Gene Therapy:
Technologies & Applications

Ryuichi Morishita
& Hironori Nakagami

Future Medicine

Gene Therapy: Technologies & Applications

Editors

Ryuichi Morishita
Osaka Graduate School of Medicine,
Osaka, Japan

Hironori Nakagami
Osaka University United Graduate
School of Child Development,
Osaka, Japan

Published by Future Medicine Ltd

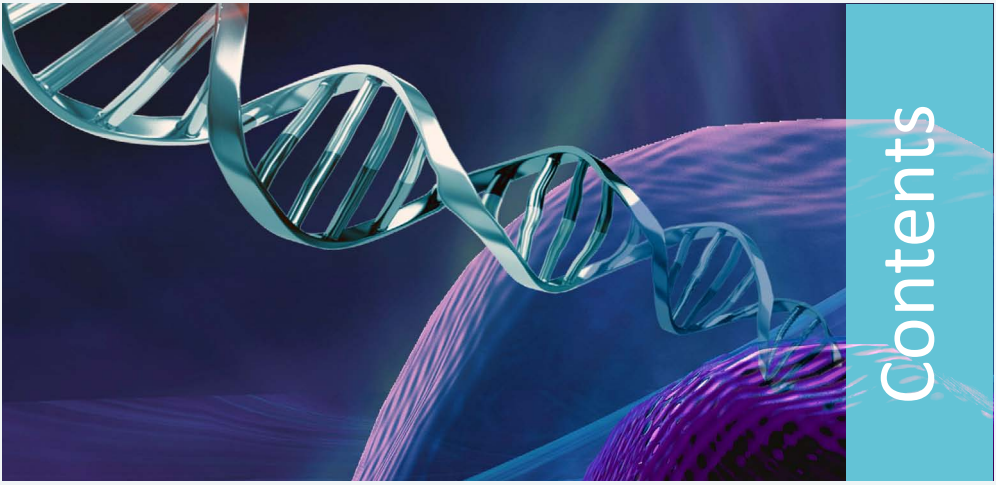
Future Medicine Ltd,
Unitec House, 2 Albert Place,
London N3 1QB, UK
www.futuremedicine.com

ISSN: 2047-332X
ISBN: 978-1-78084-215-8 (print)
ISBN: 978-1-78084-214-1 (epub)
ISBN: 978-1-78084-213-4 (pdf)

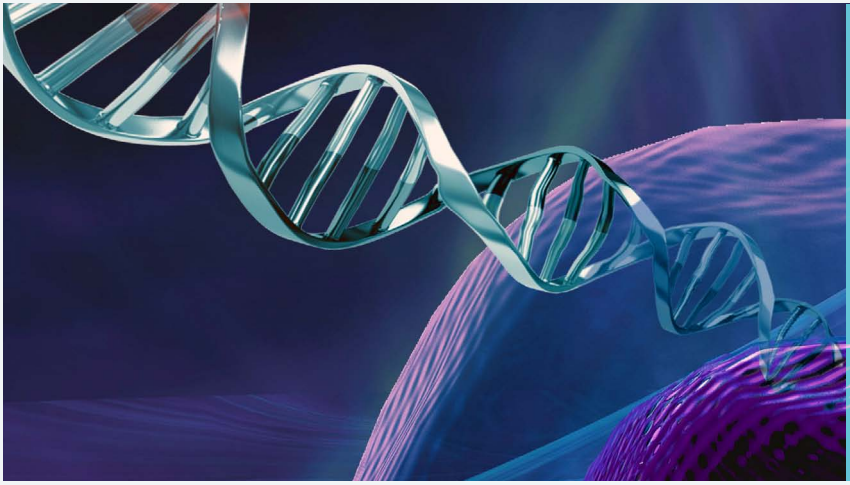
British Library Cataloguing-in-Publication Data.
A catalogue record for this book is available from the British Library.

Content Development Editor: Duc Hong Le

Senior Manager, Production & Design: Karen Rowland
Head of Production: Philip Chapman
Managing Production Editor: Harriet Penny
Production Editor: Georgia Patey
Assistant Production Editors: Gemma King & Samantha Whitham
Graphics & Design Manager: Hannah Morton



Contents



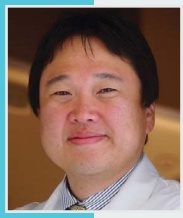
Contents

Continued

About the Editors

Hironori Nakagami

Hironori Nakagami has been Professor of the Division of Vascular Medicine and Epigenetics, Osaka University United Graduate School of Child Development since 2010. His main field of interest is vascular biology and cardiology. He is now interested in the application of DNA vaccines for cardiovascular diseases.



Ryuichi Morishita

Ryuichi Morishita has been Professor of the Department of Clinical Gene Therapy, Osaka Graduate School of Medicine since 2003. His main field of interest is cardiovascular disease, and he was the first to develop the decoy oligodeoxynuleotides for E2F or NF-κB to prevent restenosis after coronary artery intervention. He performed the clinical trial of therapeutic angiogenesis using the *HGF* gene.

Foreword

Gene therapy: technologies and applications

Hironori Nakagami & Ryuichi Morishita

In the history of human gene therapy, severe combined immunodeficiency caused by the absence of adenosine deaminase was the first monogenic disorder for which gene therapy was developed. Over 30 patients have been treated worldwide using the current protocols, and most of them have experienced clinical benefit, importantly, in the absence of any vector-related complications. Recent trials provided the first demonstration of long-term clinical efficacy of human stem cell gene therapy for severe combined immunodeficiency caused by the absence of adenosine deaminase, underlining that human stem cell gene therapy has a favorable safety profile and is effective in restoring purine metabolism and immune function.

On the website of the *Journal of Gene Medicine*, the numbers of gene therapy clinical trials approved worldwide since 1989 are shown. By January 2012, the total number of protocols reached approximately 1800 for the past 20 years, and the numbers per year have been stable, almost 100 per year, for the last 10 years. In the analysis of clinical phases, 80% of clinical trials are Phase I or I/II, which suggests that most of the trials are early stage. However, 4.3% of clinical trials (79 trials) are now at the late stages of clinical trials (Phase II/III or III). This suggests that clinical gene therapy is becoming more mature year by year. In the analysis of indications, the majority (65%) of target diseases are cancer. As the application of gene therapy may be restricted for incurable diseases, cancer has been one of the best targets for human gene therapy. Monogenic diseases are also candidate targets for

doi:10.2217/EBO.12.143

gene therapy (8%); however, the technique to correct the genome DNA needs to have a higher safety level. Of importance, cardiovascular diseases are the next target diseases (8%) and therapeutic angiogenesis has been applied to increase the neovasculization within ischemic tissue to treat peripheral arterial disease and myocardial infarction. Plasmids expressing VEGF, FGF and HGF have been reported to induce angiogenesis and clinical trials investigating this have been carried out. In the analysis of gene types, the top gene type is 'antigen'; contributing to almost 20% of all clinical trials. This indicates that, recently, the DNA vaccine has often been investigated in clinical trials. Vaccination is now a common therapy for infectious diseases or cancer, and a couple of advantages have been proposed in clinical trials of DNA vaccines. Based on the history of human clinical trials, we try to figure out the advances in technical issues, the possible application of each field, and future directions to achieve the successful goals of human gene therapy.

We have divided this book into a few parts to cover the recent progress in the field of gene therapy. In the first part, basic technologies of gene therapy will be described. In addition, the role of immunogene therapy, including vaccination, will be addressed. In the second part, the therapeutic applications will be described in specific clinical fields, neurology, hereditary diseases, cancers and cardiovascular diseases. Finally, future directions of gene therapy will be described. We hope that this book may help you share the appropriate information and motivate you to break though the hurdle of human gene therapy.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

About the Author

Seppo Ylä-Herttuala

Seppo Ylä-Herttuala is Professor of Molecular Medicine at A.I. Virtanen Institute for Molecular Sciences, University of Eastern Finland (Kuopio, Finland). He has been involved in several clinical gene therapy trials, basic research and vector development during the last 20 years. In 1995, he did the first human gene transfer in Scandinavia and was among the first researchers to use adenoviral gene transfer to blood vessels. His main interest area has been cardiovascular diseases, ischemia and angiogenesis research.

Chapter 1

General principles and approaches of gene therapy

Seppo Ylä-Herttuala

doi:10.2217/EBO.12.244

Gene therapy means the transfer of nucleic acids to somatic cells of a patient in order to obtain a therapeutic effect. Gene transfer effect can be transient or long lasting, and it is targeted to somatic cells as opposed to germline gene therapy, which is not allowed at the moment [1]. Gene transfer can be done directly *in vivo*, or *ex vivo* to cells isolated from the patient, whereafter the cells are returned back to the patient.

Potential gene therapy strategies include replacement of a defective gene, addition of a functional copy of a defective gene without interfering with the malfunctioning copy in the genome, or addition of a therapeutically useful gene in the target cells, regardless of the cause of the disease or genetic defect in the patient. At the moment, replacement or repair of a defective gene is still very challenging and most of the current gene therapy applications are based on the after two therapeutic strategies mentioned [2].

Gene transfer can be accomplished by physical, viral or nonviral methods. Examples of physical methods are electroporation and ultrasound-enhanced delivery.

Gene transfer can be done directly *in vivo* (*in vivo* gene therapy) or *ex vivo* to cells isolated from patients, whereafter the cells are returned back to the patients (*ex vivo* gene therapy). Potential treatment strategies include replacement of the defective gene, addition of a functional copy of a defective gene and addition of a therapeutically useful gene regardless of the pathogenesis of the disease. Furthermore, siRNAs and plain DNA or RNA sequences can be used for gene therapy.

Viral-based methods use genetically modified viruses, which are usually made defective in such a way that they cannot replicate or cause diseases in the patients. In addition, some replicating vectors are also currently used, mostly for cancer therapy. In nonviral methods, synthetic lipoplex, polyplex or nanoparticle-based complexes are used to deliver genetic material into the cells. In clinical trials, adenovirus, retrovirus and naked plasmid, plasmid/lipoplex, or plasmid/polyplex complexes have been most commonly used.

Gene transfer vectors

Vectors are needed to carry the transgene to the target cells in order to express the transgene or a therapeutic nucleic acid sequence. Vectors usually contain cDNA or the therapeutic sequence under a defined promoter and various elements needed for efficient expression and processing of the transgene. Since naked DNA has a very short half-life in plasma and most tissue compartments, viral or nonviral DNA carrier complexes have been utilized to aid uptake of the therapeutic constructs into the target cells and to prolong transgene expression in the treated tissues [2]. Adenoviral vectors have been widely utilized for gene therapy studies both in preclinical and clinical settings. Currently, more than 100 adenoviral serotypes have been characterized. Adenoviruses are nonenveloped viruses that contain a linear dsDNA genome. Adenoviruses are very efficient in transducing target tissues *in vivo*. However, since they cannot integrate the transgene into the genome, they only cause a transient gene transfer effect, which usually lasts from 7 to 14 days. Adenoviral vectors are relatively easy to produce in large quantities and current manufacturing processes are adequate for commercial supply of large quantities of replication-deficient adenoviral vectors. In some cancer applications, oncolytic adenoviruses, which can replicate in defined cells, have also been used in clinical applications.

Retroviral vectors can integrate the transgene stably in the target genome. Therefore, they can achieve a long-lasting gene transfer effect. Retroviruses are produced in packaging cells in quantities that are adequate for clinical trials, although manufacturing of large quantities of the vectors is still very challenging. Retroviruses also integrate randomly in the genome, which may cause significant safety issues, as has been seen in X-linked severe combined immunodeficiency trials [3]. Stability and efficacy of retroviruses can be

improved by pseudotyping. Most commonly, vascular stomatitis virus glycoprotein G is used for pseudotyping clinically used retroviral vectors.

Lentiviruses have recently gained much interest, since they can produce stable transduction of targeted cells, thus producing a long-lasting gene transfer effect, and their use may be less likely to cause significant safety problems than the use of retroviral vectors. Lentiviral vectors are produced in packaging cells with plasmid transduction or plasmid transduction combined with some lentiviral components expressed stably by the packaging cells. Lentiviruses are also frequently pseudotyped with other viral surface proteins, such as vascular stomatitis virus glycoprotein G or baculovirus GP64. Lentiviruses seem to be very efficient in transducing CNS cells and cells in the bone marrow.

Adeno-associated viruses (AAVs) have also recently gained significant interest, since they can produce a long-lasting gene transfer effect. They also seem to be very safe, as no human diseases are currently linked to the most commonly used AAV serotypes. AAV vectors can transduce both proliferating and quiescent cells, but they have a limited transgene-carrying capacity. Manufacturing of AAV vectors is currently still quite challenging, although improved manufacturing processes have been described recently.

Nonviral plasmid vectors are very easy to produce and they do not carry significant biohazards. However, gene transfer efficiency *in vivo* with nonviral vectors has been very low, and this has been the major limitation of their use in clinics. However, delivery of plasmid/polyplex or plasmid/lipoplex complexes has clearly improved their efficacy. New nanomaterials and nanoparticles will undoubtedly improve the usefulness of nonviral plasmid vectors in the future. An important application of nonviral methods is delivery of siRNAs or oligonucleotides to target tissues with the aim of downregulating target gene expression [4]. Furthermore, aptamers can be used for various biological applications *in vitro* and *in vivo*.

Gene transfer can be accomplished with nonviral and viral vectors. Of the viral vectors, adenoviruses, retroviruses, lentiviruses and adeno-associated viruses have been most commonly used for clinical gene transfer studies. Viral vectors used are usually replication deficient, although some replication-competent oncolytic viruses have also been used for cancer gene therapy.

Gene delivery methods

One of the greatest challenges for gene therapy has been efficient delivery of the constructs to target tissues. So far, systemic delivery has not been very efficient and most clinical applications have relied on either surgical, direct injection or catheter-mediated delivery methods. *Ex vivo* transduction of bone marrow cells has also been shown to be efficient for clinical

applications. Targeted homing of the vectors to diseased tissues and specific cell types has been tried by various methods, such as with antibody-conjugated vector complexes or specific receptor–ligand interaction-based vectors, but these applications have so far been relatively inefficient *in vivo*. Transfection of nontarget cells, such as liver or lymphoid tissue, has caused significant problems since this can lead to significant side effects and immune responses towards the vector and transgene. Furthermore, antibody responses generated after the first application of viral vectors have so far limited possible re-administration of currently used gene transfer vectors. Efficient targeted delivery would obviously reduce required gene drug dose and therefore reduce the possibility of side effects or immune responses towards the vector or the transgene. These problems need to be solved before gene therapy can become a widely accepted therapeutic modality for common disorders.

Current status of clinical gene therapy

After several years of intensive research and clinical testing, gene therapy has now shown potential benefits for the treatment of human diseases. Successful applications are described elsewhere in this book. As a significant landmark, the first clinical gene therapy product was approved in the EU in 2012 (Glybera®; uniQure, Amsterdam, The Netherlands) for the treatment of severe lipoprotein lipase deficiency, which shows that the regulatory pathway is now open for the approval of gene medicines for human use.

Financial & competing interests disclosure

The author has no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

Summary

- Gene transfer is a promising new alternative for the treatment of serious diseases.
- Current vector technology is already adequate for testing therapeutic applications in humans.
- Further vector development to achieve better targeting and regulation of the vectors and transgenes is still needed. Furthermore, controlling potential immunological reactions will be an important target for future research and development.
- After several years of intensive research, the first useful gene therapy applications for the treatment of severe human diseases have been published.

References

1 Ylä-Herttuala S, Rissanen TT, Vajanto I *et al.* Vascular endothelial growth factors. Biology and current status of clinical applications in cardiovascular medicine. *J. Am. Coll. Cardiol.* 49(10), 1015–1026 (2007).

2 Ylä-Herttuala S, Alitalo K. Gene transfer as a tool to induce therapeutic vascular growth. *Nat. Med.* 9(6), 694–701 (2003).

3 Hacein-Bey-Abina S, Hauer J, Lim A *et al.* Efficacy of gene therapy for X-linked severe combined immunodeficiency. *N. Engl. J. Med.* 363(4), 355–364 (2010).

4 Zhang Y, Satterlee A, Huang L. *In vivo* gene delivery by nonviral vectors: overcoming hurdles? *Mol. Ther.* 20(7), 1298–1304 (2012).

About the Author

John Nemunaitis

John Nemunaitis is Executive Medical Director of Mary Crowley Cancer Research Centers (TX, USA). He has been exploring novel targeted therapies for treating cancer patients for over 20 years. His research focus is clinical in orientation and involves determination of molecular signals in order to optimize targeted therapy, development of RNAi-based therapeutics and cancer vaccine approaches.

Chapter 2

Immunogene therapy

John Nemunaitis

doi:10.2217/EBO.12.299

The concept of immunogene therapy has dramatically advanced from initial testing in limited animal models, to large-scale, multiple Phase III trial clinical assessments. Moreover, the list of potential 'immunosensitive' malignancies, for many years previously thought to be limited to renal cell carcinoma, melanoma and possibly bladder cancer if one accepts local regional immunosensitivity to BCG, has expanded to include prostate cancer with the recent approval of Provenge® (Dendreon Corp., WA, USA), and as pointed out in this chapter, possibly non-small-cell lung cancer (NSCLC). Over the last 20 years, dozens of immunogenic approaches (those that involve DNA transfer) have been explored in clinical trials involving a variety of solid tumors. Interestingly, particular development has been observed in NSCLC. In order to review principles, rationale and up-to-date clinical advances, this chapter will focus on the microcosm of immunogene therapy in NSCLC, highlighting examples of development of this technology outside of NSCLC.

Immunogene therapy demonstrates safe and target-specific immune response, justifying this strategy as a novel, personalized, therapeutic approach in cancer management.

Historical evidence

Generally, lung cancer is identified as a nonimmunogenic disease. Nevertheless, it has been demonstrated that lung cancer cells can be converted to provide immunogenic properties. This can be achieved following cytokine stimulation or genetic modification [1]. It has also been observed that patients with immunodeficient diseases, such as AIDS, have a higher chance of developing non-small-cell lung cancer (NSCLC) and other solid tumors [2]. Tumor-infiltrating lymphocytes have also been detected in lung tumor tissue, seemingly directed against the tumor, but inhibited in function as a result of tumor-derived factors [3]. Initial studies investigating immunotherapy in NSCLC involved nonspecific immune-activating agents. Later studies involved vaccines. Several of these studies suggested limited survival advantage [4,5]. Currently, a number of vaccine trials are ongoing examining different vaccine therapeutic strategies involving advanced-stage NSCLC **(Tables 2.1 & 2.2)**. A notably large subset of these trials involve immunogene therapies.

In order to understand the rationale of immunogene therapy using NSCLC experience as a proof of principle, we need to have a better understanding of the mechanism and immune effector players **(Figure 2.1)**. Dendritic cells (DCs) are a key player in cell-mediated immunity [6–8] and have been found to play a critical role in mediating anti-tumor immunity in tumor-bearing hosts by a process of antigenic cross-presentation, and have displayed activity in NSCLC [9]. DCs efficiently display antigens on MHC class II, ultimately activating $CD4^+$ and $CD8^+$ T cells. $CD4^+$ cells further enhance the activity of natural killer (NK) cells and macrophages. In addition, NK cells amplify antigen-specific immunity through secretion of various cytokines [10–13]. Given this, DCs are thought to play a pivotal role in immunogene-based therapies involving NSCLC.

So why have previous approaches involving immunotherapy in lung cancer fail to realize the potential of this strategy? There are several hypotheses that can be generated to explain the lack of activity. These include ineffective priming of tumor-specific T cells, lack of high avidity of primed tumor-specific T cells, and physical or functional disabling of primed tumor-specific T cells by the primary host and/or tumor-related mechanism. As an example in NSCLC, many tumor-infiltrating lymphocytes have been shown to be immunosuppressive Tregs ($CD4^+CD25^+$), which secrete TGF-β and express a high level of cytotoxic T lymphocyte antigen-4 [14]. Immunosuppressive Tregs

Aa Immunogene therapy: the utilization of DNA-based technology to stimulate an immune response. In the context of cancer management, immunogene therapy is described here as inducing antigen expression, immune function enhancement and/or inhibition of cancer-induced immune inhibition.

Table 2.1. Results of gene-based vaccines in stage IIIB/IV non-small-cell lung cancer.

Study (year)	Vaccine	Stage	Patients (n)	Median survival	Ref.
Raez *et al.* (2004)	Allogeneic Ad B7.1	IIIB/IV	19	18 months (52% 1 year)	[82]
Salgia *et al.* (2003)	GM-CSF gene vaccine	IV	35	Not done	[58]
Nemunaitis *et al.* (2004)	GM-CSF gene vaccine	IIIB/IV	33	12 months (44% 1 year)	[60]
Nemunaitis *et al.* (2006)	GM-CSF gene vaccine bystander	IIIB/IV	49	7 months (31% 1 year)	[61]
Morris *et al.* (2005)	Galactosyl-transferase	IV	7	Not done	[97]
Nemunaitis *et al.* (2006)	Lucanix™ (NovaRx Corporation, CA, USA)	IIIB/IV	61	14.4 months (56% 1 year)	[98]
Nemunaitis *et al.* (2009)	Lucanix	IIIB/IV	21	15.5 months (72% 1 year)	[50]
Ramlau *et al.* (2008)	TG4010	IIIB/IV	65	14.9 months (60% 1 year)	[95]
Quoix *et al.* (2011)	TG4010	IIIB/IV	48	17.1 months	[99]

Ad: Adenocarcinoma; GM–CSF: Granulocyte–macrophage colony-stimulating factor.

have been shown to block or delay immune activation by facilitating T-cell tolerance to tumor-associated antigens, rather than cross-priming $CD8^+$ T cells. As a result, proliferation of killer T cells that recognize the tumor is suppressed [14–17].

IL-10 and TGF-β_1 and -β_2 are immunosuppressive cytokines produced by malignant cells, which have been shown to protect malignant cells against the body's immunologic defenses [18–23]. Elevated levels of IL-10 and TGF-β are found in patients with NSCLC. Our understanding of the immune system and how immunogene therapy turns it on to act against metastatic disease occurs through a series of steps. First, the cellular immunity cascade is initiated with antigen uptake, provided by the vaccine. This action is carried out through the activity of antigen-presenting cells [24]. Next, activation of $CD4^+$ T cells leads to the secretion of several cytokines, in particular IL-2 and IFN-γ, which facilitate activation of $CD8^+$ cytotoxic T cells. These cells recognize target peptide bound to MHC class I receptors [24]. These activated $CD4^+$ T cells also enhance the tumor-killing activity of NK cells and the phagocytic activity of macrophages, in addition to triggering a humoral immune response and antibody production [24]. Immunotherapy has not been effective for lung cancer in the past; however, recent

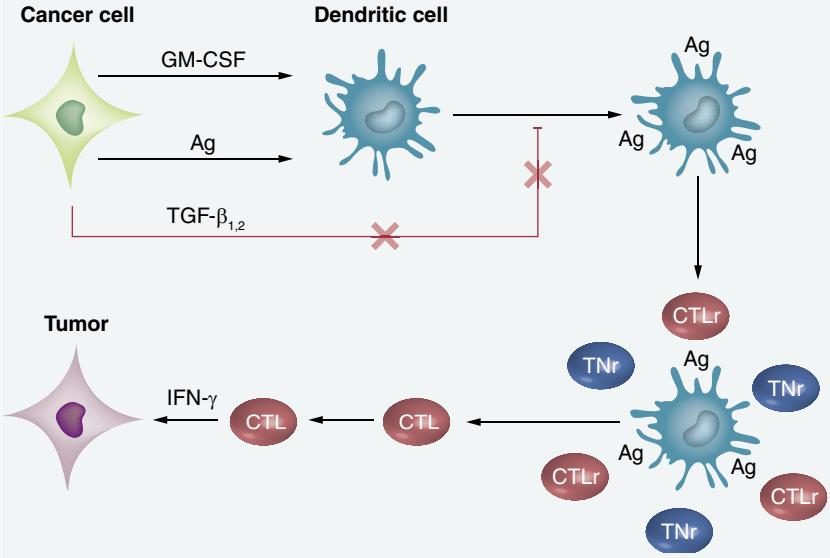
Table 2.2. Results of nongene-based vaccines in stage IIIB/IV non-small-cell lung cancer.

Study (year)	Vaccine	Stage	Patients (n)	Median survival	Ref.
O'Brien *et al.* (2004)	SRL172	IIIB/IV	210	7.3 months	[100]
Hirschowitz *et al.* (2004)	NSCLC-pulsed DCs	IA–IIIB	16	Not applicable	[9]
Morse *et al.* (2005)	Dexosomes loaded with MAGEs (Anosys Inc., CA USA)	IIIB/IV	13	Not done	[101]
Gonzalez *et al.* (2003)	CimaVax-EGF (El Centro de Inmunología Molecular, Havana, Cuba)	III, IV	40	8.2 months	[102]
Ramos *et al.* (2006)	CimaVax-EGF	IIIB, IV	43	Low dose: 6.43 months; high dose: 8.4 months	[103]
Brunsvig *et al.* (2006)	Telomerase peptide	IIIB, IV, (I,IIIA)	26	8.5 months (36% 1 year)	[104]
Butts *et al.* (2005)	Stimuvax® (EMD Serono Inc., MA, USA)	IIIB	88	17 months	[105]
Palmer *et al.* (2001)	Stimuvax	IIIB/IV	17	Low dose: 5 months; high dose: 15 months	[106]
Barve (2008)	EP2101 (Biotech Synergy, CA, USA)	IIIB/IV	135	17 months	[107]
Alfonso *et al.* (2007)	1E10 (El Centro de Inmunología Molecular, Havana, Cuba)	IIIB/IV	71	9.9 months	[108]
Hernandez *et al.* (2008)	1E10	IIIB/IV	20	10.6 months	[109]
Perroud *et al.* (2011)	Pulsed DCs	IIIB/IV	5	12 months	[110]
Zhong *et al.* (2011)	CEA-pulsed DCs	IIIB/IV	14	22 months (64% 1 year)	[111]

CEA: Carcinoembryogenic antigen; DC: Dendritic cell; MAGE: Melanoma-associated antigen; NSCLC: Non-small-cell lung cancer.

demonstrations of T-cell responses to tumor-specific antigens in patients with lung cancer provide encouraging evidence to support direction in

Figure 2.1. Vaccines providing relevant tumor antigens to local dendritic cells turn on B- and T-effector cells, which distribute systemically, seeking metastatic tumor cells containing the identified antigens.



Ag: Antigen; CTL: Cytotoxic T lymphocyte; CTLr: Cytotoxic T lymphocyte-reactive; GM-CSF: Granulocyte–macrophage colony-stimulating factor; TNr: Nonreactive T cell.

NSCLC [25]. In a move forward, the identification of lung tumor-associated antigens (TAAs) and the ability to present them in the optimal context may enable a level of control of the immune system to allow generation of clinically relevant antilung tumor effector cells [26].

