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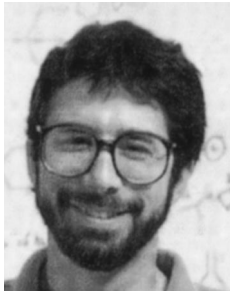
Gene Therapy for Cancer*

J. Gómez-Navarro,[1,2] D.T. Curiel[1,3] and J.T. Douglas[1,3]

[1]Gene Therapy Center; [2]Division of Hematology/Oncology; and [3]Division of Pulmonary and Critical Care Medicine, The University of Alabama at Birmingham, 1824 Sixth Avenue South, Birmingham, Alabama 35294-3300, U.S.A

Jesús Gómez-Navarro is a medical oncologist trained at the Hospital La Paz, Universidad Autonoma de Madrid, Madrid, Spain. He received further post-doctoral training in gene therapy at the Gene Therapy Center, University of Alabama at Birmingham (UAB), Birmingham, Alabama, U.S.A., where he is currently a Research Assistant Professor in the Division of Hematology/Oncology.



David T. Curiel is Director of the Gene Therapy Center at the University of Alabama at Birmingham (UAB), Alabama, U.S.A. He is also Professor of Medicine and Jeanne and Anne Griffin Chair for Women's Cancer Research at UAB.

Joanne T. Douglas received postdoctoral training in the Gene Therapy Center, University of Alabama at Birmingham, Alabama, U.S.A., where she is currently a Research Assistant Professor in the Department of Medicine.

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Correspondence to D.T. Curiel, e-mail: david.curiel@ccc.uab.edu
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WHY GENE THERAPY?

It is well established that most cancers result from a series of accumulated, acquired genetic lesions in somatic cells that are faithfully reproduced until a malignant clone is created, which is ultimately able to destroy the host. To a larger and larger extent, the genetic lesions associated with malignant transformation and progression in a wide variety of human cancers are being identified [1, 2]. Armed with this knowledge of the molecular anatomy of the cancer cell, gene therapy has emerged as a new method of therapeutic and possibly preventive intervention against cancer targeted at the level of cellular gene expression [3]. In this approach, the complex cancerous pathophysiological state is altered by delivering nucleic acids into tumoral or normal cells. These nucleic acids may be genes, portions of genes, oligonucleotides, or RNA. In conventional therapeutics, as in pharmacotherapy, a cell or tissue phenotype is altered by modifying cell physiology or metabolism at the level of protein expression. In contrast, in gene therapy this is accomplished by changing the pattern of expression of genes whose products may thus achieve the desired effect on the cellular phenotype.

In the treatment of human disease, gene therapy strategies may offer the potential to achieve a much higher level of specificity of action than conventional drug therapeutics by virtue of the highly specific control and regulatory mechanisms of gene expression that may be targeted. Additionally, interceding at an earlier, upstream step in disease pathogenesis may offer greater potential to induce fundamental changes in phenotypic parameters of disease, with a more favourable clinical outcome. The availability of gene transfer systems, or vectors, for permanent or long-term genetic modification of cells and tissues should allow definitive therapeutic or preventive interventions. Furthermore, gene transfer may be accomplished in a limited loco-regional context, producing a high concentration of therapeutic molecules in the local area. Thus, undesired systemic effects of those therapeutic molecules are avoided. Lastly, using the body to produce therapeutic proteins, potentially in only certain tissues, has practical advantages of its own [4]. Briefly, limitations associated with manufacture, stability, and duration of effect after administration of drugs based on synthetic peptides are completely avoided. From the same pharmacological point of view, designer drugs based on small molecules, currently under intensive investigation, can hardly substitute the function of complex defective proteins, such as many products of tumour suppressor genes.

In the treatment of human malignant tumours, several obstacles explain the limitations of currently available treatments for achieving definitive cures in most cases of advanced disease (Table 1). It is apparent that a combination of new chemotherapy drugs, higher doses of drugs, novel cytokines, improved regimens of radiotherapy, and more sophisticated surgery can achieve incremental improvements in cancer treatment. But these therapies do not address critical biological obstacles and, thus, probably will not bring the much-needed radical advances in the implementation and results of cancer treatment. In contrast, gene therapy offers the potential for overcoming some of these fundamental barriers (Table 1).

Table 1. *Potential contributions of gene therapy to overcome obstacles for curing cancer*

Obstacles to curing cancer	Potential contribution of gene transfer
1. All tumours are *genetically unstable* and thus they are extraordinarily adaptable to environmental changes.	Gene transfer of DNA repair or cell cycle checkpoint genes that restore DNA stability and cell susceptibility to therapeutic insults.
2. Tumours are heterogeneous in many respects, including genetic mutations, expression of oncoproteins, immunogenicity, response to environmental changes, etc.	Targeting of genetically homogeneous and stable tissues, such as the tumour vasculature and stroma; genetic immunopotentiation; chimeric vectors.
3. As a consequence of obstacles 1 and 2, tumours have, or acquire, *resistance* to cellular toxins and to many other therapeutically induced cellular insults.	Strategies above, associated with chemotherapy or radiotherapy or with the transfer of additional genes that sensitise tumour cells to drugs or radiation.
4. Tumours can have a low *cellular growth fraction*; therefore, they are less susceptible to mitotic toxins and to gene transfer vectors that require dividing cells.	Use of vectors that do not require cellular division for gene delivery and expression (adenovirus, herpesvirus, lentivirus, chimeric vectors); repeated administration of non-immunogenic vectors.
5. Tumours form *metastases*, which have to be reached systemically to eradicate the tumour completely.	Use of targetable, injectable vectors (tropism-modified viruses, cellular vehicles, liposomes); genetic immunopotentiation.
6. Tumours do not express specific tumour antigens or immune costimulatory molecules; alternatively, tumours down-regulate antigen-presentation, induce *immunological tolerance*, or inhibit the effector mechanisms of the immune response.	Transfer of genes encoding costimulatory molecules and cytokines; genetic modification of antigen-presenting cells; induce inflammatory reactions that activate antigen presentation; transfer of genes blocking tumour-secreted inhibitors of the immune response.
7. The *spontaneous* behaviour of human tumours is somewhat different from that of malignant cells *in vitro*, and from that of experimental tumours in animal models.	Development of better animal models, including tumour models in transgenic mice.
8. Tumours are diagnosed in *advanced stages*, when billions of tumour cells exist in the body, frequently widely disseminated.	Development of amplification vector systems (replicative viral vectors and exploitation of bystander effects); use of targetable, injectable vectors; genetic immunopotentiation.
9. The understanding and treatment of cancer requires the contribution of very *diverse fields* of basic knowledge, biotechnology, and medical practice.	*De facto* multidisciplinary recruitment of gene therapy researchers.

STRATEGIES

A number of strategies have been developed to accomplish cancer gene therapy. These approaches include (1) mutation compensation, (2) molecular chemotherapy, and (3) genetic immunopotentiation. For mutation compensation, gene therapy techniques are designed to correct the molecular lesions that are aetiologic of malignant transformation, or avoid the contribution to malignant growth by tumour-supporting non-malignant stromal cells. For molecular chemotherapy, methods have been developed to achieve selective delivery or expression of a toxin gene in cancer or stromal cells to induce their eradication, or alternatively to increase their sensitivity to concomitant chemotherapy or radiotherapy. Also, attempts have been made to deliver genetic sequences that protect normal bone marrow cells from the toxic effects of standard chemotherapeutic drugs, thus allowing the administration of higher drug doses without reaching otherwise limiting myelosuppression. Genetic immunopotentiation strategies attempt to achieve active immunisation against tumour-associated antigens by gene transfer methodologies. Both tumour cells and cellular components of the immune system have been genetically modified to this end. Importantly, each of these approaches has been rapidly translated into human gene therapy clinical trials [5] as summarised in Table 2.

In this review, we examine the lessons learned from the results of the first attempts to apply each gene therapy strategy in human cancers. In each section, we show both the rationale of gene therapy and the problems encountered in its development, emphasising the general biological concepts of each therapeutic strategy. Finally, we illustrate prospects for overcoming the obstacles to implementation of gene therapy by novel methods that are currently being refined.

MUTATION COMPENSATION

The knowledge of the major role that growth factors, signalling molecules, cell cycle regulators, and determinant factors of angiogenesis, invasiveness and metastasis play in neoplastic progression has positive implications for gene therapy. That is, it is possible to abrogate the malignant phenotype by correcting the underexpression of tumour suppressor genes or overexpression of oncogenes involved in these phenomena. At the level of the single cell, the inactivation of tumour suppressor genes contributes to the neoplastic phenotype by abrogating critical cell cycle checkpoints, DNA repair mechanisms, and pro-apoptotic controls. To approach this loss of function, the logical intervention is replacement of the deficient function with the wild-type gene. Mutations of more than 24 tumour suppressor genes have been described in numerous cancers. Of these, *p53*, *RB1*, and *BRCA1* are currently being administered in clinical trials as replacements for the mutated counterparts (Table 3). In all these cases, pre-clinical studies showed some expression of the wild-type protein after gene delivery and reversion of the malignant phenotype, frequently associated with the induction of apoptosis in tumour cells [6]. Of note, however, some tumours have shown persistent tumorigenicity and proliferation after successful restoration and expression of wild-type genes, a phenomenon referred to as 'tumour suppressor resistance'. Other major obstacles are mentioned later.

