by retrovirus-mediated transfer of a modified tetracycline-regulated system. *J Virol*. 1996;70(9):6054–6059.

[244] Baasner S, von Melchner H, Klenner T, Hilgard P, Beckers T. Reversible tumorigenesis in mice by conditional expression of the HER2/c-erbB2 receptor tyrosine kinase. *Oncogene*. 1996;13(5):901–911.

[245] Paulus W, Baur I, Oberer DM, Breakefield XO, Reeves SA. Regulated expression of the diphtheria toxin A gene in human glioma cells using prokaryotic transcriptional control elements. *J Neurosurg*. 1997;87(1):89–95.

[246] Yao F, Svensjo T, Winkler T, Lu M, Eriksson C, Eriksson E. Tetracycline repressor, tetR, rather than the tetR-mammalian cell transcription factor fusion derivatives, regulates inducible gene expression in mammalian cells. *Hum Gene Ther*. 1998;9(13):1939–1950.

[247] Giavazzi R, Giuliani R, Coltrini D, et al. Modulation of tumor angiogenesis by conditional expression of fibroblast growth factor-2 affects early but not established tumors. *Cancer Res*. 2001;61(1):309–317.

[248] Chong H, Ruchatz A, Clackson T, Rivera VM, Vile RG. A system for small-molecule control of conditionally replication-competent adenoviral vectors. *Mol Ther*. 2002;5(2):195–203.

[249] Hernandez-Alcoceba R, Pihalja M, Wicha MS, Clarke MF. A novel, conditionally replicative adenovirus for the treatment of breast cancer that allows controlled replication of E1a-deleted adenoviral vectors. *Hum Gene Ther*. 2000;11(14):2009–2024.

[250] Tannour-Louet M, Porteu A, Vaulont S, Kahn A, Vasseur-Cognet M. A tamoxifen-inducible chimeric Cre recombinase specifically effective in the fetal and adult mouse liver. *Hepatology*. 2002; 35(5):1072–1081.

[251] Datta R, Taneja N, Sukhatme VP, Qureshi SA, Weichselbaum R, Kufe DW. Reactive oxygen intermediates target CC(A/T)6GG sequences to mediate activation of the early growth response 1 transcription factor gene by ionizing radiation. *Proc Natl Acad Sci USA*. 1993;90(6):2419–2422.

[252] Joki T, Nakamura M, Ohno T. Activation of the radiosensitive EGR-1 promoter induces expression of the herpes simplex virus thymidine kinase gene and sensitivity of human glioma cells to ganciclovir. *Hum Gene Ther*. 1995;6(12):1507–1513.

[253] Marples B, Scott SD, Hendry JH, Embleton MJ, Lashford LS, Margison GP. Development of synthetic promoters for radiation-mediated gene therapy. *Gene Ther*. 2000;7(6):511–517.

[254] Yook JI, Kim J. Expression of p21WAF1/CIP1 is unrelated to p53 tumour suppressor gene status in oral squamous cell carcinomas. *Oral Oncol*. 1998;34(3):198–203.

[255] Codegoni AM, Nicoletti MI, Buraggi G, et al. Molecular characterisation of a panel of human ovarian carcinoma xenografts. *Eur J Cancer*. 1998;34(9):1432–1438.

[256] Clasen S, Schulz WA, Gerharz CD, Grimm MO, Christoph F, Schmitz-Drager BJ. Frequent and heterogeneous expression of cyclin-dependent kinase inhibitor WAF1/p21 protein and mRNA in urothelial carcinoma. *Br J Cancer*. 1998;77(4):515–521.

[257] Sourvinos G, Spandidos DA. Decreased BRCA1 expression levels may arrest the cell cycle through activation of p53 checkpoint in human sporadic breast tumors. *Biochem Biophys Res Commun*. 1998;245(1):75–80.

[258] Wasylyk C, Salvi R, Argentini M, et al. p53 mediated death of cells overexpressing MDM2 by an inhibitor of MDM2 interaction with p53. *Oncogene*. 1999;18(11):1921–1934.

[259] Schwerer MJ, Sailer A, Kraft K, Baczako K, Maier H. Patterns of p21(waf1/cip1) expression in non-papillomatous nasal mucosa, endophytic sinonasal papillomas, and associated carcinomas. *J Clin Pathol*. 2001;54(11):871–876.

[260] Biankin AV, Kench JG, Morey AL, et al. Overexpression of p21(WAF1/CIP1) is an early event in the development of pancreatic intraepithelial neoplasia. *Cancer Res*. 2001;61(24):8830–8837.

[261] O'Hanlon DM, Kiely M, MacConmara M, et al. An immunohistochemical study of p21 and p53 expression in primary node-positive breast carcinoma. *Eur J Surg Oncol*. 2002;28(2):103–107.

[262] Vidal MJ, Loganzo F Jr, de Oliveira AR, Hayward NK, Albino AP. Mutations and defective expression of the WAF1 p21 tumour-suppressor gene in malignant melanomas. *Melanoma Res*. 1995;5(4):243–250.

[263] Karjalainen JM, Eskelinen MJ, Kellokoski JK, Reinikainen M, Alhava EM, Kosma VM. p21(WAF1/CIP1) expression in stage I cutaneous malignant melanoma: its relationship with p53, cell proliferation and survival. *Br J Cancer*. 1999;79(5-6):895–902.

[264] Patterson LH, McKeown SR, Robson T, Gallagher R, Raleigh SM, Orr S. Antitumour prodrug development using cytochrome P450 (CYP) mediated activation. *Anticancer Drug Des*. 1999;14(6):473–486.

[265] McCarthy HO, Yakkundi A, McErlane V, et al. Bioreductive GDEPT using cytochrome P450 3A4 in combination with AQ4N. *Cancer Gene Ther*. 2003;10(1):40–48.

[266] Nuyts S, Van Mellaert L, Theys J, Landuyt W, Lambin P, Anne J. Clostridium spores for tumor-specific drug delivery. *Anticancer Drugs*. 2002;13(2):115–125.

[267] Nielsen OS, Horsman M, Overgaard J. A future for hyperthermia in cancer treatment? *Eur J Cancer*. 2001;37(13):1587–1589.

[268] Arai Y, Kubo T, Kobayashi K, et al. Control of delivered gene expression in chondrocytes using heat shock protein 70B promoter. *J Rheumatol*. 1999;26(8):1769–1774.

[269] Lin H, Blank M, Rossol-Haseroth K, Goodman R. Regulating genes with electromagnetic response elements. *J Cell Biochem*. 2001;81(1):143–148.
[270] Stein U, Walther W, Shoemaker RH. Vincristine induction of mutant and wild-type human multidrug-resistance promoters is cell-type-specific and dose-dependent. *J Cancer Res Clin Oncol*. 1996;122(5):275–282.
[271] Walther W, Wendt J, Stein U. Employment of the mdr1 promoter for the chemotherapy-inducible expression of therapeutic genes in cancer gene therapy. *Gene Ther*. 1997;4(6):544–552.
[272] Zhang L, Adams JY, Billick E, et al. Molecular engineering of a two-step transcription amplification (TSTA) system for transgene delivery in prostate cancer. *Mol Ther*. 2002;5(3):223–232.
[273] Lee EJ, Jameson JL. Cell-specific Cre-mediated activation of the diphtheria toxin gene in pituitary tumor cells: potential for cytotoxic gene therapy. *Hum Gene Ther*. 2002;13(4):533–542.
[274] Yoshimura I, Suzuki S, Hayakawa M. Application of Cre-loxP system to the urinary tract and cancer gene therapy. *Mol Urol*. 2001;5(2):81–84.
[275] Iyer M, Wu L, Carey M, Wang Y, Smallwood A, Gambhir SS. Two-step transcriptional amplification as a method for imaging reporter gene expression using weak promoters. *Proc Natl Acad Sci USA*. 2001;98(25):14595–14600.
[276] Jounaidi Y, Waxman DJ. Combination of the bioreductive drug tirapazamine with the chemotherapeutic prodrug cyclophosphamide for P450/P450-reductase-based cancer gene therapy. *Cancer Res*. 2000;60(14):3761–3769.
[277] Putzer BM, Stiewe T, Crespo F, Esche H. Improved safety through tamoxifen-regulated induction of cytotoxic genes delivered by Ad vectors for cancer gene therapy. *Gene Ther*. 2000;7(15):1317–1325.

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REVIEW ARTICLE

Gene Therapy for Cardiovascular Disease

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The last decade has seen substantial advances in the development of gene therapy strategies and vector technology for the treatment of a diverse number of diseases, with a view to translating the successes observed in animal models into the clinic. Perhaps the overwhelming drive for the increase in vascular gene transfer studies is the current lack of successful long-term pharmacological treatments for complex cardiovascular diseases. The increase in cardiovascular disease to epidemic proportions has also led many to conclude that drug therapy may have reached a plateau in its efficacy and that gene therapy may represent a realistic solution to a long-term problem. Here, we discuss gene delivery approaches and target diseases.

INTRODUCTION

Gene transfer enables the overexpression of candidate therapeutic genes either locally or systemically. Cardiovascular disease targets under investigation include therapeutic angiogenesis in ischaemic myocardium and limb muscles, treatment of hypertension, vascular bypass graft occlusion, and prevention of postangioplasty restenosis (Table 1). Cardiovascular diseases are diverse and as such have unique traits requiring precise tailoring of gene therapy strategies to a particular disease. Those features which may vary include mode of delivery, type of vector, length of gene expression, and target tissue. Unlike other inherited genetic defects which may require more long-term gene transfer, transient, nonintegrative gene expression has been shown to be sufficient to promote neovascularization in the case of angiogenesis [1]. This may also apply to antiproliferative strategies for the prevention of neointima formation postangioplasty, for the prevention of instent restenosis, or for gene therapy of coronary artery bypass graft failure [2, 3]. However, complex diseases with substantial polygenic influences such as essential hypertension will require sustained gene overexpression.

STRATEGIES FOR GENE DELIVERY

The breadth of applications for vascular gene therapy is accompanied by a need to deliver the therapeutic gene(s) to diverse vascular cell types including vascular smooth muscle (SMC), endothelium, myocardium, or tissues that influence lipid metabolism. Vector systems and gene delivery technologies must therefore be developed for individual applications. The four main modes of introducing therapeutic genes into the vasculature are *ex vivo* gene transfer to vessel segments and gene delivery using cell-based, systemic, and local delivery approaches.

Ex vivo gene therapy

Where appropriate, *ex vivo* genetic modification of vascular tissue is preferred as this allows the delivery of therapeutic genes to the target tissue in a safe and efficient manner. This is the method of choice for gene therapy of vein graft failure. During coronary artery bypass surgery (CABG), there is direct access to the vein in the clinical setting, enabling incubation and subsequent transduction with the vector before grafting. In this way, systemic release of the vector is avoided therefore decreasing potential deleterious transgene expression at distal sites. Additionally, the immune system response to the virus is diminished, since at the time of grafting all excess virus has been removed.

Cell-based genetic modification

Cell-based gene therapy involves the harvesting of cells from patients, *ex vivo* transduction to express therapeutic genes, and subsequent reimplantation of genetically-modified autologous cells. There have been very few studies of this kind. Perhaps the best example of this approach is the *ex vivo* approach used by Grossman et al [4] to treat familial hypercholesterolaemia. They transduced hepatocytes with retroviruses expressing a wild-type copy of low density lipoprotein receptor (LDL-R). Upon reimplantation, a reduction in the total cholesterol level from 671 to 608 was observed for one year [4]. Despite the partial success of this approach, there has been little enthusiasm for this cell-based strategy. In a different approach, injured rat carotid arteries were seeded with

Table 1. Gene therapy strategies for the treatment of cardiovascular diseases.

Disease	Therapeutic approach	Target
Atherosclerosis	Antiatherogenic	LCAT, apoAI, apoE mRNA
Vein graft failure, ischaemia, thrombosis	Diffusable/secreted gene products	VEGF, FGF, eNOS, antithrombotic agents, SOD, heme oxygenase
Vein graft failure	Inhibitors of smooth muscle cell migration/proliferation	p53, TIMPs, Rb, p21
Thrombosis	Prevention of thrombus formation	TFPI, tPA
Restenosis	Suicide genes	Tk
Hypertension	Antisense oligonucleotides	Angiotensinogen, AT_1 receptor, ACE
Vein graft failure	Decoys	Soluble VCAM, E2F
Hypertension	Reduction in blood pressure	Kallikrein, ANP, eNOS, endothelin

SMCs overexpressing the tissue inhibitor of matrix metalloproteinase (TIMP)-1, a significant decrease in neointimal hyperplasia was observed confirming that this technique can be used to exploit a biological effect [5].

Local gene delivery in vivo

As many target vascular tissues are inaccessible by systemic vector administration, such as the ischaemic myocardium or atherosclerotic coronary arteries, local delivery devices have achieved substantial attention and development as means of delivering vectors in a safe, selective, and efficient manner. Catheter design has rapidly evolved since its first attempts at arterial wall gene transfer [6]. Delivery catheters can be used under X-ray fluoroscopy guidance for gene delivery and by means of a contrast medium, only the lumen of the vessel is visible. Development of this process using magnetic resonance imaging (MRI) will provide valuable information on the vessel wall structure and interactions between genes and atherosclerotic lesions [7]. Another example of localised vascular gene transfer is by the use of stents [8]. Stents are commonly used devices that provide a scaffold structure to hold diseased arteries open. This can be exploited as a medium through which to deliver genes. Although stents have provided an effective treatment for stenotic saphenous aortocoronary bypass grafts, procedure-related complications have been observed in 20% of cases [9]. A recent study in a pig model demonstrated stable in vivo transgene expression in the vasculature over a 4-week period from a fibronectin-coated stent platform seeded with green fluorescent protein (GFP) transduced autologous SMC [10]. However, adherence of cells to stent surfaces under flow conditions is particularly poor with certain cell types such as endothelial cells [11]. Stents designed with a distal-embolic protection device in conjunction with a covering of therapeutic genes could be used to prevent adverse coronary events. A wide array of mechanical devices for gene delivery have been investigated for local gene therapy and include double balloon, channel balloon, and hydrogel coated catheters, microporous coated stents, microspheres, and nipple catheters (for review see [12]).