Belagenpumatucel-L

Belagenpumatucel-L is a nonviral gene-based allogeneic vaccine that incorporates the *TGF-β_2* antisense gene into a cocktail of four different NSCLC cell lines [27]. As previously described, elevated levels of TGF-β_2 are correlated with immunosuppression in cancer patients [28–32]. Moreover, TGF-β_2 expression is inversely correlated with prognosis in patients with NSCLC [33]. TGF-β_2 also has antagonistic activity against NK cells and other immune effector cells [18,20,21,34]. Using an antisense gene to inhibit TGF-β_2, several groups have demonstrated an inhibition of cellular TGF-β_2 expression resulting in an increased immunogencity of gene-modified cancer cells [35–43].

In a recent Phase II study involving 75 early- (n = 14) and late-stage (n = 61) NSCLC patients, a dose-related effect of belagenpumatucel-L was

defined [27]. Patients were randomized to one of the three dose cohorts (1.25×10^7, 2.5×10^7 or 5×10^7 cells/injection). No adverse toxicity and an impressive survival advantage at dose levels $\geq 2.5 \times 10^7$ cells/injection, with an estimated 2-year survival of 47% in response to Lucanix™ (NovaRx Corp., CA, USA) in 41 advanced-stage (IIIB and IV) patients, was found. This compared favorably with the historical 2-year survival rate of <20% of stage IIIB/IV NSCLC patients [44–49]. Induction of immune response to tumor antigens was also correlated with a positive outcome. Induced cytokine production (IFN-γ: $p = 0.006$; IL-6: $p = 0.004$; IL-4: $p = 0.007$), an antibody-mediated response to HLA antigens ($p = 0.014$) and cell-mediated response results provided a correlation trend ($p = 0.086$) towards patients achieving stable disease or better. Further Phase II testing in a second trial at the optimal dose level verified the initial clinical experience [50]. It was concluded that a further Phase III investigation of belagenpumatucel-L was justified and warranted. Phase III trial accrual involving 506 patients (STOP study), in fact, was recently completed. Pending Phase III trial analysis, the Phase II study results suggest that partial blockade of intratumoral TGF-β_2 expression mediates sufficient inhibition of immune inhibitor activity produced by the live tumor cell vaccine to mediate a beneficial anticancer immune response.

GVAX

A comparison involving multiple cytokine genes found that granulocyte–macrophage colony-stimulating factor (GM-CSF) gene-transduced vaccines demonstrated optimal induction of tumor immunity compared with other cytokine genes in relevant animal models [51]. Secretion of GM-CSF transgene products by genetically modified tumor cells was associated with tumor antigen expression of the vaccine cells and cytokine release at the vaccine site. This effect activated and attracted antigen-presenting cells and induced a tumor-specific cellular immune response [52]. Preclinical studies have also shown no significant local or systemic toxicities with GVAX (BioSante Pharmaceuticals Inc., IL, USA) at clinically relevant doses [51,53].

Results from several Phase I/II trials involving GM-CSF-secreting autologous or allogeneic tumor cell vaccines have been published [54–59]. In one Phase I/II trial involving both early- and advanced-stage NSCLC patients, GVAX was evaluated [60]. To construct GVAX, surgically harvested tumor tissue was processed to create a single-cell suspension. These cells were exposed overnight to an adenoviral *GM-CSF* gene vector supernatant and transgene expression was verified. GVAX was administered intradermally (5×10^6–100×10^6 tumor cells) every 2 weeks for a total of six vaccinations.

A total of 43 NSCLC patients (ten early-stage and 33 late-stage) were involved in the study. Vaccine-related adverse events included vaccine injection site reactions (93%), fatigue (16%) and nausea (12%). Five out of 33 patients had induced serum antibodies reactive to the autologous tumors after vaccination. A total of 32 out of 41 (78%) patients demonstrated antibody reactivity to antigens of one or more of seven allogeneic NSCLC cell lines. Statistically significant differences in immune function were not demonstrated between early- and advanced-stage patients. Three advanced-stage patients achieved durable, complete tumor regressions. Continued long-term follow-up revealed that two of these patients remained without disease more than 10 years following completion of GVAX immunogene therapy. Both had failed front- and second-line therapy prior to vaccination, and had multisite disease. One complete responder showed an *in vitro* T-cell response to autologous tumor-pulsed DCs after vaccination. Median progression-free survival of the advanced-stage patients was 4 months and the median OS was 12 months. Survival at 1 year was 44%. Extended survival was correlated with vaccine secreted GM-CSF at a rate of ≥40 ng/24 h/10^6 cells (17 months compared with 7 months for patients receiving vaccines secreting less than 40 ng/24 h/10^6 cells of GM-CSF).

Immune function: the measurement of immune response based on known assays that include assessment of induced cytokines, proportion modulation of known effector cells and measurement of the most relevant effector cells (natural killer cells, activated T cells and dendritic cells) with complex assays, such as the ELIPSOT assay.

In an attempt to reduce the requirement for genetic transduction of 'individual' tumors, a second approach called 'bystander' GVAX, a vaccine composed of autologous tumor cells mixed with an allogeneic GM-CSF-secreting cell line (K562 cells), was explored [61]. However, no evidence of efficacy was demonstrated.

Salgia *et al.* also conducted a Phase I GVAX study with 37 patients with stage IIB–IV NSCLC at three different dosages (1×10^6, 4×10^6 or 1×10^7 cells) utilizing autologous tumor tissue [58]. Toxicities were limited to grade 1–2 erythema and induration at the injection site, as well as fatigue and influenza-like symptoms. A total of 18 out of the 25 patients remained stable to receive six (every other week) vaccinations. Four patients showed prolonged stable disease at 33, 19, 12, 10 and 3 months.

Overall, results with GVAX suggest that autologous malignant tissue transfection with adenovirus-delivered GM-CSF can provide therapeutic-related immunologic evidence of patient benefit. These results

Preliminary evidence supports utilization of immune function assays to predict and/or characterize immune response. The best examples of this are demonstrated with FANG™ and TG4010 (Transgene, Illkirch Graffenstaden Cedex, France) results.

appear to correlate with functional enhancement of the immune system mediated through a GM-CSF-induced DC response.

OncoVEX

'Traditionally', melanoma is characterized as an immunosensitive malignancy. The relationship of preliminary immune responsiveness has been demonstrated with another GM-CSF DNA-based vaccine involving melanoma. OncoVEX (Amgen, Inc., CA, USA) is an immune-enhanced oncolytic herpes simplex virus type 1. It is deleted for ICP34.5 (γ_1*34.5* gene), providing tumor-selective replication, and ICP47, which otherwise blocks antigen presentation [62]. The coding sequence for human GM-CSF was inserted to replace the ICP34.5 sequence in order to enhance the immune response to tumor antigens released after virus replication and tumor cell lysis.

Safety and anti-tumor activity has been demonstrated with OncoVEX in animal studies [62]. Moreover, the Phase I trial demonstrated safety and clinical activity of OncoVEX in patients with various tumor types, including melanoma [63], and the efficacy demonstrated in a recent Phase II study in patients with unresectable metastatic melanoma [64] has provided the basis for the ongoing US FDA-approved pivotal randomized Phase III study in the same population.

Tumor cells genetically modified to secrete GM-CSF have consistently demonstrated the most potent induction of anti-tumor immunity compared with other cytokines [51]. In one study, B16 melanoma cells were engineered to secrete either GM-CSF or Flt-3 ligand and their immunologic effects were reported [65]. Although both cytokines provoked a marked expansion of DCs locally and systemically, GM-CSF stimulated greater levels of protective immunity. Three profound differences have been described, which could account for the disparity in response. First, GM-CSF induces a subset of DCs that are more efficient in the phagocytosis of apoptotic tumor cells [66,67]. Second, GM-CSF stimulates expression of high levels of costimulatory molecules associated with greater functional maturation. In particular, more efficient T-cell functional activity broadens lymphocyte effector capability [68]. Third, GM-CSF promotes DC expression of CD1d [69]. CD1d is a MHC class I molecule associated with lipid antigen presentation [70]. The CD1d lipid complex then activates NK T cell function [71]. NK T cells play a pivotal role in anticancer endogenous and therapeutic response following immune induction [72].

Based on the established safety profile, and clinical and histological results of the Phase I trial, a Phase II open-label evaluation of OncoVEX in 50 patients with stage IIIc or IV melanoma, not eligible for surgery, was

performed [64]. Eligible patients received sequential injections of OncoVEX into one or more injectable skin, subcutaneous or nodal melanoma lesions.

In total, 74% of the patients received one or more prior nonsurgical therapies. A total of 13 patients were seronegative at baseline, all of whom were seroconverted by week 7. There was no correlation of serostatus with either response or adverse effects. There were 14 objective responses (partial response [PR] and complete response [CR] by Response Evaluation Criteria In Solid Tumors criteria) to OncoVEX treatment alone [73]. All responses included changes recorded from both injected and uninjected sites. Response onset was from 2 to 10 months after the first dose with maximum (biopsy-confirmed) overall response at 12 months. Notably, but not unexpectedly [74], transient locoregional or distant progression preceded CR in four patients and PR in two patients. Two additional patients achieved CR with additional surgery; one following craniotomy and resection of a newly diagnosed brain lesion at 4 months, and one following surgery and IL-2 retreatment, which the patient had previously failed. Another patient (PR) remained showing no evidence of disease following surgical resection at 13 months. Overall, ten patients (20%) attained CR with monomodal OncoVEX and a total of 13 patients (26%) did so when combined with additional surgery. The 1-year survival for all patients was 58%. Moreover, 1-year survival of the 15 patients with CR, surgical CR and PR was 93%.

Based on the previous results, a FDA-approved, pivotal, 360-patient, Phase III, 2:1 allocation, randomized trial comparing OncoVEX to subcutaneously administered GM-CSF in patients with advanced-stage melanoma was initiated and since has recently completed accrual.

The survival results in the Phase II study compare favorably with a recently published meta-analysis of 2100 stage IV metastatic melanoma patients entered into 42 Phase II trials from 1975 through 2005 [75], where the 1-year OS rate was 25.5% with no trial providing a survival result that was statistically different from the mean (25% in 524 patients). In the same analysis, the 1-year OS for only those 1024 patients with visceral disease (stage IV M1c [melanoma has metastasized to other distant organs]) was 23.8% compared with 40% of stage IV OncoVEX-treated patients. The median survival time had not been reached at over 16 months for all patients treated with OncoVEX (58% alive), as well as for the stage IV subset (52.5% alive) at the time of publication of the Phase II results. The 28% response rate, with impressive durability of response in both injected and uninjected lesions including visceral sites in conjunction with a 58% 1-year OS rate, are compelling evidence of systemic effectiveness awaiting Phase III trial verification.

B7.1 vaccine

B7.1 ($CD80^+$) is a costimulating molecule involved in induction of T- and NK-cell activation [76,77]. Tumor cells transfected with the expressed *B7.1* gene induce a brisk immune response as a result of enhanced antigen presentation and direct activation of T cells. This activation also allows for cross-presentation [78–81]. In one Phase I trial, Raez *et al.* worked with the AD100 (NSCLC) cell line B7.1 where HLA-A1 or -A2 were transfected and CD8 cytotoxic T lymphocyte responses were generated [82]. A total of 19 patients with stage IIIB/IV NSCLC and the appropriate HLA-A allotype were treated. Most had received extensive prior chemotherapy. Each patient received three intradermal vaccinations of 5×10^7 cells every 2 weeks. Patients with stable disease and no significant toxicity were able to continue on treatment.

A total of 18 patients received three vaccinations. One patient was removed before the completion of the first course due to a serious adverse event not associated with the vaccine. Most common side effects involving four patients included minimal skin erythema.

A measurable CD8 response was detected in 17 out of the 18 evaluable patients after three vaccinations. Partial response was demonstrated in one patient for 13 months and five patients had stable disease ranging from 1.6 to >52 months [82,83]. As measured long term from the six surviving patients, the tumor vaccine demonstrated an elevated immune response for at least 150 weeks. The Kaplan–Meier estimate for the survival of all 19 patients was 18 months with a 1-year survival estimate of 52%. The low toxicity and good survival in this study suggested benefit from clinical vaccination.

L523S

L523S is a lung cancer antigen originally identified through screening of genes differentially expressed in cancer versus normal tissue [84]. L523S is shown to be expressed in approximately 80% of NSCLC cells [84]. Immunogenicity of L523S in cancer patients was initially shown via detection of antibody and helper T-cell responses to L523S antigen. Subsequently, further studies validated L523S immunogencity by showing recognition by cytotoxic T lymphocytes of autologous tumor cells and other studies which revealed cell killing associated with expression of the L523S antigen. Preclinical safety has been demonstrated following gene injection intramuscularly of plasmid pVAX/L523S and in a second study following injection of an adenovirus/L523S vector. A Phase I clinical trial involving 13 early-stage NSCLC patients

Aa Cancer antigen: identifiable target unique to malignant tissue, thereby optimizing opportunity for a targeted immune approach.

utilizing both delivery vehicles pVAX/L523S (8 mg on days 0 and 14 in all cohorts) and Ad/L523S (1, 20, 400 × 10^9 viral particles on days 28 and 56, cohorts 1, 2 and 3, respectively) was performed [85]. No safety concerns were identified. Median survival was 290 days. The majority of patients demonstrated elevation in antiadenovirus antibodies; however, a cancer-related immune response was only demonstrated in one patient. As a result of the lack of a detectable immune response, alternative formulations and/or regimens were considered. Results suggest a high level of safety, but evidence of L523S-directed immune activation was limited.

Tumor-associated glycoprotein

The TGF-β_2 antisense plus recombinant human GM-CSF (rhGM-CSF) tumor-associated glycoprotein (TAG) vaccine uses an expression plasmid that coexpresses the GM-CSF and TGF-β_2 antisense nucleotide sequences, incorporated into autologous tumor tissue [86].

In the Phase I trial of TAG, 22 patients with a mix of solid tumors were treated [86]. Patients were infused with either 1×10^7 (n = 7) or 2.5×10^7 (n = 15) cells. The median number of vials constructed per patient was 11 (range: 7–26). Cell viability was 79–99% (median: 92%). Median GM-CSF expression was 394 pg/million cells. Median TGF-β_2 knockdown was 54%, and there was minimal TGF-β_1 inhibition, as expected. The achieved GM-CSF expression and TGF-β_2 knockdown parameters were consistent with the previously explored GVAX and belagenpumatucel-L expression results. There was little evidence of adverse events, apart from minor transient injection site irritation.

Stable disease of 3 or more months in duration was observed in 15 patients and appeared to be quite prolonged in a follow-up study. One CR occurred in a patient with stage IV malignant melanoma and there was a partial response in another individual, while two patients progressed. Three of the patients were not evaluable. Median survival of all patients entered into the TAG trial was 465 days. A similar population would be expected to have a life expectancy of between 4 and 6 months.There was a correlation in this study between immune response, as determined by the ELISPOT results showing activated T-cell expression, and response to TAG. The immune response appeared to increase from day 0. An immune response was not observed in patients who did not demonstrate prolonged survival or stable disease.

To summarize the findings with TAG, no additional toxicity was observed with the combination of the two components in one vector. This suggests that it may be

The benefit of an immune response may be preceded by initial, transient tumor expansion followed by stable disease or response, and is best assessed for efficacy through measurement of regression-free survival or overall survival.

Aa RNAi-mediated knockdown: utilization of RNA interference technology to inhibit expression of immune modulatory signals relevant to generation of immune response. In the context of the FANG vaccine, a novel bifunctional RNAi technology was utilized to reduce furin expression, which is critical for expression of the cancer inhibitors TGF-β_1 and -β_2.

possible to combine vaccines as well as separate therapeutics in the same patient. There was evidence of anti-tumor activity with this vaccine.

FANG™

The FANG™ (Gradalis Inc., TX, USA) vaccine is a 'triad' vaccine with three mechanisms of immune induction involving a mosaic of tumor antigens, *GM-CSF* gene-induced activation and bifunctional RNAi-mediated knockdown of tumor inhibitors.

Furin, a regulator of TGF-β activity, is a member of the subtilisin-like proprotein convertase family. Proteolytic cleavage by furin is required for TGF-β convertase activation (i.e., pro-TGF-β to TGF-β). Both target immune modulatory TGF-β isoforms (TGF-β_1 and -β_2) contain the RXXR motif at their cleavage site, albeit with different amino acid sequences [87]. High levels of furin mRNA and furin protein are widely expressed in human tumors [88]. The presence of furin in tumor cells probably contributes significantly to the maintenance of tumor-directed TGF-β_1 peripheral immune tolerance [89].

The novelty of the FANG vaccine lies in the combined approach of depleting multiple endogenous immunosuppressive TGF-β isoforms by RNAi-mediated furin knockdown, in order to maximize the immune-enhancing effects of the incorporated *GM-CSF* transgene on autologous tumor antigen sensitization. Autologous tumor cells provide a broad array of tumor antigens relevant to the patient from which they are harvested. These tumor cells are genetically modified to secrete GM-CSF.

The capacity of FANG to deplete all TGF-β isoforms is expected to: affect silencing of a well-documented, primarily endogenous family of immunosuppressive cytokines; and specifically inhibit TGF-β suppression of GM-CSF-induced maturation of DCs, expression of MHC class II and costimulatory molecules.

The siRNA-furin component is encoded as a stem–loop composed of complete matching sequences of the passenger and guide strands [90]. Following cleavage (cleavage-dependent process) of the passenger strand by Ago 2, an endonuclease with RNase H-like activity, the guide strand is incorporated into the RNA-induced silencing complex (RISC), which binds to and cleaves complementary target mRNA (Ago 2). In distinction, the miRNA-like component of the 'bifunctional' vector incorporates mismatches between the passenger and guide strands within the encoding shRNA hairpin in order to achieve lower thermodynamic stability. This configuration forces the passenger strand to dissociate from the RISC without endonuclease-mediated

cleavage (via Ago 1, 2, 3 and 4; a cleavage-independent process) [91,92], and the RISC-incorporated guide strand then acts through mRNA degradation, translational repression and/or sequestration of the target mRNA in the cytoplasmic processing bodies.

In total, 45 advanced solid tumor patients had vaccines constructed and were evaluable for response [93]. A total of 27 patients received at least a single FANG vaccine. These were called the 'FANG'-treated group. A total of 18 patients who had successfully fulfilled surgical resection inclusion criteria and underwent debulking surgery were also followed long term for survival. These were called the 'no FANG' group.

Mean post-transfection GM-CSF expression was consistent with other GM-CSF DNA vaccines. Mean TGF-β_1 and -β_2 knockdown were 93.5 and 92.5%, respectively [93], and furin knockdown was 80–95% [94]. Mean and median survival of the FANG-treated patients from time of procurement was 469 and 554 days, respectively. Their mean and median survival from time of treatment was 336 days and not reached, respectively. Factors such as age, sex, dose level, pretreatment expression levels of TGF-β_1 and -β_2, and vaccine transgene expression or knockdown did not correlate with survival. However, breakdown of survival comparing four or more vaccines versus less than four vaccines of the FANG-treated patients was significantly different ($p = 0.018$). Difference in survival (median: 132 days for no FANG, $n = 18$ and 554 days for FANG, $n = 27$) achieved statistical significance ($p = 0.00004$). Additional analysis focusing on patients who survived only 4 or more months after procurement revealed a median survival for the no FANG patients of 255 days ($n = 10$) and for the FANG patients ($n = 25$) of 554 days ($p = 0.006$). In addition, assessment of the no FANG patients who did receive alternative therapy after vaccine harvest revealed median survival ($n = 9$) of 255 days, compared with FANG-treated patient survival of 554 days ($p = 0.019$).

Sequential ELISPOT analysis was carried out in 18 of the Phase I patients. This was done at baseline and at month 4. All patients with one exception demonstrated no autologous tumor-specific cytotoxic $CD8^+$ T-cell activity at baseline. A total of nine patients revealed a dramatic increase in immune response from a baseline mean of seven IFN-γ-producing T-cell spots to a month-4 mean of 122 IFN-γ-producing T-cell spots ($p = 0.019$), and nine showed neither reactivity nor enhancement of immune response from a baseline mean of one spot through month 4. These two populations were statistically different from each other at month 4 in the ELISPOT response (122 vs one spot; $p = 0.012$). Comparison of survival between the nine patients demonstrating a positive ELISPOT response, and those nine not demonstrating response at month 4 demonstrated a statistically

Many examples of cell-based therapeutics demonstrate manufacturing feasibility and evidence of efficacy supportive of commercial opportunity.

significant increase in survival from time of procurement (p = 0.045) and time of treatment (p = 0.025) in the former.

All patients demonstrating a positive response at month 4 who had a long-term assessment continued to show a positive ELISPOT response during vaccine treatment and after discontinuation of vaccine therapy for up to 6 months following completion of treatment. Based on the safety, immunology support data and survival response of Phase I testing, three randomized Phase II studies involving ovarian cancer, melanoma and colon cancer have been initiated.

TG4010

TG4010 (MVA–mucin 1 [MUC1]–IL-2; Transgene, Illkirch Graffenstaden Cedex, France) is a modified vaccinia virus that expresses both MUC1 antigen DNA and IL-2 DNA. A recent randomized Phase II study was conducted to evaluate the immune response induced by this vaccine in advanced-stage NSCLC patients [95]. A total of 65 patients were entered into the trial. Arm 1 patients (n = 44) received TG4010 combined with chemotherapy upfront and arm 2 patients (n = 21) received TG4010 monotherapy. TG4010 was not associated with any significant toxicity. A total of 37 patients were considered evaluable and all of them demonstrated an induced MUC-1 cellular response. The OS for arm 1 was 12.7 months and for arm 2 was 14.9 months. The 1-year survival was 53%.

A follow-up study was then performed involving 148 patients who were randomized to TG4010 weekly for 6 weeks plus chemotherapy or chemotherapy alone (1:1 randomization). Stage IIIB/IV NSCLC, $MUC1^+$ tumor patients were enrolled. They received cisplatin and gemcitabine every 3 weeks for up to six cycles, with or without TG4010 administered subcutaneously weekly for 6 weeks, then every 3 weeks until progression [96]. The primary end point of progression-free survival at 6 months was achieved (>43 vs 30%; p = 0.01 in the TG4010 arm). Toxicity was equivalent in both arms. The most frequent side effects related to TG4010 were injection site reactions, fever and abdominal pain. Overall response rate was 43 versu 27% (p = 0.03), but the time to progression was only 5.8 versus 5.2 months in the TG4010 plus chemotherapy arm and the chemotherapy-alone arm, respectively, and OS was 10.7 and 10.3, respectively. However, a subset analysis focusing on patients who had a normal blood level of activated NK cells ($CD16^+$, $CD56^+$ and $CD69^+$) at baseline demonstrated an increase of approximately 6 months of OS (17.1 months) in the TG4010 arm, compared with the control arm (11.3 months, p = 0.062). These results

suggested relevance of immune responsiveness to TG4010 stimulation in patients with normal blood levels of activated NK cells. A Phase III trial has now been initiated targeting only patients with normal baseline proportions of circulatory activated NK cells.

Financial & competing interests disclosure

J Nemunaitis cofounded Gradalis Inc. and is a shareholder. Gradalis has a novel bifunctional RNA interference technology in development. The author has no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Summary

- A successful 'triad' of mechanisms demonstrating clinically relevant end points involves antigen expression, immune activation and inhibition of immune inhibitors.
- Short- and long-term safety has been observed in clinical testing within a variety of immunogene therapies.
- Lung cancer may be an immunosensitive disease.
- Immune assays assessing activated T-cell function (i.e., ELISPOT) are contributing to management of patients undergoing immunotherapy.
- Manufacturing processes for cell-based immunotherapies have been established and enable commercial opportunity.

References

1 Kakimi K, Nakajima J, Wada H. Active specific immunotherapy and cell-transfer therapy for the treatment of non-small cell lung cancer. *Lung Cancer* 65(1), 1–8 (2009).

2 Chaturvedi AK, Pfeiffer RM, Chang L, Goedert JJ, Biggar RJ, Engels EA. Elevated risk of lung cancer among people with AIDS. *AIDS* 21(2), 207–213 (2007).

3 Yoshino I, Yano T, Murata M *et al.* Tumor-reactive T-cells accumulate in lung cancer tissues but fail to respond due to tumor cell-derived factor. *Cancer Res.* 52(4), 775–781 (1992).

4 McCracken JD, Chen T, White J *et al.* Combination chemotherapy, radiotherapy, and BCG immunotherapy in limited small-cell carcinoma of the lung: a Southwest Oncology Group Study. *Cancer* 49(11), 2252–2258 (1982).

5 Matthay RA, Mahler DA, Beck GJ *et al.* Intratumoral bacillus Calmette–Guerin immunotherapy prior to surgery for carcinoma of the lung: results of a prospective randomized trial. *Cancer Res.* 46(11), 5963–5968 (1986).

6 Gilboa E, Nair SK, Lyerly HK. Immunotherapy of cancer with dendritic-cell-based vaccines. *Cancer Immunol. Immunother.* 46(2), 82–87 (1998).

7 Timmerman JM, Levy R. Dendritic cell vaccines for cancer immunotherapy. *Annu. Rev. Med.* 50, 507–529 (1999).

8 Cranmer LD, Trevor KT, Hersh EM. Clinical applications of dendritic cell vaccination in the treatment of cancer. *Cancer Immunol. Immunother.* 53(4), 275–306 (2004).

9 Hirschowitz EA, Foody T, Kryscio R, Dickson L, Sturgill J, Yannelli J. Autologous dendritic cell vaccines for non-small-cell lung cancer. *J. Clin. Oncol.* 22(14), 2808–2815 (2004).

10 Banchereau J, Briere F, Caux C *et al.* Immunobiology of dendritic cells. *Annu. Rev. Immunol.* 18, 767–811 (2000).

11 Germain RN. MHC-dependent antigen processing and peptide presentation: providing ligands for T lymphocyte activation. *Cell* 76(2), 287–299 (1994).

12 Pulendran B, Smith JL, Caspary G *et al.* Distinct dendritic cell subsets differentially regulate the class of immune response *in vivo*. *Proc. Natl Acad. Sci. USA* 96(3), 1036–1041 (1999).

13 Akbari O, DeKruyff RH, Umetsu DT. Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen. *Nat. Immunol.* 2(8), 725–731 (2001).

14 Woo EY, Chu CS, Goletz TJ *et al.* Regulatory CD4(+)CD25(+) T cells in tumors from patients with early-stage non-small cell lung cancer and late-stage ovarian cancer. *Cancer Res.* 61(12), 4766–4772 (2001).

15 Neuner A, Schindel M, Wildenberg U, Muley T, Lahm H, Fischer JR. Cytokine secretion: clinical relevance of immunosuppression in non-small cell lung cancer. *Lung Cancer* 34(Suppl. 2), S79–S82 (2001).

16 Schwartz RH. Models of T cell anergy: is there a common molecular mechanism? *J. Exp. Med.* 184(1), 1–8 (1996).

17 Lombardi G, Sidhu S, Batchelor R, Lechler R. Anergic T cells as suppressor cells *in vitro*. *Science* 264(5165), 1587–1589 (1994).

18 Ruffini PA, Rivoltini L, Silvani A, Boiardi A, Parmiani G. Factors, including transforming growth factor beta, released in the glioblastoma residual cavity, impair activity of adherent lymphokine-activated killer cells. *Cancer Immunol. Immunother.* 36(6), 409–416 (1993).