For dominant oncogenes, it is the aberrant expression of the corresponding gene product that elicits the associated neoplastic transformation. In this context, the molecular therapeutic intervention is designed to ablate expression of the dominant oncogene. Inhibition of oncogenic function can be attempted at three levels. First, transcription of the oncogene can be inhibited. This approach uses triplex-forming oligonucleotides or other sequences that bind transcriptional start sites in the genomic DNA. An example, currently being clinically tested, is based on the adenoviral gene E1A, that inhibits transcription of the human c-*erbB-2* promoter and accordingly suppresses the tumorigenicity and metastatic potential induced by the *erbB-2* oncogene. Second, translation of the oncogene messenger RNA can be blocked using specific antisense sequences, which function by promoting degradation of the complementary message [7]. Evidence for a specific effect of antisense molecules has been particularly compelling in selected cases, and these molecules are currently undergoing clinical tests. These include antisense sequences against insulin-like growth factor 1 in glioma, K-*ras* in lung cancer, c-*myc* in breast and prostate cancer, and TGFβ in glioma (Table 3). Practical constraints have limited wide employment of this technology in protocols of human anticancer gene therapy, including the idiosyncratic efficacy of specific antisense for a given target gene and the suboptimal delivery of antisense molecules. Third, mobilisation of the nascent oncoprotein can be blocked or its function can be inhibited when in its final cell location. These last strategies involve the use of 'intracellular antibodies' that intercept and interfere with the intracellular processing of the oncoprotein, or the heterologous expression of mutant proteins that can inhibit the function of the native oncoprotein,

Table 2. Clinical trials of gene therapy for the treatment of cancer

Strategy	Clinical trials*	Molecular mechanism of anticancer effect
Mutation compensation	6	Inhibition of expression of dominant oncogenes.
	18	Augmentation of deficient tumour suppressor genes.
	2	Abrogation of autocrine growth factor loops (single chain antibodies).
Molecular chemotherapy	27	Selective delivery of toxin or toxin gene to cancer cells.
	9	Chemoprotection of normal tissues during high-dose chemotherapy.
Genetic immunopotentiation	54	*In vitro* transduction—augmentation of tropism or cell killing capacity of tumour-infiltrating lymphocytes; genetic modification of irradiated tumour cells.
	47	*In vivo* transduction—administration of costimulatory molecules or cytokines; immunisation with virus encoding tumour-associated antigens.
Viral-mediated oncolysis	2	Tumour cell lysis by viral vector replication.

*Registered in the NIH Office of Recombinant DNA Activities in December 1998 (http://www.nih.gov/od/orda/protocol.pdf).

Table 3. Mutations compensation strategies used clinically

Target*	Strategy	Vector	Tumour type
p53	Replacement of tumour suppressor gene	Adenovirus	Non-small cell lung cancer, head and neck squamous cell carcinoma, hepatic metastases of colon cancer, hepatocellular carcinoma, prostate cancer, breast cancer.
RB (Retinoblastoma)	Replacement of tumour suppressor gene	Adenovirus	Bladder cancer
BRCA-1	Replacement of tumour suppressor gene	Retrovirus	Ovarian cancer
*erb*B-2	Inhibition of promoter by E1A	Cationic liposome complex	Breast and ovarian cancers overexpressing erbB-2
Insulin-like growth factor 1	Blockade by antisense	Cationic liposome complex	Glioblastomas
k-*ras*	Blockade by antisense	Retrovirus	Non-small cell lung cancer
c-*myc*, c-*fos*	Blockade by antisense	Retrovirus	Breast and prostate cancers
TGFβ	Blockade by antisense	Plasmid and electroporation	Glioblastoma
erbB-2	scFv	Adenovirus	Ovarian cancer

*Registered in the NIH Office of Recombinant DNA Activities December of 1998 (http://www.nih.gov/od/orda/protocol.htm) scFv: single-chain intracellular antibody.

respectively. We have shown, for instance, that intracellular expression of an anti-erbB-2 single-chain antibody (scFv) results in down-regulation of cell surface erbB-2 expression and selective cytotoxicity in tumour cells expressing the oncogene target both *in vitro* and *in vivo* [8, 9].

TUMOUR PHENOMENA DEPENDENT ON MULTIPLE GENES

Angiogenesis

The development of new blood vessels is a critical step in the growth, progression, and metastatic spread of both solid and haematopoietic tumours. Despite heterogeneity in many other respects, all tumours thus share a universal feature, i.e. they depend absolutely on the vasculature not only to sustain their initial growth and dissemination but also to maintain their long-term viability. Extensive experimental data and clinical-pathological studies support this contention (reviewed in: Special Issue on Angiogenesis, *European Journal of Cancer*, Vol. **32**, issue 14, 1996). Vessel targeting, therefore, should be useful for the treatment of most kinds of cancer, either to impede the formation of tumour blood vessels, as initially proposed by Folkman (see Special Issue), or as an attempt to destroy the already formed tumour vasculature, as proposed later by Denekamp and others [10–12]. From the points of view of oncology and gene therapy, two features of this strategy seem most attractive. First, the genetic stability of endothelial cells should essentially eliminate the appearance of resistance to molecular therapeutic interventions [13], which is so pervasive in the treatment of tumour cells. This hypothesis has been confirmed in a cancer animal model that evaluated treatment with the natural angiogenesis inhibitor endostatin [14]. Second, an additional advantage of targeted killing of endothelial cells is the highly amplified killing effect of large numbers of tumour cells when deprived of their vascularisation. This can partially overcome current limitations in the number of cells modified by gene transfer *in vivo*.

In the last decade, anti-angiogenic drugs targeted to the proliferating endothelium of tumours and other diseases have been applied in the clinical setting and have entered clinical trials. In addition, the association of chemo- or radiotherapy with anti-angiogenic agents has been shown to produce an enhanced anti-tumour effect in preclinical models. Notably, combined treatments can achieve cures that are not observed with either treatment alone [15]. Thus, molecular therapeutic interventions against the tumour and its vasculature are not only strongly appealing on theoretical grounds for their use in a variety of clinical contexts, but their utility is also rapidly being tested clinically [16]. Based on this, genetic modification of the endothelium of tumour vasculature has been proposed as an alternative therapeutic modality [17, 18]. With this genetic strategy, the problems of previously explored approaches can be potentially overcome. For instance, local production of high levels of therapeutic proteins can be induced, thus obviating or diminishing the difficulties associated with systemic toxicity, and also pharmacological issues, such as largescale manufacture, bioavailability, and cost of ordinary drugs. In addition, the ability to release the gene product continuously may be relevant in certain cases, such as for the appropriate anti-angiogenic effect of interferon gamma.

Both suppression of angiogenic cellular signals and augmentation of natural inhibitors of angiogenesis have proved to be feasible strategies in *in vivo* tumour models. Examples of effective genetic interventions for the suppression of angiogenesis factors are the down-regulation of vascular endothelial growth factor (VEGF) by antisense molecules, as shown in models of glioma [19, 20], and the blockade of VEGF receptor function by delivery of mutant versions of one of its cognate membrane receptors, Flk-1 [21, 22], or of a secreted soluble version of its other receptor, Flt-1 [23, 24]. In addition, similar results have been obtained by adenoviral-mediated delivery of a soluble receptor analogous to the endothelium-specific Tie2 receptor [25], also known to play a role in tumour angiogenesis. Conversely, the replacement or supplementation of inhibitors of angiogenesis has been attempted by transfecting cells with the thrombospondin gene [26] and by using *in vivo* viral vectors that encode soluble platelet factor 4 [27] and angiostatin [28, 29]. However,

none of these strategies has been clinically tested and major issues, mostly vectorological, are still to be solved. Most obvious are the problems of assuring highly efficient gene delivery and long-term expression of the therapeutic anti-angiogenic genes to keep the tumour deprived of its growth-enabling vascularisation. In addition, the current lack of targetable, injectable vectors impedes the application of anti-angiogenesis gene-based strategies to multiple foci of tumour that characterise disseminated cancer. Lastly, different combinations of endothelial growth factors and their receptors are altered in different tumours, and may even change in single tumours during different stages of progression. Thus, despite its powerful rationale, the successful clinical implementation of anti-angiogenesis gene therapy will still require major developments.

Anti-angiogenesis seems a therapeutic manoeuvre mostly appropriate for avoiding tumour progression but, as mentioned above, alternative anti-vascular strategies have been proposed with the intention of destroying existing vasculature, thereby depriving the tumour of essential vascularisation. To date, there have been few attempts to induce direct toxicity in the vasculature of normal or tumour vasculature by gene transfer [30, 31], but development of targeted vectors should prompt immediate evaluation of such strategies.

Invasion and metastasis

Increasingly, genes and proteins involved in phenotypic aspects of tumours, other than disordered proliferation, are being described and identified as potentially useful therapeutic targets. In this regard, besides angiogenesis, one fundamental component of the metastatic cascade is the local invasion of the extracellular matrix by tumour cells. Studies in animal models have begun to show that modulation by gene transfer of molecules involved in degradation of extracellular matrix, cellular motility, and cellular adhesion, such as plasminogen activators, metalloproteinases and CD44, has the potential for inhibiting tumour cell spread [32]. To have clinical utility, however, these manoeuvres should provide long-term abrogation of the involved molecules and be used when the tumour is going through the earlier steps of the metastatic cascade in a particular patient.