Systemic gene delivery

Following in vivo administration of viral vector systems, liver sequestration predominantly occurs, precluding efficient vascular gene delivery through this route of administration [13, 14]. Due to the efficient uptake of systemically administered vectors by the liver, cardiovascular gene therapeutic strategies are limited for this mode of delivery due to the inadequacies in vector technology. However, the liver can be used to modify lipid metabolism [15] or as a factory through which to flood the bloodstream with soluble therapeutic proteins. Systemic gene therapy has been explored for the treatment of hypercholesterolaemia and hypertension (for review see [16]). As an example, systemic injection of adenovirus-apoE into the tail vein of atherosclerotic $apoE^{-/-}$ mice caused an immediate fall in plasma cholesterol levels and a sustained reduction in atherosclerosis [15].

VECTORS FOR GENE THERAPY

Viral vectors

Successful gene therapy requires the fulfilment of several criteria, namely, the appropriate therapeutic gene and most importantly a suitable vector for delivery and subsequent efficient expression of the transgene. The ideal gene transfer vector should be nonpathogenic, efficiently transduce target cells, and elicit a minimal immunogenic response whilst providing the required time in vivo. At present, no single viral vector fulfils all the criteria for use in cardiovascular gene therapy and the advantages/disadvantages of each are summarised in Table 2. Examples of the therapeutic use of vectors are given in the relevant section describing cardiovascular disease.

The vast majority of gene transfer studies, not just those pertaining to cardiovascular diseases, have focussed

Table 2. Key features of gene therapy viral vectors.

Viral vectors	Integration	Long-term gene expression	Immune response	Comments
Adenovirus	−	−	+	Broad tropism, easy to produce high titre stocks, widely characterised in vivo
Adeno-associated virus (AAV)	+	+	−	Limited cloning capacity, nonpathogenic, integrate randomly in the absence of the *rep* gene
Lentivirus	+	+	−	Retrovirus-derived, pseudotyping with heterologous coat proteins improves biosafety
Retrovirus	+	+	−	Only infects dividing cells

on the use of adenoviruses. Adenovirus (Ad) is by far the most commonly applied and well characterised of the existing viral vectors from an experimental or clinical standpoint. Indeed, of all ongoing gene therapy clinical trials almost 28% can be attributed to Ad-based protocols with less than 8% treating vascular disorders (http://www.co.uk/genmed). Ad-mediated gene therapy is dependent on the level of the coxsackie-adenovirus receptor (CAR) and integrins ($\alpha_v\beta_3$ and $\alpha_v\beta_5$) expressed on the target cell (Figure 1a). In the context of the cardiovascular system, Ad infection levels are relatively low when compared to the hepatic system, however, many successful gene therapy applications have been documented using local delivery to target tissue and clinical trials are ongoing [12]. Ad can be produced to a high titer and will infect both nondividing and dividing cells. However, Ad can result in a marked inflammatory and immunogenic response limiting gene expression, which remains relatively transient but can be overcome with gutless Ad vectors [15].

The last few years have seen an increase in studies with adeno-associated virus (AAV)-based vectors and consequently AAV has emerged as an important vector system for gene therapy. rAAV is a replication-defective virus that is unable to replicate without a helper virus (Figure 1b). It can be stripped of its viral genes to allow a virus coat that can be packaged with a transgene cassette of up to 4.5 kbp. Unlike other viral vectors, AAV has the ability to integrate into the genome therefore allowing long-term expression of the transgene, although in nondividing cells it appears that rAAV vectors can exist as high-molecular weight episomal DNA [17]. wtAAV integrates stably into chromosome 19 [18] however, rAAV vectors do not specifically integrate at this site as rep protein is required to produce this site specificity [19]. This problem can be overcome by inserting a truncated rep protein, which allows the rAAV to restore its ability to integrate at the chromosome 19 site [20]. Single intramuscular injection of AAV-*LacZ* into immunocompetent mice produced detectable expression for more than 1.5 years without any evidence of a cellular immune response [21]. The lack of immunogencity of AAV is an important feature of the vector and gives it a major advantage over adenoviruses (Table 2).

Nonviral vectors

Of the cardiovascular trials running in 2001, 47% used either plasmid DNA or liposome carriers [1]. One approach involves the use of synthetic antisense oligonucleotides, designed to attenuate detrimental proteins [22]. Cis-element double-stranded oligodeoxynucleotides (also known as decoys) have been employed to modulate gene expression through the removal of trans-acting factors from endogenous cis-elements [23]. This is not only a powerful gene therapy strategy but also enables the study of endogenous gene regulation both in vitro and in vivo. For the cardiovascular system, ultrasound can enhance gene therapy of naked plasmid DNA and has been shown to increase biological effects of therapeutic genes such as p53 [24]. Gene transfer of recombinant human VEGF cDNA in a rabbit model of arterial balloon injury led to reendothelialization which was 95% complete within 1 week [25]. In turn, intimal thickening and the occurrence of thrombotic occlusion were diminished with an associated restoration in endothelial cell-dependent vasomotor reactivity. The treatment of peripheral vascular disease has seen the intramuscular injection of plasmid DNA encoding vascular endothelial growth factor (VEGF) to increase collateral vessel development and tissue perfusion in the muscle [26]. Importantly, in a clinical trial using *ex vivo* E2F decoy oligodeoxynucleotide transfection of human vein grafts, Mann et al [42] have shown this strategy to reduce proliferation and hence the occurrence of vein graft occlusion. In addition, no clinical complications were associated with the procedure.

CARDIOVASCULAR DISEASE TARGETS FOR GENE THERAPY

Many diseases affecting the cardiovascular system are amenable to gene therapy protocols. Indeed, success has been achieved experimentally. Here, we briefly review the therapeutic strategies relating to the treatment of ischaemia, late vein graft failure, atherosclerosis, thrombosis, and hypertension.

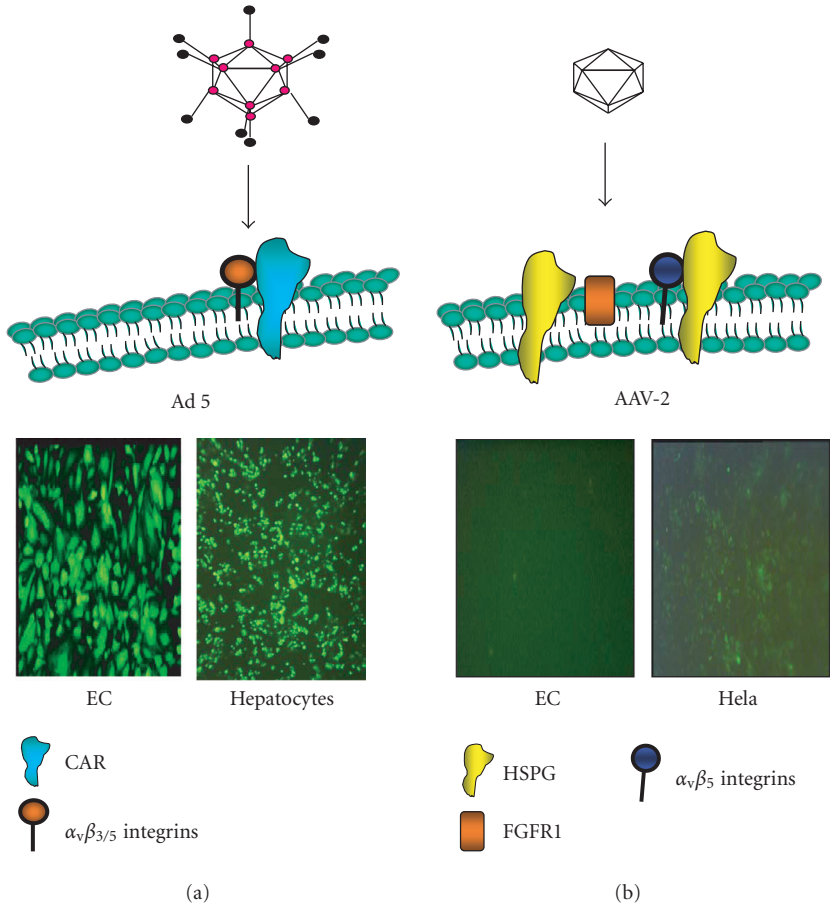

Figure 1. (a) Adenoviral (Ad) attachment and internalisation is mediated through the knob protein of the fiber binding to CAR, followed by interaction of the penton base at the base of the fiber shaft with α_v integrins on the cell surface. Following internalisation, the virus is localised within cellular endosomes which upon acidification allows the virions to escape and traffic to the nucleus. Ad-mediated infection is therefore, dependent on levels of CAR with hepatocytes being highly permissive as shown with reasonable levels of transduction in endothelial cells (EC). (b) AAV2 binds to the primary receptor heparin sulfate proteoglycan (HSPG) on the cell surface and internalization is assisted by the secondary receptors $\alpha_v\beta_5$ integrins and fibroblast growth factor receptor 1. Transduction of vascular cell, in particular EC, is very poor compared with permissive cell types such as HeLa. Transduction of both cell types with rAAV2-eGFP clearly shows the difference in transduction efficiency.

Ischaemia

Peripheral ischaemic diseases are commonly associated with the lower extremities and can be characterized by an impaired blood supply resulting from narrowed or blocked arteries, which subsequently starve tissues of the necessary nutrients and oxygen. Similarly, inadequate blood flow to the heart gives rise to myocardial ischaemia. This may occur if coronary flow is reduced by the presence of an atherosclerotic plaque, a blood clot or an artery spasm. Surgical bypassing and percutaneous revascularization have alleviated many of the symptoms but is not suitable for all patients due to the extension of arterial occlusion and microcirculation impairment. Amputation and heart transplants are the only forms of treatment for ischaemia and therefore gene therapy provides an alternative solution [27, 28]. The two main therapeutic genes under investigation are the angiogenic growth factors (VEGF) and fibroblast growth factor (FGF). Research has focused on delivering these agents to the site of ischaemia (Table 1). VEGF is a heparin binding glycoprotein, which is a principal angiogenic factor for endothelial cells. The delivery of VEGF to target cells lends itself to gene transfer since it is naturally secreted from cells and therefore can achieve its biological effect with a limited number of transfected cells (Figure 2). Gowdak et al [29] demonstrated that intramuscular injection of $AdVEGF_{121}$ resulted in significant lengthening of arterioles and capillaries of nonischaemic limbs in the rat and rabbit. Furthermore, tissue perfusion in animals receiving gene delivery two weeks prior to experimental induction of skeletal muscle ischaemia by removal of the femoral artery was preserved [29]. Clinically, Ad-mediated transfer of VEGF has been demonstrated to improve the endothelial function and to lower the extremity flow reserve in patients with peripheral arterial disease. In this case, $AdVEGF_{121}$ was delivered intramuscularly

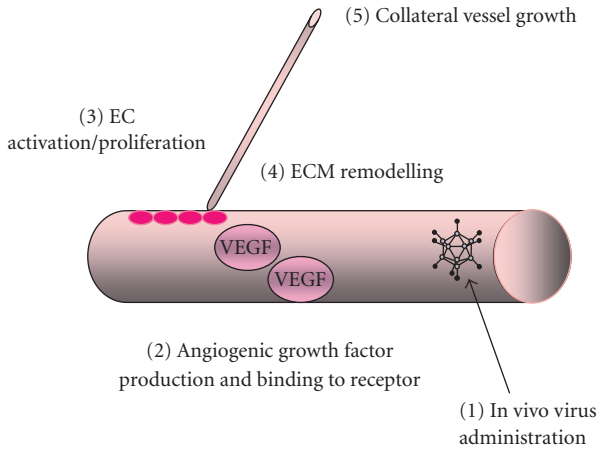

Figure 2. Angiogenesis in the ischaemic myocardium. Diseased or injured tissues produce and release angiogenic growth factors that diffuse into nearby tissues. To boost concentration of these growth factors, such as VEGF, viruses engineered to express one of the 19 existing angiogenic growth factors can be injected into the ischaemic area [1]. The angiogenic growth factors bind to specific receptors located on the endothelial cells (EC) of nearby preexisting blood vessels [2]. Activation of EC by VEGF occurs upon binding to its receptors [3]. Synthesis of new enzymes is triggered. These enzymes dissolve tiny holes in the sheath-like covering (basement membrane) surrounding all existing blood vessels. The endothelial cells proliferate and migrate out through the dissolved holes of the existing vessel [3]. Extracellular matrix (ECM)-degrading enzymes dissolve the tissue in front of the sprouting vessel tip [4]. As the vessel extends, the tissue is remoulded around the vessel and proliferating endothelial cells roll up to form a blood vessel tube [5]. Blood vessel loops are formed from individual blood vessel tubes and these are stabilized by the formation of SMC.

and endothelial function determined 30 days postinjection [30].

In a phase-I clinical trial involving a group of 21 patients given AdVEGF$_{121}$ by direct myocardial injection into the ischaemic region, no adverse effects were detected locally or systemically. Furthermore, angiography suggested an improvement in the area where the vector had been delivered and patients described alleviation in angina symptoms [31]. More recently, the AGENT trial has addressed the safety and anti-ischaemic effects of administering Ad-FGF4 in patients suffering from angina. Single intracoronary infusion of Ad-FGF4 was shown to result in improved exercise times assessed using the exercise treadmill test compared to the placebo group and no adverse side effects [32]. A single intramuscular injection of AAV-VEGF has been shown in the rat ischaemic hindlimb model to produce an increase in capillary growth, a significant increase in mean blood flow of the ischaemic limb, and a higher average skin temperature [33]. An intracardiac injection of AAV-VEGF$_{165}$ has also been shown to induce angiogenesis in the ischaemic myocardium without any evidence of angioma formation [34].