19 Tigges MA, Casey LS, Koshland ME. Mechanism of interleukin-2 signaling: mediation of different outcomes by a single receptor and transduction pathway. *Science* 243(4892), 781–786 (1989).

20 Rook AH, Kehrl JH, Wakefield LM *et al.* Effects of transforming growth factor beta on the functions of natural killer cells: depressed cytolytic activity and blunting of interferon responsiveness. *J. Immunol.* 136(10), 3916–3920 (1986).

21 Tsunawaki S, Sporn M, Ding A, Nathan C. Deactivation of macrophages by transforming growth factor-beta. *Nature* 334(6179), 260–262 (1988).

22 Fontana A, Frei K, Bodmer S *et al.* Transforming growth factor-beta inhibits the generation of cytotoxic T cells in virus-infected mice. *J. Immunol.* 143(10), 3230–3234 (1989).

23 Hirte HW, Clark DA, O'Connell G, Rusthoven J, Mazurka J. Reversal of suppression of lymphokine-activated killer cells by transforming growth factor-beta in ovarian carcinoma ascitic fluid requires interleukin-2 combined with anti-CD3 antibody. *Cell. Immunol.* 142(1), 207–216 (1992).

24 Kelly RJ, Giaccone G. Lung cancer vaccines. *Cancer J.* 17(5), 302–308 (2011).

25 Yasumoto K, Hanagiri T, Takenoyama M. Lung cancer-associated tumor antigens and the present status of immunotherapy against non-small-cell lung cancer. *Gen. Thorac. Cardiovasc. Surg.* 57(9), 449–457 (2009).

26 Raez LE, Fein S, Podack ER. Lung cancer immunotherapy. *Clin. Med. Res.* 3(4), 221–228 (2005).

27 Nemunaitis J, Dillman RO, Schwarzenberger PO *et al.* Phase II Study of Lucanix™ (belagenpumatucel) a transforming growth factor 2 (*TGF-β_2*) antisense gene modified allogeneic tumor cell vaccine in non small cell lung cancer (NSCLC). *J. Clin. Oncol.* (In Press).

28 Sporn MB, Roberts AB, Wakefield LM, Assoian RK. Transforming growth factor-beta: biological function and chemical structure. *Science* 233(4763), 532–534 (1986).

29 Massague J. The TGF-beta family of growth and differentiation factors. *Cell* 49(4), 437–438 (1987).

30 Border WA, Ruoslahti E. Transforming growth factor-beta in disease: the dark side of tissue repair. *J. Clin. Invest.* 90(1), 1–7 (1992).

31 Bodmer S, Strommer K, Frei K *et al.* Immunosuppression and transforming growth factor-beta in glioblastoma. Preferential production of transforming growth factor-beta 2. *J. Immunol.* 143(10), 3222–3229 (1989).

32 Jakowlew SB, Mathias A, Chung P, Moody TW. Expression of transforming growth factor beta ligand and receptor messenger RNAs in lung cancer cell lines. *Cell Growth Differ.* 6(4), 465–476 (1995).

33 Kong F, Jirtle RL, Huang DH, Clough RW, Anscher MS. Plasma transforming growth factor-beta1 level before radiotherapy correlates with long term outcome of patients with lung carcinoma. *Cancer* 86(9), 1712–1719 (1999).

34 Kasid A, Bell GI, Director EP. Effects of transforming growth factor-beta on human lymphokine-activated killer cell precursors. Autocrine inhibition of cellular proliferation and differentiation to immune killer cells. *J. Immunol.* 141(2), 690–698 (1988).

35 Fakhrai H, Mantil JC, Liu L *et al.* Phase I clinical trial of TGF-beta antisense-modified tumor cell vaccine in patients with advanced glioma. *Cancer Gene Ther.* 13(12), 1052–1060 (2006).

36 Liau LM, Fakhrai H, Black KL. Prolonged survival of rats with intracranial C6 gliomas by treatment with *TGF-beta* antisense gene. *Neurol. Res.* 20(8), 742–747 (1998).

37 Dorigo O, Shawler DL, Royston I, Sobol RE, Berek JS, Fakhrai H. Combination of transforming growth factor beta antisense and interleukin-2 gene therapy in the murine ovarian teratoma model. *Gynecol. Oncol.* 71(2), 204–210 (1998).

38 Tzai TS, Shiau AL, Liu LL, Wu CL. Immunization with TGF-beta antisense oligonucleotide-modified autologous tumor vaccine enhances the antitumor immunity of MBT-2 tumor-bearing mice through upregulation of MHC class I and Fas expressions. *Anticancer Res.* 20(3A), 1557–1562 (2000).

39 Tzai TS, Lin CI, Shiau AL, Wu CL. Antisense oligonucleotide specific for transforming growth factor-beta 1 inhibit both *in vitro* and *in vivo* growth of MBT-2 murine bladder cancer. *Anticancer Res.* 18(3A), 1585–1589 (1998).

40 Marzo AL, Fitzpatrick DR, Robinson BW, Scott B. Antisense oligonucleotides specific for transforming growth factor beta2 inhibit the growth of malignant mesothelioma both *in vitro* and *in vivo*. *Cancer Res.* 57(15), 3200–3207 (1997).

41 Park JA, Wang E, Kurt RA, Schluter SF, Hersh EM, Akporiaye ET. Expression of an antisense transforming growth factor-beta1 transgene reduces tumorigenicity of EMT6 mammary tumor cells. *Cancer Gene Ther.* 4(1), 42–50 (1997).

42 Kettering JD, Mohamedali AM, Green LM, Gridley DS. IL-2 gene and antisense TGF-beta1 strategies counteract HSV-2 transformed tumor progression. *Technol. Cancer Res. Treat.* 2(3), 211–221 (2003).

43 Fakhrai H, Dorigo O, Shawler DL *et al.* Eradication of established intracranial rat gliomas by transforming growth factor beta antisense gene therapy. *Proc. Natl Acad. Sci. USA* 93(7), 2909–2914 (1996).

44 Shepherd FA, Dancey J, Ramlau R *et al.* Prospective randomized trial of docetaxel versus best supportive care in patients with non-small-cell lung cancer previously treated with platinum-based chemotherapy. *J. Clin. Oncol.* 18(10), 2095–2103 (2000).

45 Fossella FV, DeVore R, Kerr RN *et al.* Randomized Phase III trial of docetaxel versus vinorelbine or ifosfamide in patients with advanced non-small-cell lung cancer previously treated with platinum-containing chemotherapy regimens. The TAX 320 Non-Small Cell Lung Cancer Study Group. *J. Clin. Oncol.* 18(12), 2354–2362 (2000).

46 Hanna N, Shepherd FA, Fossella FV *et al.* Randomized Phase III trial of pemetrexed versus docetaxel in patients with non-small-cell lung cancer previously treated with chemotherapy. *J. Clin. Oncol.* 22(9), 1589–1597 (2004).

47 Tsao MS, Sakurada A, Cutz JC *et al.* Erlotinib in lung cancer – molecular and clinical predictors of outcome. *N. Engl. J. Med.* 353(2), 133–144 (2005).

48 Shepherd FA, Rodrigues Pereira J, Ciuleanu T *et al.* Erlotinib in previously treated non-small-cell lung cancer. *N. Engl. J. Med.* 353(2), 123–132 (2005).

49 Kris MG, Natale RB, Herbst RS *et al.* Efficacy of gefitinib, an inhibitor of the epidermal growth factor receptor tyrosine kinase, in symptomatic patients with non-small cell lung cancer: a randomized trial. *JAMA* 290(16), 2149–2158 (2003).

50 Nemunaitis J, Nemunaitis M, Senzer N *et al.* Phase II trial of Belagenpumatucel-L, a *TGF-beta2* antisense gene modified allogeneic tumor vaccine in advanced non small cell lung cancer (NSCLC) patients. *Cancer Gene Ther.* 16(8), 620–624 (2009).

51 Dranoff G, Jaffee E, Lazenby A *et al.* Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. *Proc. Natl Acad. Sci. USA* 90(8), 3539–3543 (1993).

52 Scheffer SR, Nave H, Korangy F *et al.* Apoptotic, but not necrotic, tumor cell vaccines induce a potent immune response *in vivo*. *Int. J. Cancer* 103(2), 205–211 (2003).

53 Jaffee EM, Thomas MC, Huang AY, Hauda KM, Levitsky HI, Pardoll DM. Enhanced immune priming with spatial distribution of paracrine cytokine vaccines. *J. Immunother. Emphasis Tumor Immunol.* 19(3), 176–183 (1996).

54 Simons JW, Mikhak B, Chang JF *et al.* Induction of immunity to prostate cancer antigens: results of a clinical trial of vaccination with irradiated autologous prostate tumor cells engineered to secrete granulocyte–macrophage colony-stimulating factor using *ex vivo* gene transfer. *Cancer Res.* 59(20), 5160–5168 (1999).

55 Soiffer R, Lynch T, Mihm M *et al.* Vaccination with irradiated autologous melanoma cells engineered to secrete human granulocyte–macrophage colony-stimulating factor generates potent antitumor immunity in patients with metastatic melanoma. *Proc. Natl Acad. Sci. USA* 95(22), 13141–13146 (1998).

56 Simons JW, Jaffee EM, Weber CE *et al.* Bioactivity of autologous irradiated renal cell carcinoma vaccines generated by *ex vivo* granulocyte–macrophage colony-stimulating factor gene transfer. *Cancer Res.* 57(8), 1537–1546 (1997).

57 Jaffee EM, Hruban RH, Biedrzycki B *et al.* Novel allogeneic granulocyte–macrophage colony-stimulating factor-secreting tumor vaccine for pancreatic cancer: a Phase I trial of safety and immune activation. *J. Clin. Oncol.* 19(1), 145–156 (2001).

58 Salgia R, Lynch T, Skarin A *et al.* Vaccination with irradiated autologous tumor cells engineered to secrete granulocyte-macrophage colony-stimulating factor augments antitumor immunity in some patients with metastatic non-small-cell lung carcinoma. *J. Clin. Oncol.* 21(4), 624–630 (2003).

59 Soiffer R, Hodi FS, Haluska F *et al.* Vaccination with irradiated, autologous melanoma cells engineered to secrete granulocyte–macrophage colony-stimulating factor by adenoviral-mediated gene transfer augments antitumor immunity in patients with metastatic melanoma. *J. Clin. Oncol.* 21(17), 3343–3350 (2003).

60 Nemunaitis J, Sterman D, Jablons D *et al.* Granulocyte–macrophage colony-stimulating factor gene-modified autologous tumor vaccines in non-small-cell lung cancer. *J. Natl Cancer Inst.* 96(4), 326–331 (2004).

61 Nemunaitis J, Jahan T, Ross H *et al.* Phase 1/2 trial of autologous tumor mixed with an allogeneic GVAX vaccine in advanced-stage non-small-cell lung cancer. *Cancer Gene Ther.* 13(6), 555–562 (2006).

62 Liu BL, Robinson M, Han ZQ *et al.* ICP34.5 deleted herpes simplex virus with enhanced oncolytic, immune stimulating, and anti-tumour properties. *Gene Ther.* 10(4), 292–303 (2003).

63 Hu JC, Coffin RS, Davis CJ *et al.* A Phase I study of OncoVEX$^{GM\text{-}CSF}$, a second-generation oncolytic herpes simplex virus expressing granulocyte macrophage colony-stimulating factor. *Clin. Cancer Res.* 12(22), 6737–6747 (2006).

64 Senzer NN, Kaufman HL, Amatruda T *et al.* Phase II clinical trial of a granulocyte–macrophage colony-stimulating factor-encoding, second-generation oncolytic herpes virus in patients with unresectable metastatic melanoma. *J. Clin. Oncol.* 27(34), 5763–5771 (2009).

65 Mach N, Dranoff G. Cytokine-secreting tumor cell vaccines. *Curr. Opin. Immunol.* 12(5), 571–575 (2000).

66 Young JW, Inaba K. Dendritic cells as adjuvants for class I major histocompatibility complex-restricted antitumor immunity. *J. Exp. Med.* 183(1), 7–11 (1996).

67 Shen Z, Reznikoff G, Dranoff G, Rock KL. Cloned dendritic cells can present exogenous antigens on both MHC class I and class II molecules. *J. Immunol.* 158(6), 2723–2730 (1997).

68 Murtaza A, Kuchroo VK, Freeman GJ. Changes in the strength of co-stimulation through the B7/CD28 pathway alter functional T cell responses to altered peptide ligands. *Int. Immunol.* 11(3), 407–416 (1999).

69 Kawano T, Cui J, Koezuka Y *et al.* CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. *Science* 278(5343), 1626–1629 (1997).

70 Yamaguchi H, Furukawa K, Fortunato SR *et al.* Human monoclonal antibody with dual GM2/GD2 specificity derived from an immunized melanoma patient. *Proc. Natl Acad. Sci. USA* 87(9), 3333–3337 (1990).

71 Bendelac A, Rivera MN, Park SH, Roark JH. Mouse CD1-specific NK1 T cells: development, specificity, and function. *Annu. Rev. Immunol.* 15, 535–562 (1997).

72 Smyth MJ, Crowe NY, Hayakawa Y, Takeda K, Yagita H, Godfrey DI. NKT cells – conductors of tumor immunity? *Curr. Opin. Immunol.* 14(2), 165–171 (2002).

73 Senzer N, Bedell C, Nemunaitis J. OncoVEX^{GM-CSF}: an oncolytic viral immunotherapeutic in melanoma. *Drugs Fut.* 25(6), 449 (2010).

74 Wolchok JD, Hoos A, O'Day S *et al.* Guidelines for the evaluation of immune therapy activity in solid tumors: immune-related response criteria. *Clin. Cancer Res.* 15(23), 7412–7420 (2009).

75 Korn EL, Liu PY, Lee SJ *et al.* Meta-analysis of Phase II cooperative group trials in metastatic stage IV melanoma to determine progression-free and overall survival benchmarks for future Phase II trials. *J. Clin. Oncol.* 26(4), 527–534 (2008).

76 Antonia SJ, Seigne J, Diaz J *et al.* Phase I trial of a B7-1 (*CD80*) gene modified autologous tumor cell vaccine in combination with systemic interleukin-2 in patients with metastatic renal cell carcinoma. *J. Urol.* 167(5), 1995–2000 (2002).

77 Horig H, Lee DS, Conkright W *et al.* Phase I clinical trial of a recombinant canarypoxvirus (ALVAC) vaccine expressing human carcinoembryonic antigen and the B7.1 co-stimulatory molecule. *Cancer Immunol. Immunother.* 49(9), 504–514 (2000).

78 Johnston JV, Malacko AR, Mizuno MT *et al.* B7-CD28 costimulation unveils the hierarchy of tumor epitopes recognized by major histocompatibility complex class I-restricted CD8$^+$ cytolytic T lymphocytes. *J. Exp. Med.* 183(3), 791–800 (1996).

79 Liu B, Podack ER, Allison JP, Malek TR. Generation of primary tumor-specific CTL *in vitro* to immunogenic and poorly immunogenic mouse tumors. *J. Immunol.* 156(3), 1117–1125 (1996).

80 Nabel GJ, Gordon D, Bishop DK *et al.* Immune response in human melanoma after transfer of an allogeneic class I major histocompatibility complex gene with DNA-liposome complexes. *Proc. Natl Acad. Sci. USA* 93(26), 15388–15393 (1996).

81 Yamazaki K, Spruill G, Rhoderick J, Spielman J, Savaraj N, Podack ER. Small cell lung carcinomas express shared and private tumor antigens presented by HLA-A1 or HLA-A2. *Cancer Res.* 59(18), 4642–4650 (1999).

82 Raez LE, Cassileth PA, Schlesselman JJ *et al.* Allogeneic vaccination with a *B7.1 HLA-A* gene-modified adenocarcinoma cell line in patients with advanced non-small-cell lung cancer. *J. Clin. Oncol.* 22(14), 2800–2807 (2004).

83 Raez LE, Santos ES, Mudad R, Podack ER. Clinical trials targeting lung cancer with active immunotherapy: the scope of vaccines. *Exp. Rev. Anticancer Ther.* 5(4), 635–644 (2005).

84 Aguiar JC, Hedstrom RC, Rogers WO *et al.* Enhancement of the immune response in rabbits to a malaria DNA vaccine by immunization with a needle-free jet device. *Vaccine* 20(1–2), 275–280 (2001).

85 Nemunaitis JMT, Senzer N, Cunningham C *et al.* Phase I trial of sequential administration of recombinant DNA and adenoviral expressing L523S protein in early stage non small cell lung cancer (NSCLC). *Mol. Ther.* 13(6), 1185–1191 (2006).

86 Olivares J, Kumar P, Yu Y *et al.* Phase I trial of TGF-beta2 antisense *GM-CSF* gene-modified autologous tumor cell (TAG) vaccine. *Clin. Cancer Res.* 17(1), 183–192 (2011).

87 Kusakabe M, Cheong PL, Nikfar R, McLennan IS, Koishi K. The structure of the TGF-beta latency associated peptide region determines the ability of the proprotein convertase furin to cleave TGF-betas. *J. Cell Biochem.* 103(1), 311–320 (2008).

88 Page RE, Klein-Szanto AJ, Litwin S *et al.* Increased expression of the pro-protein convertase furin predicts decreased survival in ovarian cancer. *Cell Oncol.* 29(4), 289–299 (2007).

89 Pesu M, Watford WT, Wei L *et al.* T-cell-expressed proprotein convertase furin is essential for maintenance of peripheral immune tolerance. *Nature* 455(7210), 246–250 (2008).

90 Rao DD, Maples PB, Senzer N *et al.* Enhanced target gene knockdown by a bifunctional shRNA: a novel approach of RNA interference. *Cancer Gene Ther.* 17(11), 780–791 (2010).

91 Wu L, Fan J, Belasco JG. Importance of translation and nonnucleolytic ago proteins for on-target RNA interference. *Curr. Biol.* 18(17), 1327–1332 (2008).

92 Wang B, Li S, Qi HH, Chowdhury D, Shi Y, Novina CD. Distinct passenger strand and mRNA cleavage activities of human Argonaute proteins. *Nat. Struct. Mol. Biol.* 16(12), 1259–1266 (2009).

93 Senzer N, Barve M, Kuhn J *et al.* Phase I trial of 'bi-shRNAi(furin)/GMCSF DNA/autologous tumor cell' vaccine (FANG) in advanced cancer. *Mol. Ther.* 20(3), 679–686 (2012).

94 Kumar P, Phalon C, Pappen BO *et al.* Expression of GMCSF, TGFβ1, TGFβ2 and correlation to FURIN expression in culture supernatants from pre- and post-FANG™ plasmid transfected autologous tumor cells. *Mol. Ther.* 20(Suppl. 1), S197 (2012) (Abstract 511).

95 Ramlau R, Quoix E, Rolski J *et al.* A Phase II study of Tg4010 (Mva-Muc1-Il2) in association with chemotherapy in patients with stage III/IV non-small cell lung cancer. *J. Thorac. Oncol.* 3(7), 735–744 (2008).

96 Rossi A, Maione P, Schettino C *et al.* Non-small-cell lung carcinoma vaccines in clinical trials. *Expert Rev. Vaccin.* 10(6), 887–897 (2011).

97 Morris JC, Vahanian N, Janik JE. Phase I study of an antitumor vaccination using α (1,3)galactosyltransferase expressing allogeneic tumor cells in patients (Pts) with refractory or recurrent non-small cell lung cancer (NSCLC). *J. Clin. Oncol. ASCO Annu. Meet. Proc.* 23(Suppl.), 2586 (2005).

98 Nemunaitis J, Dillman RO, Schwarzenberger PO *et al.* Phase II study of belagenpumatucel-L, a transforming growth factor β-2 antisense gene-modified allogeneic tumor cell vaccine in non-small-cell lung cancer. *J. Clin. Oncol.* 24(29), 4721–4730 (2006).

99 Quoix E, Ramlau R, Westeel V *et al.* Therapeutic vaccination with TG4010 and first-line chemotherapy in advanced non-small-cell lung cancer: a controlled phase 2B trial. *Lancet Oncol.* 12(12), 1125–1133 (2011).

100 O'Brien ME, Anderson H, Kaukel E *et al.* SRL172 (killed *Mycobacterium vaccae*) in addition to standard chemotherapy improves quality of life without affecting survival, in patients with advanced non-small-cell lung cancer: Phase III results. *Annu. Oncol.* 15(6), 906–914 (2004).

101 Morse MA, Garst J, Osada T *et al.* A Phase I study of dexosome immunotherapy in patients with advanced non-small cell lung cancer. *J. Transl. Med.* 3(1), 9 (2005).

102 Gonzalez G, Crombet T, Torres F *et al.* Epidermal growth factor-based cancer vaccine for non-small-cell lung cancer therapy. *Annu. Oncol.* 14(3), 461–466 (2003).

103 Ramos TC, Vinageras EN, Ferrer MC *et al.* Treatment of NSCLC patients with an EGF-based cancer vaccine: report of a Phase I trial. *Cancer Biol. Ther.* 5(2), 145–149 (2006).

104 Brunsvig PF, Aamdal S, Gjertsen MK *et al.* Telomerase peptide vaccination: a Phase I/II study in patients with non-small cell lung cancer. *Cancer Immunol. Immunother.* 55(12), 1553–1564 (2006).

105 Butts C, Murray N, Maksymiuk A *et al.* Randomized Phase IIB trial of BLP25 liposome vaccine in stage IIIB and IV non-small-cell lung cancer. *J. Clin. Oncol.* 23(27), 6674–6681 (2005).

106 Palmer M, Parker J, Modi S *et al.* Phase I study of the BLP25 (MUC1 peptide) liposomal vaccine for active specific immunotherapy in stage IIIB/IV non-small-cell lung cancer. *Clin. Lung Cancer* 3(1), 49–57; Discussion 58 (2001).

107 Barve M, Bender J, Senzer N *et al.* Induction of immune responses and clinical efficacy in a Phase II trial of IDM-2101, a 10-epitope cytotoxic T-lymphocyte vaccine, in metastatic non-small-cell lung cancer. *J. Clin. Oncol.* 26(27), 4418–4425 (2008).

108 Alfonso S, Diaz RM, de la Torre A *et al.* 1E10 anti-idiotype vaccine in non-small cell lung cancer: experience in stage IIIb/IV patients. *Cancer Biol. Ther.* 6(12), 1847–1852 (2007).

109 Hernandez AM, Toledo D, Martinez D *et al.* Characterization of the antibody response against NeuGcGM3 ganglioside elicited in non-small cell lung cancer patients immunized with an anti-idiotype antibody. *J. Immunol.* 181(9), 6625–6634 (2008).

110 Perroud MW Jr, Honma HN, Barbeiro AS *et al.* Mature autologous dendritic cell vaccines in advanced non-small cell lung cancer: a Phase I pilot study. *J. Exp. Clin. Cancer Res.* 30, 65 (2011).

111 Zhong R, Teng J, Han B, Zhong H. Dendritic cells combining with cytokine-induced killer cells synergize chemotherapy in patients with late-stage non-small cell lung cancer. *Cancer Immunol. Immunother.* 60(10), 1497–1502 (2011).

About the Authors

Zhenghong Xu

Zhenghong Xu is a postdoctoral Research Associate, Division of Molecular Pharmaceutics in the Eshelman School of Pharmacy, the University of North Carolina at Chapel Hill (NC, USA). Her research is mainly focused on multifunctional nanoparticles, such as gene-/drug-delivery systems for cancer therapy. She has published seven first-author and nine coauthor research articles in *Biomaterials*, and the *Journal of Controlled Release*, among others.

Leaf Huang

Leaf Huang is a Fred Eshelman Distinguished Professor, Division of Molecular Pharmaceutics in the Eshelman School of Pharmacy, the University of North Carolina at Chapel Hill. He conducts research in the area of gene therapy and targeted drug delivery. He pioneered the liposome nonviral and cationic lipid vectors for the first nonviral clinical trials. Currently, his focus is on nanoparticle vectors for gene transfer in treating cancer and infectious diseases. In 2004, he received the Alec D Bangham MD FRS Achievement Award. His *h*-index is 79 having been the author of more than 320 peer-reviewed papers, and more than 130 reviews and book chapters.

Chapter 3

Chemical vectors for gene delivery

Zhenghong Xu & Leaf Huang

Gene therapy provides great hope for the treatment of many acquired and inherited life-threatening diseases. Nucleic acid drugs, however, must be delivered to the interior of the target cell while surviving an array of biological barriers, including reticuloendothelial system uptake and endosome entrapment. Successful gene therapy is thus dependent on the development of an efficient delivery vector. Nonviral vectors, as an attractive alternative to viral vectors, have been intensively investigated for decades; advantages such as a potentially improved safety profile, flexibility in the size of the delivered gene and lower immunogenicity have all been demonstrated. The first-generation nonviral vectors are mainly simple structures assembled with cationic lipids or polymers. To achieve better efficiency, several 'intelligent' multifunctional nonviral vectors have been rationally designed, representing a new generation of nonviral gene-delivery systems.

doi:10.2217/EBO.12.304

Gene therapy using macromolecular therapeutics, such as antisense oligonucleotides, siRNA and plasmid DNA, offers the possibility of a cure for a wide variety of inherited and acquired genetic disorders, viral infections and cancers [1]. The efficacy of nucleic acid drugs requires the molecule to be delivered to the proper compartment of the target cells. However, this delivery involves multiple challenges, as naked nucleic acids are often prone to degradation before reaching their target site or sometimes do not reach their target site at all. Therefore, efficient delivery has caused a bottleneck in the successful implementation of therapeutic nucleic acids as medical agents. Currently, there are two major categories of gene-delivery systems: viral vectors and nonviral vectors. Although viral vectors offer greater efficiency, nonviral vectors, which are typically based on cationic lipids or polymers, have been intensively investigated for decades as an attractive alternative. Nonviral vectors have advantages such as a potentially improved safety profile, flexibility in the size of the delivered gene and lower immunogenicity [2].

Lipid-based delivery systems

Lipid-based gene-delivery systems, especially cationic liposomes, are the most established materials for nucleic acid packaging. A variety of cationic lipid formulations, such as Lipofectamine™ 2000 and Lipofectin® (Life Technologies, NY, USA) are commercially available and have been used widely as transfection reagents. Several lipid formulations are already under clinical trials for gene therapy, treating cancers, cystic fibrosis and virus infections. Phospholipids (e.g., 1,2-dioleoyl-3-trimethylammoniumpropane and 1,3-dioleoyloxy-*N*-[2-(sperminecarboxamido)ethyl]-*N*,*N*-dimethyl-1-propanaminiumtrifluoroacetate) that have a hydrophilic polar head group and a hydrophobic hydrocarbon tail can form spherical bilayered structures, called liposomes, when dispersed in an aqueous medium. Formation of some liposomes may also require the addition of 'helper lipids', such as 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) or cholesterol. To deliver the nucleic acids using these systems, the genetic material can either be encapsulated into the internal aqueous phase of the liposomes or bound to their surface through electrostatic interactions. Cationic, anionic and synthetically modified lipids have all been investigated individually and in combination; however, higher encapsulation efficiencies and better transfection efficacies of cationic liposomes indicate that they could become one of the most popular gene-delivery systems [3]. Upon mixing, cationic liposomes can spontaneously form lipoplexes with negatively charged nucleic acids through electrostatic interactions, resulting in lipid bilayers with the DNA sandwiched between them. The lipoplexes not only protect

the nucleic acids from degradation by nucleases in the surrounding environment, but also enhance cellular uptake and transfection. The efficacy of lipoplexes depends on components of the cationic liposomes, the type of cell being targeted, nucleic acid chemistry and properties, and the characteristics of the lipoplexes themselves. Functional cationic lipids responding to *in vivo* environment stimuli have been rationally designed and investigated for gene delivery. The ionizable cationic lipid 1,2-dilinoleyloxy-3-dimethylaminopropane and its derivatives have proven to be highly effective and have been extensively tested in rodents and nonhuman primates [4]. 1,2-dilinoleyloxy-3-dimethylaminopropane is now being evaluated in human clinical trials. Recently, a new lipid-based delivery system, called lipidoids, was synthesized and evaluated [5]. Some of them showed superior efficiency than the traditional cationic lipids. Therapeutic efficacy was observed *in vivo* in the liver, lung and peritoneal macrophages. However, their toxicity profile has not been carefully evaluated.