Apoptosis

The highly orchestrated form of cell death known as apoptosis goes awry to some extent in most cancers. Increasingly, a general theme in cancer pathophysiology is the development of a defect in the function of pro-apoptotic molecules, such as p53, that commonly prepare the cell for apoptosis whenever cell proliferation or DNA damage is induced, their absence thus depriving the cell of a critical safety mechanism [33]. Alternatively, a functional excess of anti-apoptotic molecules, such as Bcl-2, may also occur in tumours. In each case, the result is an imbalance that favours the inappropriate survival of tumour cells. The mechanisms involved are attractive therapeutic targets because the tumour cell is totally dependent on them for its survival, and appears to have a higher sensitivity to the induction of apoptosis than normal tissues [33]. In addition, restoring or enhancing the capacity to undergo apoptosis may, in some cases, be a crucial event which renders tumours sensitive to classical anticancer agents, such as chemotherapy [34, 35] and radiotherapy [36, 37].

With the increasing recognition of the molecular basis of the apoptotic pathway [33, 38–40], and the description of several of its components acting as oncogenes or tumour suppressor genes, gene therapy has emerged as a rational strategy for the modulation of apoptosis. Therefore, the genetic modification of tumour cells and their supportive stroma with genes that modulate the apoptotic process has been recently proposed for the gene therapy of cancer [6, 41–43]. Three general requirements for the successful therapeutic application of genetic modulation of apoptosis in cancers are apparent. First, significantly better gene transfer vectors may be needed to modify and trigger apoptosis in most malignant cells in any given tumour. Current vectors are far from achieving *in vivo* the requisite high levels of tumour cell modification. Alternatively, mechanisms may be implemented regionally to amplify the effects of the expression of transferred genes, i.e. by inducing a bystander apoptosis. Second, given the ubiquity of the numerous cellular proteins involved in apoptotic pathways, selective activation in cancer cells of the lethal processes may also be a critical requirement of therapeutic manoeuvres. The lower threshold for undergoing apoptosis that characterises tumour cells could, however, offer an advantageous therapeutic window that makes this requirement less stringent. Third, given the complexity and redundancy of the signalling circuits involved, modulation of several components of the apoptotic pathways may be needed to provoke cell death. Interventions downstream in the circuits might also be preferable to avoid regulatory counterbalances.

Despite the theoretical constraints just mentioned, preliminary attempts to explore the therapeutic modulation of apoptosis against cancer by gene transfer have already begun, driven by encouraging preclinical data in animal models. Clinical trials are currently ongoing evaluating the value of pro-apoptotic *p53* and adenoviral E1A, and a growing number of other candidate genes are being considered and tested preclinically (Table 4).

Obstacles to mutation compensation

Although the strategies currently used for the restoration of normal genes and ablation of mutant genes have offered in-depth insights into the molecular biology of carcinogenesis and tumour progression, they face critical problems that restrict their clinical application. Human tumours are remarkably heterogeneous in the patterns of expression of relevant oncogenes. Thus, therapeutic targeting of a single molecular abnormality may have only an inconsequential impact on the clinical management of the disease, considering both the population and individual patients. In addition, several mutated genes produce molecules with transdominant effects, thus necessitating the blocking of their effects and not merely their supplementation with a wild-type version of the gene. Furthermore, because these strategies mostly modulate intracellular responses, nearly every tumour cell might have to be targeted for these approaches to be clinically effective. The current state of development of gene therapy vectors, both viral and non-viral, makes this feat unachievable within non-toxic margins of vector dose. Clearly, breakthrough developments in vector technology are needed for these obstacles to be overcome. A better understanding of the tumour-supportive micro-environment and of multicellular tumour phenomena may also suggest genetic interventions that, even with a limited gene transfer, can elicit widespread effects in the tumour. In addition, approaches such as molecular chemotherapy or immune system augmentation that exhibit an amplified regional or systemic effect hold the promise of tackling some of the aforementioned limitations.

Table 4. *Genetic modulation of apoptosis for cancer therapy*

Strategy	Target genes or molecules
Add or restore pro-apoptotic molecules	
Induce exogenous death signals (ligand/death receptor)	Granzyme B/Perforin, FasL (CD95L)/Fas (CD95), TNF/TNFR1, Apo3L/DR3, Apo2L(TRAIL)/DR4 or DR5
Induce endogenous triggers of apoptosis	Cytochrome c, *TP53*, *E1A*
Induce pro-apoptosis regulators	bax, bcl-xs, apoptin
Re-link pro-apoptotic signals with apoptosis effectors	Apoptosome components (cytochrome c, Apaf-1, CARD, caspase-9), others
Activate directly apoptosis effectors	Caspase recruitment domain (CARD)
Suppress anti-apoptotic molecules	
Inhibit exogenous survival signals	?
Inhibit inhibitors of exogenous death signals	NF-kB
Inhibit anti-apoptosis regulators	*bcl-2* Inhibitors of apoptosis (IAPs), such as survivin, XIAP, IAP-1, and IAP-2

Genes used or targeted in clinical trials appear in italics.

MOLECULAR CHEMOTHERAPY

A number of distinct approaches to molecular chemotherapy for cancer have been developed. These include the administration of (1) toxin genes to eliminate tumour cells and the stromal cells that support them, (2) drug resistance genes to protect the bone marrow from myelosuppression induced by chemotherapy, and (3) genes that enhance the effect of conventional anticancer treatments. Initially, the approach of molecular chemotherapy was designed to achieve selective eradication of carcinoma cells via expression of a toxin gene. This is similar to conventional chemotherapy, where pharmacological agents are employed. However, in the latter approach, toxicity of the drug is often manifested both in malignant and non-malignant cells. Therefore, in order to effect a reduction in the burden of neoplastic cells, the patient's normal tissues and organs have to be exposed to potentially harmful quantities of the drug. Molecular chemotherapy is designed to circumvent this limitation by selectively targeting toxin delivery or expression to cancer cells on the basis of more specific tissue- or transformation-associated markers, thereby reducing the potential for non-specific toxicity. Commonly, a non-toxic pro-drug is administered that requires activation in genetically modified cells in order to be transformed into a toxic metabolite that ultimately leads to cell death [44–49].

Toxin genes

Thymidine kinase. The most common molecular chemotherapy system utilised to date to accomplish cell killing has been the herpes simplex virus thymidine kinase (HSV-*tk*) gene given in combination with the pro-drug ganciclovir (GCV) [50]. The selectivity of the HSV-*tk* system is based on the fact that, contrary to normal mammalian thymidine kinase, HSV-*tk* preferentially monophosphorylates GCV, rendering it toxic to the cell. GCV is then further phosphorylated by cellular kinases to produce triphosphates that are incorporated into cellular DNA. The incorporation of the triphosphate form of GCV causes inhibition of DNA synthesis and of RNA polymerase, leading to cell death [44]. Thus, tumour cells (or any other cell undergoing mitosis) transduced to express the viral *tk* gene have enhanced sensitivity to cell killing after exposure to GCV. Somewhat unexpectedly, normal cells transduced with HSV-*tk* after intravenous (i.v.) [51] or intrahepatic [52] administration of adenoviral HSV-*tk* vector have also shown high sensitivity to GCV, leading to liver degeneration and low survival in mice. The absence of toxicity of GCV after i.v. administration of a control adenovirus or subcutaneous administration of an adenovirus encoding HSV-*tk* suggests that the toxicity is specifically liver-associated. The relationship between toxicity and the status of liver parenchymal cells with respect to the cell cycle remains to be determined. The toxicity and efficacy of the transfer of HSV-*tk* are currently being tested in more than two dozen phase I human clinical trials, including tumours of the ovary, brain, prostate, head and neck, mesothelioma, multiple myeloma, leukaemia, and liver metastasis of colon cancer (for an updated list of protocols visit the Office of Recombinant DNA activities website at http://www.nih.gov/od/orda/protocol.htm).

Bystander effect. Whilst the benefits of selectively eradicating tumour cells are obvious, an important limitation associated with molecular chemotherapy is the inability to genetically modify 100% of the tumour cells with the toxin gene. However, this has proved not to be as severe a limitation as initially thought due to the phenomenon known as the 'bystander effect', whereby the eradication of HSV-*tk* transduced cells elicits a killing effect upon the surrounding non-transduced tumour cells. That not all of the tumour cells need to contain the HSV-*tk* gene to obtain complete eradication of the tumour was an observation of early experiments employing the relatively inefficient retroviral vectors in brain tumours [53, 54]. This occurrence was later confirmed in a variety of other tumour model systems [55–58].

Other toxins. Several additional combinations of enzyme/pro-drug have been developed to improve the efficacy of molecular chemotherapy and overcome the limitations of *tk*/GCV. For example, some of the enzyme/pro-drug combinations induce toxic effects not only in cycling but also in non-cycling cells (carboxypeptidase G2, nitroreductase, purine nucleoside phosphorylase). With others, the bystander effect is stronger (purine nucleoside phosphorylase) or does not require cell contact (cytosine deaminase, nitroreductase).

With some exceptions, single drugs in standard chemotherapy do not cure cancer. Historically, effective cancer treatments were developed when drugs with different mechanisms of action were used in combination. Extending

this concept to molecular chemotherapy, several combinations of enzyme/pro-drug have been shown to induce synergistic killing effects *in vitro* [59, 60]. Combination schemes have achieved also higher rates of tumour regression and cure in animal models [61, 62]. Thus, the application of classical chemotherapy principles for designing drug combinations would recommend the use of pro-drug/enzymes that target both dividing and non-dividing cells, that elicit different mechanisms of bystander effect, and that have non-overlapping toxicities.