Although VEGF has positive effects on the promotion of angiogenesis, there are pertinent safety considerations. It has been shown that VEGF may enhance atherosclerotic plaque development through an increase in focal macrophage levels [35]. Macrophages then induce those growth factors and cytokines, which mediate intimal hyperplasia and contribute to plaque instability through enhancing levels of matrix metalloproteinases (MMPs) and other hydrolytic enzymes. Other potential risks of therapeutic angiogenesis include the production of nonfunctional, leaky vessels, and stimulation of angiogenesis in tumours [36]. Animal studies in mice highlight the need for regulated expression of VEGF as persistent unregulated VEGF expression following intraventricular injection resulted in the formation of intramural vascular tumours at the site of myoblast implantation [37]. The development of tissue-specific vectors and promoters may help to minimise the risks from these adverse reactions.

Protection from reperfusion injury

Reperfusion of ischaemic myocardium resulting from dissolution of the blockage by clinical intervention, may in turn further injure the damaged tissue as a result of reperfusion injury. Reperfusion leads to oxidative stress in the tissue and hence may itself require intervention. Hypoxic regulatable elements and overexpression of agents, which scavenge free radicals or reduce oxidative stress, have been targeted using gene therapy protocols (Table 1). To produce long-term myocardial protection Melo et al [38] used AAV to deliver the cytoprotective heme oxygenase gene by intramyocardial injection into rat hearts. They found that eight weeks after administration of the AAV-hHO-1 when acute coronary artery ligation was performed the treated rats had a dramatic reduction in myocardial infarction size [38]. Phillips et al [39] also devised a cardioprotective strategy using a "vigilant vector" AAV construct. This involves a heart specific promoter, MLC2v, which only expresses mRNA in the heart. The vector also includes a hypoxia regulatory element (HRE) which can act as an "on" switch so that production of the transgene antisense AT1R only occurs when ischaemia is detected. This would result in long-term protection of cardiac function during bouts of ischaemia [39]. In an acute model of oxidative stress, the effects of expression of superoxide dismutase (SOD) from adenoviral vectors was investigated [40]. High doses of Ad-SOD3 (3×10^{10} pfu) resulted in a 3-fold elevation of serum SOD activity and was protective against hepatic ischaemia-reperfusion injury.

Late vein graft failure

As one of the most commonly performed surgical procedure at some 400,000 cases worldwide each year, coronary artery bypass grafts (CABG) are effective at relieving symptoms of angina and prolong life for those patients with multiple vessel disease. Vein grafts are inserted into the arterial circulation and undergo a sequence of

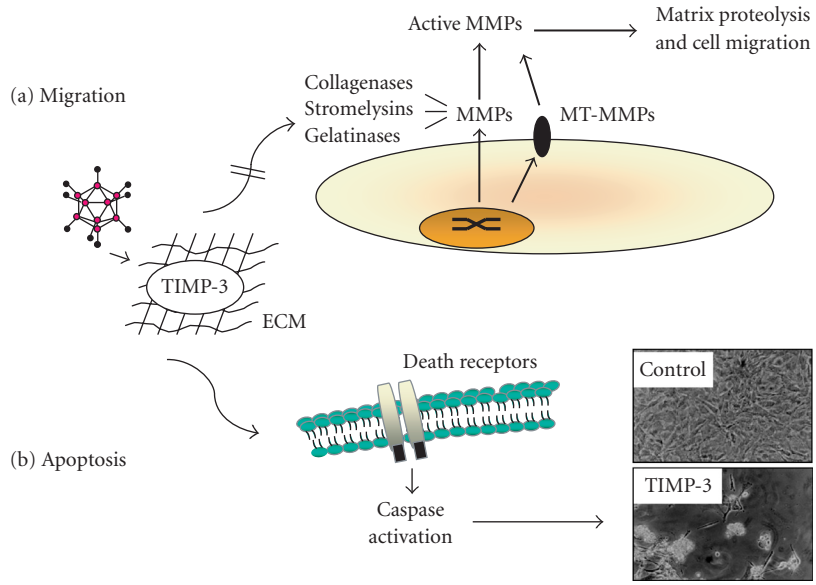

FIGURE 3. Gene therapy by overexpression of TIMP-3. Following Ad-mediated gene delivery to vascular smooth muscle cells, TIMP-3 is secreted and is found located with the extracellular matrix (ECM). From here, TIMP-3 is available to exert two distinctly different phenotypes through its metalloproteinase inhibitory effects. (a) Matrix metalloproteinases (MMPs, including collagenases, stromelysins, gelatinases, and membrane-type metalloproteinases [MT-MMPs]) are upregulated following vascular injury. TIMPs, through their native MMP inhibitory activity, are able to bind to and retard pro-MMP-to-active enzyme conversion and combined with the ability to block active MMP activity, matrix proteolysis and hence cell migration is inhibited [45]. (b) TIMP-3, uniquely amongst the TIMP family, is also able to promote smooth muscle cell death through death receptor-induced caspase activation and induction of apoptosis [46]. Micrographs courtesy of Mark Bond, Bristol Heart Institute, UK.

adaptive physiological changes. Early thrombotic occlusion occurs in 10% of grafts with patency rates of 50% over 10 years due to the onset of intimal thickening and atheromas [3]. Early thrombosis occurs in the first few weeks after grafting, particularly at the distal anastomosis due to vessel wall injury. Drug treatments include aspirin and other antiplatelet agents, which reduce but do not eliminate early occlusions but are often associated with hemorrhagic side effects. Late vein graft failure is characterized by progressive medial thickening and neointima formation. Therefore, vein graft failure limits the clinical success of coronary bypass grafting in terms of symptoms and mortality.

Remodelling of the vascular wall by MMPs promotes SMC migration and proliferation, ultimately leading to neointima formation and a narrowing of the vessel lumen [3]. The main therapeutic targets in the context of late vein graft failure are those affecting SMC migration and proliferation. Vein graft lends itself ideally to gene therapy, as there is a clinical therapeutic window whereby surgically prepared vein can be genetically modified prior to grafting. Many candidate therapeutic target genes have been studied experimentally with the aim to prevent the formation of neointimal lesions associated with late vein graft failure (Table 1). SMC proliferation and migration and matrix degradation are integral to lesion formation and hence, among those classes of gene investigated are antiproliferative, proapoptotic, antiinflammatory, or antimigratory agents (for review see [41]).

The PREVENT clinical trial [42] aimed to assess the efficacy of intraoperative gene therapy in patients receiving bypass vein grafts. By blockading the cell transcription factor E2F with decoy oligodeoxynucleoties at the time of grafting, they were able to significantly reduce the incidence of post-operative occlusion. E2F upregulates up to a dozen cell cycle genes and its inhibition inhibits target cell cycle gene expression and DNA synthesis. The success of this trial in reducing bypass graft failure in a high risk cohort underlines the important role that gene therapy could play in the prevention of vein graft failure.

MMPs have been shown to be an integral part of neointima formation, and overexpression of the naturally occurring tissue inhibitors of MMPs (TIMPs) is a possible approach. Ad-mediated transfer of TIMPs has been demonstrated in a number of models of vein graft failure. In a mouse model, local delivery of Ad-TIMP-2 was found to reduce vein graft diameter [43]. In contrast, using the pig saphenous vein-carotid artery interposition graft model, George et al [46] demonstrated that while Ad-mediated TIMP-2 delivery was ineffective at reducing vein graft neointima formation, TIMP-3 had a profound inhibitory effect on lesion formation (Figure 3). This was attributed, in part, to its proapoptotic effect in the medial and neointimal layers. Encouragingly, the results from the porcine model were translated into a human *ex vivo* model of vein graft failure with an 84% reduction in neointima formation following local delivery of Ad-TIMP-3 [46]. Similarly, TIMP-1 was also shown to

have an inhibitory effect on lesion formation in this model [47].

Endothelial nitric oxide synthase (eNOS) is important to vascular homeostasis and plays a vasoprotective role by inhibiting platelet and leukocyte adhesion, inhibiting SMC proliferation and migration, and in turn promoting endothelial survival. Local eNOS delivery would, in theory, arrest the proliferative response to vascular injury. Nitric oxide (NO) bioactivity is substantially reduced postbypass graft surgery whilst levels of NO scavenging superoxide are increased. It is therefore likely that the loss of NO may contribute to vascular remodelling events in the vein graft [48]. West et al [49] showed that Ad-nNOS gene transfer in a rabbit vein graft model favourably affected vein graft remodelling by inhibiting the early inflammatory changes and reducing late intimal hyperplasia [49]. They observed an increase in NOS activity, a reduction in adhesion molecule expression and inflammatory cell infiltration, and a reduction in basal superoxide generation.

Targeting SMC proliferation has also been shown to be effective using gene therapy protocols. In a rabbit model of vein grafting, Ad-mediated expression of a constitutively active form of the retinoblastoma gene product (AdΔRb) reduced neointima formation four weeks after surgery by 22% [50]. In the human saphenous vein model of vein graft, targeting SMC by overexpression of wild-type p53 both induced apoptosis and inhibited SMC migration resulting in a reduction in lesion formation [51]. Likewise, overexpression of C-type natriuretic peptide, which inhibits SMC growth by Ad resulted in accelerated graft re-endothelialisation and reduced thrombosis and neointima formation [52]. Kibbe et al [53] inhibited intimal hyperplasia in porcine vein grafts by incubation with Ad-iNOS for 30 minutes prior to surgery. This effect was sustained up to 21 days postgrafting [53]. While these strategies all show short-term effects, long-term studies are fundamentally important owing to the long-term nature of bypass graft failure.

Thrombosis

Defects in the vessel wall, namely, endothelial cell dysfunction can result in a reduction of antithrombotic activity leading to clot formation. The two main groups of anti-thrombotic genes are those with antiplatelet or anticoagulant activity. Prostacylin (PGI_2), nitric oxide (NO) and thrombin inhibitors all act through the inhibition of platelet adhesion and aggregation, in conjunction with the prevention of vascular SMC proliferation and vasoconstriction. The anti-thrombotic treatment, tissue plasminogen activator (tPA), which has anticoagulant properties and is used to lyse existing clots, may be a useful therapeutic gene for antithrombotic therapy (Table 1). The short half-life of tPA could be overcome with sustained overexpression from a gene therapy vector. Other anticoagulant gene products include hirudin, thrombomodulin, antistasin and, tissue factor pathway inhibitor (TFPI). Hirudin is perhaps the most potent inhibitor of thrombin, the enzyme responsible for fibrinogen cleavage, platelet activation, and SMC proliferation [54]. The advantage of local expression of antithrombotic therapeutic genes is that not only is thrombolysis promoted at specific sites in the artery, but also the side effects of the conventional anticoagulant are avoided. Clinical conditions amenable to antithrombotic gene therapy include CABG, percutaneous transluminal coronary angioplasty, peripheral artery angioplasty, and intravascular stenting. Intravascular clot formation is a major cause of acute myocardial infarction and contributes to the majority of sudden deaths in patients with coronary artery disease. Successful thrombolysis for acute thrombosis is dependent on prompt treatment and delays in vector administration and expression of antithrombotic factors suggests that antithrombotic gene therapy is more likely to play a role in the prevention of reocclusion and chronic arterial narrowing. Cyclo-oxygenase-1 (COX-1), the rate limiting enzyme in the synthesis of PGI_2, was overexpressed by local delivery of Ad to porcine carotid arteries immediately postangioplasty. This was shown to increase the levels of PGI_2 and, in turn, inhibit thrombosis in injured vessels [55]. By manipulating the coagulation cascade integral in thrombus formation, a number of positive gene therapy studies have been reported. In a rabbit stasis/injury model of arterial thrombosis, local overexpression of thrombomodulin using Ad was assessed. In addition to reducing thrombosis, the vector did not induce inflammatory damage at the site of delivery [56]. Work by the same group targeting tissue-type plasminogen activator (tPA) also demonstrated effective prevention of thrombus formation [57]. Local gene transfer of TFPI into rabbit carotid arteries using an Ad vector prior to experimental thrombosis induction completely inhibited the formation of thrombi without affecting systemic coagulation status [58]. Similar data were described in the porcine carotid artery model following local delivery of Ad-TFPI [59].

Atherosclerosis

Due to the complexity and interplay of genetic and environmental factors in the development of atherosclerosis, it is unlikely that localized gene therapy will be a useful approach in the primary prevention of the disease. Inefficient intravascular gene transfer efficiency through atherosclerotic lesions and lipid-rich atheromas has been attributed to the very low numbers of transfectable cells and the high connective tissue content [60]. An extensive list of therapeutic genes exists for the treatment of atherosclerosis including LDL- or VLDL-receptor gene transfer to overcome LDL-receptor efficiency, a major inherited genetic defect and a determinant of atherosclerosis. Patients with defective enzymes vital for lipoprotein metabolism such as lipoprotein and hepatic lipases would benefit from gene transfer of DNA producing the correct enzymes. A reduction in the level of atherogenic

apolipoprotein (apo) B100 is possible after gene transfer of the apoB mRNA editing enzyme, whilst lipoprotein A could be lowered with synthesis inhibiting ribozymes. Apolipoprotein AI (apoAI) and lecithin-cholesterol acyltransferase (LCAT) are important factors in the removal of excess cholesterol and the subsequent reduction in the incidence of atherosclerotic lesions (Table 1). Through in vitro bicistronic expression of these two genes from AAV plasmid vectors, it was shown that increased synthesis of apoAI and LCAT could play a role in reducing atherosclerotic risk [61].

The apoE$^{-/-}$ transgenic mouse is a well-established experimental model for atherosclerosis as it develops severe hypercholesterolaemia and atherosclerotic lesions similar to humans. Harris et al (2002) found that they could detect apoE mRNA in the muscle from a single intramuscular injection into the apoE$^{-/-}$ mouse, but could not detect circulating recombinant apoE in the plasma [62]. However circulating antibodies were detected against the human apoE. The most significant finding of this study was three months after administration of the AAV-apoE they found a significant reduction (approximately 30%) in atherosclerotic plague density in the aortas of treated animals compared to the controls. These results suggest that only low levels of apoE are required to produce protection against atherosclerosis.