Aa Ionizable cationic lipid: lipids with pKa < pH 7.0 that can efficiently compact and formulate nucleic acids at low pH and maintain a neutral or low cationic surface charge density at pH 7.4. The low surface charge should reduce nonspecific interaction with serum proteins and disruption of plasma membranes, providing better control of the circulation properties. As positive charge density increases substantially in the acidic environment of the endosome, the membrane-destabilizing property of the cationic lipids could be activated to facilitate endosome release.

Lipidoid: lipid-like materials; a structurally diverse library of amino–alkyl–acrylate and amino–alkyl–acrylamide materials that are generated by the conjugated addition of primary or secondary amines to alkyl–acrylates or alkyl–acrylamides.

Polymer-based delivery systems

Cationic polymers, which form polyplexes with nucleic acids through self-assembly, constitute another major category of nonviral vectors [6]. Research has been conducted to explore the potential efficiency of cationic polymers, such as polyethyleneimine (PEI), poly(L-lysine), polyamidoamine, polyallylamine, chitosan and poly(amidoamine) dendrimers, as gene carriers. While diethylaminoethyl-dextran can be considered a predecessor of cationic polymers used for gene transfection, PEI and its derivatives are probably the most common polycations investigated for nucleic acid delivery. Polycations can vary greatly in chemical composition, as well as in the number of repeating units. This variation allows for the easy assembly of a wide range of different polyplexes. Combinatorial and multiple parallel syntheses, along with the high-throughput approach, have also been employed to generate and identify those cationic polymers that are the most promising for nucleic acid delivery. To lower the nondesirable accumulation and toxicity, research focus has been placed on biocompatible and biodegradable polymers, such as polylactic acid and poly(lactic-*co*-glycolic acid) [7], as well as the biodegradable derivatives of cationic polymers, such

as bioreducible cross-linked PEI–disulfide bond [8]. Cyclodextrin-containing polymers have also drawn considerable attention in targeted delivery of nucleic acids due to their lack of immunogenicity, and unique geometric and structural features [9].

Challenges & strategies

Rational design and the ability to synthesize a wide variety of lipids and polymers have resulted in a highly adaptable and flexible system capable of gene delivery. Successful delivery of DNA and RNA to various cell types has been reported, including tumor cells, airway epithelial cells, endothelial cells, hepatocytes, muscle cells and lymphocytes. However, the efficiency of nonviral vectors and their further *in vivo* applications are still restricted by both extra- and intra-cellular barriers. Major extra- and intra-cellular barriers include reticuloendothelial system (RES) uptake and endosome entrapment, respectively. Mechanisms and strategies to prevent RES recognition and facilitating endosome release have been extensively investigated.

RES evasion

In systemic applications, charged complexes will interact nonspecifically with serum proteins, resulting in rapid clearance from the bloodstream by the RES. For several decades, coating particles with polyethylene glycol (PEG) has been considered to be the gold standard for reducing the protein adsorption, avoiding opsonization and recognition by macrophages, and prolonging the circulation time [10]. In the case of cationic liposomes, PEG–lipid (e.g., PEG–1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine [DSPE]) is usually inserted to form a hydrated layer on the surface. The efficiency of the protection is determined by the density and conformation of PEG covering the particles. For PEG–DSPE-stabilized liposomes, PEG takes a mushroom conformation at low density and will shift to a brush conformation as the content of PEG–DSPE is increased [11]. The brush mode is believed to be the ideal configuration for protecting nanoparticles. PEGylation of cationic polymers can be achieved through the synthesis of copolymers (e.g., PEI–PEG and poly(L-lysine)–PEG) with different grafting ratios [12]. PEGylated particles with reduced RES uptake are believed to be the prerequisite for enhanced tumor targeting via the enhanced permeability and retention effect.

Aa Enhanced permeability and retention effect: accumulation of certain sizes of molecules (usually liposomes, nanoparticles and macromolecular drugs) in the tumor tends to be much higher than that in normal tissues because of the impaired blood vessels and ineffective lymphatic drainage.

The presence of hydrophilic polymers on the surface of particles prevents their aggregation and nonspecific interaction with extracellular components. However, unfortunately, it also prevents the close

interactions between particles and target cell membranes, inhibiting the cellular uptake and, subsequently, preventing endosomal escape. All of these factors combine to decrease the biological efficacy of PEGylated particles. To overcome the 'PEG dilemma', specific targeting moieties, such as sugars, antibodies, peptides and small molecules (e.g., folate and anisamide), are conjugated to the particle surface to confer tissue specificity and enhance the intracellular uptake. Another strategy is to incorporate cleavable or sheddable PEG, meaning that the particles lose their protective shield at or in the target cells. Exploiting the microenvironment of target tissue (e.g., tumor tissues) and the acidic nature of endosomes, PEG–lipids and polyplexes with degradable spacers are used to prepare liposomes and cationic polymer vectors with cleavable PEG. pH-sensitive and degradable bonds, such as orthoester, hydrazone, vinyl ethers or acetals, are the most favorable. Enzyme-cleavable PEG has also been introduced into liposome-based delivery systems [13]. With the exception of external stimuli-triggered PEG shedding, conjugates of lipids and hydrophilic polymers are generally able to diffuse off membranes (spontaneous shedding) at a rate dependent on the strength of the anchorage or the anchor chain composition [14]. When particles (liposomes or lipoplexes) are coated with sheddable, stealth PEG–lipid, the PEG–lipid will continuously shed from particles (de-PEGylation) within certain microenvironments (stimuli-triggered shedding) or when particles circulate in the blood (spontaneous shedding). De-PEGylation eventually exposes the shielded cationic lipids and enables membrane destabilization for nanoparticle uptake and endosome release. In this case, the rate of de-PEGylation is a critical parameter that must be addressed when designing a sheddable PEG coating.

Another challenge for attaching PEG to the surface is accelerated blood clearance, where a second or subsequent injection of PEGylated particles results in a rapid clearance from the circulation [15]. Following the first dose, antibodies against the PEG are formed and these are responsible for the rapid clearance. Opsonization of the second dose of PEGylated particles and uptake by the Kupffer cells are also direct results of the antibody formation. However, in some cases of empty PEG liposomes, the half-life does not change after repeated administration. Therefore, it has been proposed that the CpG motifs in DNA, which can be used as an adjuvant, may be critical for the production of antibodies against PEG chains. Alternative hydrophilic polymers (e.g., polyvinylpyrrolidone and poly[hydroxyethyl-L-asparagine]) or polysaccharides have been suggested for use as shielding polymers instead of PEG. Incorporation of rapidly sheddable PEG–lipids (e.g., PEG–CerC14) in liposomes and lipoplexes is also shown to significantly reduce the induction of antibodies and their subsequent rapid elimination [16].

Endosome escape

The delivery of nonviral gene vehicles almost invariably involves endocytosis. Therefore, nonviral vectors must escape from endosomes before they are transported into lysosomes to avoid enzymatic degradation. Several mechanisms and strategies involving enhanced endosome release have been proposed.

Through a possible mechanism proposed by Xu and Szoka, cationic liposomes are able to destabilize the endosome membrane to facilitate the endosome escape [17]. Cationic lipids from lipoplexes can bind to anionic lipids on the endosome membrane to form neutral ion pairs. These ion pairs lead to formation of the inverted hexagonal (H_{II}) phase in the endosome membrane, promoting disassembly of the lipoplex and releasing nucleic acids into the cytoplasm. According to this mechanism, some protonatable groups that are charged at acidic pH but less charged at neutral pH could be an alternative choice to promote endosome release. The design rationale lies in the pKa of the ionizable cationic lipid and their ability to induce the H_{II} phase structure with the anionic lipids of the endosomal membrane when protonated in the acidic endosome. Gordon *et al.* demonstrated, however, that anionic lipids in the endosomal membrane may not be necessary to trigger the release [18], while neutral helper lipid such as DOPE could play an important role by promoting the transition from the lamellar phase to the H_{II} phase and therefore destabilizing the endosomal membrane. In this case, the addition of DOPE in a liposome formulation can be considered as a crucial trigger of endosomal escape and release of the nucleic acids into the cytoplasm. An important aspect of lipoplex-mediated delivery is that the nucleic acids are released into the cytoplasm without being required to dissociate from their carrier. This may serve as an advantage for the delivery of antisense oligonucleotides, siRNA/miRNA and mRNA, of which the final target destination is the cytoplasm. For DNA molecules, however, this type of release does not account for susceptibility to cytoplasmic nucleases.

As for polyplexes, some polycations have an intrinsic endosomolytic activity, while others need the 'helper molecules' to induce endosomal escape. Polymers with an intrinsic endosomolytic activity, such as PEI, poly(amidoamine) dendrimers and polydimethylaminoethylmethacrylate, usually have a high charge density, a highly branched structure and the potential to be protonated at weakly acidic pH [19]. Upon the acidification of the endosome, these polymers could buffer the compartment by becoming extensively protonated. The acidification causes more protons and chloride ions to migrate into the endosomes, leading to an increased osmotic pressure that ruptures the endosome. When the endosome

ruptures, the polyplexes are released into the cytosol of the cells. This process is called the 'proton sponge effect'. Some peptides containing the cationic amino group (lysine and arginine) and the imidazole group (histidine) [20], are also believed to trigger endosome release through the proton sponge effect.

Aa GALA: synthetic peptide that mimics the viral fusogenic peptides. The 30-amino acid GALA contains a glutamic acid–alanine–leucine–alanine sequence that is repeated four times. Since the carboxyl groups of glutamic acid are negatively charged at physiological pH, electric repulsion between these groups forces GALA to form a random coil structure. However, at acidic endosomal pH, protonation of the carboxyl group side chains of the glutamic acids dissipates electric repulsion, so the GALA structure changes into an α-helix, a structure that tends to induce membrane fusion.

Cationic polymers without intrinsic endosomal escape properties require the addition or covalent coupling of fusogenic or endosome molecules to induce their endosomal escape. These endosome disruptive agents, which can be either synthetic or virus derived, mostly depend on the lower pH for their activity. In this case, they expose more hydrophobic regions for interaction with the endosome lipid bilayers to finally destabilize the endosome. Polyethylacrylic acid and polypropylacrylic acid are examples of these molecules, as well as some virus-derived fusogenic peptides, such as hemagglutinin HA-2 from the influenza virus. Some amphipathic peptides, for example, glutamic acid–alanine–leucine–alanine (GALA) and lysine–alanine–leucine–alanine (KALA), are synthesized to mimic the viral fusogenic peptides [21].

Depending on the endosomal escape mechanism, the nucleic acids reach the cytosol in either 'naked' form or still encapsulated within the particle. In the latter, the protection against enzymatic degradation is maintained, but an additional step of dissociation is required to induce the therapeutic effects. Some studies demonstrate that the unpacking of particles in the cytoplasm is another potential barrier for polyplex-mediated nucleic acid delivery. In general, low-molecular-weight cationic polymers are expected to release the therapeutic nucleic acids more easily than high-molecular-weight polymers because there are fewer interactions between nucleic acids and a single polymer chain. However, the reduced affinity also implies the poor stability of the polyplexes in physiological conditions. Therefore, high-molecular-weight polycations are designed to be degradable into their low-molecular-weight building blocks under intracellular conditions (e.g., an acidic environment in the endosome or a reducing environment in the cytoplasm), thereby facilitating the release of nucleic acids.

Recent progress & prospects

The cationic liposomes and polymer-based gene-delivery systems discussed above represent the first generation of nonviral vectors, which are mainly

Aa Physicochemical properties: chemical, electronic and optical properties of nanoparticles; the parameters usually include particle composition, size, shape, surface charge and modification, drug encapsulation efficiency and loading capacity, and particle stability, among others.

characterized by a bottom-to-top approach. The approach begins with the synthesis of a new material and then tests for the physicochemical properties and transfection efficiency. This bottom-to-top approach has made us aware of the delivery problems at the top phase (e.g., *in vivo* application) and how these correlate to the physicochemical properties of the developed materials. Based on the theories and hypotheses from this approach, we are able to develop the next-generation vectors guided by a top-to-bottom approach, starting with the identification of the delivery barriers, followed by the rational design of vectors equipped with materials of the appropriate characteristics.

According to the mechanisms proposed from the bottom-to-top approach, an ideal vector should perform all of the following functions: compacting nucleic acids into the particle and protecting them from degradation; shielding the particles from undesired interaction with opsonins; and enhancing the cellular uptake and intracellular trafficking from the endosome into the cytoplasm or the nucleus. Obviously, a single material is unable to carry out all of the above tasks. To achieve an efficient delivery and biological efficacy, some lessons could be learned from the virus. A viral particle is composed of a RNA or DNA genome wrapped by a protein shell called a capsid. Enveloped viruses also have a bilayer lipid membrane that protects the capsid and promotes fusion with the plasma membrane or the endosome membrane. Viruses adopt many different strategies to infect a host cell, including hijacking endocytic routes, and subsequently escaping the endosomal compartment with the assistance of the lower pH in the early and/or the late endosomes. Structural changes usually occur during the trafficking, in response to changes in pH, reductive environment, Ca^{2+} concentration or enzymatic activity (e.g., phosphorylation), then exposing new layers of the particles.

Recently, a virus-like structure was developed with condensed nucleic acids located inside the lipid membranes [22]. It was initially prepared by condensing nucleic acids with protamine into a compact complex and coating it with cationic liposome to obtain liposome polycation/DNA (LPD) nanoparticles. The compact complex formed by DNA, mRNA and/or siRNA and protamine constitutes the core of the LPD. PEG-conjugated lipids were inserted into the outer lipid membrane after LPD formation to stabilize the particles. A targeting ligand (anisamide, a sigma-1 receptor ligand) was also conjugated to the distal end of PEG for targeting sigma receptor-expressing tumor cells. The stealth LPD nanoparticles

were characterized by lower liver uptake and efficient delivery of nucleic acids to tumors. It was hypothesized and further verified that PEG on the LPD surface was arranged in a brush conformation and thus prevented serum opsonization and improved the chances of reaching the tumor via the enhanced permeability and retention effect. Pharmacokinetics data also showed a rapid distribution phase and a short circulation time of injected particles, indicating that the brushed PEG shed quickly, resulting in the reduction of the PEG content in the nanoparticles and further blood clearance. However, PEG shedding, is believed to be critical and favorable for further endosome escape and cargo release after endocytosis. Several efficacy studies further demonstrated that, compared with lipoplexes (cationic liposome/nucleic acid complex), LPD offers nucleic acids better protection against enzymatic digestion and higher efficiency of silencing or gene expression in mice via intravenous administration. For efficient siRNA delivery, a lipid–calcium–phosphate nanoparticle (LCP) sharing a similar structure with LPD was developed [23]. The LCP entraps siRNA in a biodegradable nano-sized calcium phosphate precipitate core wrapped by lipid bilayers. The LCP de-assembly occurs in the endosomes due to the dissolved calcium phosphate precipitate core in acidic conditions, which in turn increases the osmotic pressure and finally causes the endosome to swell and burst, releasing the entrapped siRNA into the cytoplasm. LCP can be further modified and optimized for different applications by changing the precipitate core and the coating lipids.

This design concept has also been employed by a multifunctional nanodevice (MEND) for systemic gene delivery [24]. Similar to LPD, MEND consists of a nucleotide core condensed with polycations and lipid membranes with various surface modifications (e.g., PEG for prolonged circulation and specific ligands for targeting). An enzyme-cleavable PEG system, PEG–peptide–DOPE conjugate, was used to avoid the PEG dilemma. In this strategy, dePEGylation of MEND can be achieved via cleavage by a matrix metalloproteinase, which is specifically expressed in tumor tissues. To promote the endosome release, MEND was further modified with a fusogenic peptide GALA (WEAALAEALAEALAEHLAEALAEALEALAA), which contains a glutamic acid–alanine–leucine–alanine sequence that is repeated four times. A shorter version of GALA (shGALA) was developed and masked by the PEG layer to avoid the recognition by biomacromolecules *in vivo*. The shGALA-modified MEND showed significant gene silencing and tumor inhibition.

To achieve efficient gene delivery *in vivo*, nonviral vectors consisting of multifunctional components, which can overcome a series of extra- and

intra-cellular barriers, are desired. Furthermore, the vectors should also undergo programmed structural changes including deshielding and disassembling in a proper microenvironment to eliminate potential interference among the components and to make the vector more compatible at each step of the delivery process. Moreover, considering the cytotoxicity, biocompatible and degradable biomaterials would be preferred. In other words, an ideal nonviral vector should be an 'intelligent' multifunctional system that is assembled by biocompatible, nontoxic components and able to undergo environment-specific structural changes to activate the required functions to deliver the gene, in a manner similar to that of natural viruses.

Acknowledgements

The authors would like to thank Kelly Racette for editing the manuscript.

Financial & competing interests disclosure

The original work in this laboratory was supported by NIH grants CA129835, CA129421, CA149363, CA151652 and CA151455. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Summary

- Successful gene therapy is dependent on the development of an efficient delivery vector. Nonviral vectors, typically based on cationic lipids or polymers, have been intensively investigated for decades as an attractive alternative to viral vectors.
- The efficiency of nonviral vectors and their further *in vivo* applications are hampered by both extra- and intra-cellular barriers, among which reticuloendothelial system uptake and endosome entrapment are the two major ones, respectively.
- Coating particles with polyethylene glycol (PEG) can prevent reticuloendothelial system uptake, but it also affects the cellular uptake and endosome escape of particles. To overcome this PEG dilemma, specific targeting moieties can be conjugated on to the tip of the PEG chain to achieve enhanced target cell uptake. Cleavable and sheddable PEG derivatives are alternative options.
- Cationic liposomes are able to destabilize the endosome membrane to facilitate the endosomal escape. Some polycations have an intrinsic endosomolytic activity, while others need the 'helper molecules' to induce the endosomal escape.
- 'Intelligent' multifunctional gene-delivery systems, which are assembled by multiple components and able to undergo environment-specific structural changes, represent the next generation of efficient gene-delivery systems.

References

1 Verma IM, Somia N. Gene therapy – promises, problems and prospects. *Nature* 389, 239–242 (1997).

2 Ogris M, Wagner E. To be targeted: is the magic bullet concepta viable option for synthetic nucleic acid therapeutics? *Hum. Gene Ther.* 22, 799–807 (2011).

3 Simoes S, Filipe A, Faneca H *et al.* Cationic liposomes for gene delivery. *Expert Opin. Drug Deliv.* 2, 237–254 (2005).

4 Semple SC, Akinc A, Chen J *et al.* Rational design of cationic lipids for siRNA delivery. *Nat. Biotechnol.* 28(2), 172–176 (2010).

5 Akinc A, Zumbuehl A, Goldberg M *et al.* A combinatorial library of lipid-like materials for delivery of RNAi therapeutics. *Nat. Biotechnol.* 26(5), 561–569 (2008).

6 Jeong JH, Kim SW, Park TG. Molecular design of functional polymers for gene therapy. *Prog. Polym. Sci.* 32, 1239–1274 (2007).

7 Kumari A, Yadav SK, Yadav SC. Biodegradable polymeric nanoparticles based drug delivery systems. *Colloids Surf. B Biointerfaces* 75(1), 1–18 (2010).

8 Kim TI, Lee M, Kim SW. A guanidinylated bioreducible polymer with high nuclear localization ability for gene delivery systems. *Biomaterials* 31, 1798–1804 (2010).

9 Boe SL, Longva AS, Hovig E. Cyclodextrincontaining polymer delivery system for light-directed siRNA gene silencing. *Oligonucleotides* 20, 175–182 (2010).

10 Klibanov AL, Maruyama K, Torchilin VP *et al.* Amphipathic polyethylene glycols effectively prolong the circulation time of liposomes. *FEBS Lett.* 268, 235–237 (1990).

11 Huang L, Liu Y. *In vivo* delivery of RNAi with lipid-based nanoparticles. *Annu. Rev. Biomed. Eng.* 15(13), 507–530 (2011).

12 Park TG, Jeong JH, Kim SW. Current status of polymeric gene delivery systems. *Adv. Drug Deliv. Rev.* 58(4), 467–486 (2006).

13 Hatakeyama H, Akita H, Ito E *et al.* Systemic delivery of siRNA to tumors using a lipid nanoparticle containing a tumor specific cleavable PEG–lipid. *Biomaterials* 32, 4306–4316 (2011).

14 Romberg B, Hennink WE, Storm, G. Sheddable coatings for long circulating nanoparticles. *Pharm. Res.* 25, 55–71 (2008).

15 Dams ET, Laverman P, Oyen WJ *et al.* Accelerated blood clearance and altered biodistribution of repeated injections of sterically stabilized liposomes. *J. Pharmacol Exp. Ther.* 292 (3), 1071–1079 (2000).

16 Judge A, McClintock K, Phelps JR *et al.* Hypersensitivity and loss of disease site targeting caused by antibody responses to PEGylated liposomes. *Mol. Ther.* 13, 328–337(2006).

17 Xu Y, Szoka FC. Mechanism of DNA release from cationic liposome/DNA complexes used in cell transfection. *Biochemistry* 35(18), 5616–5623 (1996).

18 Gordon SP, Berezhna S, Scherfeld D *et al.* Characterization of interaction between cationic lipid–oligonucleotide complexes and cellular membrane lipids using confocal imaging and fluorescence correlation spectroscopy. *Biophys. J.* 88, 305–316 (2005).

19 Bieber T, Meissner W, Kostin S *et al.* Intracelullar route and transcriptional competence of polyethylenimine–DNA complexes. *J. Control. Release* 82, 441–454 (2002).

20 Fella C, Walker GF, Ogris M *et al.* Amine-reactive pyridylhydrazone based PEG reagents for pH-reversible PEI polyplex shielding. *Eur. J. Pharm. Sci.* 34, 309–320 (2008).

21 Li W, Nicol F, Szoka FC. GALA: a designed synthetic pH-responsive amphipathic peptide with applications in drug and gene delivery. *Adv. Drug Deliv. Rev.* 56 (7), 967–985 (2004).

22 Li SD, Chono S, Huang L. Efficient gene silencing in metastatic tumor by siRNA formulated in surface-modified nanoparticles. *J. Control. Release* 126, 77–84 (2008).

23 Li J, Chen YC, Tseng YC *et al.* Biodegradable calcium phosphate nanoparticle with lipid coating for systemic siRNA delivery. *J. Control. Release* 142, 416–421 (2010).

24 Sakurai Y, Hatakeyama H, Sato Y *et al.* Endosomal escape and the knockdown efficiency of liposomal-siRNA by the fusogenic peptide shGALA. *Biomaterials* 32, 5733–5742 (2011).

About the Authors

Munehisa Shimamura

Munehisa Shimamura has been Associate Professor of the Division of Vascular Medicine and Epigenetics, Osaka University United Graduate School of Child Development (Japan) since 2010. His main field of interest is neurology and the post-ischemic immune system, inflammation, neuritogenesis and neurogenesis.

Hironori Nakagami

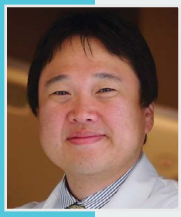
Hironori Nakagami has been Professor of the Division of Vascular Medicine and Epigenetics, Osaka University United Graduate School of Child Development since 2010. His main field of interest is vascular biology and cardiology. He is now interested in the application of DNA vaccines for cardiovascular diseases.

Hitomi Kurinami

Hitomi Kurinami has been Assistant Professor of the Division of Vascular Medicine and Epigenetics, Osaka University United Graduate School of Child Development since 2012. Her main field of interest is neurobiology. She studies inflammatory responses to neuronal damage.

Naoyuki Sato

Naoyuki Sato has been Associate Professor of the Division of Clinical Gene Therapy, Graduate School of Medicine, Osaka University (Japan) since 2006. His main field of interest is neurology and geriatrics. He is now interested in the relationship between lifestyle diseases, especially diabetes mellitus and Alzheimer's disease.



Ryuichi Morishita

Ryuichi Morishita has been Professor of the Department of Clinical Gene Therapy, Osaka Graduate School of Medicine since 2003. His main field of interest is cardiovascular disease, and he was the first to develop the decoy oligodeoxynuleotides for E2F or NF-κB to prevent restenosis after coronary artery intervention. He performed the clinical trial of therapeutic angiogenesis using the *HGF* gene.

Chapter 4

Plasmid DNA-based gene therapy in neurological disorders

Munehisa Shimamura, Hironori Nakagami, Hitomi Kurinami, Naoyuki Sato & Ryuichi Morishita

Therapeutic approaches utilizing plasmid DNA have been sought for nontreatable neurological disorders, such as ischemic stroke, Parkinson's disease, Alzheimer's disease and multiple sclerosis. One strategy is to induce overexpression of growth factors that can promote neuroprotection, neurogenesis or neuritogenesis in diabetic neuropathy, ischemic stroke and Parkinson's disease. Another therapeutic strategy includes the use of a 'DNA vaccine' to induce immunity against amyloid Aβ in Alzheimer's disease or to induce immune tolerance in multiple sclerosis. In this chapter, the authors discuss the advantages and problems associated with the use of plasmid DNA-mediated gene therapy in neurological disorders.

Aa Immune tolerance: the unresponsiveness to an antigen that is induced by previous exposure to that same antigen. When specific lymphocytes encounter antigens, the lymphocytes may be activated and produce an immune response, or the cells may be inactivated or eliminated and produce immune tolerance.

VEGF: a 45-kDa basic heparin that binds homodimeric glycoprotein and has been the most extensively studied growth factor in the field of gene therapy. There are four isoforms of VEGF, including VEGF-A, -B, -C and -D. VEGF-A also has isoforms, including $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$. The receptors for VEGF, FLT-1 and FLK-1, activate intracellular tyrosine kinase.

Although neurological disorders, such as stroke, Alzheimer's disease (AD), Parkinson's disease (PD) and multiple sclerosis (MS), are commonly associated with a decreased quality of life in patients, effective treatments are quite limited. Plasmid DNA (pDNA) has been investigated as a new treatment option for many neurological diseases in both experimental animals and clinical trials. Naked pDNA is easy to work with and safer than viral vectors, although its low transfection efficiency in the CNS poses a problem. To overcome this disadvantage, researchers have attempted to improve the structure of pDNA, vectors and injection devices, such as liposomes, polyethylene glycolylated immunoliposomes, ultrasound microbubbles, nanoparticles and electroporation.