Drug-resistance genes

In a second molecular chemotherapy approach, the host tolerance to higher doses of standard chemotherapeutic drugs is increased by transducing bone marrow cells, known to be highly sensitive to chemotoxicity, with genes that confer drug resistance [63–65]. Some potential problems with this strategy are, however, apparent. These include the absence of clear cut evidence demonstrating that higher chemotherapy doses translate into improved patient survival, very low transduction efficiency of the target human haematopoietic cells with retrovirus vectors, the dose-limiting effects determined by other non-haematological toxicities, and the fact that contaminating cancer cells in the marrow could be transduced with the drug-resistance gene, which could rapidly give rise to clones of treatment-resistant tumour cells.

Chemosensitisation and radiosensitisation

A third approach of molecular chemotherapy seeks to modulate the level of expression of a variety of genes that influence the sensitivity of the cell to toxic stimuli, including conventional chemotherapeutic drugs and radiotherapy. Genetic chemosensitisation can be achieved by inducing apoptosis, by inhibiting molecules involved in tumour cell resistance, or by enhancing intratumoral production of cytotoxic drugs. To facilitate apoptosis, genes such as *p53* may be administered to tumour cells to enhance the mechanisms of apoptosis induced by chemotherapeutic agents [66]. Our group has shown that down-regulation of Bcl-2 protein levels by an intracellular anti-Bcl-2 single-chain antibody increases drug-induced cytotoxicity [67]. Analogously, genetic down-regulation of cellular factors related to chemoresistance has been shown to enhance chemosensitivity [68]. Alternatively, genes can be administered intratumorally to enhance metabolic conversion of conventional chemotherapeutic agents. For example, transfer of a liver cytochrome P450 gene, *CYP2B1*, into human breast cancer cells greatly sensitised these cells to the cancer chemotherapeutic agent cyclophosphamide as a consequence of the acquired capacity for intratumoral drug activation. This effect produced a substantially enhanced antitumour activity *in vivo* [69]. Lastly, combinations of conventional chemotherapeutic agents and molecular chemotherapy can serve the established rule of administering cytotoxic drugs with different mechanisms of action and toxicities. For example, one ongoing clinical trial is evaluating the association of adenovirus-mediated transduction of ovarian cancer cells with the *tk* gene followed by administration of acyclovir and the chemotherapeutic drug topotecan (http://www.nih.gov/od/orda/protocol.pdf).

Several drugs are proven radiosensitisers, a fact that is commonly exploited in the clinic. One of these drugs is 5-fluorouracil (5-FU), which can be produced by the cytosine deaminase (CD) suicide gene. In this regard, molecular chemotherapy based on CD has been shown to enhance the effects of radiation therapy in animal models of gliosarcoma and cholangiocarcinoma [70]. Thus, strategies to alter both chemosensitivity and radiosensitivity by gene transfer appear to have potentially wide applicability in many tumour contexts.

Obstacles to molecular chemotherapy. With all its promise, molecular chemotherapy also bears some practical limitations. To date, the strategy of molecular chemotherapy has been mainly used in loco-regional disease models to overcome the lack of targeted vector systems. In these *in situ* schemes, a vector encoding the toxin gene is administered intratumorally or into an anatomic compartment containing the tumour mass. The goals of this delivery method are to achieve high local vector concentration in order to favour tumour transduction and to limit vector dissemination. However, transduction efficiencies of presently available vectors have been shown to be inadequate. Even in the context of closed compartment delivery, it has not been possible to modify a sufficient number of tumour cells to achieve a clinically relevant tumoral response [71]. Furthermore, although transduction with HSV-*tk* followed by ganciclovir treatment reduces tumour burden and prolongs survival in various model systems, including those utilising intratumoral and intraperitoneal (i.p.) administration, the elevated doses of viral vector needed to obtain transduction of the majority of the tumour cells are associated with limiting toxicity. In fact, substantial toxicity and experimental animal death have been noted [51, 52, 72]. Thus, the small therapeutic index of currently available vectors in the context of *in situ* administration is a critical limiting factor for the purpose of gene therapy of cancer. Furthermore, and most importantly, a well-known limitation of conventional chemotherapy is also to be expected with the use of molecular chemotherapy, i.e. the appearance of drug-resistant tumour subpopulations (Table 1). In conclusion, vector limitations and well-known barriers to classical cytotoxic manoeuvres impede the full exploitation of the promise of a more selective eradication of carcinoma cells via the expression of toxin or protective genes.

GENETIC IMMUNOPOTENTIATION

The development of clinically evident tumours implies the obvious failure of the host immune system to recognise and eliminate tumour antigen(s), a hypothetical role suggested by Thomas and embodied by Burnet under the name of 'immune surveillance of neoplasia' [73]. Genetic immunopotentiation strategies attempt to achieve active immunisation against tumour-associated antigens by gene transfer methodologies applied either to tumour cells, to enhance their immunogenicity, or to cellular components of the immune system, to enhance their anti-tumour prowess.

Genetic modification of immune effector cells

Cells of the immune system have been modified to augment their capacity to recognise and reject tumour antigens [74]. To this end, gene therapy offers the possibility of genetically modifying effector cells and, importantly, this intervention can be performed *ex vivo*, thus avoiding the toxicity that characterises most biological response modifiers when administered systemically.

Tumour infiltrating lymphocytes (TILs). TILs are derived from mononuclear cells obtained from leucocytes infiltrating resected specimens of solid tumours. In the early 1990s, it was hypothesised that TILs could be an enriched source of

natural killer (NK) cells and cytotoxic T-lyphocytes (CTLs) specific for tumour antigens, and could also have tropism towards systemic tumour foci. On this basis, technology for their expansion in culture was developed, and TILs were the first immune cells to be genetically modified and applied in a human gene therapy clinical trial against cancer [75]. It was soon observed that while TILs do include CTL and NK activated cells, only a few of these cells in these mixed populations are specific against the tumour from which they are isolated. Furthermore, reinfused TILs localised poorly into tumours, and their required expansion *in vivo* using IL-2 was rather toxic. Although several strategies have been applied to improve treatments based on TILs and other lymphocytes, including an elegant re-engineering of their tropism [76, 77], a modest localisation of TILs in tumours remains a limitation for the efficacy of this poorly tolerated and expensive therapy.

Genetic modification of tumour cells

An alternative strategy for trying to augment the antitumour immune response is to genetically modify tumour cells, or to manipulate their components, to facilitate the start of a robust immune response. Thus, it has been hypothesised that a formerly tolerant host may revert its immune status, characterised by tolerance or anergy, and thus ultimately experience tumour rejection. In other words, it is hypothesised that the host can be 'vaccinated' against the tumour by exposing tumour antigens to the immune system in a more favourable context [78–81]. Most clinical experience with antitumour vaccines to date has been obtained in melanoma patients. For years, irradiated tumour cells, either autologous or allogeneic, were administered in combination with different adjuvants, such as BCG. Later, the molecular definition of tumour-associated antigens allowed the testing of vaccines based on individual antigenic determinants delivered to the patient in the form of peptides or DNA. More recently, tumour cells themselves have been genetically modified to increase their immunogenicity by transfer of a variety of genes, including cytokines such as GM-CSF, costimulatory molecules such as B7, and MHC molecules. Clinical responses have been occasionally observed in melanoma, but not in colon or renal cancer [82]. A common requirement, not adequately accomplished routinely yet, is to introduce the gene of interest in tumour explants or cultured cells with high efficiency. A more fundamental problem has been observed in experimental models using tumours naturally arising in transgenic mice. In these spontaneous tumours, a clear lack of efficacy of vaccines called into question the relevance of previously observed responses in animal models of grafted, syngeneic tumours [83].

Obstacles to genetic immunopotentiation

The main advantage of genetic immunopotentiation is the possibility of enlisting physiological mechanisms for a potentially vast amplification of the therapeutic manoeuvre. To this end, even modest levels of gene transfer were initially expected to be followed by clonal expansion and systemic spread of effector immune cells and mediators. Thus, efficiency of gene transfer would be not critical, given the relatively low amounts of cells and gene products needed to obtain a potentially powerful response from the immune system.

The level of gene transfer into tumour and immune effector cells observed clinically has been limited [82], and this has been thought to partly explain the poor results obtained by tumour immunotherapy in humans. However, there are other, probably more important, obstacles. Factors that can explain the failure of the immune system in the cancer patient are legion, and it is not clear which of them are critical in the clinical context. Some of these factors may similarly explain the failure of previous immune therapeutic attempts. In general, a lack of an immune response can be due to inadequate immunogenicity of the tumour or to a deficiency of the immune system to recognise, respond and reject tumour antigens. Reduced tumour immunogenicity can be related to the absence, heterogeneity and plasticity of tumour-specific antigens or the loss of MHC class I molecules on the tumour cells, which are essential for presentation of cellular antigens to effector $CD8^+$ T lymphocytes. Alternatively, it may well be that the lack of costimulatory molecules, such as B7, in tumour cells and the lack of other 'danger' signals in the tumour site establishes immune tolerance or ignorance, which keeps the tumour from being rejected. In effect, current knowledge of tumour immunobiology establishes that T cells able to recognise tumour-associated antigens can be found *in vivo* and are inducible *in vitro*. Thus, the lymphocyte repertoire against these epitopes has not been deleted. However, either tolerance to these (tumour) self-antigens has been induced or, in the absence of costimulatory signals, peripheral T cells simply have ignored these antigens or become tolerant ([84] and discussion below on the 'danger' model). In this regard, induction of tumour antigen-specific T cell anergy in adoptively transferred cells has recently been shown in experimental models to be an early event in the course of tumour progression [85]. In addition, studies with transgenic mice that develop spontaneous tumours have shown that vaccination with tumour cells transduced with cytokines fails to inhibit tumour onset and progression, whereas the same cells are able to immunise non-transgenic mice subsequently grafted with tumours [83]. Thus, the failure of naturally established tumours to present antigens efficiently, and to attract and activate tumour-specific T cells at the tumour site, may impede successful vaccination against cancer antigens. Of note, ignorance by the immune system can abort most of the immunotherapy manoeuvres being tested and discussed above. An obvious consequence is that cancer vaccines should be able by design to break down tolerance to tumour antigens.