Attenuation of lesion development has been demonstrated using Ad-mediated overexpression of heme oxygenase-1 [63], TIMP-1 [64], platelet-activating factor acetylhydrolase (PAF-AH)—the enzyme responsible for the inactivation of PAF [65] and apoE itself, administered intravenously [66]. Of particular significance, Kim et al [15] recently described the lifetime correction of hypercholesterolaemia in apoE$^{-/-}$ mice following a single intravenous injection of a helper-dependent Ad vector. Follow-up of 2.5 years old mice demonstrated 100% coverage of the aorta with atherosclerotic plaques in control mice with almost no lesion development in treated animals [15].

Hypertension

Systemic hypertension is a common multifactorial disorder primarily manifesting itself as chronic high blood pressure and is a major risk factor for atherosclerosis, peripheral vascular disease stroke, and many other complications associated with structural damage to the cardiovascular system. Drugs for controlling high blood pressure are effective over a 24-hour period, are nonspecific and cause side effects. It is well established that the hyperactive renin-angiotensin system (RAS) is a key factor in primary hypertension and gene therapy strategies have concentrated on those genes in the RAS controlling regulation of blood pressure.

Due to its multifactorial nature, gene therapy for hypertension has yet to be demonstrated clinically. Many gene therapy interventions have, however, been employed successfully in the laboratory (for review, see [67, 68, 69]). Targeting elements involved in the oxidative stress pathways (Table 1), Alexander et al [70] and Fennell et al [71] have demonstrated an improvement of endothelial function in the SHRSP with local delivery of Ad-eNOS [70] or Ad-extracellular SOD [71]. Angiotensin II-induced hypertension and accompanying endothelial dysfunction were studied by Nakane et al [72]. Gene transfer of Ad-eNOS but not SOD (copper/zinc or extracellular SOD) was shown to restore endothelial function *ex vivo* in aortic rings from treated rabbits [72]. Ad-eNOS delivery into the rostral ventrolateral medulla of SHRSP and WKY rats resulted in a significant reduction in blood pressure in both rats [73]. A continuous supply of tissue kallikrein by a single intramuscular injection of Ad produced a significant delay of elevated blood pressure for five weeks in the SHR [74]. An increase in vasodilator proteins such as kallikrein, eNOS, and atrial natriuretic peptide (ANP) in animal models has correlated with a reduction in blood pressure [75].

Antisense targeting angiotensinogen and the angiotensin type-1 (AT1) receptor attempted to decrease those genes responsible for vasoconstriction [76]. AAV-plasmids have been used to deliver antisense AT1-R to SHR rats [76]. They showed that a single intracardiac injection was sufficient to reduce blood pressure by 30 mm Hg when compared to the controls over a five-week period [76]. The effect of AAV-AGT-AS on the development of hypertension in SHR rats has also been examined. The rats were injected with AAV-AGT-AS five days after birth as the development of hypertension in SHR rats commences between the eighth and tenth week after birth. A significant slowing of the development of hypertension for six months was observed but there was no complete inhibition of the rise in blood pressure [77].

THE FUTURE OF GENE THERAPY IN THE TREATMENT OF CARDIOVASCULAR DISEASE

Due to the complexity of cardiovascular disorders, a major stumbling block may be the identification of the best gene to treat the disease. It is most likely that a cocktail of therapeutic genes rather than a single particular gene will be the most effective treatment of certain cardiovascular diseases such as hypertension and atherosclerosis. In order for gene therapy to become a reality in the cardiovascular clinic, effective therapeutic genes and suitable vectors must be identified and developed. Results from the first clinical trials have indicated that vascular gene transfer is not only safe but may have therapeutic benefits when administered intravascularly or intramuscularly.

REFERENCES

[1] Isner JM, Vale PR, Symes JF, Losordo DW. Assessment of risks associated with cardiovascular gene therapy in human subjects. *Circ Res.* 2001;89(5): 389–400.

[2] Kingston PA, Sinha S, David A, Castro MG, Lowenstein PR, Heagerty AM. Adenovirus-mediated gene transfer of a secreted transforming growth factor-β type II receptor inhibits luminal loss and constrictive remodeling after coronary angioplasty and enhances adventitial collagen deposition. *Circulation*. 2001;104(21):2595–2601.

[3] Baker AH, Mehta D, George SJ, Angelini GD. Prevention of vein graft failure: potential applications for gene therapy. *Cardiovasc Res*. 1997;35(3):442–450.

[4] Grossman M, Rader DJ, Muller D, et al. A pilot study of *ex vivo* gene therapy for homozygous familial hypercholesterolaemia. *Nat Med*. 1995;1(11):1148–1154.

[5] Forough R, Koyama N, Hasenstab D, et al. Overexpression of tissue inhibitor of matrix metalloproteinase-1 inhibits vascular smooth muscle cell functions *in vitro* and *in vivo*. *Circ Res*. 1996;79(4):812–820.

[6] Nabel EG, Plautz G, Nabel GJ. Site-specific gene expression *in vivo* by direct gene transfer into the arterial wall. *Science*. 1990;249(4974):1285–1288.

[7] Yang X, Atalar E, Li D, et al. Magnetic resonance imaging permits *in vivo* monitoring of catheter-based vascular gene delivery. *Circulation*. 2001; 104(14):1588–1590.

[8] Klugherz BD, Jones PL, Cui X, et al. Gene delivery from a DNA controlled-release stent in porcine coronary arteries. *Nat Biotechnol*. 2000;18(11):1181–1184.

[9] Baim DS, Wahr D, George B, et al. Randomized trial of a distal embolic protection device during percutaneous intervention of saphenous vein aorto-coronary bypass grafts. *Circulation*. 2002;105(11): 1285–1290.

[10] Panetta CJ, Miyauchi K, Berry D, et al. A tissue-engineered stent for cell-based vascular gene transfer. *Hum Gene Ther*. 2002;13(3):433–441.

[11] Vassalli G, Dichek DA. Gene therapy for arterial thrombosis. *Cardiovasc Res*. 1997;35(3):459–469.

[12] Yla-Herttuala S, Martin JF. Cardiovascular gene therapy. *Lancet*. 2000;355(9199):213–222.

[13] Jaffe HA, Danel C, Longenecker G, et al. Adenovirus-mediated in vivo gene transfer and expression in normal rat liver. *Nat Genet*. 1992;1(5):372–378.

[14] Koeberl DD, Alexander IE, Halbert CL, Russell DW, Miller AD. Persistent expression of human clotting factor IX from mouse liver after intravenous injection of adeno-associated virus vectors. *Proc Natl Acad Sci USA*. 1997;94(4):1426–1431.

[15] Kim IH, Jozkowicz A, Piedra PA, Oka K, Chan L. Lifetime correction of genetic deficiency in mice with a single injection of helper-dependent adenoviral vector. *Proc Natl Acad Sci USA*. 2001;98(23): 13282–13287.

[16] Ponder KP. Systemic gene therapy for cardiovascular disease. *Trends Cardiovasc Med*. 1999;9(6):158–162.

[17] Flotte TR, Afione SA, Zeitlin PL. Adeno-associated virus vector gene expression occurs in nondividing cells in the absence of vector DNA integration. *Am J Respir Cell Mol Biol*. 1994;11(5):517–521.

[18] Kotin RM, Siniscalco M, Samulski RJ, et al. Site-specific integration by adeno-associated virus. *Proc Natl Acad Sci USA*. 1990;87(6):2211–2215.

[19] Young SM Jr, McCarty DM, Degtyareva N, Samulski RJ. Roles of adeno-associated virus Rep protein and human chromosome 19 in site-specific recombination. *J Virol*. 2000;74(9):3953–3966.

[20] Rinaudo D, Lamartina S, Roscilli G, Ciliberto G, Toniatti C. Conditional site-specific integration into human chromosome 19 by using a ligand-dependent chimeric adeno-associated virus/Rep protein. *J Virol*. 2000;74(1):281–294.

[21] Xiao X, Li J, Samulski RJ. Efficient long-term gene transfer into muscle tissue of immunocompetent mice by adeno-associated virus vector. *J Virol*. 1996; 70(11):8098–8108.

[22] Phillips MI, Wielbo D, Gyurko R. Antisense inhibition of hypertension: a new strategy for renin-angiotensin candidate genes. *Kidney Int*. 1994;46(6): 1554–1556.

[23] Morishita R, Aoki M, Kaneda Y. Oligonucleotide-based gene therapy for cardiovascular disease: are oligonucleotide therapeutics novel cardiovascular drugs? *Curr Drug Targets*. 2000;1(1):15–23.

[24] Taniyama Y, Univ O, Jsapan S, et al. Local delivery of naked plasmid DNA of p53 gene into rat balloon injured carotid artery using low voltage ultrasound with contrast microbubbles (optison): development of novel non-viral transfection system into blood vessels. *Circulation*. 2000;102(suppl II):II–164 abstract.

[25] Asahara T, Chen D, Tsurumi Y, et al. Accelerated restitution of endothelial integrity and endothelium-dependent function after phVEGF$_{165}$ gene transfer. *Circulation*. 1996;94(12):3291–3302.

[26] Tsurumi Y, Takeshita S, Chen D, et al. Direct intramuscular gene transfer of naked DNA encoding vascular endothelial growth factor augments collateral development and tissue perfusion. *Circulation*. 1996;94(12):3281–3290.

[27] Losordo DW, Vale PR, Symes JF, et al. Gene therapy for myocardial angiogenesis: initial clinical results with direct myocardial injection of phVEGF$_{165}$ as sole therapy for myocardial ischemia. *Circulation*. 1998;98(25):2800–2804.

[28] Baumgartner I, Pieczek A, Manor O, et al. Constitutive expression of phVEGF$_{165}$ after intramuscular gene transfer promotes collateral vessel development in patients with critical limb ischemia. *Circulation*. 1998;97(12):1114–1123.

[29] Gowdak LH, Poliakova L, Wang X, et al. Adenovirus-mediated VEGF$_{121}$ gene transfer stimulates angiogenesis in normoperfused skeletal muscle and preserves tissue perfusion after induction of ischemia. *Circulation*. 2000;102(5):565–571.

[30] Rajagopalan S, Shah M, Luciano A, Crystal R, Nabel EG. Adenovirus-mediated gene transfer of $VEGF_{121}$ improves lower-extremity endothelial function and flow reserve. *Circulation*. 2001;104(7):753–755.

[31] Rosengart TK, Lee LY, Patel SR, et al. Angiogenesis gene therapy: phase I assessment of direct intramyocardial administration of an adenovirus vector expressing $VEGF_{121}$ cDNA to individuals with clinically significant severe coronary artery disease. *Circulation*. 1999;100(5):468–474.

[32] Grines CL, Watkins MW, Helmer G, et al. Angiogenic Gene Therapy (AGENT) trial in patients with stable angina pectoris. *Circulation*. 2002;105(11): 1291–1297.

[33] Shimpo M, Ikeda U, Maeda Y, et al. AAV-mediated VEGF gene transfer into skeletal muscle stimulates angiogenesis and improves blood flow in a rat hindlimb ischemia model. *Cardiovasc Res*. 2002; 53(4):993–1001.

[34] Su H, Lu R, Kan YW. Adeno-associated viral vector-mediated vascular endothelial growth factor gene transfer induces neovascular formation in ischemic heart. *Proc Natl Acad Sci USA*. 2000;97(25):13801–13806.

[35] Celletti FL, Waugh JM, Amabile PG, Brendolan A, Hilfiker PR, Dake MD. Vascular endothelial growth factor enhances atherosclerotic plaque progression. *Nat Med*. 2001;7(4):425–429.

[36] Springer ML, Chen AS, Kraft PE, Bednarski M, Blau HM. VEGF gene delivery to muscle: potential role for vasculogenesis in adults. *Mol Cell*. 1998;2(5): 549–558.

[37] Lee RJ, Springer ML, Blanco-Bose WE, Shaw R, Ursell PC, Blau HM. VEGF gene delivery to myocardium: deleterious effects of unregulated expression. *Circulation*. 2000;102(8):898–901.

[38] Melo LG, Agrawal R, Zhang L, et al. Gene therapy strategy for long-term myocardial protection using adeno-associated virus-mediated delivery of heme oxygenase gene. *Circulation*. 2002;105(5):602–607.

[39] Phillips MI, Tang Y, Schmidt-Ott K, Qian K, Kagiyama S. Vigilant vector: heart-specific promoter in an adeno-associated virus vector for cardioprotection. *Hypertension*. 2002;39(pt 2):651–655.

[40] Wheeler MD, Katuna M, Smutney OM, et al. Comparison of the effect of adenoviral delivery of three superoxide dismutase genes against hepatic ischemia-reperfusion injury. *Hum Gene Ther*. 2001; 12(18):2167–2177.

[41] Kibbe MR, Billiar TR, Tzeng E. Gene therapy for restenosis. *Circ Res*. 2000;86(8):829–833.

[42] Mann MJ, Whittemore AD, Donaldson MC, et al. *Ex-vivo* gene therapy of human vascular bypass grafts with E2F decoy: the PREVENT single-centre, randomised, controlled trial. *Lancet*. 1999; 354(9189):1493–1498.

[43] Hu Y, Baker AH, Zou Y, Newby AC, Xu Q. Local gene transfer of tissue inhibitor of metalloproteinase-2 influences vein graft remodeling in a mouse model. *Arterioscler Thromb Vasc Biol*. 2001;21(8):1275–1280.

[44] Baker AH, Zaltsman AB, George SJ, Newby AC. Divergent effects of tissue inhibitor of metalloproteinase-1, -2, or -3 overexpression on rat vascular smooth muscle cell invasion, proliferation, and death in vitro. TIMP-3 promotes apoptosis. *J Clin Invest*. 1998;101(6):1478–1487.

[45] Bond M, Murphy G, Bennett MR, Newby AC, Baker AH. Tissue inhibitor of metalloproteinase-3 induces a Fas-associated death domain-dependent type II apoptotic pathway. *J Biol Chem*. 2002;277(16): 13787–13795.

[46] George SJ, Lloyd CT, Angelini GD, Newby AC, Baker AH. Inhibition of late vein graft neointima formation in human and porcine models by adenovirus-mediated overexpression of tissue inhibitor of metalloproteinase-3. *Circulation*. 2000;101(3):296–304.