One application for pDNA is to overexpress growth factors because several neurotrophic factors have the potential to promote blood flow through angiogenesis and confer neuronal protection, neuritogenesis and/or neurogenesis. This overexpression technique has been examined in diabetic neuropathy, ischemic stroke and PD. The use of pDNA has also been examined as a 'DNA vaccine' because pDNA itself has potential as a 'built-in adjuvant' that can stimulate dendritic cells and promote the acquisition of immunity [1]. Furthermore, this pDNA technique was tested as a vaccine for AD. By contrast, pDNA also has the potential of inducing immune tolerance in autoimmune diseases, including MS. Here, the authors review the use of pDNA as a treatment for various neurological disorders.

The promotion of neuroprotection by pDNA-based gene therapy

Diabetic neuropathy

The use of pDNA-based gene therapy in diabetic neuropathy has been intensively studied because of the relative ease of transferring genes. One of the pathophysiological features of diabetic neuropathy is an ischemic microangiopathy that leads to damage of the axon and nerve sheath. To reverse this damage, strategies that supply neuroprotective and angiogenic factors, such as VEGF or HGF, have been investigated. Although pDNA could theoretically be transfected directly into peripheral nerves, muscles transfected with pDNA encoded for growth factors can produce and secrete these proteins to support the adjacent peripheral nerves. First,

exogenous supplementation of VEGF using pDNA was demonstrated to have beneficial effects in the treatment of experimental models of diabetic neuropathy by promoting angiogenesis and neuronal protection. A randomized, double-blind clinical trial of VEGF pDNA transfection was performed in 50 patients with diabetic neuropathy (average glycated hemoglobin [HbA1c]: 7.6) who did not have cancer or retinopathy [2]. In this trial, pDNA was injected intramuscularly in eight standardized sites adjacent to the sciatic, peroneal and tibial nerves of one leg every 2 weeks for a total of 6 weeks. Although the total examination score and nerve conduction test results were not significantly different between the treated and untreated groups, the symptom score and the distribution of sensory disturbances were significantly improved in patients that were treated. Adverse effects, such as leg edema, were equally observed in both groups.

Another candidate gene for use in pDNA therapy is *HGF*. The coupling of HGF and its receptor, c-Met, enhanced the survival of neurons in primary culture and induced neurite outgrowth during neuronal development *in vitro*. Other studies suggest that HGF prevents apoptosis in neurons via the PI3-kinase/Akt pathway. pDNA encoding the *HGF* gene was examined in an experimental diabetic neuropathy model and showed recovery of peripheral nerve function [3]. Recently, a clinical trial (Phase I/II) was initiated using a plasmid HGF gene, *VM202*, which encodes two isoforms of HGF, one consisting of 728 amino acids (HGF) and the other consisting of 723 amino acids (deleted HGF; ClinicalTrials.gov NCT01002235) [4]. Thus, the data suggest that plasmid VEGF or HGF gene therapy will be a promising therapy for diabetic neuropathy; however, further evaluation in clinical trials is still required.

Ischemic stroke

Although thrombolysis and controlled hypothermia are effective treatments in the acute stages of ischemic stroke, an effective treatment to enhance neuroprotection, neuritogenesis, neurogenesis and angiogenesis for the ischemic brain has not yet been established. Growth factors, such as VEGF, GDNF, BDNF and HGF, are ideal molecules for use in pDNA therapy because of their ability to promote neuroprotection, neuritogenesis, neurogenesis and angiogenesis. Previously, the effect of growth factors on cerebral ischemia were demonstrated in experimental stroke models using recombinant proteins; however, recent studies also demonstrated the efficacy of these growth factors using pDNA. Wang *et al.* demonstrated that an intraventricular injection of a human $VEGF_{165}$-expressing plasmid mixed with liposomes after transient middle cerebral artery occlusion produced an increase in angiogenesis and reduced infarct volume in the brain 2 weeks

after transient middle cerebral artery occlusion. In addition, *VEGF* overexpression significantly increased cell proliferation in the ipsilateral subventricular zone and striatum [5]. The effects of HGF pDNA were examined in several models of cerebral ischemia. When HGF pDNA was injected into the cisterna magna using hemagglutinating virus of Japan liposomes, the decreased cerebral blood flow induced by bilateral occlusion of the common carotid arteries was reversed through an increase in angiogenesis. Additionally, overexpression of *HGF*, using a combination of HGF pDNA and a hemagglutinating virus of Japan envelope vector system, attenuated brain ischemic injury in a rat model through angiogenic and neuroprotective actions without producing cerebral edema. Improvement of functional recovery was also observed when the HGF treatment was started 7 days after the induction of cerebral ischemia, as shown by the enhancement of neurite outgrowth, increased synapses and inhibition of astrogliosis [6].

Thus, HGF or VEGF pDNA gene therapy administered directly into the brain was shown to have the potential to treat cerebral ischemia. Yet, the invasiveness of this procedure, such as intracisternal injection for gene transfer, is a major disadvantage for its application to the clinic. A recent study examined the effects of intravenously injected human umbilical cord blood that contained $CD34^+$ cells transfected with GDNF pDNA in cerebral ischemia [7]. When the cells were administered 6 h after transient middle cerebral artery occlusion, the transplanted cells migrated to the peri-infarct area and produced an increase in GDNF and a reduction in infarct volume. 28 days after transplantation of *GDNF* gene-modified $CD34^+$ cells, the peri-infarct area was populated with significantly more GFP-positive cells, neurons and astrocytes, probably derived from the grafted cells [7]. Although the exact mechanism of functional improvement was not demonstrated, the authors speculated that *GDNF* gene-modified $CD34^+$ cells could maintain a high level of GDNF after transplantation and that elevated GDNF secretion might have facilitated neuroprotection.

Although clinical use of pDNA has not yet been attempted in the field of ischemic stroke, the development of a practical delivery system is expected to lead to the clinical application of this therapy.

Parkinson's disease

PD is a largely sporadic, progressive neurodegenerative disease. Its histopathological hallmarks consist of intracellular inclusions called 'Lewy bodies' and dopaminergic striatal insufficiency that is secondary to a loss of dopaminergic neurons in the substantia nigra pars compacta. All currently available symptomatic treatments for PD have significant limitations and new therapeutic approaches are needed. Researchers have attempted to

establish gene therapies using GDNF, BDNF or HGF. Some studies were conducted using recombinant protein or viral vectors encoding these growth factors, while other groups have tested the use of naked pDNA because of concerns as to the safety of gene therapy. For example, the effects of HGF pDNA were demonstrated in an experimental 6-hydroxydopamine model of PD in mice and monkeys [8]. Stereotaxic transfection of naked pDNA encoding human HGF cDNA into the striatum resulted in the significant prevention of abnormal behaviors and improvement in immunohistochemical, HPLC and PET scan analyses [8]. Another trial was conducted to determine the prolongation of survival for transplanted cells that contained GDNF pDNA and that were compacted into DNA nanoparticles with 10-kDa polyethylene glycol-substituted lysine 30-mers [9]. Tissue chunk grafts pretreated with pGDNF DNA nanoparticles and placed into the striatum showed a 16-fold increase in the number of surviving tyrosine hydroxylase-positive cells within tissue chunk grafts when compared with saline-pretreated grafts. Behaviorally, a larger improvement was observed in the grafts treated with pGDNF DNA nanoparticles [9]. This study shows the potential of pDNA to support the survival of grafted cells.

Thus, pDNA has been demonstrated to have the capacity for the treatment of PD, although no clinical trials have been initiated. Further studies are needed to shed light on the development of new pDNA treatments for PD.

DNA vaccine

DNA vaccines were first developed in the field of infectious diseases because the transgene expression of viral components resulted in the production of antibodies against viruses. In general, an intramuscular injection of pDNA induces a Th1-type immune response by activation of the Toll-like receptor through cytidine–phosphate–guanosine (CpG) motifs in the pDNA. By contrast, an intradermal injection administered by a gene gun, a needle-free device that delivers gold beads coated with DNA vaccine plasmids into the epidermal layer of the skin, induced a Th2-type immune response. The differences observed between the two routes of administration are probably due to differences in the cell type that was transfected. Myocytes and a few antigen-presenting cells were transfected by intramuscular injection, whereas Langerhans cells were transected by the intradermal injection [10].

Interestingly, pDNA can also induce immune tolerance [10]. Different forms of the same antigen may induce an immune response

Although plasmid DNA-based gene therapy has not been examined in clinical trials involving Parkinson's disease patients, a double-blind, randomized, controlled trial of intraputaminal AAV2-neurturin was performed. Even though this clinical trial showed no beneficial effects on clinical outcome when assessed 12 months after treatment, the possibility of a benefit with additional targeting of the substantia nigra and longer term follow-up is under investigation.

(immunogens) or tolerance (tolerogens or tolerogenic antigens). Although the mechanism by which injected pDNA induces immunity or immune tolerance is still controversial, it is believed to be dependent on multiple factors, such as signals that T cells receive from antigen-presenting cells or environmental factors during their activation process [10].

Alzheimer's disease

The main strategy for the use of a DNA vaccine in AD is to reduce amyloid Aβ by immunization without causing an autoimmune response in the brain. Previously, active immunization using AN-1792, a mixture of AB1-42 peptide and adjuvant QS21, was clinically examined [11]. Although AN-1792 cleared senile plaques, it caused autoimmune encephalitis and the clinical trial was halted [11]. This autoimmune encephalitis was presumed to be induced by T-cell-mediated and/or Fc-mediated immune responses, and a Th1 immune response was shown to be involved in the induction of autoimmune encephalitis [12]. In general, Th1 cells produce proinflammatory cytokines, such as IFN-γ and induce cellular immunity. By contrast, Th2 cells produce cytokines, such as IL-4 and IL-10, and promote humoral immunity. In this regard, the potential of a 'DNA vaccine' that could induce a Th2-predominant immune response has attracted the attention of AD researchers.

Gene gun delivery of pDNA is an attractive tool to induce a Th2-predominant immune response and has been used in several experiments. For example, gene gun delivery of the mouse *Aβ42* dimer gene induced a significant humoral immune response and no evident cytotoxic T-lymphocyte response in wild-type mice after three vaccinations at 10-day intervals [13]. This same research group tested gene gun immunization in transgenic mice using the *Aβ42* gene in a bacterial plasmid with a pSP72-E3L-Aβ42 construct. In the treated group, a high titer of anti-Aβ42 antibody with a Th2-type predominant-immune response was observed. Furthermore, the level of Aβ42 in the treated transgenic mouse brain was reduced by 60–77.5% [14]. Another study examined the modification of pDNA to express a fusion protein that could induce a Th2 immune response. Movsesyan *et al.* designed pDNA expressing a fusion protein that consisted of three copies of the self-B-cell epitope of Aβ42 (Aβ[1–11]), a nonself T-helper cell epitope (PADRE) and a macrophage-derived chemokine (MDC/CCL22) as a molecular adjuvant to promote a strong anti-inflammatory Th2 phenotype [15]. When it was given to a 3xTg-AD mouse starting

A DNA vaccine inducing immune tolerance for myelin basic protein, proteolipid protein and myelin oligodendrocyte glycoprotein was demonstrated to be effective in animal models, but clinical trials using plasmid DNA encoding myelin basic protein have only recently been completed.

at 3–4 months of age, prophylactic immunizations with the DNA epitope vaccine generated a robust Th2 immune response that induced a high titer of anti-Aβ antibody. In addition, the DNA vaccine inhibited the pathological accumulation of Aβ in the brain of older mice. The vaccination also reduced glial activation and prevented the development of behavioral deficits in aged animals, without increasing the incidence of microhemorrhages [15].

Thus, animal experiments have shown that intradermal gene delivery using a gene gun or structural development of pDNA can stimulate Th2 immune responses without activating Th1 immune responses. The DNA vaccine could be an ideal treatment for AD, although clinical trials have not yet commenced.

Multiple sclerosis

MS is a chronic inflammatory disease characterized by demyelination, axonal damage and perivascular inflammation in the CNS [10]. Autoimmunity could play a major role in the susceptibility to and development of the disease. Autoreactive T cells against myelin basic protein (MBP), proteolipid protein (PLP), and myelin oligodendrocyte glycoprotein (MOG) were suggested to be immunological antigens in animal models of MS, such as experimental autoimmune encephalomyelitis (EAE). Several studies have shown that immunomodulation using pDNA encoding these antigens or epitopes improved the outcome in an EAE model. For example, Lewis rats were vaccinated with pDNA encoding an encephalitogenic T-cell epitope, guinea pig MBP68-85 peptide, prior to induction of EAE with MBP68-85 in complete Freund's adjuvant [16]. The vaccination suppressed clinical and histopathological signs of EAE and reduced T-cell activity against MBP and IFN-γ production after challenge with MBP68-85 [16]. With regard to PLP, the effect of a naked DNA construct encoding proteolipid protein (pRc/CMV-PLP) was examined [17]. After vaccination with pDNA, the SJL/J mice strain were sensitized to EAE with a PLP139-151 peptide in the adjuvant. Early sensitization to EAE (4 weeks after DNA vaccination) showed a severe, exacerbated form of disease through induction of a Th1-type cytokine response, while late sensitization (>10 weeks) resulted in a milder, ameliorated form of EAE, as shown by a decrease in T-cell proliferation and cytotoxic T-cell response, no Th2 response and no increase in apoptosis [17]. These data suggest a differential effect of DNA vaccination based on the timing of sensitization.

In clinical practice, a randomized, double-blind, placebo-controlled trial (Phase I/II) was performed with BHT-3009, a tolerizing DNA vaccine encoding full-length human MBP, in patients with MS [18]. Thirty patients with relapsing–remitting or secondary progressive MS who were not taking

any other disease-modifying drugs, were enrolled in this clinical trial [18]. BHT-3009 was administered intramuscularly at weeks 1, 3, 5 and 9 after randomization into the trial, with or without atorvastatin calcium, a compound reported to have potent immunomodulatory effects in MS [19]. As a result, BHT-3009 was safe and well tolerated, provided favorable trends in brain MRI findings and produced beneficial antigen-specific immune changes [18]. A marked decrease in the proliferation of IFN-γ-producing, myelin-reactive $CD4^+$ T cells in peripheral blood and a reduction in the titer of myelin-specific autoantibodies in the cerebral spinal fluid were also observed. Based on these results, the same group reported a placebo-controlled Phase II trial in which 289 relapsing–remitting or secondary progressive MS patients were enrolled [20]. When compared with the placebo, the median 4-week rate of new enhancing lesions during weeks 28–48 was 50% lower and during weeks 8–48 was 61% lower with 0.5 mg BHT-3009 [20]. Although the mean volume of enhancing lesions at week 48 was 51% lower with 0.5 mg BHT-3009, no significant improvement in MRI lesion parameters was observed with 1.5 mg BHT-3009. A marked reduction in myelin-specific autoantibodies was observed in the 0.5-mg BHT-3009 group, but it was not observed with the placebo or 1.5-mg BHT-3009 group. Immunological data in a preselected subgroup of patients also indicated that treatment with 0.5 mg BHT-3009 induced antigen-specific immune tolerance, although it was ineffective in the group of patients given 1.5 mg BHT-3009 [20]. A possible explanation for the lack of efficacy with high-dose BHT-3009 plasmid is that it contains some residual consensus immunostimulatory CpG motifs. Because CpG sequences bind to TLR9, and thereby induce IFN-γ production, the 1.5-mg dose might have delivered sufficient numbers of CpG motifs to overcome the tolerogenic effect [20]. However, no significant difference was noted in the disability assessment parameters even in patients with the dose of 0.5 mg BHT-3009 [20]. The authors speculated that the reason for the lack of effects on clinical outcome was that the trial was short and did not have sufficient statistical power [20]. Although a lack of improvement in the tested clinical outcome is a disadvantage, this clinical trial showed that pDNA has the potential to induce immune tolerance in MS and optimization of the dose of pDNA is an important factor for inducing successful immune tolerance in autoimmune diseases.

Thus, a DNA vaccine might be a promising approach to treat neurodegenerative diseases and autoimmune diseases. Importantly, the structure of pDNA, the delivery method and the dose of pDNA should be carefully determined in view of the complex immune system response.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

Summary

- There are two main strategies in plasmid DNA (pDNA)-mediated gene therapy, including the supplementation of growth factors and the DNA vaccine.
- Mild effectiveness was observed in a randomized, double-blind trial using VEGF pDNA transfection in diabetic neuropathy.
- Although animal experiments using pDNA-based gene therapy for growth factor genes showed positive results in ischemic stroke and Parkinson's disease, no clinical studies have been completed.
- In Alzheimer's disease animal models, a pDNA vaccination was shown to reduce amyloid Aβ accumulation without causing a cytotoxic immune response.
- A randomized, double-blind, placebo-controlled trial in patients with MS showed that immune tolerance and the reduction of new lesions could be achieved, but the clinical outcome was not significant.
- Scientists continue to research with the aim to improve the effectiveness and reduce the side effects of pDNA-based gene therapy.

References

1 Coban C, Koyama S, Takeshita F, Akira S, Ishii KJ. Molecular and cellular mechanisms of DNA vaccines. *Hum. Vaccin.* 4(6), 453–456 (2008).

2 Ropper AH, Gorson KC, Gooch CL *et al.* Vascular endothelial growth factor gene transfer for diabetic polyneuropathy: a randomized, double-blinded trial. *Ann. Neurol.* 65(4), 386–393 (2009).

3 Koike H, Morishita R, Iguchi S *et al.* Enhanced angiogenesis and improvement of neuropathy by cotransfection of human hepatocyte growth factor and prostacyclin synthase gene. *FASEB J.* 17(6), 779–781 (2003).

4 Saeed M, Martin A, Ursell P *et al.* MR assessment of myocardial perfusion, viability, and function after intramyocardial transfer of VM202, a new plasmid human hepatocyte growth factor in ischemic swine myocardium. *Radiology* 249(1), 107–118 (2008).

5 Wang YQ, Guo X, Qiu MH, Feng XY, Sun FY. VEGF overexpression enhances striatal neurogenesis in brain of adult rat after a transient middle cerebral artery occlusion. *J. Neurosci. Res.* 85(1), 73–82 (2007).

6 Shimamura M, Sato N, Waguri S *et al.* Gene transfer of hepatocyte growth factor gene improves learning and memory in the chronic stage of cerebral infarction. *Hypertension* 47(4), 742–751 (2006).

7 Ou Y, Yu S, Kaneko Y *et al.* Intravenous infusion of GDNF gene-modified human umbilical cord blood $CD34^+$ cells protects against cerebral ischemic injury in spontaneously hypertensive rats. *Brain Res.* 1366, 217–225 (2010).

8 Koike H, Ishida A, Hayashi T *et al.* Injection of HGF plasmid cDNA to prevent manifestation of Parkinson disease: a preclinical study using a primate model. *Open Gene Ther. J.* 2, 38–44 (2009).

9 Yurek DM, Flectcher AM, Kowalczyk TH, Padegimas L, Cooper MJ. Compacted DNA nanoparticle gene transfer of GDNF to the rat striatum enhances the survival of grafted fetal dopamine neurons. *Cell Transplant* 18(10), 1183–1196 (2009).

10 Fissolo N, Montalban X, Comabella M. DNA-based vaccines for multiple sclerosis: current status and future directions. *Clin. Immunol.* 142(1), 76–83 (2010).

11 Nicoll JA, Wilkinson D, Holmes C *et al.* Neuropathology of human Alzheimer disease after immunization with amyloid-beta peptide: a case report. *Nat. Med.* 9(4), 448–452 (2003).

12 Monsonego A, Zota V, Karni A *et al.* Increased T cell reactivity to amyloid beta protein in older humans and patients with Alzheimer disease. *J. Clin. Invest.* 112(3), 415–422 (2003).

13 Qu B, Rosenberg RN, Li L, Boyer PJ, Johnston SA. Gene vaccination to bias the immune response to amyloid-beta peptide as therapy for Alzheimer disease. *Arch. Neurol.* 61(12), 1859–1864 (2004).

14 Qu B, Boyer PJ, Johnston SA, Hynan LS, Rosenberg RN. Abeta42 gene vaccination reduces brain amyloid plaque burden in transgenic mice. *J. Neurol. Sci.* 244(1–2), 151–158 (2006).

15 Movsesyan N, Ghochikyan A, Mkrtichyan M *et al.* Reducing AD-like pathology in 3xTg-AD mouse model by DNA epitope vaccine – a novel immunotherapeutic strategy. *PLoS ONE* 3(5), e2124 (2008).

16 Weissert R, Lobell A, de Graaf KL *et al.* Protective DNA vaccination against organ-specific autoimmunity is highly specific and discriminates between single amino acid substitutions in the peptide autoantigen. *Proc. Natl Acad. Sci. USA* 97, 1689–1694 (2000).

17 Selmaj K, Kowal C, Walczak A, Nowicka J, Raine CS. Naked DNA vaccination differentially modulates autoimmune responses in experimental autoimmune encephalomyelitis. *J. Neuroimmunol.* 111(1–2), 34–44 (2000).

18 Bar-Or A, T Vollmer, J Antel *et al.* Induction of antigen-specific tolerance in multiple sclerosis after immunization with DNA encoding myelin basic protein in a randomized, placebo-controlled Phase 1/2 trial. *Arch. Neurol.* 64(10), 1407–1415 (2007).

19 Neuhaus O, Hartung HP. Evaluation of atorvastatin and simvastatin for treatment of multiple sclerosis. *Expert Rev. Neurother.* 7(5), 547–556 (2007).

20 Garren H, Robinson WH, Krasulová E *et al.* Phase 2 trial of a DNA vaccine encoding myelin basic protein for multiple sclerosis. *Ann. Neurol.* 63(5), 611–620 (2008).

About the Authors

Salima Hacein-Bey-Abina

Salima Hacein-Bey-Abina is Full Professor of Immunology at Paris Descartes University, and Head of the Gene Therapy Unit in the Biotherapy Department at Necker Hospital (Paris, France). After a residency in Medical Biology, she obtained a PhD degree in 1998. Since then, and after joining the Biotherapy Department, she set up many gene therapy trials with the first success in the field related to patients affected with X-linked severe combined immunodeficiency.

Alain Fischer

Alain Fischer has been Full Professor of Pediatrics since 1986 and Director of the Department of Pediatric Immunology and Hematology at Necker Hospital, University Paris Descartes. He is also Head of a National Institute for Health and Medical Research Laboratory and has been Director of the Institute of Genetic Diseases 'Imagine' at Necker Hospital since 2009. His main research interests are development and genetic diseases of the immune system, and gene therapy.

Marina Cavazzana-Calvo

Marina Cavazzana-Calvo is Full Professor in Hematology at Paris Descartes University, Head of the Biotherapy Department and Director of the Biotherapy Clinical Investigation Center at Necker Hospital. She studied medicine in Padua, Italy, Pediatrics and Life Sciences in Paris, France and dedicated her entire career to setting up innovative strategies to treat inherited disorders of the hematological system that, when conventional treatments are used, have unsatisfying clinical results.

Chapter 5

Gene therapy for X-linked severe combined immunodeficiency and other hereditary diseases

Salima Hacein-Bey-Abina, Alain Fischer & Marina Cavazzana-Calvo

doi:10.2217/EBO.12.156

The long-term follow-up (up to 12 years) of the seminal clinical studies of human stem cell gene therapy for severe combined immunodeficiency provided the proof of concept of the efficacy of this new therapeutic approach. These studies based on first-generation retroviral vectors showed a good long-term immune system reconstitution in most treated patients despite the occurrence of vector-related leukemia in a few of them. Improvement of vectors safety will allow a broader application of gene therapy.

The dawn of the age of genetics

Gene therapy is not a new concept. The notion that genetic material could be used to cure human disease emerged several decades ago. Since the 1950s, discoveries in molecular genetics foreshadowed and then enabled the emergence of this new therapeutic option. These achievements were founded on a critical discovery made at the Rockefeller Institute of Medical Research (NY, USA) in 1944; Oswald Avery, Colin McLeod and Maclyn McCarthy showed that pneumococci could be modified by nucleic acid transfer and thus identified for the first time the molecular nature of the material responsible for changing bacterial characteristics. Viral gene transfer was first demonstrated with bacteriophages and then with animal viruses. In the mid to late 1960s, James Sambrook and Miroslav Hill demonstrated that in the course of transforming a cell from a normal phenotype to a neoplastic phenotype, the papovavirus SV40 and the polyomavirus integrated their genetic information covalently, stably and heritably into the target cell's genome. The expression of viral transforming regions was shown to induce new phenotypic characteristics in transformed cells. It had then become clear that viruses could be considered as 'carriers' of genetic material. However, the advent of recombinant DNA technology constituted the true birth of gene therapy. Gene cloning became possible and it was shown *in vitro* that foreign genes could correct genetic defects in mammalian cells.

In the meantime, greater knowledge of the retroviral life cycle had prompted the development of the retroviral vectors. Then, in the early 1980s, Temin, Scolnick, Weinberg and their colleagues reported on techniques that enabled the modification of retroviral genetic information by adding potentially therapeutic genes. The resulting vectors were capable of highly efficient infection of human cells. After several disease-related genes had been transferred into various cells *in vitro*, the concept of gene therapy via efficient *in vivo* or *ex vivo* gene transfer into mammalian cells became widely discussed [1].

However, the emergence of gene therapy was also accelerated by the huge progress made in the field of stem cell biology in general, and the biology and manipulation of hematopoietic stem cells (HSCs) in particular.

The first gene therapy clinical trials

By the late 1980s, the foundations for the first clinical trials of gene therapy had been laid. The first ever protocol of this kind was a 'gene-marking' study of tumor-infiltrating T cells performed in 1989 by Steven Rosenberg's group. However, the first truly therapy-focused trial was that initiated in 1990 at the US NIH (MD, USA) by R Michael Blease's group. The researchers

treated patients suffering from adenosine deaminase (ADA) deficiency, a form of severe combined immunodeficiency (SCID) of purine metabolism. Since then, the number of clinical trials has grown steadily, reflecting the remarkable increase in the identification of disease-associated genes and the development of new systems (both viral and nonviral) for gene transfer. The completion of the Human Genome Project in 2003 – yielding knowledge of the sequence of nearly all genes in the human genome – also played a major role in this expansion, of course.

Adenosine deaminase (ADA) deficiency was the first severe combined immunodeficiency condition for which a genetic and molecular cause was identified. The absence of ADA leads to accumulation of (de)adenosine compounds that induce apoptosis (mostly of lymphoid progenitors) and then a near-total absence of lymphocytes. Given that this enzyme is ubiquitously expressed, this deficiency is considered as a metabolic disease that induces other abnormalities. Hence, enzyme replacement therapy with repeated injections of PEGylated ADA led to significant improvements in lymphopoiesis and survival, although the patients remained dependent on supplementation and the disease correction was only partial. For gene therapy approach, it was reasoned that enzyme replacement therapy should be removed to maintain the transduced progenitor cells' growth advantage. Over the last decade, 27 patients with ADA deficiency underwent *ex vivo* gene therapy by using retroviral vectors. Among them, 19 have corrected their immunodeficiency (at least partially) without any adverse events.