Immune system deficiencies can, in turn, be either generalised or regional, including in the latter case the active suppression by the tumour of host antigen presentation and of effector cells in the local micro-environment by expression of a variety of molecules. (For reviews on the mechanisms involved in tumour escape see refs. [86, 87].) Clearly, the presence of immunosuppressive factors in tumours suggests the need to complement any immunotherapy strategy with manoeuvres explicitly addressing the intratumoral presence of inhibitors of the immune system response, a combined strategy which to our knowledge is yet to be directly tested. An additional general feature of the immune response to consider when designing gene-based immunotherapy is the redundant phenomenology of the immune system. Its destructive power, occasionally needed in its entire exuberance, requires a complex network of balances and counterbalances to control the pathways of activation and termination of the immune response. Interventions directed to supplement or inhibit single mediators will most probably yield partial physiological and therapeutic results in the best case, may frequently yield no result at all, and occasionally

will produce effects opposed to those desired. Thus, combinations of cytokines are increasingly being used to try to control the complexity of the immune response against tumours. In the field of organ transplantation, successful induction of tolerance to prolong organ survival has been achieved by blocking multiple effector cells and mediators of the adaptive and innate immune systems. Similarly, it is conceivable that breaking the tolerance to tumours will require a strategy of multiple interventions including several target cells and cytokines.

NOVEL STRATEGIES TO OVERCOME CURRENT LIMITATIONS

As we have reviewed above, gene transfer therapies are remarkably successful in *in vitro* and *in vivo* animal model systems. In effect, we already know that the malignant phenotype can be reverted in tumour cell lines by 'knocking-out' or adding certain genes; that tumours can be eradicated by delivery of cytotoxic genes followed by treatment with appropriate pro-drugs; and that tumours can be cured in murine models by making the tumour cells either more immunogenic or by making the immune system cells more responsive, via the expression of cytokines, or by induction of costimulatory and immunogenic molecules. However, overriding limitations have been made apparent in pre-clinical experiments and in the first human gene therapy clinical trials against cancer, as emphasised by the Orkin-Motulsky report to the NIH [88] and the first published clinical results. Most difficulties in obtaining clinically relevant benefits come from the inefficiency of current gene vectors in transducing tumour or immune cells and their inability to access in a selective way target cells distributed systemically. Several avenues for improvement have been proposed, and some will be succinctly reviewed in this section.

Mutation compensation requires quantitative gene transfer

For mutation compensation strategies to work successfully, it seems that every tumour cell would have to be corrected in its genetic defect to achieve a therapeutic outcome. Thus, quantitative transduction of therapeutic genes into the tumour after *in situ* administration of the gene therapy vector may be an essential requirement. To this end, a variety of vector amplification strategies are being explored, including replicative [89, 90] and integrative [91] viral systems.

Replicative vector systems. One method to circumvent suboptimal tumour transduction of therapeutic genes *in vivo* would be the use of conditionally replicative viral vectors: a replication-competent virus would be employed to replicate selectively within infected tumour cells, leaving normal tissues unaffected. Production of progeny virions from the infected tumour cells would then allow infection of neighbouring tumour cells. Thus, the intratumoral viral inoculum would increase, improving the tumour transduction efficiency. In addition, the use of viruses that display a lytic life cycle would allow virus-mediated oncolysis. This effect would occur irrespective of the delivered transgene. In both cases, an amplification of the antitumour effect would be achieved [90, 92]. The limitations of non-replicative vectors already observed in human trials have facilitated rapidly increasing acceptance of this experimental strategy, once considered an eccentric endeavour.

For clinical application of this strategy, a virus with *in vivo* stability and the capacity for conditional replication within tumour cells is mandated [93]. Lack of integration of the viral genome into the cell chromosome seems also desirable. In this regard, both recombinant adenoviruses and herpes viruses have the potential to provide the required properties. Not only do they display high efficiency and stability *in vivo*, but also their replication can be controlled. In the case of adenoviruses, replication can be restricted to tumour cells by placement of genes needed for viral replication under the control of tumour- or tissue-specific transcriptional control elements, such as the promoter of the prostate-specific antigen (PSA) [94]. Alternatively, mutant adenoviruses have been designed to replicate selectively in cells lacking functional *p53*. Because *p53* is absent in many tumours, the replication of this lytic adenovirus would be selective in tumours, and a therapeutic strategy for cancer based on this concept has been proposed [95]. Clinical trials using this virus are currently ongoing, and encouraging preliminary results have been presented [96]. However, extensive studies in a variety of cell lines and animal tumour models have to date failed to confirm the selective properties of the virus to replicate only in *p53* mutant tumour cells [97, 98].

Herpes viruses have also been developed that replicate conditionally in dividing or tumour cells. This selectivity is based on several possible mutations engineered in the viral genome that prevent it from replicating unless the infected cell provides for a substituting molecular activity [99]. These properties have established brain tumours, which are surrounded by non-mitotic cells, as an ideal therapeutic model for testing replication-conditional herpes vectors. Notably, clinical trials have already begun to test both adenovirus and herpes virus-based replicative vector systems for the treatment of human cancer.

A small, non-pathogenic virus called parvovirus went through human trials of viral oncolysis several years ago. The ability of this virus to replicate depends on factors associated with proliferation and differentiation, and as a consequence the virus preferentially displays a cytopathic effect in transformed cells. However, the capacity of the virus to replicate and spread robustly within a solid tumour, and subsequently to induce tumour lysis, appears to be limited.

As another intriguing example, the capacity of human reovirus to replicate selectively in tumour cells having an activated Ras signalling pathway has recently been described in an *in vivo* model [100].

Further refinements in replicative vectors are anticipated that can significantly enhance the possibilities for the realisation of a practical clinical benefit in the context of virus-mediated cancer treatment. A systematic analysis of the life cycle of a replicative virus reveals four areas where further engineering of vectors can bring the required improvements. Thus, better vectors would have increased infectious capacity, would replicate with tight selectivity in target tumours or tissues, would have an enhanced replicative 'burst', and would modulate the local immune response allowing unimpeded regional dissemination throughout the tumour to the required extent. Efforts to realise each of these features have already begun in several laboratories [101–103]. As early examples, our group is developing defective adenoviral vectors that replicate selectively under the stimulus of the cytokine interleukin-6 [104], or under the controlled addition of second vectors carrying replication-enabling DNA sequences [105, 106].

Prolonged transgene expression: integrative vector systems. Lack of stability *in vivo* has confined the use of retroviruses to the *ex vivo* modification of target cells. For *in situ* gene delivery, vectors with high efficiency and stability *in vivo* are needed. Of vector systems with both characteristics, adenoviruses have been most extensively characterised and used (Table 5). However, adenoviruses also have important limitations. In addition to a significant inflammatory and immune response, an additional basis for the limited transgene expression associated with adenoviral vectors derives from their non-integrative nature, such that vector sequences are not retained in the host genome and are not inherited by progeny cells. In this regard, after adenoviral-mediated gene transfer, the recombinant genome is present as an episome in infected cells. Thus, with the proliferation of transduced cells, vector sequences are lost, with the consequence of limited duration of transgene expression. For utility in mutation compensation, and in other gene therapy strategies it thus would be desirable to develop methods to achieve integration of adenoviral vector-delivered transgene sequences in infected cells. As a novel approach to meet this need, we and others have developed a chimeric viral vector system that exploits favourable aspects of both adenoviral and retroviral vectors. In this scheme, adenoviral vectors induce target cells to function as transient retroviral producer cells *in vivo*. The progeny retroviral vector particles can then effectively achieve stable transduction of neighbouring cells [107, 108]. Thus, the principle of combining selected features of available vectors into novel chimeric vectors is being explored in the development of virus-based gene transfer systems [109].

Lentiviruses are retroviruses that, in contrast to other members of the family, can infect both dividing and non-dividing cells. This fundamental feature has driven significant efforts for the development of recombinant lentiviral vectors, although practical issues related to the production and safety have to date limited its widespread use. The recent development of novel vector packaging systems can significantly facilitate availability [110], and new self-inactivating lentiviral vectors can allow safer use [111, 112]. Efficiency of transduction of potential cellular targets by pseudotyped lentiviral vectors and *in vivo* utility are intriguing, and have begun to be described [112, 113].

Prolonged transgene expression: immune tolerance to viral vectors. Gene delivery via adenoviral vectors has been associated *in vivo* with the induction of characteristically intense inflammatory and immunological responses. A number of specific cellular and humoral immune effector mechanisms, together with non-specific innate defence factors, eliminate the infecting virus [114–117]. This process, refined over the course of millennia for maximal efficiency, has been associated with attenuation of expression of the transferred therapeutic gene due, at least in part, to loss of the vector-transduced cells. Based on an understanding of the biology of this phenomenon, specific strategies have been developed to mitigate the process [103]. Of note, the recent development of replicative viral vector systems will mandate the effective modulation of the anti-viral immune response.