[47] George SJ, Johnson JL, Angelini GD, Newby AC, Baker AH. Adenovirus-mediated gene transfer of the human TIMP-1 gene inhibits smooth muscle cell migration and neointimal formation in human saphenous vein. *Hum Gene Ther*. 1998;9(6):867–877.

[48] Buttery LD, Chester AH, Springall DR, et al. Explanted vein grafts with an intact endothelium demonstrate reduced focal expression of endothelial nitric oxide synthase specific to atherosclerotic sites. *J Pathol*. 1996;179(2):197–203.

[49] West NE, Qian H, Guzik JJ, et al. Nitric oxide synthase (nNOS) gene transfer modifies venous bypass graft remodeling: effects on vascular smooth muscle cell differentiation and superoxide production. *Circulation*. 2001;104(13):1526–1532.

[50] Schwartz LB, Moawad J, Svensson EC, et al. Adenoviral-mediated gene transfer of a constitutively active form of the retinoblastoma gene product attenuates neointimal thickening in experimental vein grafts. *J Vasc Surg*. 1999;29(5):874–881.

[51] George SJ, Angelini GD, Capogrossi MC, Baker AH. Wild-type p53 gene transfer inhibits neointima formation in human saphenous vein by modulation of smooth muscle cell migration and induction of apoptosis. *Gene Ther*. 2001;8(9):668–676.

[52] Ohno N, Itoh H, Ikeda T, Ueyama K, et al. Accelerated reendothelialization with suppressed thrombogenic property and neointimal hyperplasia of rabbit jugular vein grafts by adenovirus-mediated gene transfer of C-type natriuretic peptide. *Circulation*. 2002;105(14):1623–1626.

[53] Kibbe MR, Tzeng E, Gleixner SL, et al. Adenovirus-mediated gene transfer of human inducible nitric oxide synthase in porcine vein grafts inhibits intimal hyperplasia. *J Vasc Surg*. 2001;34(1):156–165.

[54] Mehta SR, Eikelboom JW, Rupprecht HJ, et al. Efficacy of hirudin in reducing cardiovascular events in patients with acute coronary syndrome undergoing early percutaneous coronary intervention. *Eur Heart J*. 2002;23(2):117–123.

[55] Zoldhelyi P, McNatt J, Xu XM, et al. Prevention of arterial thrombosis by adenovirus-mediated transfer of cyclooxygenase gene. *Circulation*. 1996;93(1):10–17.

[56] Waugh JM, Yuksel E, Li J, et al. Local overexpression of thrombomodulin for in vivo prevention of arterial thrombosis in a rabbit model. *Circ Res*. 1999;84(1):84–92.

[57] Waugh JM, Kattash M, Li J, et al. Gene therapy to promote thromboresistance: local overexpression of tissue plasminogen activator to prevent arterial thrombosis in an in vivo rabbit model. *Proc Natl Acad Sci USA*. 1999;96(3):1065–1070.

[58] Nishida T, Ueno H, Atsuchi N, et al. Adenovirus-mediated local expression of human tissue factor pathway inhibitor eliminates shear stress-induced recurrent thrombosis in the injured carotid artery of the rabbit. *Circ Res*. 1999;84(12):1446–1452.

[59] Zoldhelyi P, McNatt J, Shelat HS, Yamamoto Y, Chen ZQ, Willerson JT. Thromboresistance of balloon-injured porcine carotid arteries after local gene transfer of human tissue factor pathway inhibitor. *Circulation*. 2000;101(3):289–295.

[60] Laitinen M, Makinen K, Manninen H, et al. Adenovirus-mediated gene transfer to lower limb artery of patients with chronic critical leg ischemia. *Hum Gene Ther*. 1998;9(10):1481–1486.

[61] Fan L, Drew J, Dunckley MG, Owen JS, Dickson G. Efficient coexpression and secretion of anti-atherogenic human apolipoprotein AI and lecithin-cholesterol acyltransferase by cultured muscle cells using adeno-associated virus plasmid vectors. *Gene Ther*. 1998;5(10):1434–1440.

[62] Harris JD, Schepelmann S, Athanasopoulos T, et al. Inhibition of atherosclerosis in apolipoprotein-E-deficient mice following muscle transduction with adeno-associated virus vectors encoding human apolipoprotein-E. *Gene Ther*. 2002;9(1):21–29.

[63] Juan SH, Lee TS, Tseng KW, et al. Adenovirus-mediated heme oxygenase-1 gene transfer inhibits the development of atherosclerosis in apolipoprotein E-deficient mice. *Circulation*. 2001;104(13):1519–1525.

[64] Rouis M, Adamy C, Duverger N, et al. Adenovirus-mediated overexpression of tissue inhibitor of metalloproteinase-1 reduces atherosclerotic lesions in apolipoprotein E-deficient mice. *Circulation*. 1999;100(5):533–540.

[65] Quarck R, De Geest B, Stengel D, et al. Adenovirus-mediated gene transfer of human platelet-activating factor-acetylhydrolase prevents injury-induced neointima formation and reduces spontaneous atherosclerosis in apolipoprotein E-deficient mice. *Circulation*. 2001;103(20):2495–2500.

[66] Harris JD, Graham IR, Schepelmann S, et al. Acute regression of advanced and retardation of early aortic atheroma in immunocompetent apolipoprotein-E (apoE) deficient mice by administration of a second generation [E1(-), E3(-), polymerase(-)] adenovirus vector expressing human apoE. *Hum Mol Genet*. 2002;11(1):43–58.

[67] Phillips MI. Is gene therapy for hypertension possible? *Hypertension*. 1999;33(1):8–13.

[68] Phillips MI. Gene therapy for hypertension: the preclinical data. *Methods Enzymol*. 2002;346:3–13.

[69] Gelband CH, Katovich MJ, Raizada MK. Current perspectives on the use of gene therapy for hypertension. *Circ Res*. 2000;87(12):1118–1122.

[70] Alexander MY, Brosnan MJ, Hamilton CA, et al. Gene transfer of endothelial nitric oxide synthase but not Cu/Zn superoxide dismutase restores nitric oxide availability in the SHRSP. *Cardiovasc Res*. 2000;47(3):609–617.

[71] Fennell JP, Brosnan MJ, Frater AJ, et al. Adenovirus-mediated overexpression of extracellular superoxide dismutase improves endothelial dysfunction in a rat model of hypertension. *Gene Ther*. 2002;9(2):110–117.

[72] Nakane H, Miller FJ Jr, Faraci FM, Toyoda K, Heistad DD. Gene transfer of endothelial nitric oxide synthase reduces angiotensin II-induced endothelial dysfunction. *Hypertension*. 2000;35(2):595–601.

[73] Kishi T, Hirooka Y, Ito K, Sakai K, Shimokawa H, Takeshita A. Cardiovascular effects of overexpression of endothelial nitric oxide synthase in the rostral ventrolateral medulla in stroke-prone spontaneously hypertensive rats. *Hypertension*. 2002;39(2):264–268.

[74] Zhang JJ, Wang C, Lin KF, Chao L, Chao J. Human tissue kallikrein attenuates hypertension and secretes into circulation and urine after intramuscular gene delivery in hypertensive rats. *Clin Exp Hypertens*. 1999;21(7):1145–1160.

[75] Chao J, Chao L. Kallikrein gene therapy: a new strategy for hypertensive diseases. *Immunopharmacology*. 1997;36(2-3):229–236.

[76] Phillips MI, Mohuczy-Dominiak D, Coffey M, et al. Prolonged reduction of high blood pressure with an in vivo, nonpathogenic, adeno-associated viral vector delivery of AT1-R mRNA antisense. *Hypertension*. 1997;29(1 Pt 2):374–380.

[77] Kimura B, Mohuczy D, Tang X, Phillips MI. Attenuation of hypertension and heart hypertrophy by adeno-associated virus delivering angiotensinogen antisense. *Hypertension*. 2001;37(pt 2):376–380.

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REVIEW ARTICLE

Targeting of Synthetic Gene Delivery Systems

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Safe, efficient, and specific delivery of therapeutic genes remains an important bottleneck for the development of gene therapy. Synthetic, nonviral systems have a unique pharmaceutical profile with potential advantages for certain applications. Targeting of the synthetic vector improves the specificity of gene medicines through a modulation of the carriers' biodistribution, thus creating a dose differential between healthy tissue and the target site. The biodistribution of current carrier systems is being influenced to a large extent by intrinsic physicochemical characteristics, such as charge and size. Consequently, such nonspecific interactions can interfere with specific targeting, for example, by ligands. Therefore, a carrier complex should ideally be inert, that is, free from intrinsic properties that would bias its distribution away from the target site. Strategies such as coating of DNA carrier complexes with hydrophilic polymers have been used to mask some of these intrinsic targeting effects and avoid nonspecific interactions. Preexisting endogenous ligand-receptor interactions have frequently been used for targeting to certain cell types or tumours. Recently exogenous ligands have been derived from microorganisms or, like antibodies or phage-derived peptides, developed de novo. In animal models, such synthetic vectors have targeted remote sites such as a tumour. Furthermore, the therapeutic proof of the concept has been demonstrated for fitting combinations of synthetic vectors and therapeutic gene.

INTRODUCTION

The efficient and specific delivery of therapeutic genes to a target site is a challenge that will need to be overcome in order to tap into the promise and potential of gene medicines [1]. Over the last decade a number of promising synthetic, nonviral systems gene delivery systems have been developed and a profile of their potential advantages and disadvantages has emerged. Synthetic vectors have advantages relating to pharmaceutical issues, safety, and ease of use but tend to be less efficient than some viral systems [2, 3].

One of the critical issues that determine efficacy and safety of a therapeutic approach is its specificity, which is based on the recognition and exploitation of differentials between the diseased site and healthy tissue. As these differentials exist on different levels—molecular to systemic—it is crucial that each element of a potential gene medicine is selected with a view to exploit potential differences. The basic modules of a gene medicine, namely carrier, gene, and effector protein, each contribute to the overall activity and specificity profile. Further levels of specificity may be added through the use of, for example, prodrugs, which the effector protein then acts upon.

Targeting provides a generic strategy to improve the specificity of a pharmaceutical formulation independently of the specificity of the drug or gene itself, primarily by creation of a dose differential between healthy and diseased tissue. This review will examine strategies and specific challenges relating to the targeting of synthetic gene vector systems.

SYNTHETIC GENE DELIVERY VECTORS

Viral and nonviral synthetic, nonviral systems gene delivery vectors are characterised by a profile of potential pharmaceutical advantages and disadvantages, which need to be matched to the therapeutic strategy [3]. While short-term expression of the gene, for example, with a synthetic vector, may be acceptable for immunisation, an integrating viral vector may provide a more sustained expression suitable for gene replacement therapy.

Important advantages of synthetic vector systems are their safety, lack of immunogenicity, very low frequency of integration, and relative ease of large-scale production, which makes them more akin to conventional pharmaceutical excipients. These systems are also very flexible with regards to the therapeutic nature of size of the gene, as even mammalian artificial chromosomes of 60 mega bases have been transfected successfully [4].

The potential disadvantage of lower efficiency presents the flip side of the coin. However, one needs to bear in mind that therapeutic efficacy will ultimately depend on the suitable combination of vector and gene. For a number of synthetic systems therapeutic potential has been demonstrated, for example, in tumour models in vivo [5]. The systems also allow repeat dosing which potentially greatly improves efficacy [6]. The duration of

gene expression can be increased significantly by genetically optimising of the expression plasmid [7].

Packaging

One of the key concepts for the use of drug carriers in general is "packaging": delivery systems fulfil a number of generic functions analogous to a mail package, such as, for example, protection of content, ease of handling, and an address for delivery. The pharmaceutical properties of the package/delivery system are determined by the box/carrier and are largely independent from the content, which only becomes relevant once the package has been delivered.

In the case of the synthetic vector systems, the active principle (pharmacophore) is the plasmid DNA. While (naked) plasmid DNA could be regarded as a macromolecular drug, its vulnerability to enzymatic degradation (nucleases) in biological fluids ($t_{1/2}$ plasma = 1.2 min [8]) makes a protective packaging mandatory for most applications.

"Packaging" systems based on synthetic, nonviral vectors fall into two main groups, water-soluble polymers and cationic lipids/vesicles. (Another group of synthetic carrier materials is based on the use of peptides [9] which usually contain a group of charged amino acids (eg, lysine) to complex DNA.)

Complex formation and physicochemistry

The principles that govern the formation of the different complexes are similar, but some physicochemical differences exist, which affect some of the system's biological properties.

The complex formation between plasmid DNA and the carrier is initially electrostatic, that is, induced by the attraction between the anionic DNA and the cationic carrier material. This attraction leads to the formation of a DNA-carrier aggregate, so called polyplex, in the case of polymers, or lipoplexes for cationic lipids, respectively.

The stability of these complexes depends on the strength of the electrostatic interaction and thus on the total charge and the charge density of the carrier molecule. Complex formation is not always easily controlled, as the process is influenced by stoichiometric as well as kinetic factors [10]. The resulting complexes are particles with a size ranging from less than 100 nm to over 1 μm.

The total charge of the carrier molecule also depends on the number of attachment points per molecule: in the case of a polymer binding to DNA it is a many-to-many relationship, that is, each molecule provides multiple interaction opportunities; the cationic lipids on the other hand will normally be monovalent leading to a many-to-one relationship. Therefore, the noncovalent self-assembly processes are central to stabilising these types of systems. For amphiphilic carrier material such as cationic lipids, an additional stabilising factor is based on the phase separation, which leads to the sandwiching of the DNA between lipid layers.

In general, the size of the complex will be greater than that of the starting material, for example, vesicles, and may in fact continue to increase in size and ultimately lead to precipitation if the formulation is not colloidally stable.