The rationale for gene therapy of X-linked SCID

Limits of X-linked SCID treatment by HSC transplantation

X-linked SCID (SCID-X1) is the most common SCID and is characterized by a complete lack of T and natural killer (NK) cells, although B-cell development is normal [2]. The gene responsible for this disease (*IL2RG*) was identified in 1993 [3]. It encodes a common γ-chain (γc) shared by the cell surface receptors for six interleukin cytokines (IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21) [4]. The pathophysiological features in SCID-X1 are explained by the absence of the survival and proliferation signals normally provided by IL-7 and IL-15 in lymphoid progenitors [5]. These two cytokines play a critical role in the development of T cells and NK cells, respectively.

As with other SCIDs, allogeneic HSC transplantation (HSCT) has been the standard curative treatment for SCID-X1 for approximately the last 40 years. Without curative treatment, SCID-X1 patients die from uncontrolled infections within the first year of life. In fact, HSCT constitutes an emergency treatment that does not leave enough time to search for an unrelated, matched donor (which usually takes at least 3 months). Parental (i.e., haploidentical) transplants are associated with a 72% likelihood of survival at 5 years [6]. Mortality is mainly related to the occurrence of graft-versus-host disease and uncontrolled severe infections. However, this overall survival

Hematopoietic stem cell transplantation: a graft procedure using autologous or allogeneic hematopoietic stem cells collected from bone marrow or mobilized peripheral stem cells. Hematopoietic stem cell transplantation is used in multiple clinical conditions to repopulate the hematopoietic system with normal and functional cells.

Aa Graft-versus-host disease: is a frequent complication of allogeneic hematopoietic stem cell transplantation, in which the donor's T cells attacks the patient's tissues and organs, because the latter harbor HLA markers identified as 'nonself'. There are two forms of graft-versus-host disease: acute and chronic. Patients may develop one, both or neither. Acute and chronic graft-versus-host disease differ in their clinical signs and time of onset.

rate declines to less 50% when patients are diagnosed after 6 months of age and are severely affected by opportunistic agents (cytomegalovirus and adenovirus). These limitations accelerated (at least in part) the application of gene therapy to SCID-X1.

The biological and clinical knowledge generated in HSCT has been exploited in the development of gene therapy protocols; this explains why the first genetic diseases to have been 'corrected' by gene transfer were immunodeficiencies. The goal of *ex vivo* gene transfer of a functional copy of the defective gene into a patient's HSCs with a viral vector is to correct progenitor cells and enable the subsequent, stable production of differentiated cells. These circumstances suggest that gene therapy is a treatment option for SCID-X1 patients.

Criteria making the SCID-X1 an ideal model for gene transfer into HSCs

- The fact that the γc is constitutively expressed in all hematopoietic lineages prompted researchers to hope that no adverse effects would be observed (even in the absence of tight regulation of the transgene by its own promoter). Moreover, the γc is involved in di- or tri-meric complex receptors, within which its function is controlled by the other subunits;
- There is no risk of an immune response against the transgene;
- The restoration of γc expression should confer a selective growth advantage on the lymphoid precursors. Indeed, intense proliferation of lymphocyte precursors and their subsequent differentiation into a very large number of long-lived T cells should correct the disease phenotype for an extended period. This selective growth advantage has been observed in many cases of somatic mosaicism in patients with immunodeficiencies, some of which result from *in vivo* reversion of an inherited mutation. This latter phenomenon (referred to as 'natural gene therapy') has been reported in patients with ADA deficiency, SCID-X1 and Wiscott–Aldrich syndrome [7,8]. Following reversion of a single cell *in vivo*, the reverted cells expand with the selective growth advantage provided by corrected gene expression. These findings have had a significant impact on the prospects for stem-cell gene therapy of immunodeficiencies. The presence of a selective advantage may be an important success factor because only small numbers of gene-corrected

cells may have to be infused in SCID gene therapy trials.

Aa Long terminal repeat: repeated and inverted identical DNA sequences present at both ends of proviral DNA and transposons and essential for their integration into the host genome. Long terminal repeats also contain regulatory sequences that control gene expression using transcriptional enhancers, promoters, transcription initiation, termination and polyadenylation sites.

Optimization of gene transfer methods

In addition to favorable disease characteristics, improvements in gene transfer methods have made it possible to perform this type of clinical trial. Various combinations of cytokines induce the activation but not differentiation of HSCs and suitable combinations improve stem cell gene transduction [9]. Furthermore, the use of a recombinant fragment of fibronectin helps the target cells and vector to co-localize [10]. Significant improvements in retroviral vector design and production methods have also raised the efficiency of gene expression and transduction. Transgene expression in lymphoid populations has been optimized and vectors have been improved by deleting sequences in the long terminal repeat (LTR) that inhibit gene expression [11,12]. Furthermore, the earliest techniques were improved by the construction of helper-free packaging cell lines capable of producing high-titer vectors.

SCID-X1 clinical trial results

Immune reconstitution

After having performed a large number of fundamental studies on SCID-X1 stem cell gene therapy, we initiated the first clinical trial of gene therapy for SCID-X1 in 1999. Ten children under the age of 1 year and lacking an HLA-identical sibling were enrolled. No conditioning regimen was applied. They received an average dose of 9×10^6 of $CD34^+$ cells/kg transduced with a γ-retroviral vector within which the γc transgene was under the transcriptional control of the LTR.

Of the nine evaluable patients, seven had a stably corrected immunological phenotype [13]. T-cell counts rose to normal-for-age levels between 3 and 6 months post-therapy and remained stable over time in all patients (except those who have developed monoclonal lymphoproliferation: patients 4, 5, 7 and 10 [see later]). T cells proliferated *in vitro* in response to mitogens (phytohemagglutinin and anti-CD3) and vaccine antigens. The cells provided effective protection against infection *in vivo*, as evidenced by recovery from infections during the first few months post-treatment and (in patient 7) chicken pox that occurred 1 year after gene therapy.

The T cells developed a polyclonal T-cell receptor repertoire. Moreover, 10 years after treatment, thymopoiesis is still ongoing in all but one of the patients, as evidenced by the continued detection of circulating 'recent

thymic emigrants' – even in patients who subsequently developed leukemia and received chemotherapy. Normal NK cell differentiation was also observed after the infusion of genetically modified progenitors. However, the NK cell count fell rapidly to subnormal values after 1 year. This phenomenon (which is also observed after allogeneic HSCT) could be attributed to the weaker proliferative and/or survival capacity of NK cell progenitors, relative to that of T-cell progenitors. Despite the absence of genetically corrected B cells in the patients' peripheral blood, four of the seven patients are still able to produce normal levels of immunoglobulins. Moreover, the $CD19^+/CD27^+$ memory B-cell subpopulation was poorly represented, except for normal levels in patient 2. We have shown that the B cells were able to produce immunoglobulins via γc-independent pathways. However, the development of memory B cells and persistence of a strong humoral response against recall antigens required the involvement of the γc-dependent cytokine IL-21 [13]. These results (which are similar to those observed after allogeneic HSCT in the absence of myeloablation) indicate that despite the absence of the IL-21-inducible activation pathway, γc-independent cytokines are able to produce some of the responses mediated by B lymphocytes (e.g., T-independent antibody responses and extrafollicular B-cell activation). However, the development of full humoral responses and the long-term maintenance of normal levels of memory B cells (associated with stable production of high-affinity antibodies against specific antigens) can only be obtained by stable engraftment of genetically modified stem cells. This requires access to the hematopoietic niches by conditioning the patient prior to transplantation [13].

In conclusion, genetic correction of the immunodeficiency restored the patients' general health status and has enabled them to lead a normal life.

Insertional genotoxicity

Four of the aforementioned patients developed T-cell leukemia between 30 and 68 months after gene therapy and required chemotherapy [14,15]. Three of the patients recovered after treatment and are doing well, thanks to a good immunological recovery by the residual γc-transduced $CD34^+$ cells. Genetic analysis of the malignant cells showed that the retroviral vector had integrated within or near tumor-promoting genes mainly *LMO2* locus causing their transcriptional activation. Since these events, large-scale analysis of retroviral integration patterns has been performed on peripheral blood samples provided by patients included in all the various retrovirus-based gene therapy trials. These studies clearly showed that retroviral vectors integrate semirandomly throughout the genome in the

coding region of highly expressed genes involved in proliferation and cell survival [16,17].

A similar trial was performed in the UK [18]. In all, 17 of the 20 treated SCID-X1 patients are alive and display sustained correction of the immunodeficient phenotype [13,18]. These trials have taught us that retroviral vector-based γc gene transfer into hematopoietic progenitor cells enables the long-term correction of the patients' immune system defect. The trials have also highlighted the significant toxicity associated with the LTR's strong enhancer effect on the Moloney leukemia virus-based retroviral vector, which was responsible for the five cases of leukemia in the two SCID-X1 trials.

The next generation of SCID-X1 gene therapy trials

Many research groups have devoted significant efforts to the design of new vectors that are just as effective but less toxic [19]. One of the first optimizations (in retroviral and lentiviral vectors) was the deletion of the LTR enhancer sequences and the introduction of an internal, 'weak' promoter capable of inducing transcription of the therapeutic gene alone [20]. A new international trial in SCID-X1 has been jointly initiated by groups in the USA and the UK and our group in France. The trial is based on a self-inactivating retroviral vector and is restricted to patients with severe infections, in whom the prognosis following allogeneic HSCT is poor. To date, we have included four patients (three of whom have severe, disseminated bacille Calmette–Guérin infection). The three patients showed a significant improvement in their clinical condition after transplantation of genetically-modified $CD34^+$ cells, which was associated with functional T-cell recovery. The full safety assessment of this new vector requires long-term patient monitoring.

Second-generation vector technology

The clinical success achieved in SCID-X1 and in the other form of SCID, ADA deficiency [21], set the stage for a remarkable resurgence in support for gene therapy. These seminal clinical trials established the feasibility of gene therapy in humans and helped overcome a variety of regulatory and political barriers. Development of lentivirus-derived vectors represented a major advance in this field, since they are capable of infecting nondividing cells [22]. The vectors' first clinical application in gene therapy involved an *ex vivo* bone marrow strategy in a lethal pediatric, neurodegenerative disease called adrenoleukodystrophy (ALD). In contrast to previous work with SCID-X1, genetically corrected HSCs do not have a selective advantage in ALD. Successful reconstitution with corrected cells in the ALD patients

Aa Homologous recombination: DNA recombinant technology used to manipulate the genome by exchange between two similar or identical DNA sequences present in two separate chromosomes or DNA vectors. This technology is based on DNA sequence-specific nucleases able to induce the integration of an exogenous sequence in a specific region of the targeted genome for gene correction.

therefore requires myeloablation of the recipient's marrow associated with efficient *ex vivo* transduction. In fact, this pilot study was a resounding scientific and clinical success. Patients were repopulated with high counts of transduced cells of virtually all hematopoietic lineages and the disease was stabilized [23].

Moving towards genome engineering

The ultimate goal in gene therapy would be to achieve DNA recombination in specific, safe sites and thus correct *in situ* mutations without the more-or-less random introduction of an additional copy of the gene into the host genome. Today's genome engineering technologies are mainly based on two DNA sequence-specific nucleases: zinc-finger nuclease (ZNF) and transcriptional activator-like effector nuclease, to create a double-strand break and thus significantly increase the efficiency of homologous recombination [24,25]. If these systems can be further improved, they may eventually circumvent the issue of insertional mutagenesis and will constitute a breakthrough in gene therapy. Genetic modifications could also consist of introducing a nucleic acid coding for a specific antisense or interfering RNA, in order to establish (for example) cellular immunity to a viral infection [26]. Although some promising steps towards these objectives have been reported, safety issues (regarding potential off-target effects, for example) will have to be well documented.

The status of targeted cell population impacts on cell therapy processes development timelines

The design and development of scalable, reproducible processes capable of maintaining the biological features and functions of this new therapeutic class is the ultimate goal of ongoing R&D programs in this new field. The first gene therapy trials were related to immunodeficiencies in which the characteristics of the bone marrow and the most immature hematopoietic progenitors were similar to those of a normal marrow, which are the underlying defect had no impact on the distribution of hematopoietic lineages. Gene therapy has now been extended to diseases in which the underlying defect causes hyperplasia of a particular hematopoietic lineage and a commitment bias in immature progenitors. Gene transfer efficiency and final product quality will then depend not only on the characteristics of the vector, but also on those of the target cell population. In the context of pharmaceutical-grade processes, cell

products should be made consistently and reproducibly according to a set of predefined criteria that outline the quality parameters of the product. It is essential to be able to control all the parameters that define the initial product and thus fulfil the final product's batch release specifications. Well-defined cell requirements are used to create a staged process development strategy that enables adequate clinical-phase production and minimizes the risk of altering the product's safety or efficacy during scale-up.

The next frontier: renewed commitment by the biopharmaceutical industry?

The technical challenges in cell and gene therapy have been overcome after three decades of basic and translational research – most of which has been performed by academic laboratories. The pilot clinical trials have been successful and many exciting new applications are emerging; nearly 2000 gene therapy clinical trials have been initiated over the last 5 years, with major breakthroughs in the treatment of hemophilia, solid tumors, leukemia and various types of eye diseases. These positive clinical trial results and the upswing in public support have prompted renewed interest and commitment from the biopharmaceutical industry. Virtually all the major biopharmaceutical firms and venture capital funds are examining or re-examining the value of cell and gene therapy products. These 'live' biologics still present unique challenges in manufacturing, characterization and delivery; the companies supplying the enabling technology mainly have experience of cell lines for biotech products and vector production, rather than clinical cell-based therapy. We are entering a new era in which the clinical development of cell-based therapeutics could be performed collaboratively by academic labs, capable of providing state-of-art designs and know-how in cell therapy technology, and biopharmaceutical companies, capable of fulfilling regulatory requirements through good manufacturing practice and good tissue practice, and providing the infrastructure for commercial-scale cell therapy.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

Summary

- Gene therapy based on living cellular products has exploited the steady advances in stem cell manipulation and gene transfer technologies and has started to change the way medicine is practiced.
- The field is now booming with approvals for gene and cell therapy trials rising exponentially and extending to a whole range of diseases.
- What will now ensure the clinical development of these cell-based therapies is mainly establishment of scalable technology platforms that allow for varying levels of production throughout the different stages of clinical testing.
- As these pharmaceutical-grade processes development marry with the growing capacity for targeting viral integration, these new forms of designer drug might fulfil the promise of efficient and safe therapies for currently untreatable diseases.

References

1 Friedmann T. A brief history of gene therapy. *Nat. Genet*. 2(2), 93–98 (1992).

2 Buckley RH. Primary immunodeficiency diseases: dissectors of the immune system. *Immunol. Rev.* 185, 206–219 (2002).

3 Noguchi M, Yi H, Rosenblatt HM *et al.* Interleukin-2 receptor γ chain mutation results in X-linked severe combined immunodeficiency in humans. *Cell* 73(1), 147–157 (1993).

4 Sugamura K, Asao H, Kondo M *et al.* The interleukin-2 receptor γ chain: its role in the multiple cytokine receptor complexes and T cell development in XSCID. *Ann. Rev. Immunol*. 14, 179–205 (1996).

5 Leonard WJ. The molecular basis of X-linked severe combined immunodeficiency: defective cytokine receptor signaling. *Ann. Rev. Med.* 47, 229–239 (1996).

6 Gennery AR, Slatter MA, Grandin L *et al.* Transplantation of hematopoietic stem cells and long term survival for primary immunodeficiencies in Europe: entering a new century, do we do better? *J. Allergy Clin. Immunol.* 126(3), 602–610 (2010).

7 Bousso P, Wahn V, Douagi I *et al.* Diversity, functionality, and stability of the T cell repertoire derived *in vivo* from a single human T cell precursor. *Proc. Natl Acad. Sci. USA* 97(1), 274–278 (2000).

8 Speckmann C, Pannicke U, Wiech E *et al.* Clinical and immunologic consequences of a somatic reversion in a patient with X-linked severe combined immunodeficiency. *Blood* 112(10), 4090–4097 (2008).

9 Dao MA, Hannum CH, Kohn DB *et al.* FLT3 ligand preserves the ability of human $CD34^+$ progenitors to sustain long-term hematopoiesis in immune-deficient mice after *ex vivo* retroviral-mediated transduction. *Blood* 89(2), 446–456 (1997).

10 Hanenberg H, Xiao XL, Dilloo D *et al.* Colocalization of retrovirus and target cells on specific fibronectin fragments increases genetic transduction of mammalian cells. *Nat. Med*. 2(8), 876–882 (1996).

11 Halene S, Wang L, Cooper RM *et al.* Improved expression in hematopoietic and lymphoid cells in mice after transplantation of bone marrow transduced with a modified retroviral vector. *Blood* 94(10), 3349–3357 (1999).

12 Robbins PB, Skelton DC, Yu XJ *et al.* Consistent, persistent expression from modified retroviral vectors in murine hematopoietic stem cells. *Proc. Natl Acad. Sci. USA* 95(17), 10182–10187 (1998).

13 Hacein-Bey-Abina S, Hauer J, Lim A *et al.* Efficacy of gene therapy for X-linked severe combined immunodeficiency. *N. Engl. J. Med.* 363(4), 355–364 (2010).

14 Hacein-Bey-Abina S, Von Kalle C, Schmidt M *et al. LMO2*-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science* 302(5644), 415–419 (2003).

15 Hacein-Bey-Abina S, Garrigue A, Wang GP *et al.* Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1. *J. Clin. Invest.* 118(9), 3132–3142 (2008).

16 Deichmann A, Hacein-Bey-Abina S, Schmidt M *et al.* Vector integration is nonrandom and clustered and influences the fate of lymphopoiesis in SCID-X1 gene therapy. *J. Clin. Invest*. 117(8), 2225–2232 (2007).

17 Wang GP, Berry CC, Malani N *et al.* Dynamics of gene-modified progenitor cells analyzed by tracking retroviral integration sites in a human SCID-X1 gene therapy trial. *Blood* 115(22), 4356–4366 (2010).

18 Gaspar HB, Cooray S, Gilmour KC *et al.* Long-term persistence of a polyclonal T cell repertoire after gene therapy for X-linked severe combined immunodeficiency. *Sci. Transl. Med.* 3(97), 97ra79 (2011).

19 Montini E, Cesana D, Schmidt M *et al.* The genotoxic potential of retroviral vectors is strongly modulated by vector design and integration site selection in a mouse model of HSC gene therapy. *J. Clin. Invest*. 119(4), 964–975 (2009).

20 Modlich U, Navarro S, Zychlinski D *et al.* Insertional transformation of hematopoietic cells by self-inactivating lentiviral and γ retroviral vectors. *Mol. Ther.* 17(11), 1919–1928 (2009).

21 Aiuti A, Cattaneo F, Galimberti S *et al.* Gene therapy for immunodeficiency due to adenosine deaminase deficiency. *N. Engl. J. Med.* 360(5), 447–458 (2009).

22 Naldini L, Blomer U, Gallay P *et al. In vivo* gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* 272(5259), 263–267 (1996).

23 Cartier N, Hacein-Bey-Abina S, Bartholomae CC *et al.* Hematopoietic stem cell gene therapy with a lentiviral vector in X-linked adrenoleukodystrophy. *Science* 326(5954), 818–823 (2009).

24 Hockemeyer D, Wang H, Kiani S *et al.* Genetic engineering of human pluripotent cells using TALE nucleases. *Nat. Biotechnol.* 29(8), 731–734 (2011).

25 Urnov FD, Rebar EJ, Holmes MC *et al.* Genome editing with engineered zinc finger nucleases. *Nat. Rev. Genet.* 11(9), 636–646 (2010).

26 Burnett JC, Rossi JJ, Tiemann K. Current progress of siRNA/shRNA therapeutics in clinical trials. *Biotechnol. J.* 6(9), 1130–1146 (2011).

About the Authors

Shunsuke Kagawa

Shunsuke Kagawa is Senior Assistant Professor at the Department of Gastroenterological Surgery in Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences (Japan). He is a leading proponent of minimally invasive surgical approaches to gastric cancer. He has been working on the proapoptotic cancer gene therapy and genetically modified oncolytic virotherapy.

Toshiyoshi Fujiwara

Toshiyoshi Fujiwara is Professor and Chairman of the Department of Gastroenterological Surgery in Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences (Japan). He has completed the first *p53* gene therapy trial for lung cancer in Japan. He is currently developing telomerase-specific oncolytic adenoviruses for diagnostic and therapeutic application.

Chapter 6

Gene therapy for cancer treatment

Shunsuke Kagawa & Toshiyoshi Fujiwara

Advances in knowledge and techniques for manipulating genes have lead scientists to alter genetic information to treat or prevent disease. Gene therapy for cancer is a novel and experimental treatment that involves transferring nucleic acids into cells to treat cancer. Gene therapy has been studied in clinical trials for different types of cancers but has not yet been integrated into standard treatments. Some hurdles must be overcome before the clinical application of this novel treatment becomes realistic. This chapter discusses the current advances and trends in cancer gene therapy and its clinical outlook through recent Phase III trials.

doi:10.2217/EBO.12.279

The broad definition of cancer includes malignancies such as leukemias and lymphomas, which are derived from blood-forming tissues, and carcinomas and sarcomas, which are derived from solid organs. Here, we use the term cancer in a narrow sense as a malignancy derived from solid organs. Despite developments in the prevention, early diagnosis and treatment of cancer, there has been a steady rise in the occurrence of cancer along with the extension of life expectancy. Thus, cancer remains a serious worldwide health problem. Owing to the asymptomatic nature of cancer, it is often diagnosed at an advanced stage, which limits the possibility of a complete resection and increases the possibility of a recurrence after treatment. Although surgery, chemotherapy and radiotherapy constitute the conventional cancer treatment modality, complete surgical resection remains the most effective treatment and the only way to cure cancer. However, in cases of advanced-stage cancer, daughter cells from the primary tumor have metastasized to distant locations in the body, and the metastatic disease may not necessarily be visible by imaging studies. The majority of advanced cancers remain resistant to conventional chemotherapy and radiotherapy, thus novel treatments are needed.

Gene therapy emerged as a promising alternative for the treatment of cancer more than two decades ago, and numerous clinical trials of gene therapies have been conducted. Gene therapy approaches to cancer treatment include the replacement of mutant or defective genes, enhancement of the immune response, targeted killing of cancer cells and inhibition of angiogenesis. This chapter focuses only on representative cancer gene therapies that have reached Phase III clinical studies.

Gene replacement or addition strategy with nonreplicative virus vectors

Ad-*p53*

The direct application of the initial concept of gene therapy for genetic disease is to replace mutant or defective genes with wild-type genes. Because missing or altered genes, such as *p53*, may cause cancer, the substitution of these genes may be used to treat cancer. This intuitive approach was initially promising, and several clinical trials have been conducted. The most widely studied application of this approach is *p53* gene therapy. *p53* gene is a tumor suppressor gene that is crucial to the regulation of the cell cycle and the control of apoptotic cell death [1,2]. *p53* functions to maintain the genetic integrity of the cell and to induce apoptosis when DNA damage is too severe to produce normal progeny cells. It controls cell-cycle regulation, apoptosis and DNA repair. Thus,

abnormalities in *p53* cause deregulations of the cell cycle and apoptotic pathways, which are among the most common and fundamental molecular mechanisms of cancer pathogenesis and treatment resistance [3]. These observations formed the rationale for developing *p53* cancer therapy [4–6].

Head and neck squamous cell cancer (HNSCC) frequently exhibits inactivation of the tumor suppressor gene *p53* by mutation [7], overexpression of the primary negative regulator of *p53* [8], inactivation of the inhibitor of the negative regulator [9,10] and interference with *p53* posttranslational modifications that may be necessary for the gene to function [11]. Ad-*p53* is a replication-defective adenoviral vector that consists of the cytomegalovirus promoter, wild-type human *p53* cDNA, and a SV40 polyadenylation signal inserted into the E1-deleted region of modified adenovirus-5 [12,13]. Ad-*p53* treatment, in combination with radiation and/or chemotherapy, results in dramatic apoptosis in *p53*-deficient cancer cell lines [14,15]. Extensive animal studies revealed significant efficacy after intratumoral injection with Ad-*p53* in human cancer xenograft models. Repeated intratumoral injections of Ad-*p53* were well tolerated in Phase I trials, resulted in *p53* transgene expression, and were associated with antitumor activity [16]. The main side effects were transient fever and local inflammatory responses [17]. Phase II trials of Ad-*p53* have been conducted in various types of cancers including unresectable recurrent HNSCC [18].

Encouraging results from Phase I and II trials lead to the development of two Phase III trials, which compared Ad-*p53* with methotrexate and analyzed the results for their correlation with *p53* biomarkers in advanced recurrent HNSCC [18]. Vast majority of responded tumors to Ad-*p53* therapy had wild-type *p53* that was inactivated by upregulation of the *p53* inhibitors Mdm-2 or Mdm-4, or had low expression of mutated *p53*. Patients with this favorable *p53* profile showed a significant increase in survival compared with those with an unfavorable *p53* profile (7.2 vs 2.7 months). The Phase III trial demonstrated a lower toxicity profile of Ad-*p53*, supporting the data of Phase I and II trials. However, application of Ad-*p53* as a local regional therapeutic did not have the expected effectiveness in patients with HNSCC. Therefore, further clinical development of Ad-*p53* for the treatment of HNSCC was stopped.

In ovarian cancer, promising preclinical and clinical data also led to the initiation of an international randomized Phase II and III trial of Ad-*p53* that was intraperitoneally administered in combination with standard chemotherapy to patients with ovarian cancers containing *p53* mutations. However, the study was closed after the first interim analysis due to the lack of adequate therapeutic benefit.

Ad-*p53* gene therapy failed, although the high frequency of *p53* mutations and the central role of *p53* in regulating growth and apoptosis suggested that the *p53* gene would be an ideal target for gene replacement therapy. There are several possible reasons for its failure. The repair of a single gene might not be a suitable strategy for the treatment of cancers, which have multiple genetic changes and epigenetic dysregulations. There is also a substantial problem in targeting tumors with adenovirus. The heterogeneity or lack of expression of receptors and cofactors in tumors and the presence of adenovirus-neutralizing antibodies in patients are inevitable [19].

TNFerade™

TNF-α is a soluble cytokine and mediator of the cellular immune response with potent anticancer activities [20]. Its anticancer activities are exerted through apoptosis, necrosis, antiangiogenesis, immunomodulation and direct antitumor toxicity. The most critical anticancer mechanism of TNF-α would be the production of hydroxyl radicals, which lead to DNA damage. Radiation therapy also produces cell damage by free-radical formation, thus a synergistic interaction between TNF-α and radiation is anticipated.

TNFerade™ biologic (GenVec Inc., MD, USA) is an adenoviral vector that contains the gene for TNF-α under the control of a radiation-inducible promoter [21]. Administration of TNFerade biologic and subsequent activation of the *TNF-*α gene by radiation provides spatial and temporal control of the expression of *TNF-*α in tumors [22]. In Phase I and II studies, TNFerade biologic was injected into locally advanced pancreatic carcinomas by using endoscopic ultrasound or percutaneous administration once a week for 5 weeks together with radiation and 5-fluorouracil. Dose-limiting toxicities were pancreatitis and cholangitis. The antitumor activities among 50 patients consisted of one complete response, three partial responses and 12 patients with stable disease [23].