Manoeuvres to minimise the immune response against viral vectors include manipulations of both the vector and the host. Firstly, recombinant viral vectors can be genetically engineered to delete viral genes encoding highly immunogenic or cytotoxic viral proteins. However, viral vectors with most of their genomes deleted are more difficult to propagate and purify, transgene expression tends to be unstable, and the vectors are still not totally devoid of immunogenic properties. However, the most recent versions of these vectors may provide adequate production and non-toxic, sustained expression of encoded genes for several months [118]. Alternatively,

Table 5. *Gene transfer systems used clinically against cancer*

Type	Vector system	Duration of expression	Clinical trials (No.)	Distinguishing features
Nonviral	Liposomes	Transient	30	Repetitive and safe administration feasible, inefficient gene delivery, transient expression.
	Naked DNA or RNA (injection, gene gun, electroporation)	Transient	5	Easy preparation, inefficient gene delivery, transient expression.
	Molecular conjugates	Transient	–	Flexible design, inefficient gene delivery, transient expression, unstable *in vivo*.
	Bacteria		–	Useful as vaccines for gene delivery into antigen presenting cells
Viral	Retrovirus	Prolonged	63	Integrates into the chromosome of dividing cells, unstable *in vivo*.
	Adenovirus	Transient	34	Highly efficient *in vivo*, production in high titre, tropism can be modified, induces potent inflammation and immunity, replicative vectors available.
	Poxvirus (vaccinia)	Transient	15	Extensive clinical experience with parent virus, large insert capacity, induces potent inflammation and immunity.
	Adeno-associated virus	Prolonged	–	Non pathogenic, low insert capacity, difficult to scale-up.
	Herpes simplex virus	Transient	1	Highly efficient *in vivo*, large insert capacity, cytotoxic, replicative vectors available.
	Chimeric vectors (e.g. ad/retro)	Prolonged	–	Combine features of component genetic vectors.
	Lentivirus	Prolonged	–	Integrates into the chromosome of *both* dividing and non-dividing cells, well-characterised production system not yet established.

*Registered in the NIH Office of Recombinant DNA Activities in December of 1998 (http://www.nih.gov/od/orda/protocol.pdf).

different serotypes and species of adenoviruses have been proposed to minimise the stimulus for an immune response. Secondly, vectors have been modified to express immunomodulatory molecules. It has been hypothesised that this could create a locally privileged environment for the vector. Some of these engineered molecules are viral genes that interfere with the apparatus of antigen presentation [119], such as the adenoviral glycoprotein 19 K, the herpes simplex virus (HSV) immediate early protein ICP47, or the viral interleukin 10 [120]. Others are recombinant molecules designed to imitate the viral proteins mentioned, such as antisense oligonucleotides or single-chain antibodies against MHC class I and II proteins, or to block costimulation, such as CTL4Ig [103].

Interventions on the immune system of the host have been adopted from common practices in the field of organ transplantation. In this regard, virally transduced cells have been considered to behave, to some extent, as allogeneic cell transplants. Thus, drugs are employed that inhibit the cellular immune response, such as anti-CD4 antibodies, cyclosporine, dexamethasone, and FK 506. In addition, drugs that decrease the humoral immune response, such as cyclophosphamide and deoxyspergualin, have been used. Recently, several groups have demonstrated transient and more specific immune blockade with inhibitors of T cell costimulation, such as anti-CD40 ligand, CTL4Ig, and anti-LFA-1. Furthermore, interventions aimed to decrease the innate response have recently been attempted. For instance, a soluble tumour necrosis factor receptor has been shown to greatly reduce the early adenovirus-induced inflammatory response, and to prolong expression of encoded genes [121]. Unfortunately, the required chronic administration of these immunosuppressive drugs affects systemic immune function and could lead to a number of potential complications, such as infection and malignancy. This makes them less attractive in principle for clinical application, although short-term treatment in cancer patients should be feasible. Lastly, a more specific intervention, induction of tolerance to adenovirus vectors, has been induced by several manoeuvres, including intrathymic injection of adenovirus [122], oral ingestion of adenoviral antigens [123], and infusion of antigen-presenting cells infected with adenovirus and expressing Fas ligand [124]. Thus, although inflammatory and immunological issues have limited the overall utility of adenoviral vectors for gene therapy applications, many of the aforementioned strategies appear promising, and may ultimately allow these limitations to be overcome, at the very least in the context of cancer treatments.

Molecular chemotherapy requires selectivity and amplification

Any approach to cancer gene therapy involving either molecular chemotherapy or mutation compensation requires a high level of efficiency of gene transfer specifically to the tumour cells. Selective gene delivery is necessary because the number of vector particles available for delivery to the cancer cells would be decreased by sequestration by normal, nontarget cells. This would then allow ectopic expression of the delivered therapeutic gene, with possibly deleterious consequences for the normal cells [125].

To date, *in vivo* cancer gene therapy strategies have been restricted to the treatment of compartmentalised tumours in an attempt to achieve high local vector concentrations and relatively efficient tumour transduction. Thus, molecular chemotherapy has been employed in a number of animal models and clinical trials in which adenoviral or retroviral vectors or retroviral vector-producing cells expressing a toxin gene have been directly injected into localised neoplasms confined within body cavities [72, 126–129]. The tumours treated in this manner include glioblastoma, mesothelioma and ovarian carcinomas.

However, these attempts to restrict expression of the therapeutic gene to the target cancer cells merely by confining vector administration have proved inadequate. In this regard, locally administered adenoviral vectors carrying the HSV-*tk* gene have been shown to disseminate, probably as a result of leakage into the blood stream, resulting in a high level of liver-associated toxicity [51]. Substantial hepatic toxicity related to the absence of tumour cell-specific targeting has also been demonstrated in adenovirus-mediated transfer of the HSV-*tk* gene in an ascites model of human breast cancer [72]. In addition, *in situ* injection of adenoviral vectors has been associated with a low level of efficiency of gene transfer to the disease cells in human clinical trials [71]. This phenomenon has been correlated with a paucity of primary receptors on the cancer cells [101, 130]. Hence, it is apparent that there is a need to develop a vector which will achieve a high efficiency of gene transfer selectively to target tumour cells following compartmentalised administration in order to increase the therapeutic index and realise the full potential of gene therapy as a safe approach to the treatment of cancer. Moreover, it is clear that the presently available vectors are inadequate for the treatment of metastatic disease. In order to achieve gene delivery to disseminated cancer cells, the vector must be administered i.v. In this context, there is a stringent demand for specificity of gene delivery to the tumour cells, both in order to avoid vector wastage following transduction of nontarget cells and, more importantly, to prevent toxicity associated with expression of the therapeutic genes in normal cells [125]. Therefore, a means must be developed to modify the gene delivery vehicle to permit efficient gene expression specifically in target cancer cells.

Targeting. Targeted gene therapy for cancer can be accomplished at different levels [131]. In one approach, the tumour cell can be targeted at the level of transduction to achieve the selective delivery of the therapeutic gene. This involves the derivation of a vector that binds selectively to the target cancer cell. Alternatively, the therapeutic gene can be placed under the control of tumour-specific transcriptional regulatory sequences that are activated in tumour cells but not in normal cells and, therefore, target expression selectively to the tumour cell. In addition, targeted cancer gene therapy can exploit the unique physiology of solid tumours.

Transductional targeting. The ability to alter the binding tropism of viral vectors is based on an understanding of the basic biology of viral entry. In this regard, attempts to modify the tropism of adenoviral vectors have been facilitated by the fact that the entry of adenoviruses into susceptible cells requires two sequential steps involving the interaction of two distinct viral capsid proteins with specific receptors on the surface of the target cell. The initial high affinity binding of the adenovirus to the primary cellular receptor (designated the coxsackievirus and adenovirus receptor, CAR [132, 133], occurs via the carboxy-terminal knob domain of the fibre [134, 135]. The next step in infection is internalisation of the virion by receptor-mediated endocytosis potentiated by the interaction of Arg-Gly-Asp (RGD) peptide sequences in the penton base with secondary host cell receptors, integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ [136, 137].

Therefore, strategies to alter adenoviral tropism are based on modification of the viral capsid proteins, fibre and penton base, to permit the recognition of alternative cell-specific receptors. To this end, we have shown that it is possible to redirect adenoviral infection by employing the Fab fragment of a neutralising anti-knob monoclonal antibody (MAb) chemically conjugated to a cell-specific ligand [138–144]. When complexed with preformed adenoviral vector particles, the bispecific conjugate simultaneously ablates endogenous viral tropism and introduces novel tropism, thereby resulting in a truly targeted adenoviral vector. We have employed a number of targeting ligands, including folate, basic fibroblast growth factor, and an antibody directed against the epidermal growth factor receptor. In this manner, we have demonstrated that tropism-modified adenoviral vectors can infect cells that are refractory to transduction by the native vector; that tropism-modified adenoviral vectors can enhance gene transfer to target cells; and that this enhancement in infection can be translated into a therapeutic benefit *in vivo*. Wickham and colleagues have similarly retargeted adenoviral vectors by means of bispecific antibodies, in this case comprising a MAb to an epitope engineered in the penton base and a MAb to a cell surface receptor [145, 146]. However, this approach to the generation of tropism-modified adenoviral vectors suffers from a number of limitations. In particular, since the targeting ligand is not covalently coupled to the adenovirus particle, there is the potential for the bispecific conjugate to become dissociated from the vector.