In general, colloidal stability is being achieved by working with an excess of cationic charge, that is, carrier material, to ensure complete coating of the DNA to create charged complexes that are stabilised by the electrostatic repulsion between the particles. The majority of synthetic gene delivery systems carry a positive charge and are thus prone to interact with negatively charged molecules and surfaces. (While DNA "packaging" in the overwhelming majority of systems is based on charge-charge interactions, a few systems (eg, for DNA vaccination) aim to encapsulate DNA in the aqueous core of a vesicle [11, 12].)

This nonspecific interaction of positively charged complexes with cell surface molecules such as glycosaminoglycans is in fact an important factor in determining overall uptake and downstream transfection efficiency [13, 14, 15]. While this nonspecific binding and uptake effect may be useful for in vitro transfection and locoregional applications, it also introduces the potential, for a number of interactions in vivo that will bias the biodistribution and may compromise the stability of the delivery systems [2].

The adsorption of serum proteins can induce a number of effects such as complex destabilisation, aggregation, or retargeting. The binding of cellular elements such as erythrocytes [16] and platelets [17] can lead to extensive aggregation and potentially acute toxicity.

TARGETING

In the context of drug and gene delivery systems, we define "*targeting*" as any strategy that increases specificity primarily through a modulation of the carriers' biodistribution.

Given the complexity of the biological barriers that need to be overcome for the targeting to a remote site, locoregional administration represents a very pragmatic alternative to the targeting after systemic administration.

Locoregional administration

The feasibility of locoregional administration as an approach to improve specificity depends very much on the disease and the therapeutic strategy. For situations where one aims to treat a small number of easily accessible disease sites, it will in fact often be the method of choice. Furthermore, it is not necessarily required for the gene complex itself to reach the diseased site: local production of a remotely acting effector protein may be more efficient (eg, local production of growth factors which act on a tumour or APC stimulation for immunotherapy).

In a mouse model of intrahepatic hepatocellular carcinoma, the advantages of localised application become

evident; transgene expression and toxicity were clearly superior after intratumour application of the gene medicine compared to either administration into the tail vein or the portal vein [18].

Anatomical barriers, such as the blood-brain-barrier, are another reason for the use of locoregional administration, which separate the organ from the systemic circulation. The blood-brain barrier seals the brain off from the systemic circulation and local administration of gene medicines is a practical way of circumventing this barrier. The intrathecal administration of a lipoplex with anti-apoptotic transgenes into the cerebrospinal fluid alleviated postischemic damage in a model of ischemic brain damage [19].

One of the most important parenteral administration routes is through the oral/nasal mucosa and the lung. Local delivery using the lung is the first choice for the therapy of cystic fibrosis, that is, typically aiming to replace the faulty CFTR gene to restore the faulty transport of salt in the lung epithelium. Clinical trials for cystic fibrosis using lipoplexes were among the first to establish the safety of these vectors [20] and the efficacy of repeat dosing [6] in a clinical setting.

Although locoregional administration offers many advantageous route-specific barriers, for example, sputum [21] may need to be taken into consideration and carriers optimised accordingly [22].

Targeting after systemic administration

Targeting in drug and gene delivery usually has a narrower context than suggested in the above definition and refers to approaches, which aim to achieve a differential in drug concentration between a remote target site and the body in general.

When trying to target drugs or DNA complexes to a remote site in the body, the simplest starting point is an intravenous injection; any other means of administration (eg, intramuscular or subcutaneous injection) leads to a greater complexity of the system as additional barriers will have to be overcome to gain access to the systemic circulation.

Conventionally, targeting strategies have been categorized as either "active" or "passive." We suggest a more useful way of thinking about targeting of delivery systems may be to distinguish between targeting properties *intrinsic* to the carrier system, which are largely based on the systems' physicochemistry, and independent, *extrinsic* targeting functions, for example, ligands (cf. Figure 1).

To allow specific ligand-directed distribution to a target site, a carrier/complex should ideally be inert, that is, free from inherent properties that would bias the biodistribution. (Coming back to the picture of the "package," one could say that the site of delivery should be determined only by the address label and that the nature of the box should not influence the way the parcel is being processed.)

INTRINSIC TARGETING

Because of the complexity of biological systems and the plethora of potential interactions, it could be argued that there is no such thing as an untargeted delivery system: for any set of intrinsic physicochemical carrier properties there are specific biological interactions and effects that will bias its biodistribution. In order to be able to specifically target synthetic gene delivery systems, it is therefore important to understand the bias of biodistribution inherent to the systems' physicochemistry. In the context of a suitable therapeutic strategy, the intrinsic properties of selected systems can actually facilitate an accumulation at a remote target site.

Charge

While the cationic charge of DNA complexes complicates the systemic administration of such gene medicines, it is clear that this interaction produces a distinctive pattern of biodistribution [17] which may potentially be used with an advantage to target sites of increased vascular growth such as tumours. Some cationic vesicles have been shown to have a 15–33-fold higher uptake in angiogenic endothelial cells of the tumour neovasculature than in corresponding normal endothelial cells [23].

After intravenous administration of DNA complexes, expression in the lung tends to be one to two orders of magnitude higher than in other organs. The mechanisms behind the lung targeting effect are not entirely clear [24] but have frequently been linked to carrier charge [25]. There is also evidence that the nature of the carrier materials plays a role: lung endothelial cells show signs of active transport of PEI complexes [26] with a potential involvement of an endogenous polyamine transporter [27].

While charge is an intrinsic property of most synthetic vectors, it may also be utilised as an extrinsic targeting function, for example, for the retargeting of adenovirus to the lung [28].

Size

Complex size is a potentially important property of synthetic gene delivery vectors. DNA complexes tend to form particulate systems (ie, size range of 0.05–1 μm) with the exact size depending on a number of factors such as, for example, DNA to carrier ratio, total concentrations, ionic strength of the buffer, and kinetics of mixing. The lower limit of the particles' size is not easily adjustable and is thus intrinsic to any given formulation.

For many gene therapy applications, the target cells will form a part of the parenchyma or interstitium of the organ and the access to these cells is restricted for particulate drug carriers after vascular administration. This is because macromolecules and particulate carriers can only extravasate from the vasculature at specialised sites, for example, the liver or spleen, where the endothelial lining has suitable gaps, so called fenestrae, which allow particles of around 200 nm or smaller to pass [29].

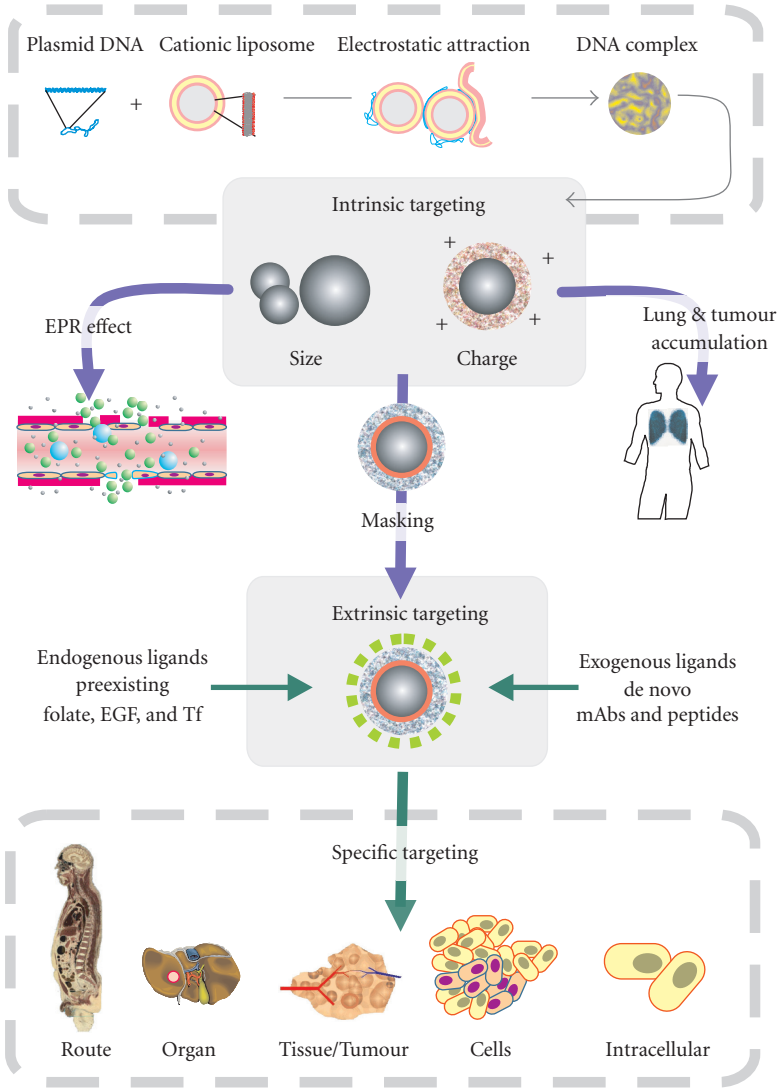

FIGURE 1. Targeting of synthetic vector systems. Schematic summary of factors important for the specific targeting of synthetic gene delivery systems. The mechanism of DNA packaging by cationic vectors, for example, vesicle, leads to the formation of condensed particles, which carry a positive charge. Particle size and charge are intrinsic physicochemical properties of these DNA complexes that, in interaction with the body, result in a modified biodistribution (eg, lung or tumour accumulation). In order to allow the use of more specific ligands, intrinsic targeting functions need to be masked, for example, by coating with hydrophilic polymers. These more inert carriers can then be directed specifically to a remote target site through the use of endogenous or exogenous ligands to allow targeting of differentials between target site and healthy tissue on various levels.

Thus, size can become a potentially important intrinsic property of synthetic gene delivery systems that will limit organ access and modulate biodistribution at the cellular level [17, 30, 31, 32].

While many studies compare the relative transfection efficiencies of formulations using "bulk reporter assays" (eg, based on organ lysates using luminescence), there is less information available about the histological distribution of the complexes and transgene expression within an organ [33]. In fact, most assays do not provide a straightforward link between quantitation and distribution of transgene expression.

The lung and liver tend to be the main organs of biodistribution after systemic administration and parenchymal expression has been reported in both of these organs. In the case of the liver, the fenestrations may play an important role in allowing gene medicines access to the parenchyma and increased hydrostatic pressure has been used to widen those gaps and thus improve delivery [34].

For the lung, it is not clear what factors are involved in overcoming the endothelial barriers but an active transcellular transport has been observed for polyethylenimine (PEI) polyplexes in the lung [26].

The bias of biodistribution introduced by complex

size however, can also potentially be used for targeting: solid tumours growing over a size of a few millimetres recruit additional blood supply in the process of neovascularisation [35] which produces rapidly sprouting vessels with a "leaky" endothelial lining [36, 37]. Drug carriers can extravasate this "leaky" tumour vasculature and accumulate in perivascular clusters within the tumour. This "enhanced penetration and retention" (EPR) effect [38] allows the targeting of long circulating macromolecular or particulate drug carriers to tumours [39, 40, 41] and could potentially also be used with suitable synthetic gene delivery systems.

Both, charge and size of the carrier thus represent important intrinsic properties of a formulation but many other aspects of physicochemistry will potentially also influence the biodistribution. This intrinsic bias will often interfere with more specific targeting efforts. However, the example of the EPR effect-based targeting to tumours demonstrates that these effects could potentially also be used for the targeting of a suitable gene medicine.

Targeting based on the physicochemical properties of the package is not necessarily based only on the intrinsic properties: the addition of cationic charges to uncharged virus particles demonstrates the utility of charge as an extrinsic factor for the retargeting of adenovirus to the lung [28].

Minimizing biodistribution bias

Strategies to minimise bias from these intrinsic effects have been predominately focused on charge shielding and steric stabilisation.

In drug targeting, the method of using water-soluble PEG chains which create a steric barrier around the drug carrier is well established [42] and has since been applied to various gene delivery systems. PEG-phospholipid conjugates have been used to stabilise lipoplexes [43] and polyplexes. Polyplex stabilisation with PEG was achieved through the use of polylysine block [44] or comb type copolymers [45, 46], also after adsorption [47] or covalent coupling [16] to PEI polyplexes, and by covalent coupling to chitosan nanoparticles [48].

Other water-soluble materials that have helped to reduce the effects of charge-based interactions are HPMA, which has been used in conjunction with polylysine [49] or PEI polyplexes [50] and block copolymers of ethylene oxide and propylene oxide (Pluronic) [51]. Interestingly, these polymers can also be used for the retargeting of adenovirus [52].

Alternatively, the encapsulation of cationic complexes in standard liposomes can be used to reduce nonspecific interactions [53].

In addition to polymers, the coating of complexes with protein could be used to mask positive charges, although the protein itself may also introduce a bias of biodistribution. Transferrin (Tf), a popular ligand for targeting to the transferrin receptor, also can be used to create negatively charged complexes [54] or to mask the positive charge of PEI Tf polyplexes [55].

To achieve high-level transgene expression after successful minimisation of the nonspecific interactions, it is necessary to introduce an extrinsic functionality that mediates efficient targeting and cellular uptake and replaces the nonspecific binding and uptake.

EXTRINSIC TARGETING

In general, extrinsic targeting functions can be most efficiently utilised when DNA-complexes are sufficiently inert, that is, their intrinsic, nonspecific interactions do not unfavorably bias the biodistribution and interfere with the targeting.

Such vectors can in fact be considered "detargeted," as the lack of nonspecific interactions leads to a reduced cellular uptake and thus frequently makes these complexes less efficient in vitro than standard complexes. However, once such complexes have been complemented by an extrinsic targeting function they are well suited for specific, high-resolution targeting in vivo. For the majority of applications, the extrinsic targeting functionality is based on ligand-receptor interactions but other approaches such as, for example, the direction of paramagnetic complexes through the use of an external magnetic field are also being explored [56].

The binding of a "ligand" to a "receptor" (in the broadest sense) provides the basis for most specific biological interactions and provides the blueprint for ligand-based targeting strategies.

It is from this group of preexisting,"natural" ligands or receptor substrates, many of the candidates used to target synthetic gene delivery systems have initially been selected.

Preexisting ligands

Endogenous

The most widely used preexisting targeting ligands are based on endogenous molecules, which are already present in the body. Preexisting ligands are often relatively easily available and the receptor and its distribution are fairly well studied.