These results led to a Phase III study. The Pancreatic Cancer Clinical Trial was a multicenter, randomized, active and controlled study of 330 patients designed to evaluate the safety and efficacy of TNFerade plus standard of care versus standard of care alone in patients with locally advanced pancreatic cancer. This gene therapy was injected directly into pancreatic cancer and was studied in combination with standard chemoradiation [23]. However, the study was stopped after an interim analysis because the trial could not demonstrate clinically relevant evidence of effectiveness. The data did not yield the statistical significance required for the approval of a biological license application and thereby warranted discontinuation of the trial.

The goal of this gene therapy strategy was to improve overall survival in patients with advanced cancer. Like Ad-*p53*, TNFerade biologic is a nonreplicating adenoviral vector, and the main effect of this strategy would be local disease control by direct tumor cell killing. Therefore, this kind of therapy needs to target locally advanced but limited diseases. Otherwise, advanced disease in which the symptoms are relieved by local control should be targeted, and the end point would be improved quality of life, rather than extended survival.

ProstAtak™

Suicide gene therapy is based on the transduction of a viral or bacterial gene that encodes an enzyme able to convert a nontoxic prodrug into a lethal drug. The herpes simplex virus thymidine kinase gene (*HSV-tk*) with ganciclovir (GCV) [24] and the cytosine deaminase gene of *Escherichia coli* with 5-fluorocytosine [25] are the most extensively studied suicide gene therapies. In the *HSV-tk*/GCV system, the expression of viral thymidine kinase metabolizes GCV to ganciclovir monophosphate, which is then converted to ganciclovir triphosphate by cellular kinases. The phosphorylated compounds are nucleotide analogs that are incorporated into DNA during cell division, leading to the termination of DNA replication and cell death [26,27]. The number of cells killed significantly exceeds the number of cells transduced with the *HSV-tk* gene, a phenomenon known as the bystander effect [27]. In addition to the local bystander effect, a systemic bystander effect that generates protection against tumor rechallenge was observed [28].

The promising results in the preclinical studies with the *HSV-tk*/GCV system led to clinical trials in various cancers including prostate cancer [29,30]. This approach has now entered a Phase III trial in which the local administration ProstAtak™ (Advantagene, MA, USA), an adenovirus expressing the *HSV-tk* gene, is followed by valacyclovir, a valine-ester of acyclovir as an oral formulation, in combination with standard external beam radiation therapy with or without hormonal therapy for localized prostate cancer. The results of this ongoing trial are highly anticipated.

Replication-selective oncolytic virotherapy

OncoVEX$^{GM\text{-}CSF}$

Tumor-killing oncolytic viruses are lytic viruses that replicate selectively in cancer cells and lyze them before spreading to adjacent cells [31]. JS1/34.5-/47-/granulocyte–macrophage colony-stimulating factor (GM-CSF), namely OncoVEX$^{GM\text{-}CSF}$, is an immune-enhanced oncolytic herpes simplex virus type 1. It is deleted for neurovirulence factor ICP34.5, which provides tumor-selective replication, and ICP47, which promotes antigen

Aa Oncolytic virus: naturally occurring or genetically engineered viruses that can selectively proliferate in and kill infected cancer cells. This selective replication in a tumor theoretically increases the therapeutic index of this agent. Oncolytic virus can also be modified as a vector to carry various therapeutic genes encoding toxic proteins or cytokines including granulocyte–macrophage colony-stimulating factor (GM-CSF).

GM-CSF: a cytokine that stimulates the differentiation of hematopoietic progenitor cells into dendritic cells, which are potent antigen-presenting cells. This property may enhance the presentation of tumor antigens in dendritic cells after immunization with tumors that express GM-CSF.

presentation [32]. The gene for GM-CSF was inserted to maximize the immune response generated following the release of tumor antigens by virus replication. The virus was tested *in vitro* in human tumor cell lines and *in vivo* in mice and demonstrated significant anti-tumor effects [33]. *In vivo*, both injected and noninjected tumors showed significant shrinkage or clearance and mice were protected against rechallenge with tumor cells [33]. The virus would therefore be expected to have a potent oncolytic anti-tumor effect and also function as a patient-specific tumor vaccine.

A Phase I study has established safety and clinical activity in various tumor types, including melanoma [32]. In a Phase II clinical trial, the direct injection of OncoVEX$^{GM\text{-}CSF}$ into melanoma lesions resulted in a 26% objective response rate [34]. Responding patients demonstrated regression of both injected and uninjected lesions, indicating that OncoVEX$^{GM\text{-}CSF}$ has both a direct oncolytic effect in injected tumors and an immune-mediated anti-tumor effect on distant tumors. Based on these preliminary results, a prospective, randomized Phase III clinical trial in patients with unresectable stage IIIb or IIIc and stage IV melanoma, the OncoVEX Pivotal Trial in Melanoma has been initiated and is now recruiting patients [35].

Reolysin®

Reoviruses (respiratory enteric orphan viruses) are cytoplasmically replicating viruses comprised of two concentric protein capsids surrounding a genome consisting of ten segments of dsRNA [36]. Studies of human volunteers in the 1960s indicated that reoviruses possibly play an etiologic role in the generation of minor respiratory/enteric illnesses, but in general reovirus infections are asymptomatic [37]. Thus, they were initially classified as orphan viruses, indicating a virus that is not associated with any known severe human disease. There are three serotypes of reoviruses that are based on their hemagglutination activity. Reovirus type 3 Dearing, a naturally occurring virus, exerted significant antitumor effects in preclinical *in vitro* and *in vivo* studies and has been developed as oncolytic viral agent, Reolysin® (Oncolytics Biotech Inc., Alberta, Canada) [38]. Hashiro *et al.* reported that transformed cell lines were susceptible to reovirus infection, whereas normal human cells were spared [39]. Transformed cells with oncogenes such as *Ras*, *Sos*, *v-ervB* and *c-myc* were susceptible to reovirus infection [40–42]. Reovirus can also

activate both innate and adaptive immune responses against murine and human tumors [43,44]. Collectively, these observations suggested that reovirus has an innate anticancer potential, which led to its use as a powerful anticancer agent against *Ras* oncogenic tumors.

Intravenous administration of Reolysin had favorable toxicity profiles with preliminary evidence of antitumor activity [45]. Interestingly, despite the presence of neutralizing antibodies, viral localization and replication in tumors were confirmed by biopsies in some patients. In addition, Reolysin combined with radiotherapy or chemotherapy has showed feasibility and safety, with a number of patients showing disease responses [46–48].

Systemic rather than intralesional administration of Reolysin enables the virus to reach metastatic sites and makes this agent more generally applicable for clinical development. Based on the results of Phase I and Phase II studies, the agent is now in a Phase III trial with paclitaxel and carboplatin that was started in 2010 for patients with relapsed or metastatic HNSCC.

Immune vaccine gene therapy

PROSTVAC®

Prostate-specific antigen (PSA) is a serine protease secreted by prostatic epithelial cells that is widely used as a marker for prostate cancer [49]. The tissue specificity of PSA makes it a potential target for specific immunotherapy, especially in prostate cancer patients after prostatectomy and in whom the PSA-expressing tissue exists only in metastatic sites. Initial clinical studies with a recombinant vaccinia viral vector expressing PSA demonstrated an immune response and clinical efficacy [50]. PSA-targeted pox viral vaccines for prostate cancer were developed in subsequent preclinical studies. PROSTVAC® (BN ImmunoTherapeutics Inc., CA, USA) is a sequentially dosed combination of two different poxviruses which each encode PSA plus three immune-enhancing costimulatory molecules, B7.1 (CD80), ICAM-1 (CD45) and Lfa-3 (CD58), which are designated as TRICOM [51]. The first poxvirus is replication competent and is good for immune priming, which was termed Vaccinia-PSA-TRICOM. Fowlpox-PSA-TRICOM is the second poxvirus, a nonreplicating virus, which is good for repetitive immune boosting. The PROSTVAC is given as monthly injections starting with a Vaccinia-PSA-TRICOM priming dose and followed by 6-monthly Fowlpox-PSA-TRICOM boosts.

The latest trial, a randomized (2:1) Phase II placebo-controlled study of 125 patients with metastatic prostate cancer showed that patients receiving PROSTVAC had significantly longer overall survival by an average of 8.5 months as compared with the control group and that PROSTVAC had an adequate safety and tolerability profile [52]. Patients are being recruited for

a Phase III study to determine whether PROSTVAC alone or in combination with GM-CSF is effective in prolonging overall survival in men with few or no symptoms from metastatic, castration-resistant prostate cancer.

Perspectives of gene therapy for cancer

The Phase III trials described above are summarized in **Table 6.1**. Two of the trials have already failed, but the ongoing Phase III trials appear to be promising and are expected to yield good results. Most subjects in clinical trials of cancer gene therapy have not responded to standard cancer treatments, meaning that they have advanced-stage disease with an overwhelming cancer burden. In such cases, gene therapy can only provide a small improvement, if any, to the survival period, which must be difficult to demonstrate. Additionally, the patients in these trials have systemic disease that is not localized. There are two strategies to destroy cancer cells, direct cell killing by transduction of the gene of interest into cancer cells and indirect tumor suppression by eliciting an immune reaction against cancer by vaccination or by changing the microenvironment through, for instance, inhibition of angiogenesis. The former strategy represents *p53* gene therapy, which is expected to mainly kill the cancer cells in which it is expressed. *TNF-*α gene therapy is also a strategy to treat cancer cells reached by the gene therapy in combination with radiation. The lesson learned from the frontier gene therapy strategy with *p53* or *TNF-*α with replication-incompetent vectors is that local treatment for advanced cancer might rarely extend the limited period of survival, although it might bring some relief of cancer-related symptoms.

Overall, cancer gene therapy is shifting away from the local treatment model toward more systemic approaches. The recent trends are immunotherapy and oncolytic viruses that utilize the gene transfer and vector technology developed in numerous gene therapy studies. Suicide gene therapy by ProstAtak is expected to yield a systemic bystander effect, and OncoVEX$^{GM\text{-}CSF}$ and PROSTVAC are also expected to induce immune-mediated anti-tumor effects. In addition to conditionally replicative oncolytic virus, the development of intravenous, systemic administration would be a significant advance, because, so far, the existence of immunity has prevented the systemic administration of a viral agent. It will be interesting to see if Reolysin can demonstrate clinical efficacy against metastatic tumors.

In addition to the gene therapies that have reached Phase III trials, other promising gene therapies are also emerging. JX-594 is a Wyeth strain vaccine-derived oncolytic virus modified to inactivate the viral thymidine kinase gene and to express the GM-CSF and β-galactosidase genes [53]. Selective replication in cancer cells is driven by the EGFR/Ras pathway, thymidine kinase elevation and type I interferon resistance. A Phase I trial of intratumoral injection of

Table 6.1. Gene therapy agents in clinical trials

Product	Company	Composition	Indication	Phase
ADVEXIN®	Introgen Therapeutics	Adenoviral vector expressing the *p53* tumor suppressor gene	Squamous cell carcinoma of the head and neck	III
TNFerade™	GenVec	Adenoviral vector expressing the *TNF-α* gene	Pancreatic cancer	III
ProstAtak™	Advantagene	Adenovirus expressing the *HSV/tk* gene, followed by valacyclovir	Prostate cancer	III
OncoVEX^{GM-CSF}	BioVex	HSV-1 with deletions in ICP34.5 and ICP47 modified for expression of US11 and GM-CSF	Melanoma	III
Reolysin®	Oncolytics Biotech	Reovirus serotype 3 Dearing	Squamous cell carcinoma of the head and neck	III
PROSTVAC®	BN ImmunoTherapeutics	PROSTVAC-V-TRICOM: replication competent poxvirus with PSA plus three costimulatory molecules, B7.1, ICAM-1 and Lfa-3 PROSTVAC-F-TRICOM: nonreplicating virus with PSA plus B7.1, ICAM-1 and Lfa-3	Prostate cancer	III
JX-594	Jennerex	Thymidine kinase gene-inactivated oncolytic vaccinia virus expressing *GM-CSF* and *LacZ* genes	Liver cancer, melanoma, colorectal cancer	II

GM-CSF: Granulocyte–macrophage colony-stimulating factor.

JX-594 into primary or metastatic liver tumors has shown a good toxicology profile and signs of activity [54]. Another Phase I trial showed that JX-594 could selectively infect, replicate and express transgene products in metastatic tumors after intravenous infusion [55]. As well as the above described oncolytic viruses, a great deal of attention should be given to exploring the feasibility of systemically administered oncolytic viruses to treat metastatic disease [56].

Conclusion

The goal of cancer therapy is to eradicate not only the primary tumor but also any systemic metastasis that may reside in organs and tissues. Gene therapy

has the potential to act as highly specific, personalized medicine in cancer therapy. Vector systems and therapeutic genes have been considerably improved, and some promising strategies have finally reached Phase III trials. Earlier work has focused on vector and gene selection and the improvement of gene expression. The benefits of this work can be enhanced by adding other approaches, such as immunotherapy and chemotherapy. We hope that the current approach provides clinical success in the future.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

Summary

- Several approaches for cancer gene therapy have been extensively studied, but none of them has been integrated into standard cancer therapy.
- Cancer gene therapy strategy is shifting away from the local treatment model toward more systemic approaches.
- Use of oncolytic virus and immunotherapy may heighten the therapeutic potency and cancer selectivity of gene therapy.
- Some of the gene therapeutic agents have entered the definitive clinical testing Phase III stage.

References

1 Hartwell LH, Kastan MB. Cell cycle control and cancer. *Science* 266(5192), 1821–1828 (1994).

2 Kastan MB, Canman CE, Leonard CJ. P53, cell cycle control and apoptosis implications for cancer. *Cancer Metastasis Rev.* 14(1), 3–15 (1995).

3 Levine AJ, Momand J, Finlay CA. The *p53* tumour suppressor gene. *Nature* 351(6326), 453–456 (1991).

4 Clayman GL, el-Naggar AK, Lippman SM *et al.* Adenovirus-mediated *p53* gene transfer in patients with advanced recurrent head and neck squamous cell carcinoma. *J. Clin. Oncol.* 16(6), 2221–2232 (1998).

5 Swisher SG, Roth JA, Komaki R *et al.* Induction of *p53*-regulated genes and tumor regression in lung cancer patients after intratumoral delivery of adenoviral *p53* (INGN 201) and radiation therapy. *Clin. Cancer Res.* 9(1), 93–101 (2003).

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

7 Nigro JM, Baker SJ, Preisinger AC *et al.* Mutations in the *p53* gene occur in diverse human tumour types. *Nature* 342(6250), 705–708 (1989).

8 Ladanyi M, Cha C, Lewis R *et al. MDM2* gene amplification in metastatic osteosarcoma. *Cancer Res.* 53(1), 16–18 (1993).

9 Kamijo T, Weber JD, Zambetti G *et al.* Functional and physical interactions of the ARF tumor suppressor with *p53* and Mdm2. *Proc. Natl Acad. Sci. USA* 95(14), 8292–8297 (1998).

10 Pomerantz J, Schreiber-Agus N, Liégeois NJ *et al.* The Ink4a tumor suppressor gene product, p19Arf, interacts with MDM2 and neutralizes MDM2's inhibition of *p53*. *Cell* 92(6), 713–723 (1998).

11 Shono T, Tofilon PJ, Schaefer TS *et al.* Apoptosis induced by adenovirus-mediated *p53* gene transfer in human glioma correlates with site-specific phosphorylation. *Cancer Res.* 62(4), 1069–1076 (2002).

12 Zhang WW, Alemany R, Wang J *et al.* Safety evaluation of Ad5CMV-*p53 in vitro* and *in vivo*. *Hum. Gene Ther.* 6(2), 155–164 (1995).

13 Zhang WW, Fang X, Mazur W *et al.* High-efficiency gene transfer and high-level expression of wild-type *p53* in human lung cancer cells mediated by recombinant adenovirus. *Cancer Gene Ther.* 1(1), 5–13 (1994).

14 Parker LP, Wolf JK, Price JE. Adenoviral-mediated gene therapy with Ad5CMV*p53* and Ad5CMV*p21* in combination with standard therapies in human breast cancer cell lines. *Ann. Clin. Lab. Sci.* 30(4), 395–405 (2000).

15 Spitz FR, Nguyen D, Skibber JM *et al.* Adenoviral-mediated wild-type *p53* gene expression sensitizes colorectal cancer cells to ionizing radiation. *Clin. Cancer Res.* 2(10), 1665–1671 (1996).

16 Fujiwara T, Tanaka N, Kanazawa S *et al.* Multicenter Phase I study of repeated intratumoral delivery of adenoviral *p53* in patients with advanced non-small-cell lung cancer. *J. Clin. Oncol.* 24(11), 1689–1699 (2006).

17 Clayman GL, el-Naggar AK, Lippman SM *et al.* Adenovirus-mediated *p53* gene transfer in patients with advanced recurrent head and neck squamous cell carcinoma. *J. Clin. Oncol.* 16(6), 2221–2232 (1998).

18 Nemunaitis J, Clayman G, Agarwala SS *et al.* Biomarkers Predict *p53* Gene Therapy Efficacy in Recurrent Squamous Cell Carcinoma of the Head and Neck. *Clin. Cancer Res.* 15(24), 7719–7725 (2009).

19 Zeimet AG, Marth C. Why did *p53* gene therapy fail in ovarian cancer? *Lancet Oncol.* 4(7), 415–422 (2003).

20 Old LJ. Tumor necrosis factor (TNF). *Science* 230(4726), 630–632 (1985).

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

22 Senzer N, Mani S, Rosemurgy A *et al.* TNFerade biologic, an adenovector with a radiation-inducible promoter, carrying the human tumor necrosis factor alpha gene a Phase I study in patients with solid tumors. *J. Clin. Oncol.* 22(4), 592–601 (2004).

23 Hecht JR, Farrell JJ, Senzer N *et al.* EUS or percutaneously guided intratumoral TNFerade biologic with 5-fluorouracil and radiotherapy for first-line treatment of locally advanced pancreatic cancer a Phase I/II study. *Gastrointest. Endosc.* 75(2), 332–338 (2012).

24 Fillat C, Carrió M, Cascante A *et al.* Suicide gene therapy mediated by the herpes simplex virus thymidine kinase gene/ganciclovir system fifteen years of application. *Curr. Gene Ther.* 3(1), 13–26 (2003).

25 Fischer U, Steffens S, Frank S *et al.* Mechanisms of thymidine kinase/ganciclovir and cytosine deaminase/5-fluorocytosine suicide gene therapy-induced cell death in glioma cells. *Oncogene* 24(7), 1231–1243 (2005).

26 Balfour HH. Antiviral drugs. *N. Engl. J. Med,* 340(16), 1255–1268 (1999).

27 Eastham JA, Chen SH, Sehgal I *et al.* Prostate cancer gene therapy herpes simplex virus thymidine kinase gene transduction followed by ganciclovir in mouse and human prostate cancer models. *Hum. Gene Ther.* 7(4), 515–523 (1996).

28 Hall SJ, Mutchnik SE, Chen SH *et al.* Adenovirus-mediated herpes simplex virus thymidine kinase gene and ganciclovir therapy leads to systemic activity against spontaneous and induced metastasis in an orthotopic mouse model of prostate cancer. *Int. J. Cancer* 70(2), 183–187 (1997).

29 Duarte S, Carle G, Faneca H *et al.* Suicide gene therapy in cancer Where do we stand now? *Cancer Lett.* 324(2), 160–170 (2012).

30 Nasu Y, Saika T, Ebara S *et al.* Suicide gene therapy with adenoviral delivery of *HSV-tK* gene for patients with local recurrence of prostate cancer after hormonal therapy. *Mol. Ther.* 15(4), 834–840 (2007).

31 Kishimoto H, Kojima T, Watanabe Y *et al. In vivo* imaging of lymph node metastasis with telomerase-specific replication-selective adenovirus. *Nat. Med.* 12(10), 1213–1219 (2006).

32 Hu JC, Coffin RS, Davis CJ *et al.* A Phase I study of OncoVEX$^{GM\text{-}CSF}$, a second-generation oncolytic herpes simplex virus expressing granulocyte macrophage colony-stimulating factor. *Clin. Cancer Res.* 12(22), 6737–6747 (2006).

33 Liu BL, Robinson M, Han ZQ *et al.* ICP34.5 deleted herpes simplex virus with enhanced oncolytic, immune stimulating, and anti-tumour properties. *Gene Ther.* 10(4), 292–303 (2003).

34 Senzer NN, Kaufman HL, Amatruda T *et al.* Phase II clinical trial of a granulocyte-macrophage colony-stimulating factor-encoding, second-generation oncolytic herpesvirus in patients with unresectable metastatic melanoma. *J. Clin. Oncol.* 27(34), 5763–5771 (2009).

35 Kaufman HL, Bines SD. OPTIM trial a Phase III trial of an oncolytic herpes virus encoding GM-CSF for unresectable stage III or IV melanoma. *Future Oncol.* 6(6), 941–949 (2010).

36 Kim M, Chung YH, Johnston RN. Reovirus and tumor oncolysis. *J. Microbiol.* 45(3), 187–192 (2007).

37 Rosen L, Evans HE, Spickard A. Reovirus infections in human volunteers. *Am. J. Hyg.* 77, 29–37 (1963).

38 Karapanagiotou EM, Roulstone V, Twigger K *et al.* Phase I/II trial of carboplatin and paclitaxel chemotherapy in combination with intravenous oncolytic reovirus in patients with advanced malignancies. *Clin. Cancer Res.* 18(7), 2080–2089 (2012).

39 Hashiro G, Loh PC, Yau JT. The preferential cytotoxicity of reovirus for certain transformed cell lines. *Arch. Virol.* 54(4), 307–315 (1977).

40 Strong JE, Coffey MC, Tang D *et al.* The molecular basis of viral oncolysis usurpation of the *Ras* signaling pathway by reovirus. *EMBO J.* 17(12), 3351–3362 (1998).

41 Strong JE, Lee PW. The *v-erbB* oncogene confers enhanced cellular susceptibility to reovirus infection. *J. Virol.* 70(1), 612–616 (1996).

42 Coffey MC, Strong JE, Forsyth PA *et al.* Reovirus therapy of tumors with activated Ras pathway. *Science* 282(5392), 1332–1334 (1998).

43 Prestwich RJ, Ilett EJ, Errington F *et al.* Immune-mediated antitumor activity of reovirus is required for therapy and is independent of direct viral oncolysis and replication. *Clin. Cancer Res.* 15(13), 4374–4381 (2009).

44 Errington F, Steele L, Prestwich R *et al.* Reovirus activates human dendritic cells to promote innate antitumor immunity. *J. Immunol.* 180(9), 6018–6026 (2008).

45 Vidal L, Pandha HS, Yap TA *et al.* A Phase I study of intravenous oncolytic reovirus type 3 Dearing in patients with advanced cancer. *Clin. Cancer Res.* 14(21), 7127–7137 (2008).

46 Lolkema MP, Arkenau HT, Harrington K *et al.* A Phase I study of the combination of intravenous reovirus type 3 Dearing and gemcitabine in patients with advanced cancer. *Clin. Cancer Res.* 17(3), 581–588 (2011).

47 Comins C, Spicer J, Protheroe A *et al.* REO-10 a Phase I study of intravenous reovirus and docetaxel in patients with advanced cancer. *Clin. Cancer Res.* 16(22), 5564–5572 (2010).

48 Harrington KJ, Karapanagiotou EM, Roulstone V *et al.* Two-stage Phase I dose-escalation study of intratumoral reovirus type 3 dearing and palliative radiotherapy in patients with advanced cancers. *Clin. Cancer Res.* 16(11), 3067–3077 (2010).

49 Wang MC, Valenzuela LA, Murphy GP *et al.* Purification of a human prostate specific antigen. 1979 [classical article]. *J. Urol.* 167(3), 1226–1230 (2002).

50 Eder JP, Kantoff PW, Roper K *et al.* A Phase I trial of a recombinant vaccinia virus expressing prostate-specific antigen in advanced prostate cancer. *Clin. Cancer Res.* 6(5), 1632–1638 (2000).

51 DiPaola RS, Plante M, Kaufman H *et al.* A Phase I trial of pox PSA vaccines (PROSTVAC-VF) with B7–1, ICAM-1, and LFA-3 co-stimulatory molecules (TRICOM) in patients with prostate cancer. *J. Transl. Med.* 4, 1 (2006).

52 Kantoff PW, Schuetz TJ, Blumenstein BA *et al.* Overall survival analysis of a Phase II randomized controlled trial of a Poxviral-based PSA-targeted immunotherapy in metastatic castration-resistant prostate cancer. *J. Clin. Oncol.* 28(7), 1099–1105 (2011).

53 Mastrangelo MJ, Maguire HC, Eisenlohr LC *et al.* Intratumoral recombinant GM-CSF-encoding virus as gene therapy in patients with cutaneous melanoma. *Cancer Gene Ther.* 6(5), 409–422 (1999).

54 Park BH, Hwang T, Liu TC *et al.* Use of a targeted oncolytic poxvirus, JX-594, in patients with refractory primary or metastatic liver cancer a Phase I trial. *Lancet Oncol.* 9(6), 533–542 (2008).

55 Breitbach CJ, Burke J, Jonker D *et al.* Intravenous delivery of a multi-mechanistic cancer-targeted oncolytic poxvirus in humans. *Nature* 477(7362), 99–102 (2011).

56 Liu TC, Kirn D. Systemic efficacy with oncolytic virus therapeutics clinical proof-of-concept and future directions. *Cancer Res.* 67(2), 429–432 (2007).

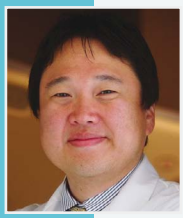
About the Authors

Mariana Kiomy Osako

Mariana Kiomy Osako is Assistant Professor at Hironori Nakagami's laboratory and has been conducting research on vascular biology and DNA vaccines.

Hironori Nakagami

Hironori Nakagami has been Professor of the Division of Vascular Medicine and Epigenetics, Osaka University United Graduate School of Child Development since 2010. His main field of interest is vascular biology and cardiology. He is now interested in the application of DNA vaccines for cardiovascular diseases.



Ryuichi Morishita

Ryuichi Morishita has been Professor of the Department of Clinical Gene Therapy, Osaka Graduate School of Medicine since 2003. His main field of interest is cardiovascular disease, and he was the first to develop the decoy oligodeoxynuleotides for E2F or NF-κB to prevent restenosis after coronary artery intervention. He performed the clinical trial of therapeutic angiogenesis using the *HGF* gene.

Chapter 7

Human gene therapy in cardiovascular diseases

Mariana Kiomy Osako, Hironori Nakagami & Ryuichi Morishita

Cardiovascular disease is the leading cause of mortality worldwide and has been frequently targeted in clinical trials of gene therapy. Despite promising preclinical research and several clinical trials, few candidates succeed to progress through the clinical Phases. In this chapter, some of those clinical trials and targets for treating cardiovascular disease are discussed.

doi:10.2217/EBO.12.144

Cardiovascular diseases are the second target for gene therapy lagging far away behind cancer diseases, in an approximate ratio of 1:10. Further studies in cardiovascular field are necessary to support a broader application of gene therapy.