The drawbacks inherent in any strategy to redirect adenovirus tropism by complexing the vector particles with bispecific targeting conjugates could be avoided by the direct genetic engineering of the viral capsid proteins to contain cell-targeting ligands. In this regard, the carboxy terminus of the adenovirus fibre protein can be modified to incorporate targeting motifs with specificity for cellular receptors [147–150]. In an alternative approach, it has also been reported that targeting ligands can be incorporated within the so-called HI loop of the fibre knob [101, 151]. Adenoviral vectors which have been engineered to incorporate either a polylysine motif at the carboxy terminus of the fibre [147, 150] or an RGD motif at the carboxy terminus [149] or in the HI loop [101] have demonstrated significantly enhanced infection of cancer cell lines and primary tumour cells which express low levels of the primary adenovirus receptor. Thus, these genetic modifications to the fibre protein have resulted in expanded tropism by successfully redirecting adenovirus binding to alternative cellular receptors.

The next challenge in this field will be to employ genetic methods to engineer adenoviral vectors with specificity for a single target cell type. In addition to recognising novel receptors, such vectors should also lack the ability to bind to the native primary adenovirus receptor. This could be accomplished by site-directed mutagenesis of the fibre knob domain to eliminate the cell-binding site. An important consequence of the ablation of native adenovirus tropism is that it will not be possible to propagate these vectors on standard cell lines that express the fibre receptor. However, we have recently developed a novel artificial primary receptor that can be recognised by adenovirus vectors that fail to bind the native fibre receptor [152]. This technology should be useful in the propagation of genetically modified, truly targeted adenoviral vectors.

In contrast to adenoviruses, retroviruses employ a single envelope glycoprotein to accomplish both binding to the cellular receptor and the subsequent step of membrane fusion. As a consequence, modification of retroviral tropism has proven problematic, with few reports of modified envelope proteins which retain these two functions of binding and fusion [153]. A number of molecules, including single-chain antibodies, growth factors and cytokines, can be genetically incorporated into the retroviral envelope glycoprotein, whereupon they confer novel binding specificities into the engineered viral particles. However, some of these surface displayed polypeptides failed to mediate retroviral infection; rather, they proved inhibitory to gene delivery by the modified vectors. In an elegant approach to overcome this obstacle, Russell has incorporated a protease cleavage site into the design of the retargeted vector. Thus, upon contact with proteases expressed on the surface of the target cell, the inhibitory polypeptide is cleaved from the viral surface, thereby restoring infectivity. To date, tropism-modified retroviral vectors have suffered from significantly lower viral titres than the parental vectors and it is therefore not yet proven possible to employ targeted retroviruses *in vivo*.

A key factor in any transductional targeting scheme is the availability of appropriate specific molecules on the target cells that can be exploited. To date, a somewhat restricted range of targeting moieties have been chosen either for proof of principle or for their ability to bind to the relatively short list of previously identified cellular receptors. However, a number of groups have described systems which fundamentally share the similarity of examining libraries of peptides displayed on the surface of bacteriophage for their ability to bind to specific cell types, both *in vitro* and *in vivo* [154, 155]. Thus, a powerful new technology has been developed which allows the rapid isolation and screening of potential tumour-specific ligands, without requiring that the target of the ligand be identified. This approach should, therefore, prove to be a high throughput method to permit the derivation of transductionally targeted vectors for cancer gene therapy.

Transcriptional targeting. Transcriptional targeting has found wide application in the area of molecular chemotherapy where tumour- or tissue-specific regulatory sequences have been employed to restrict expression of the prodrug-converting enzyme specifically to the target cancer cells. For example, transcriptionally targeted adenoviral vectors expressing toxin genes under the control of the tumour-specific alpha-fetoprotein promoter have been employed in molecular chemotherapy approaches to hepatocellular carcinoma [156, 157]. The selective expression of the therapeutic gene in the target hepatomas suggests that transcriptionally targeted adenoviral vectors would be of clinical utility in other diseases. However, it has been reported that certain tumour-specific regulatory elements lose their specificity in the context of an adenoviral vector. Further limitations come from the prohibitively large size of many regulatory sequences, which exceed the capacity of certain current vectors. However, novel gene transfer systems with larger capacity are being developed and could be employed to overcome this limitation—these vectors include gutless adenoviral vectors [158] and reviewed in [117] and recombinant herpes virus [159].

To date, targeted gene therapy has been attempted by employing either transductional targeting or transcriptional targeting alone. However, it should be possible to enhance the overall level of specificity by combining the complementary

approaches of transductional and transcriptional targeting, each of which might be imperfect or 'leaky' by itself [131].

Targeting strategies exploiting tumour physiology. As described above, current approaches to targeted gene therapy for cancer have exploited cellular and molecular differences between normal and malignant cells. However, the physiology of solid tumours at the micro-environmental level provides a unique and selective target for cancer treatment [160, 161]. The regions of hypoxia and necrosis within solid tumours present opportunities for targeted, tumour-selective gene therapy. For example, the hypoxic environment of solid tumours provides a selective means to control gene transcription based on lower oxygen levels compared with normal tissues. Gene therapy strategies activated by hypoxia could include the transcriptional control of a prodrug-activating enzyme by a hypoxia-responsive element. Of course, this approach will still require a means of delivering the constructs specifically to the tumours. Gene therapy strategies could similarly be designed to exploit tumour necrosis. In this regard, certain species of anaerobic bacteria of the genus *Clostridium* can selectively germinate and grow in hypoxic/necrotic regions of solid tumours after i.v. injection of spores [162]. Thus, it might prove possible to exploit clostridia as gene therapy vectors engineered to express therapeutic genes, e.g. a prodrug-activating enzyme.

Modulation of the bystander effect. Limitations of current vectors preclude direct genetic modification of a significant proportion of malignant cells in tumours. It is, therefore, of paramount importance for obtaining clinically relevant results to extend the effects of therapeutic gene expression from the transduced cells to neighbour non-modified cells. Several manoeuvres may be undertaken to extend the magnitude of this required bystander effect. First, survival of genetically modified cells can be prolonged. By doing this, modified cells can sustain longer the expression of the therapeutic gene, thus enhancing the exposure of bystander cells to its protein product. For example, the expression of the cyclin-dependent kinase inhibitor p27 inhibits DNA synthesis and, thus, renders the cells resistant to concomitant herpes simplex virus thymidine kinase/ganciclovir (HSV-*tk*/GCV) treatment. These cells with augmented survival are thus, allowed to prolong the time during which they can pump out cytotoxic metabolites, and hence the bystander effect is increased [163]. However, this intervention should not compromise the capacity for eradication of the genetically modified cells, which could dangerously equate this strategy to the genetic induction of resistance to treatment. Second, the definition of the molecular basis of the bystander effect allows novel interventions to increase directly its magnitude. The intercellular gap junctions, for instance, are known to mediate at least in part the bystander effect of HSV-*tk*/GCV treatment. Retinoic acid and the drugs apigenin and lovastatin upregulate the function of the gap junctions, and have recently been shown to increase considerably the killing effect of HSV-*tk*/GCV both *in vitro* and *in vivo* [164, 165]. Conceivably, genes that encode gap junction molecules can also be transferred into tumour cells for increasing the bystander effect. Third, it is possible to employ therapeutic genes that can be secreted and exert their function in an autocrine and paracrine manner, thus extending regionally their effects against the tumour or its supporting stroma. For instance, the secretion of a soluble receptor for an essential angiogenesis factor can compete regionally for the natural receptor. This blockade limits binding of the angiogenic growth factor to the natural receptor, and consequently restricts the development of the tumour vasculature, thus leading to tumour suppression [23, 24]. There is, therefore, accumulating evidence that the modulation of the bystander effect can regionally amplify the effects of therapeutic gene transfer, and can contribute to overcoming the limitations of current vector systems.

Cellular vehicles. Vectors with the capacity for targeted systemic gene delivery have not been available, and this fact has limited the overall efficacy of gene therapy in cancer, including molecular chemotherapy strategies. As an alternative to viral and other nonviral vectors, cells have been employed for gene delivery. In this approach, the cells are removed from the body and therapeutic genes are transferred into them extracorporally, followed by autologous re-implantation into the patient. In this manner, the genetically modified cell becomes itself the ultimate vector for gene delivery. Examples of primary cells commonly used in this context, so-called 'cellular vehicles', are T lymphocytes, hepatocytes, and fibroblasts.

For application of cell vehicles in the context of disseminated diseases, a cellular vector should possess the attributes of systemic distribution and appropriate tropism, and should be readily available. In this regard, circulating endothelial progenitors have recently been described [166, 167]. Phenotypically, these cells are characterised by the expression of the cellular surface markers CD34 and Flk-1, a receptor for vascular endothelial growth factor. A very intriguing aspect of their behaviour, originally described in animal models of limb ischaemia, is their capacity to localise into areas of angiogenesis after their systemic administration. A variety of genes could conceivably be introduced in these cells, and expression of genetic payloads could be obtained in the environment where these cells ultimately localise. A locoregional effect subsequent to the expression of the therapeutic gene would thus be achieved in areas otherwise poorly accessible to gene transfer. Therefore, endothelial progenitors may represent a novel cellular vector approach with unique features, based on their capacity for systemic circulation and their peculiarly advantageous natural tropism to areas of active angiogenesis. To be exploitable in a gene therapy context, however, it is critical for these endothelial progenitors to be primarily amenable to efficient and safe genetic modification for delivery of the payload therapeutic genes. Unfortunately, genetic modification of human and non-human primate $CD34^+$ cells with a variety of viral vectors has been persistently hampered by very low efficiency. Efforts are currently undergoing in several laboratories, including ours, to improve gene transfer into $CD34^+$ cells without unduly compromising their phenotype and function by using novel vectors *ex vivo*, and for evaluating the potential of endothelial progenitors for systemic gene delivery into metastatic cancer.