Their use for targeting is based on the fact that a differential of expression levels exists between the target site, for example, tumour, and healthy tissue. A potential disadvantage of these ligands is the background expression of the receptor in healthy tissues and the interference of circulating endogenous ligand molecules.

The use of the vitamin *folic acid* as a ligand is an examples of the effective use of the natural receptor substrate for targeting. The development of targeted synthetic vectors based on folate is a natural extension of the use of this ligand for the targeting of drugs and imaging agents [57]. The receptor for the vitamin, folic acid, is overexpressed on a number of human tumors, including cancers of the ovary, kidney, uterus, testis, brain, colon,

lung, and myelocytic blood cells [57]. In gene delivery, folic acid has been used for the targeting of liposome-encapsulated complexes [53], polylysine polyplexes [58], and polyethylenimine [59] polyplexes to receptor-positive cells.

A recent report [60] illustrates the effect of intrinsic bias on the targeting of folate lipoplexes in vivo: the lung accumulation of the cationic complexes was successfully reduced using masking with PEG polymer but targeting of these complexes with folate failed to increase the accumulation of the complexes in receptor-positive tumours, probably because other intrinsic factors were overriding the specific effects. However, the folate ligand did have a positive effect on transfection efficiency in cells that had taken up the complex.

For targeting of poly-L-lysine DNA-complexes to the liver, *asialoorosomucoid* [61, 62] and *galactose* [63, 64] have been used as ligands. While uptake in hepatocytes has been demonstrated for such systems, some of the liver targeting may be caused by the tendency of the liver, and specifically cells of the reticuloendothelial system (Kupfer cells), to "filter" out particulate drug carriers.

Since its early use for the targeting to erythroblasts [65] *transferrin* has become one of the most widely used ligands for targeting of synthetic vectors. Transferrin receptors are found on the surface of most proliferating cells and, in elevated numbers, on erythroblasts and on many tumours [66, 67]. Its presence on various cell types potentially limits the specificity of transferrin-targeted vectors. There have been suggestions that the effects of transferrin on gene delivery may be nonspecific and not related to targeting [68]. It has been used in conjunction with lipoplexes [69], polyplexes prepared from polylysine [70], PEI [71], or chitosan [48], and with DNA-gelatin nanoparticles and DNA-binding peptides [72].

Another important potential ligand for tumour targeting is the *epidermal growth factor* (EGF) [73], which has been used to target polylysine complexes [74], liposomes [75], PEI polyplexes [74], and adenovirus-derived peptides [76] to receptor-positive cells. There is currently only limited experience with the use of EGF-targeted vectors for targeting of synthetic vectors in vivo [77].

Exogenous

Many pathogenic organisms have coevolved with their hosts and developed sophisticated targeting capabilities that allow them to home on specific tissues and infect a particular cell type [78]. If this targeting functionality can be isolated from the organism's pathogenicity, such ligands would potentially be useful for targeting.

The malaria circumsporozoite (CS) protein, which coats the entire surface of sporozoites of malaria parasites and has been shown to bind specifically to the basolateral surface of hepatocytes after intravenous injection and also to target polylysine polyplexes to these cells [79]. The papilloma virus capsid [80] is an example of a virus-derived targeting ligand optimised by coevolution.

De novo identified ligands

The development of targeted gene delivery vectors tends to mirror the progress in drug delivery but with a focus on the adaptation of techniques to the specific challenges of synthetic gene delivery vectors. A number of technologies for the de novo selection of potential binding ligands such as, for example, antibodies, phage display, combinatorial peptide, or nucleotide (aptamer) libraries, have recently become widely available and recombinant technology has greatly accelerated the discovery process. (Currently, phage display [81] drives the move to smaller targeting moieties such as antibody fragments [82, 83] and peptides [84, 85].) Ligands derived from these technologies have a number of advantages, in particular with respect to pharmaceutical, regulatory, and production issues, and some have already been tested in the clinic.

Antibodies to the transferrin receptor [82], the anti-platelet endothelial cell adhesion molecule PECAM [86], the polymeric immunoglobulin receptor pIgR [83], anti-CD5 [87], and the ErbB-2 receptor [88] have been used for the targeting of liposomes [11, 89], polylysine polyplexes [90], PEI polyplexes [86], and DNA-peptide complexes [91]. These systems in general mediated an increased uptake or expression in vivo compared to the untargeted vector.

SUMMARY AND CONCLUSIONS

Safe, efficient, and specific delivery remains a potential bottleneck for the further development of gene therapy. Each of the current systems has advantages and disadvantages and the selection of a suitable vector needs to be seen in context with the therapeutic strategy.

Targeting strategies aim to increase the specificity of a gene delivery formulation primarily through a modulation of the carriers' biodistribution, so that a dose differential is created between healthy tissue and the target site. In its simplest form, this can often be achieved by locoregional administration of the formulation, for example, intratumoural injection or inhalation for treatment of the lung. Systemic, intravenous administration of gene delivery system potentially allows targeting to (multiple) remote sites. To allow specific distribution to the target site, a carrier/complex should ideally be inert, that is, free of intrinsic properties that would bias its biodistribution.

Synthetic vector systems package the therapeutic DNA in order to protect it from degradation, deliver it to the target cells, and finally shuttle it to the nucleus to allow expression of the transgene. Current systems protect the DNA through the formation of electrostatic complexes between cationic carrier and anionic DNA. The complexes are particulate systems and tend to be positively charged. Intrinsic physicochemical characteristics of the complex such as size and charge can strongly influence the biological properties.

The bias of biodistribution introduced by the intrinsic physicochemical properties of synthetic delivery systems frequently interferes with other more specific means of targeting such as ligands. However, when used in a controlled manner, these intrinsic properties can also provide a means of targeting in their own right, for example, the accumulation of particulate and macromolecular systems in tumours due to the enhanced penetration and retention effect.

Strategies such as coating of complexes with hydrophilic polymers have been used successfully to camouflage some of the interfering intrinsic properties of synthetic vectors. As nonspecific interactions form an important part of the cellular binding and uptake mechanism, the masked charge needs to be replaced in order to maintain efficient uptake and retain efficacy.

Specific targeting is most frequently achieved by using preexisting endogenous ligand-receptor interactions such as folate-folate receptor and transferrin-transferrin receptor. A potential drawback of endogenous ligand-receptor interactions for targeting is the background from soluble receptors, receptors in nontarget tissue, and the presence of circulating ligand. Antibodies are exogenous ligands, which avoid some of these problems. Furthermore, recent technologies for the identification of ligands (eg, phage display) have given access to a whole range of novel ligands such as antibodies, antibody fragments, and peptides.

A number of studies have recently demonstrated targeting of synthetic delivery systems (eg, using folate [60]) to remote sites such as a tumour in animal models. More importantly, tumour-targeted synthetic vectors in combination with a properly selected therapeutic gene can produce therapeutic effects [92].

The difficulty of separating intrinsic and extrinsic targeting effects represents a significant challenge for the further development of this field. The influence of intrinsic effects and nonspecific interactions becomes more difficult to predict in the complex in vivo environment. Consequently, the correlation between in vitro and in vivo efficacy of a delivery system is notoriously fragile. This means that well-controlled in vitro experiments are only of limited utility. The systematic optimisation of potentially important targeting parameters such as affinity/avidity, chemistry of conjugation, and steric situation of the coupling in vivo, however, is extremely challenging.

It is important to pursue the rapid technology transfer into the clinic with intelligent combinations of synthetic vector and therapeutic gene in order to validate the use of such carriers in vivo, create clinical safety profiles, and provide first indications how animal models correlate with clinical experience. Bearing in mind the limitations of current systems, it is however equally important to gain a deeper understanding of the complex correlation of physicochemistry and biology in order to be able to rationally design vectors that overcome systemic, cellular, and molecular barriers to genetic therapy.

REFERENCES

[1] Anderson WF. Human gene therapy. *Nature*. 1998; 392(Suppl 6679):25–30.

[2] Schätzlein AG. Non-viral vectors in cancer gene therapy: principles and progress. *Anticancer Drugs*. 2001;12(4):275–304.

[3] Verma IM, Somia N. Gene therapy—promises, problems and prospects. *Nature*. 1997;389(6648): 239–242.

[4] de Jong G, Telenius A, Vanderbyl S, Meitz A, Drayer J. Efficient in-vitro transfer of a 60-Mb mammalian artificial chromosome into murine and hamster cells using cationic lipids and dendrimers. *Chromosome Res*. 2001;9(6):475–485.

[5] Anwer K, Meaney C, Kao G, et al. Cationic lipid-based delivery system for systemic cancer gene therapy. *Cancer Gene Ther*. 2000;7(8):1156–1164.

[6] Hyde SC, Southern KW, Gileadi U, et al. Repeat administration of DNA/liposomes to the nasal epithelium of patients with cystic fibrosis. *Gene Ther*. 2000;7(13):1156–1165.

[7] Yew NS, Zhao H, Przybylska M, et al. CpG-depleted plasmid DNA vectors with enhanced safety and long-term gene expression in vivo. *Mol Ther*. 2002;5(6):731–738.

[8] Houk BE, Hochhaus G, Hughes JA. Kinetic modeling of plasmid DNA degradation in rat plasma. *AAPS PharmSci*. 1999;1(3):E9.

[9] Sparrow JT, Edwards VV, Tung C, et al. Synthetic peptide-based DNA complexes for nonviral gene delivery. *Adv Drug Deliv Rev*. 1998;30(1–3):115–131.

[10] Huebner S, Battersby BJ, Grimm R, Cevc G. Lipid-DNA complex formation: reorganization and rupture of lipid vesicles in the presence of DNA as observed by cryoelectron microscopy. *Biophys J*. 1999;76(6):3158–3166.

[11] Wang CY, Huang L. Highly efficient DNA delivery mediated by pH-sensitive immunoliposomes. *Biochemistry*. 1989;28(24):9508–9514.

[12] Perrie Y, Frederik PM, Gregoriadis G. Liposome-mediated DNA vaccination: the effect of vesicle composition. *Vaccine*. 2001;19(23-24):3301–3310.

[13] Mislick KA, Baldeschwieler JD. Evidence for the role of proteoglycans in cation-mediated gene transfer. *Proc Natl Acad Sci USA*. 1996;93(22):12349–12354.

[14] Ruponen M, Yla-Herttuala S, Urtti A. Interactions of polymeric and liposomal gene delivery systems with extracellular glycosaminoglycans: physicochemical and transfection studies. *Biochim Biophys Acta*. 1999;1415(2):331–341.

[15] Miller CR, Bondurant B, McLean SD, McGovern KA, O'Brien DF. Liposome-cell interactions in vitro: effect of liposome surface charge on the binding and endocytosis of conventional and sterically stabilized liposomes. *Biochemistry*. 1998;37(37):12875–12883.

[16] Ogris M, Brunner S, Schuller S, Kircheis R, Wagner E. PEGylated DNA/transferrin-PEI complexes:

reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery. *Gene Ther*. 1999;6(4):595–605.
[17] McLean JW, Fox EA, Baluk P, et al. Organ-specific endothelial cell uptake of cationic liposome-DNA complexes in mice. *Am J Physiol*. 1997;273(pt 2):H387–H404.
[18] Mohr L, Yoon SK, Eastman SJ, et al. Cationic liposome-mediated gene delivery to the liver and to hepatocellular carcinomas in mice. *Hum Gene Ther*. 2001;12(7):799–809.
[19] Cao YJ, Shibata T, Rainov NG. Liposome-mediated transfer of the bcl-2 gene results in neuroprotection after in vivo transient focal cerebral ischemia in an animal model. *Gene Ther*. 2002;9(6):415–419.
[20] Caplen NJ, Alton EWFW, Middleton PG, et al. Liposome-mediated CFTR gene transfer to the nasal epithelium of patients with cystic fibrosis. *Nat Med*. 1995;1(1):39–46.
[21] Sanders NN, Van Rompaey E, De Smedt SC, Demeester J. On the transport of lipoplexes through cystic fibrosis sputum. *Pharm Res*. 2002;19(4):451–456.
[22] Sanders NN, De Smedt SC, Cheng SH, Demeester J. Pegylated GL67 lipoplexes retain their gene transfection activity after exposure to components of CF mucus. *Gene Ther*. 2002;9(6):363–371.
[23] Thurston G, McLean JW, Rizen M, et al. Cationic liposomes target angiogenic endothelial cells in tumors and chronic inflammation in mice. *J Clin Invest*. 1998;101(7):1401–1413.
[24] Uyechi LS, Gagne L, Thurston G, Szoka FC Jr. Mechanism of lipoplex gene delivery in mouse lung: binding and internalization of fluorescent lipid and DNA components. *Gene Ther*. 2001;8(11):828–836.
[25] Barron LG, Gagne L, Szoka FC Jr. Lipoplex-mediated gene delivery to the lung occurs within 60 minutes of intravenous administration. *Hum Gene Ther*. 1999;10(10):1683–1694.
[26] Goula D, Becker N, Lemkine GF, et al. Rapid crossing of the pulmonary endothelial barrier by polyethylenimine/DNA complexes. *Gene Ther*. 2000;7(6):499–504.
[27] Sokol PP, Longenecker KL, Kachel DL, Martin WJ 2nd. Mechanism of putrescine transport in human pulmonary artery endothelial cells. *J Pharmacol Exp Ther*. 1993;265(1):60–66.
[28] Ma Z, Mi Z, Wilson A, et al. Redirecting adenovirus to pulmonary endothelium by cationic liposomes. *Gene Ther*. 2002;9(3):176–182.
[29] Torchilin VP. Affinity liposomes in vivo: factors influencing target accumulation. *J Mol Recognit*. 1996;9(5-6):335–346.
[30] Griesenbach U, Chonn A, Cassady R, et al. Comparison between intratracheal and intravenous administration of liposome-DNA complexes for cystic fibrosis lung gene therapy. *Gene Ther*. 1998;5(2):181–188.
[31] Liu Y, Mounkes LC, Liggitt HD, et al. Factors influencing the efficiency of cationic liposome-mediated intravenous gene delivery. *Nat Biotechnol*. 1997;15(2):167–173.
[32] Hofland HE, Nagy D, Liu JJ, et al. In vivo gene transfer by intravenous administration of stable cationic lipid/DNA complex. *Pharm Res*. 1997;14(6):742–749.
[33] Zhu N, Liggitt D, Liu Y, Debs R. Systemic gene expression after intravenous DNA delivery into adult mice. *Science*. 1993;261(5118):209–211.
[34] Liu F, Song Y, Liu D. Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. *Gene Ther*. 1999;6(7):1258–1266.
[35] Eatock MM, Schätzlein AG, Kaye SB. Tumour vasculature as a target for anticancer therapy. *Cancer Treat Rev*. 2000;26(3):191–204.
[36] Jain RK. Delivery of molecular and cellular medicine to solid tumors. *Adv Drug Deliv Rev*. 1997;26(2-3):71–90.
[37] Hashizume H, Baluk P, Morikawa S, et al. Openings between defective endothelial cells explain tumor vessel leakiness. *Am J Pathol*. 2000;156(4):1363–1380.
[38] Maeda H. The tumor blood vessel as an ideal target for macromolecular anticancer agents. *J Control Release*. 1992;19(1–3):315–324.
[39] Yuan F, Leunig M, Huang SK, Berk DA, Papahadjopoulos D, Jain RK. Microvascular permeability and interstitial penetration of sterically stabilized (stealth) liposomes in a human tumor xenograft. *Cancer Res*. 1994;54(13):3352–3356.
[40] Gabizon A, Catane R, Uziely B, et al. Prolonged circulation time and enhanced accumulation in malignant exudates of doxorubicin encapsulated in polyethylene-glycol coated liposomes. *Cancer Res*. 1994;54(4):987–992.
[41] Vasey PA, Kaye SB, Morrison R, et al. Phase I clinical and pharmacokinetic study of PK1 [N-(2-hydroxypropyl)methacrylamide copolymer doxorubicin]: first member of a new class of chemotherapeutic agents-drug-polymer conjugates. Cancer Research Campaign Phase I/II Committee. *Clin Cancer Res*. 1999;5(1):83–94.
[42] Winterhalter M, Frederik PM, Vallner JJ, Lasic DD. Stealth(R) liposomes: from theory to product. *Adv Drug Deliv Rev*. 1997;24(2-3):165–177.
[43] Hong K, Zheng W, Baker A, Papahadjopoulos D. Stabilization of cationic liposome-plasmid DNA complexes by polyamines and poly(ethylene glycol)-phospholipid conjugates for efficient in vivo gene delivery. *FEBS Lett*. 1997;400(2):233–237.
[44] Katayose S, Kataoka K. Remarkable increase in nuclease resistance of plasmid DNA through supramolecular assembly with poly(ethylene glycol)-poly(L-lysine) block copolymer. *J Pharm Sci*. 1998;87(2):160–163.
[45] Choi YH, Liu F, Kim JS, Choi YK, Park JS, Kim SW. Polyethylene glycol-grafted poly-L-lysine as

polymeric gene carrier. *J Control Release*. 1998;54(1): 39–48.

[46] Brown MD, Schätzlein AG, Brownlie A, et al. Preliminary characterization of novel amino acid based polymeric vesicles as gene and drug delivery agents. *Bioconjug Chem*. 2000;11(6):880–891.

[47] Blessing T, Kursa M, Holzhauser R, Kircheis R, Wagner E. Different strategies for formation of pegylated EGF-conjugated PEI/DNA complexes for targeted gene delivery. *Bioconjug Chem*. 2001;12(4):529–537.

[48] Mao HQ, Roy K, Troung-Le VL, et al. Chitosan-DNA nanoparticles as gene carriers: synthesis, characterization and transfection efficiency. *J Control Release*. 2001;70(3):399–421.

[49] Toncheva V, Wolfert MA, Dash PR, et al. Novel vectors for gene delivery formed by self-assembly of DNA with poly(L-lysine) grafted with hydrophilic polymers. *Biochim Biophys Acta*. 1998;1380(3):354–368.

[50] Oupicky D, Ogris M, Howard KA, Dash PR, Ulbrich K, Seymour LW. Importance of lateral and steric stabilization of polyelectrolyte gene delivery vectors for extended systemic circulation. *Mol Ther*. 2002;5(4): 463–472.

[51] Ochietti B, Guerin N, Vinogradov SV, et al. Altered organ accumulation of oligonucleotides using polyethyleneimine grafted with poly(ethylene oxide) or pluronic as carriers. *J Drug Target*. 2002;10(2): 113–121.

[52] Fisher KD, Stallwood Y, Green NK, Ulbrich K, Mautner V, Seymour LW. Polymer-coated adenovirus permits efficient retargeting and evades neutralising antibodies. *Gene Ther*. 2001;8(5):341–348.

[53] Lee RJ, Huang L. Folate-targeted, anionic liposome-entrapped polylysine-condensed DNA for tumor cell-specific gene transfer. *J Biol Chem*. 1996;271(14): 8481–8487.

[54] Simoes S, Slepushkin V, Gaspar R, de Lima MC, Duzgunes N. Gene delivery by negatively charged ternary complexes of DNA, cationic liposomes and transferrin or fusigenic peptides. *Gene Ther*. 1998; 5(7):955–964.

[55] Kircheis R, Wightman L, Schreiber A, et al. Polyethylenimine/DNA complexes shielded by transferrin target gene expression to tumors after systemic application. *Gene Ther*. 2001;8(1):28–40.

[56] Scherer F, Anton M, Schillinger U, et al. Magnetofection: enhancing and targeting gene delivery by magnetic force in vitro and in vivo. *Gene Ther*. 2002;9(2):102–109.

[57] Wang S, Low PS. Folate-mediated targeting of antineoplastic drugs, imaging agents, and nucleic acids to cancer cells. *J Control Release*. 1998;53(1–3):39–48.

[58] Mislick KA, Baldeschwieler JD, Kayyem JF, Meade TJ. Transfection of folate-polylysine DNA complexes: evidence for lysosomal delivery. *Bioconjug Chem*. 1995;6(5):512–515.

[59] Guo W, Lee RL. Receptor-targeted gene delivery via folate-conjugated polyethylenimine. *AAPS PharmSci*. 1999;1(4):E19.

[60] Hofland HE, Masson C, Iginla S, et al. Folate-targeted gene transfer in vivo. *Mol Ther*. 2002;5(6): 739–744.

[61] Wu CH, Wilson JM, Wu GY. Targeting genes: delivery and persistent expression of a foreign gene driven by mammalian regulatory elements in vivo. *J Biol Chem*. 1989;264(29):16985–16987.

[62] Stankovics J, Crane AM, Andrews E, Wu CH, Wu GY, Ledley FD. Overexpression of human methylmalonyl CoA mutase in mice after in vivo gene transfer with asialoglycoprotein/polylysine/DNA complexes. *Hum Gene Ther*. 1994;5(9):1095–1104.

[63] Hisayasu S, Miyauchi M, Akiyama K, Gotoh T, Satoh S, Shimada T. In vivo targeted gene transfer into liver cells mediated by a novel galactosyl-D-lysine/D-serine copolymer. *Gene Ther*. 1999;6(4):689–693.

[64] Hashida M, Takemura S, Nishikawa M, Takakura Y. Targeted delivery of plasmid DNA complexed with galactosylated poly(L-lysine). *J Control Release*. 1998;53(1–3):301–310.

[65] Stavridis JC, Deliconstantinos G, Psallidopoulos MC, Armenakas NA, Hadjiminas DJ, Hadjiminas J. Construction of transferrin-coated liposomes for in vivo transport of exogenous DNA to bone marrow erythroblasts in rabbits. *Exp Cell Res*. 1986; 164(2):568–572.

[66] Phylchenkov AA, Slukvin I, Kudryavets YI, et al. Expression and functional-activity of transferrin receptor in human tumor-cell of different histogenesis. *Eksperimentalnaya Onkologiya*. 1992;14(6):22–27.

[67] Goding J. *CD71*. NCBI, 1998. http://www.ncbi.nlm.nih.gov/PROW/guide/1445562251_g.htm.

[68] Simoes S, Slepushkin V, Pires P, Gaspar R, de Lima MP, Duzgunes N. Mechanisms of gene transfer mediated by lipoplexes associated with targeting ligands or pH-sensitive peptides. *Gene Ther*. 1999;6(11):1798–1807.

[69] Cheng PW. Receptor ligand-facilitated gene transfer: enhancement of liposome-mediated gene transfer and expression by transferrin. *Hum Gene Ther*. 1996;7(3):275–282.

[70] Cotten M, Langle-Rouault F, Kirlappos H, et al. Transferrin-polycation-mediated introduction of DNA into human leukemic cells: stimulation by agents that affect the survival of transfected DNA or modulate transferrin receptor levels. *Proc Natl Acad Sci USA*. 1990;87(11):4033–4037.

[71] Kircheis R, Kichler A, Wallner G, et al. Coupling of cell-binding ligands to polyethylenimine for targeted gene delivery. *Gene Ther*. 1997;4(5):409–418.

[72] Plank C, Tang MX, Wolfe AR, Szoka FC Jr. Branched cationic peptides for gene delivery: role of type and number of cationic residues in formation and in vitro activity of DNA polyplexes. [published erratum appears in *Hum Gene Ther*. 1999;10(13):2272] *Hum Gene Ther*. 1999;10(2):319–332.

[73] Voldborg BR, Damstrup L, Spang-Thomsen M, Poulsen HS. Epidermal growth factor receptor (EGFR) and EGFR mutations, function and possible role in clinical trials. *Ann Oncol*. 1997;8(12):1197–1206.

[74] Cristiano RJ, Roth JA. Epidermal growth factor mediated DNA delivery into lung cancer cells via the epidermal growth factor receptor. *Cancer Gene Ther*. 1996;3(1):4–10.

[75] Kikuchi A, Sugaya S, Ueda H, et al. Efficient gene transfer to EGF receptor overexpressing cancer cells by means of EGF-labeled cationic liposomes. *Biochem Biophys Res Commun*. 1996;227(3):666–671.

[76] Medina-Kauwe LK, Maguire M, Kasahara N, Kedes L. Nonviral gene delivery to human breast cancer cells by targeted Ad5 penton proteins. *Gene Ther*. 2001;8(23):1753–1761.

[77] Liu X, Tian P, Yu Y, Yao M, Cao X, Gu J. Enhanced antitumor effect of EGF R-targeted p21WAF-1 and GM-CSF gene transfer in the established murine hepatoma by peritumoral injection. *Cancer Gene Ther*. 2002;9(1):100–108.

[78] Mammen M, Choi SK, Whitesides GM. Polyvalent interactions in biological systems: implications for design and use of multivalent ligands and inhibitors. *Angew Chem Int Ed Engl*. 1998;37(20):2754–2794.

[79] Ding ZM, Cristiano RJ, Roth JA, Takacs B, Kuo MT. Malarial circumsporozoite protein is a novel gene delivery vehicle to primary hepatocyte cultures and cultured cells. *J Biol Chem*. 1995;270(8):3667–3676.

[80] Muller M, Gissmann L, Cristiano RJ, et al. Papillomavirus capsid binding and uptake by cells from different tissues and species. *J Virol*. 1995;69(2):948–954.

[81] Nilsson F, Tarli L, Viti F, Neri D. The use of phage display for the development of tumour targeting agents. *Adv Drug Deliv Rev*. 2000;43(2-3):165–196.

[82] Xu L, Tang WH, Huang CC, et al. Systemic p53 gene therapy of cancer with immunolipoplexes targeted by anti-transferrin receptor scFv. *Mol Med*. 2001;7(10):723–734.

[83] Gupta S, Eastman J, Silski C, Ferkol T, Davis PB. Single chain Fv: a ligand in receptor-mediated gene delivery. *Gene Ther*. 2001;8(8):586–592.

[84] Vaysse L, Guillaume C, Burgelin I, Gorry P, Ferec C, Arveiler B. Proteolipidic vectors for gene transfer to the lung. *Biochem Biophys Res Commun*. 2002;290(5):1489–1498.

[85] Harbottle RP, Cooper RG, Hart SL, et al. An RGD-oligolysine peptide: a prototype construct for integrin-mediated gene delivery. *Hum Gene Ther*. 1998;9(7):1037–1047.

[86] Li S, Tan Y, Viroonchatapan E, Pitt BR, Huang L. Targeted gene delivery to pulmonary endothelium by anti-PECAM antibody. *Am J Physiol Lung Cell Mol Physiol*. 2000;278(3):L504–L511.

[87] Merwin JR, Carmichael EP, Noell GS, et al. CD5-mediated specific delivery of DNA to T lymphocytes: compartmentalization augmented by adenovirus. *J Immunol Methods*. 1995;186(2):257–266.

[88] Uherek C, Fominaya J, Wels W. A modular DNA carrier protein based on the structure of diphtheria toxin mediates target cell-specific gene delivery. *J Biol Chem*. 1998;273(15):8835–8841.

[89] Wang CY, Huang L. pH-sensitive immunoliposomes mediate target-cell-specific delivery and controlled expression of a foreign gene in mouse. *Proc Natl Acad Sci USA*. 1987;84(22):7851–7855.

[90] Trubetskoy VS, Torchilin VP, Kennel SJ, Huang L. Use of N-terminal modified poly(L-lysine)-antibody conjugate as a carrier for targeted gene delivery in mouse lung endothelial cells. *Bioconjug Chem*. 1992;3(4):323–327.

[91] Fominaya J, Wels W. Target cell-specific DNA transfer mediated by a chimeric multidomain protein. Novel non-viral gene delivery system. *J Biol Chem*. 1996;271(18):10560–10568.

[92] Kircheis R, Wightman L, Kursa M, Ostermann E, Wagner E. Tumor-targeted gene delivery: an attractive strategy to use highly active effector molecules in cancer treatment. *Gene Ther*. 2002;9(11):731–735.

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