In addition to autologous cells, gene therapy based on genetic modification of non-autologous cells has been attempted. Protection within immuno-isolating devices would allow implantation of well-established recombinant cell lines in different hosts, offering a cost-effective approach to gene therapy of cancer when long-term treatment is required [168].

Genetic immunopotentiation to break immune tolerance to tumours

Cancer immunotherapy is yet to be realised as a therapeutic approach in the oncologist's armamentarium. New

ways to consider the immune response against tumours are probably needed if gene transfer is going to be applied in a clinically relevant way. Novel gene therapy approaches that exploit the accumulating knowledge on cytokines and cells involved in the immune response are mounting. They have been reviewed extensively [169, 170]. We would rather first emphasise a novel conceptual framework developed in recent years that can offer new insights on the entire approach of cancer immunotherapy. Secondly, we will focus on gene therapy strategies that, within this theoretical framework, seem particularly apt for offering useful biological information and therapeutic potential.

Danger versus tolerance. The classical paradigm of tumour immunology considers the responses of the immune system to follow a model of discrimination between 'self' and 'non-self' antigens. According to this paradigm, cancers, as microbes, are 'non self' and a major function of the immune system is to seek out and destroy new cancers as they arise. The practical corollary has been a very intense effort to develop tumour 'vaccines'. However, an alternative theoretical model has been proposed by Polly Matzinger to explain and modulate the relationship between the immune system and a genetic disease [171]. In this view, termed the 'danger' model, the need to defend the organism against exogenous lethal pathogens and the need to avoid lethal auto-immunity are equally balanced. According to this new paradigm, to avoid auto-immunity the default reaction of T cells to antigens on non-haematopoietic tissues is tolerance, and it is the role of the antigen-presenting cells to detect and report to T cells situations of dangerous tissue distress (for instance, the beginning of either an inflammatory reaction or tissue damage) that are worth its activation into cytotoxic T cells [84, 172]. If tissue cells normally induce tolerance in susceptible T cells, it is predicted that the default immune response to tumour antigens occurring in those tissues is tolerance as well.

This model can change the emphasis applied in certain immunotherapy strategies. In the classical model, importance is given to the identification of tumour antigens and elaboration of vaccines based on these antigens. Furthermore, it is expected that once activated, the immune response against cells bearing tumour antigens will proceed until their complete elimination. In contrast, the danger model would suggest potentially more relevant new goals such as the orchestration of inflammatory processes in tumour foci, the activation of dendritic cells and other antigen presenting cells, and the direction of T lymphocytes towards the tumour. In other words, the aim should be to recruit not only the adaptive immune response but also and most importantly the cells (macrophages, neutrophils, NK cells) and mediators (cytokines, chemokines) of the innate immune system [84] that establish the immune response in the context of activating 'danger', and make it distinct from tolerogenic immune responses. Importantly, these manoeuvers, including vaccination, should be maintained until elimination of the tumour to avoid its default tolerogenic effects.

Polynucleotide immunisation. Pursuant to the successful application of the strategies of mutation compensation and molecular chemotherapy, obtaining vector targeting and amplification is a critical goal. In contrast, for some genetic immunopotentiation strategies, it may appear that a sophisticated vector is not absolutely needed to facilitate the otherwise inefficient transfer of DNA into tumour or immune system cells.

The possibility exists for eliciting potent, prolonged, and specific immune responses through the intramuscular injection of fragments of nucleic acid encoding tumour-associated antigens [173, 174]. This so-called 'polynucleotide immunisation' (PNI) approach offers several advantages with respect to classic protein immunisation. First, synthesis of the antigen (or antigens) in eukaryotic cells *in vivo* is more likely to result in a protein that is correctly folded and with its antigenic domains adequately presented. Second, PNI elicits a $CD8^+$ cytotoxic T lymphocyte response in addition to a humoral response. Third, long term expression of the encoded antigen may favour long-lived immunity. Of note, the danger model would recommend that, to avoid toleration, repetitive immunisations that involve local inflammatory responses should be administered to keep the association between danger signals and the encoded antigens. Fourth, several nucleotides could easily be combined for induction of responses against multiple relevant antigens. Fifth, safety concerns related to virus-derived or cell derived vaccines are obviated. Sixth, manufacturing and use of recombinant DNA may have economical and logistic advantages with respect to standard vaccines. Polynucleotides in the form of both DNA and RNA can be used. For example, plasmid DNA encoding carcinoembryonic antigen, a non-transforming tumour-associated antigen, has shown prolonged humoral and lympho-proliferative responses in non-human primates [174], and is being tested in a clinical protocol for colorectal cancer patients. Transforming tumour-associated antigens, such as erbB-2, may be encoded by RNA constructs that avoid the risk of integration of a potentially oncogenic sequence and are expressed only transiently. Once the antigen is expressed in myofibres, its presentation to the effector cells follows an unknown pathway, but is known to induce antibody production, T cell proliferation, lymphokine release, generation of CTL, and delayed hypersensitivity reactions. Importantly, encouraging results in animal models have been followed by clinical trials for both immune protection and therapeutic applications. Although tumours are antigenically heterogeneous, the hypothesis is that immune responses against the polynucleotide-encoded antigens can break immune tolerance for the tumour via a single epitope, which, in turn, would alert the immune system to the existence of the tumour as a foreign entity, provoking a systemic response.

Enhanced antigen presentation by genetically modified dendritic cells. As we reviewed above, most tumours are ignored by the immune system. Thus, tumour antigen-specific T lymphocytes, which are certainly present in the immune repertoire, are not activated and migrate systemically without showing any special tropism towards its cognate antigens present in the tumour sites. This has been partly attributed to a lack of activation and antigen presentation by dendritic cells (DCs) in tumours [175, 176]. Indeed, DCs infiltrating several tumours lack B7-1 and B7-2 molecules, which reveals a non-stimulatory status and impedes the encounter by T lymphocytes of the required 'signal 2' on DCs for antigen-specific activation. However, when autologous DCs are expanded and exposed *ex vivo* to tumour antigens and these DCs are then reinfused, activation of tumour-specific cytotoxic T lymphocytes ensues. In animal models, this intervention achieves a protective effect against subsequent exposure to tumours and also can induce a therapeutic effect in tumours already present [175]. This strategy is currently being explored in patients [177].

Multiple vectors are being tested for delivering tumour antigens into DCs, including viral vectors, naked DNA, RNA, tumour lysates, and peptides [178, 179]. It is possible that methods that maximise exposure of DCs to a variety of tumour antigens may have an advantage by overcoming the expected emergence of antigen-loss variants as well as natural immunovariation of tumours [180]. Importantly, fusion of DCs and tumour cells have also shown the capacity to revert established immune tolerance [181]. This concept has been tested in transgenic animals tolerant to the antigen MUC1, and refractory to vaccination with irradiated MUC-1 positive cells. Immunisation with the dendritic cell fusion that express MUC1 resulted in the rejection of established metastases and there was no apparent autoimmunity against normal tissues. These findings demonstrate that tolerance to tumour-associated antigens can be reversed, and suggest that immunisation with hybrids of dendritic and carcinoma cells may be a powerful methodology for whole cell vaccination against cancer.

Reversal of tumour-induced immunosuppression. DCs are conceived as a powerful way to stimulate tumour-specific T cells. It may be, however, that a robust generation of such cytotoxic T cells is not enough for rejecting established tumours, as has been shown in elegant animal models [182]. Indeed, this can be predicted from the anatomy of the T cell response, whereby it is critical not only for DCs to uptake tumour antigens in the tumour site, mature and migrate to lymph nodes, and present antigens to T lymphocytes. But it is additionally needed for stimulated antigen-specific T lymphocytes to home assertively into the possibly widespread tumour sites, and keep their activation and proliferation therein, despite numerous immunosuppressive signals. Further strategies may be needed, therefore, for (1) attracting and activating the tumour-specific T cells into various tumour sites [183, 184]; (2) inhibiting the local production of immunosuppressive molecules, such as TGF beta [185], interleukin-10, VEGF, and fas ligand; and (3) counteracting the antigen variation and down-regulation of antigen presentation. It can be concluded that a systematic intervention with multiple targets at the different pathophysiological levels mentioned seems a reasonable programme to achieve a meaningful antitumour immune response.

CONCLUSION

The delineation of the molecular basis of cancer allows the possibility of specific intervention at the molecular level for therapeutic purposes. To this end, three main approaches have been developed: mutation compensation, molecular chemotherapy, and genetic immunopotentiation. For each of these conceptual approaches, human clinical protocols have entered testing in phases I and II to assess dose escalation, safety and toxicity issues, and more recently to evaluate efficacy, respectively. However, major problems remain to be solved before these approaches can become effective and common place strategies for cancer. Principle among these is the basic ability to deliver therapeutic genes quantitatively, and specifically, not only into tumour cells but also into tumour-supporting tissues and effector cells of the immune system. As vector technology fulfils these stringent requirements, it is anticipated that the promising results already observed in pre-clinical studies will translate quickly into the clinic for amelioration of life-threatening malignant diseases.

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