Cardiovascular disease is the leading cause of mortality worldwide despite therapeutic developments. Many of the therapeutic interventions eventually fail because the interventions themselves injure the vessel and cause, for example, restenosis following angioplasty and vascular bypass graft occlusion. In face of the necessity for new strategies to treat cardiovascular diseases, gene therapy has posed as an attractive strategy because in most cases, only a transient expression of the therapeutic gene or transient action of the oligonucleotide (ODN) is required to achieve a beneficial biological effect. Following this, cardiovascular diseases represent the second-most popular target for clinical gene therapy.

Gene therapy consists of introducing nucleic acids to somatic cells to correct genetic defects or to express gene products that are therapeutically beneficial for acquired disorders. In general, gene modification can be achieved by replacement, correction or augmentation. This latter method, gene augmentation, is the most promising approach for the treatment of cardiovascular diseases and consists in transferring nucleic acid in the form of plasmid or ODNs to increase local expression of therapeutic factors.

Only a small amount of naked plasmid or ODNs can be transferred into the cells, therefore the use of virus as vector or carrier molecule have been used to improve the transfer efficiency. For cardiovascular applications, recombinant viruses have been used in most clinical trials: retrovirus, adenovirus, adeno-associated virus or the hemagglutinating virus of Japan (Sendai virus); however, the use of viruses imposes some limitations. Retroviruses enter into the cells via specific receptors, and the genomic RNA is reverse transcribed to DNA that is integrated into the host genome, which confers a long-lasting gene expression. However, the integration may occur in proximity to proto-oncogenes and be deleterious to the cell, and consequently for the patient. Another limitation is that a retrovirus can deliver its genetic material only to proliferating cells. On the other hand, adenoviruses do not lead to stable integration of its genetic material into the host genome, where it remains extrachromosomal and is transiently expressed; and adenoviruses can transfect both proliferating and quiescent cells. However, adenoviruses elicit immunological responses despite efforts in modifying this vector [1]. Carrier molecules such as cationic liposomes and nanoparticles to improve the transfer efficiency have also been investigated, and although current *in vivo* methods for cardiovascular gene transfer are still limited by lack of efficiency and potential toxicity, we are going to discuss in this chapter

recent clinical trials of gene therapy that may provide an opportunity to treat cardiovascular diseases.

Targets for cardiovascular gene therapy

Most approaches have focused on inducing gene transfer into the vascular wall or into the myocardium, and mainly target the excessive proliferation of vascular cells (restenosis, vein graft occlusion), ischemia after reperfusion, improvement in cell function (myocardial infarction) and angiogenesis (peripheral artery disease).

Restenosis & vein graft occlusion

Arteries are composed of an endothelial cell monolayer (intima) surrounded by basement membrane proteins that, together with elastic fibers, maintain multilayers of smooth muscle cells (media) in their contractile state and inhibit their proliferation. Atherosclerotic diseases are multifactorial disorders characterized by stenosis or total occlusion of large arteries caused by the development of plaques that eventually block the blood flow. It is usually managed by different types of percutaneous evascularization: stenting, the artery narrowing is pushed against by a hollow stainless steel mesh tube (stent), which can be coated with drugs or nucleic acids to limit cell proliferation (restenosis) and improve the reendothelization [1]; artery balloon angioplasty, the artery narrowing is pushed against by a catheter with a tiny balloon that is inflated to widen the lumen and allow blood flow, and a stent is usually placed in the region to sustain the artery [2]; atherectomy, the plaque is removed by laser or little rotating blades on a catheter [3]; artery bypass, vein is grafted above and below the obstructed area, allowing the blood flow around the atherosclerotic area [4].

Although percutaneous revascularization succeeds in reestablishing the blood flow, the vessel intima is disrupted, and denuded intima may lead to thrombosis. In addition, the limited ability of the endothelium to regenerate allows the smooth muscle cell migration, proliferation and extracellular matrix deposition, leading to neointima formation (intimal hyperplasia) and repeated narrowing of the vessel (restenosis).

Despite consistent evidences in several animal models, the Kuopio Angiogenesis Trial (KAT) study is still the unique example of a clinical study of gene therapy using plasmids to treat restenosis. Patients submitted to angioplasty (n = 103 patients), in which stents were introduced in 90% of the patients: VEGF-A_{165}-expressing adenovirus

Aa VEGF: induces the proliferation of endothelial cells, the cells lining the intima layer of vessels, and its main action is to induce angiogenesis. However, during sprouting, the VEGF-induced vessels are leaky.

were delivered to the coronary artery wall by an intracoronary balloon catheter at the site of stent implantation in 37 patients, 28 patients received VEGF plasmid in liposomes, and 38 control patients received Ringer's lactate. After a follow-up of 6 months, however, angiographic restenosis was unaffected in the treated group compared with placebo [2].

In contrast to plasmids that express therapeutic genes, ODNs can also be transferred to the injured area but the mechanism of action is quite distinct. The decoy ODNs are used to inhibit gene expression by acting on the pathway of DNA transcription into mRNA. ODNs are synthetic and short dsDNA sequences composed of the exact same binding sequence for the targeted transcription factor. Thus, once within the cell, the ODNs act as a decoy for the transcription factors that inhibits the binding to the promoter or enhancer region, and therefore inhibits the expression of a set of genes [3]. The transcription factor NF-κB is a convergent point for the pathways of different stimulants, with a key role in cell growth and differentiation, inflammatory responses and apoptosis. Based on successful evidence from animal models [4,5], the open-label, multicenter Phase I/IIa clinical trial INDOR evaluated the safety and efficacy of NF-κB ODNs to assess the inhibitory effects of NF-κB decoy ODNs on restenosis after stenting in the coronary artery. Seventeen patients were treated with NF-κB decoy ODNs after angioplasty using bare metal stents. As a result, the stenosis improved to 1.4 ± 5.9% after the intervention, and significant restenosis was found in only one of the 17 patients 6 months after, and the average restenosis rate was 39.6 ± 22.3%. No in-stent thrombosis was found and no significant systemic adverse effect occurred in any of the patients in this observation period [6]. In a case report, the NF-κB ODNs suppressed the development of neointima formation at the site of stenting in a 4-year follow-up study [7].

E2F has attracted attention in the process of intimal hyperplasia because it is a pivotal cell-cycle transcription factor that coordinates transactivation of cell cycle-regulatory genes, such as *c-myc*, *cdc2* and the gene encoding the proliferating cell nuclear antigen. Preclinical studies targeting E2F with ODNs showed to be effective in inhibiting restenosis [8,9]. However, as shown in the PREVENT III/IV randomized controlled trial, E2F ODNs failed to show significant amelioration of bypass graft disease, which may be partially explained by the complexity of mechanism involved in the intimal hyperplasia development [10].

The chimeric ODNs targeting both E2F and NF-κB has been developed [11] and proved

The KAT and INDOR study are unique examples of gene therapy trials to prevent restenosis. In KAT, no therapeutic effect was found after delivery of VEGF-expressing plasmids, while the INDOR study showed significant reduction in restenosis by targeting NF-κB using the oligo decoy strategy.

to successfully inhibit anastomotic hyperplasia in a rabbit model [12]. Further clinical studies are required to evaluate this promising strategy.

Aa FGF: has a wide variety of effects, including angiogenesis by inducing the proliferation of endothelial and mural cells.

HGF: similar to FGF, besides angiogenesis, it also induces enlargement of vascular wall structures (arteriogenesis) by acting on mural cells. The HGF-induced vessels are completely developed and functional.

Ischemia

Therapeutic angiogenesis has been applied to increase the number of small vessels within ischemic tissue. Plasmids expressing VEGF, FGF and HGF have been reported to induce angiogenesis *in vivo*, and have been mostly used to treat peripheral arterial disease and myocardial ischemia.

Peripheral arterial disease

In the previously mentioned KAT study, significant increase in vascularization distal to the gene transfer site was seen in both VEGF-treated groups (VEGF-A_{165}-expressing adenovirus and VEGF plasmids with liposomes) compared with controls. Despite this, there were no differences in limb salvage or restenosis rates between groups [2]. In another Phase II randomized clinical trial, the RAVE trial, (VEGF-A_{165})-expressing adenovirus were intramuscularly delivered for the treatment of unilateral claudication, and similarly, no significant improvement could be detected in the treated [13]. In the Groningen study, a Phase II randomized clinical trial of intramuscular VEGF-A plasmid treatment in diabetic patients, there was no statistically significant difference in the primary end point of amputation rate, but improvements in ulcer healing and overall clinical status were reported [14]. Limb edema was reported in all these three studies. In a 10-year safety follow-up in patients with local *VEGF* gene transfer to ischemic lower limb, despite no significant differences in the number of amputations or causes of death, the *VEGF* transfer proved to not increase the incidence of cancer, diabetes, retinopathy or any other diseases during the 10-year follow-up time [15].

The TALISMAN trial enrolled 125 patients with critical limb ischemia unsuitable for surgical revascularization and associated with nonhealing ischemic ulcers. The patients received plasmid expressing *FGF* (NV1FGF) intramuscularly four times in a period of 6 weeks. Although the trial did not meet its primary end point (ulcer healing), the risk of major amputation was reduced by as much as half in patients who received NV1FGF [16]. Following this, the Phase III TAMARIS clinical trial delivered the same agent used in the TALISMAN trial, and enrolled 490 patients. However, in the TAMARIS study, there were no significant decreases in all amputations, death, ulcer healing and pain relief in the NV1FGF group [17].

The VEGF- and FGF-expressing plasmid-based gene therapies for peripheral artery diseases showed no clinical therapeutic effect up to now. By contrast, hopes on therapy using HGF-expressing plasmids arise from STAT trial, and results using a larger population are awaited.

Plasmids expressing *HGF* have also been evaluated in peripheral arterial disease. Our group has completed a Phase I open-label clinical trial using intramuscular injection of a HGF-expressing vector (Collategene™; AnGes MG Inc., Osaka, Japan) in 22 patients with either critical limb ischemia or Buerger's disease in Japan. The study was conducted with 4 mg of HGF plasmid divided between two dosing sessions 4 weeks apart. The study found no safety concerns. Systemic HGF protein levels were unchanged during the study. Although the study population was small, there was a suggestion of efficacy. The ankle brachial pressure index increased by 0.1 in all patients, and a decrease in ulcer size of >25% occurred in eight out of 11 ulcers. In following Phase I/II studies (HGF-STAT and HGF0205 trials), *HGF* gene transfer improved limb perfusion as measured by changes in transcutaneous oxygen tension TcPo2. Values >30 mmHg have been shown to be associated with wound healing, and 80% of patients who received the high-dose HGF plasmid had a TcPo2 >30 mmHg at the 6-month time point compared with 39% in the placebo group. There was no difference between treatment groups observed in other secondary end points such as change in toe pressure index, wound healing, limb salvage or survival; however, that study was not powered to address differences in these secondary end points [18]. A Japanese Phase III study randomized 40 patients to treatment with a total of 8 mg HGF-expressing plasmid or placebo. The primary end point of rest pain and ischemic ulcer size was significantly improved without adverse effect in the treatment group at 12-week follow-up [19]. Currently, a global Phase III study to recruit 560 patients is being designed and the development of Collategene has been granted fast-track status by the US FDA.

Myocardial infarction

Intramuscular injection of naked plasmid encoding VEGF into ischemic myocardium through a minithoracotomy, and similar to the human trials in peripheral artery disease, transfection of VEGF resulted in a marked increase in blood flow, collateral growth, myocardial contractile function and improved the clinical symptoms without apparent toxicity [20,21]. To date, there are three published Phase II/III trials for cardiac angiogenesis: the KAT, AGENT and EUROINJECT ONE trials, and all of them with positive treatment effects. Although VEGF-A_{165} showed no significant effect in the restenosis rate in the KAT trial [2], there was a significant improvement in the myocardial perfusion of patients treated with VEGF-A_{165}-expressing plasmid at 6 months, without any adverse effect in a 10-year follow-up [2].

In the AGENT trial, FGF-expressing adenovirus was given by intracoronary injection, and the primary end point was effects on treadmill exercise capacity as an index of improved coronary perfusion [22,23]. In the EUROINJECT ONE trial, patients were treated with intramyocardial injection of plasmid expressing $VEGF_{165}$ or with placebo plasmid, both myocardial perfusion (primary end point) and local wall motion (secondary end point and estimate of myocardial function) improved in the treated group [24,25].

Despite the use of HGF for myocardial infarction, it has not reached clinical trial phases, its role has been positively evaluated in animal models: overexpression of HGF was reported to stimulate angiogenesis and collateral vessel formation in a rat myocardial infarction model [26]. In addition to angiogenesis, an antifibrotic action of HGF has been identified, and prevention of fibrosis by HGF was confirmed by previous studies in which administration of human rHGF or gene transfer of human HGF prevented and/or regressed fibrosis in liver and pulmonary injury models [27,28]. HGF may also provide a new therapeutic strategy for fibrotic cardiovascular disease (i.e., cardiomyopathy). A human gene therapy protocol for HGF-expressing plasmid injected intracardiacally during surgery has been proposed.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

Summary

- The objectives of gene therapy protocols for cardiovascular disease are generally to evaluate the *in vivo* efficacy of the gene transfer method, the safety of the gene transfer method and the possible therapeutic efficacy.
- Remarkable progress in the field and in the understanding of the pathophysiological mechanism of cardiovascular disorders brought into light several potential candidates to be targeted or manipulated by gene therapy.
- However, we learned that besides successful preclinical data, few candidates demonstrated clear clinical benefit.
- Although few, those successful cases underline the importance in innovations in gene transfer methods and the selection of the right gene and cell-specific targeting strategies, and suggest that gene therapy is not so far from reality in the cardiovascular field.

References

1 Williams PD, Ranjzad P, Kakar SJ, Kingston PA. Development of viral vectors for use in cardiovascular gene therapy. *Viruses* 2(2), 334–371 (2010).

2 Hedman M, Hartikainen J, Syvänne M *et al.* Safety and feasibility of catheter-based local intracoronary vascular endothelial growth factor gene transfer in the prevention of postangioplasty and in-stent restenosis and in the treatment of chronic myocardial ischemia: Phase II results of the Kuopio Angiogenesis Trial (KAT). *Circulation* 107(21), 2677–2683 (2003).

3 Osako MK, Tomita N, Nakagami H *et al.* Increase in nuclease resistance and incorporation of NF-κB decoy oligodeoxynucleotides by modification of the 3′-terminus. *J. Gene Med.* 9(9), 812–819 (2007).

4 Yamasaki K, Asai T, Shimizu M *et al*. Inhibition of NFκB activation using cis-element 'decoy' of NFκB binding site reduces neointimal formation in porcine balloon-injured coronary artery model. *Gene Ther.* 10(4), 356–364 (2003).

5 Yoshimura S, Morishita R, Hayashi K *et al*. Inhibition of intimal hyperplasia after balloon injury in rat carotid artery model using cis-element 'decoy' of nuclear factor-κB binding site as a novel molecular strategy. *Gene Ther.* 8(21), 1635–1642 (2001).

6 Egashira K, Suzuki J, Ito H, Aoki M, Isobe M, Morishita R. Long-term follow up of initial clinical cases with NF-κB decoy oligodeoxynucleotide transfection at the site of coronary stenting. *J. Gene Med.* 10(7), 805–809 (2008).

7 Suzuki J *et al*. An initial case of suppressed restenosis with nuclear factor-κB decoy transfection after percutaneous coronary intervention. *J. Gene Med.* 11(1), 89–91 (2009).

8 Kaneda Y, Morishita R, Dzau VJ. Prevention of restenosis by gene therapy. *Ann. NY Acad. Sci.* 811, 299–308 (1997).

9 Hashiya N, Aoki M, Tachibana K *et al.* Local delivery of E2F decoy oligodeoxynucleotides using ultrasound with microbubble agent (Optison) inhibits intimal hyperplasia after balloon injury in rat carotid artery model. *Biochem. Biophys. Res. Commun.* 317(2), 508–514 (2004).

10 Conte MS, Bandyk DF, Clowes AW *et al*. Results of PREVENT III: a multicenter, randomized trial of edifoligide for the prevention of vein graft failure in lower extremity bypass surgery. *J. Vasc. Surg.* 43(4), 742–751 (2006).

11 Miyake T, Aoki M, Nakashima H *et al.* Prevention of abdominal aortic aneurysms by simultaneous inhibition of NFκB and ets using chimeric decoy oligonucleotides in a rabbit model. *Gene Ther.* 13(8), 695–704 (2006).

12 Miyake T, Aoki M, Morishita R. Inhibition of anastomotic intimal hyperplasia using a chimeric decoy strategy against NFκB and E2F in a rabbit model. *Cardiovasc. Res.* 79(4), 706–714 (2008).

13 Rajagopalan S, Mohler ER 3rd, Lederman RJ *et al.* Regional angiogenesis with vascular endothelial growth factor in peripheral arterial disease: a Phase II randomized, double-blind, controlled study of adenoviral delivery of vascular endothelial growth factor 121 in patients with disabling intermittent claudication. *Circulation* 108(16), 1933–1938 (2003).

14 Kusumanto YH, Tio RA, Loef BG, Sluiter WJ, Mulder NH, Hospers GA. Systemic VEGF levels after coronary artery bypass graft surgery reflects the extent of inflammatory response. *Acute. Card. Care* 8(1), 41–45 (2006).

15 Muona K, Mäkinen K, Hedman M, Manninen H, Ylä-Herttuala S. 10-year safety follow-up in patients with local VEGF gene transfer to ischemic lower limb. *Gene Ther.* 19(4), 392–395 (2012).

16 Nikol S, Baumgartner I, Van Belle E *et al.* Therapeutic angiogenesis with intramuscular NV1FGF improves amputation-free survival in patients with critical limb ischemia. *Mol. Ther.* 16(5), 972–978 (2008).

17 Belch J, Hiatt WR, Baumgartner I *et al.* Effect of fibroblast growth factor NV1FGF on amputation and death: a randomised placebo-controlled trial of gene therapy in critical limb ischaemia. *Lancet* 377(9781), 1929–1937 (2011).

18 Morishita R, Aoki M, Hashiya N *et al.* Safety evaluation of clinical gene therapy using hepatocyte growth factor to treat peripheral arterial disease. *Hypertension* 44(2), 203–209 (2004).

19 Shigematsu H, Yasuda K, Iwai T *et al*. Randomized, double-blind, placebo-controlled clinical trial of hepatocyte growth factor plasmid for critical limb ischemia. *Gene Ther.* 17(9), 1152–1161 (2010).

20 Losordo DW, Vale PR, Symes JF *et al*. Gene therapy for myocardial angiogenesis: initial clinical results with direct myocardial injection of phVEGF165 as sole therapy for myocardial ischemia. *Circulation* 98(25), 2800–2804 (1998).

21 Symes JF, Losordo DW, Vale PR *et al*. Gene therapy with vascular endothelial growth factor for inoperable coronary artery disease. *Ann. Thorac. Surg*. 68(3), 830–836 (1999).

22 Grines CL, Watkins MW, Mahmarian JJ *et al*. A randomized, double-blind, placebo-controlled trial of Ad5FGF-4 gene therapy and its effect on myocardial perfusion in patients with stable angina. *J. Am. Coll. Cardiol.* 42(8), 1339–1347 (2003).

23 Grines CL, Watkins MW, Helmer G *et al*. Angiogenic gene therapy (AGENT) trial in patients with stable angina pectoris. *Circulation* 105(11), 1291–1297 (2002).

24 Kastrup J, Jørgensen E, Rück A *et al*. Direct intramyocardial plasmid vascular endothelial growth factor-A165 gene therapy in patients with stable severe angina pectoris A randomized double-blind placebo-controlled study: the Euroinject One trial. *J. Am. Coll. Cardiol.* 45(7), 982–988 (2005).

25 Stewart DJ, Hilton JD, Arnold JM *et al*. Angiogenic gene therapy in patients with nonrevascularizable ischemic heart disease: a Phase 2 randomized, controlled trial of AdVEGF(121) (AdVEGF121) versus maximum medical treatment. *Gene Ther.* 13(21), 1503–1511 (2006).

26 Aoki M, Morishita R, Taniyama Y *et al*. Angiogenesis induced by hepatocyte growth factor in non-infarcted myocardium and infarcted myocardium: up-regulation of essential transcription factor for angiogenesis, ets. *Gene Ther.* 7(5), 417–427 (2000).

27 Ueki T, Kaneda Y, Tsutsui H *et al*. Hepatocyte growth factor gene therapy of liver cirrhosis in rats. *Nat. Med.* 5(2), 226–230 (1999).

28 Nakamura T, Sakata R, Ueno T, Sata M, Ueno H. Inhibition of transforming growth factor β prevents progression of liver fibrosis and enhances hepatocyte regeneration in dimethylnitrosamine-treated rats. *Hepatology* 32(2), 247–255 (2000).

About the Author

Yasufumi Kaneda

Yasufumi Kaneda graduated from Osaka University School of Medicine (Japan) in 1980 and presented his medical doctoral thesis there in 1984. He was the first Full Professor of Division of Gene Therapy Science, Graduate School of Medicine, Osaka University (Japan), a position started in 1998. He has developed unique gene delivery vectors, such as hemagglutinating virus of Japan (HVJ) liposomes and the HVJ envelope vector using inactivated Sendai virus (HVJ) particles. He has also promoted clinical trials of gene therapy targeting angiogenesis using the *HGF* gene and cancer treatment using the HVJ envelope. He has been Chairman of the Board of Directors of the Japan Society of Gene Therapy since 2009.

Chapter 8

Future directions for gene therapy

Yasufumi Kaneda

Although a lot of difficulties have been encountered with gene therapy since human gene therapy started in 1990, we have gradually developed some solutions to overcome these difficulties, as well as possible approaches to promote gene therapy. The safety and efficacy of existing vectors have been evaluated and optimized, although they are still far from ideal technologies. Recently, some successful cases of human gene therapy were reported especially in the treatment of hereditary diseases. Guidelines related to gene therapy are now more standardized and have been improved, and organizations to support gene therapy are being started. The current progress in gene therapy may suggest that there will soon be a breakthrough in gene therapy. As the final chapter of this book, from the viewpoints of science, practical medicine and administration, I hereby summarize what should be done in the future in regard to gene therapy.

doi:10.2217/EBO.12.155

Human gene therapy was first developed for the treatment of hereditary diseases. The first patient was a 4-year-old female suffering from an immunodeficiency caused by adenosine deaminase deficiency. The patient was cured after gene therapy, but the trials of the same treatment were not always successful after that. The reason was that there was only transient gene expression due to the limited survival of peripheral lymphocytes after gene transfer in spite of the stable integration of the transgene into the host genome by a retroviral vector. Subsequently, the target cells were changed to hematopoietic stem cells because of their long-term survival as a result of self-renewal. From 2000 to 2002, X-chromosome-linked severe combined immunodeficiency gene therapy was performed using hematopoietic stem cells transduced with IL-2 receptor gene-loaded retroviral vectors in France. Seven out of 11 patients appeared to recover from the immunodeficiency in the first 3 years. However, leukemia occurred in two patients due to retrovirus-mediated insertional mutagenesis of the *LMO2* gene, which induces the proliferation of lymphocytes. These results were a great blow to gene therapy worldwide.

From the viewpoint of science

Gene correction therapy for hereditary disease

Currently, gene therapy is focused on the replenishment of cellular functions with genes without correction of the underlying genomic mutations. For hereditary diseases resulting in monogenic disorders, the supplementation compensates by transiently relieving the symptoms, but this is not the ultimate therapy. Using a lentiviral vector, retroviral vector or transposon–transposase system, transgenes are inserted into genomic DNA with the possibility of long-term gene expression [1]. However, after the insertion of a viral genome into host chromosomal DNA, abnormal proliferation may be induced, as was seen in the case of X-chromosome-linked severe combined immunodeficiency gene therapy [2]. Safer insertion sites in the human genome, such as the AAV-S1 site on the short arm of human chromosome 19, into which adeno-associated virus is integrated, are also being investigated. Site-specific integration into a safe location is desired. However, epigenetic silencing may suppress the gene expression by insertion-mediated alteration of the genome structure. To avoid the risk of the insertion mutagenesis, it has been expected that mutation sites should be replaced with normal genes. Numerous trials have been reported, but at present the most promising method for replacement is homologous recombination after the digestion of mutation sites by a ZNF [3].

The breakthrough regarding this technology was the design of zinc-finger proteins that recognize target sequences in the endogenous genome. When the zinc-finger protein is connected with some restriction enzyme, the DNA regions around the mutation site can be digested. Then, when a template DNA with a normal sequence at the mutation site is introduced into the nucleus at the same time, the mutation site in the host DNA can be corrected by homologous recombination. The frequency of gene correction by this method is higher than that by previous methods in cultured cells.

This technology has already been used for *ex vivo* gene therapy, and a mutated gene in a mouse model of hemophilia was corrected in the liver *in vivo* using this technology [4]. However, by a precise analysis of the

whole-genome sequence after the gene correction, several genome alterations were detected in regions other than the original mutation sites [5]. Therefore, there may be a problem avoiding the off-target effects of the ZNF. There is a limit to the ability of the zinc-finger protein itself to recognize the specific genomic element. However, if the designed zinc-finger protein is combined with another method to provide better recognition, the specificity of the sequence recognition may be enhanced to avoid potential off-target effects.

It has been considered that gene therapy for hereditary diseases may be impossible. However, with the progress in gene transfer vectors, such as adeno-associated viral and lentiviral vectors, some successful clinical trials of gene therapy for hereditary diseases have been reported. These include Leber's congenital amaurosis, which causes blindness due to the abnormal development of photoreceptor cells, and β-thalassemia, which causes anemia due to a variety of mutation of the β-globin gene. Clinicians and researchers were encouraged by the successful results, which helped to promote further gene therapy trials for other hereditary diseases.

More research for cancer gene therapy

Genes that can induce cancer cell-specific apoptosis

p53 is the most famous tumor suppressor gene, and at the beginning of human gene therapy, an adenoviral vector containing the *p53* gene was used for cancer treatment. A single therapeutic strategy using *p53* gene transfer was not sufficiently powerful to induce remission in cancer patients. Further investigation revealed that melanoma differentiation associated gene-7 (so-called IL-24) was silenced in many cancer cells, and that overexpression of the gene induced apoptosis in cancer cells [6]. Recently, the overexpression of the *REIC/Dkk-3* gene, a member of the human Dickkopf (Dkk) family, was found to cause cancer cell-specific apoptosis due to the induction of endoplasmic reticulum stress [7]. *REIC/Dkk-3* is silenced in cancer cells, probably as a result of DNA methylation. Thus, through the epigenomic analyses, we may identify target genes that can distinguish cancer cells from normal cells. Another hint may be present in virus–cell interactions. Virotherapy has been of interest since the discovery of cancer remission in cancer patients after they suffered from a viral infection [8]. Various viruses have been used for cancer treatment, but their efficacy was relatively low, probably owing to the suppression of the viruses by the host immune response. Remarkable progress was produced in cancer virotherapy by the development of an oncolytic virus that enabled viral replication selectively in cancer cells [9]. Attenuated virus strains are also available for cancer cell-specific replication. In addition to these natural mutant viruses, artificial mutant viruses have been developed by viral gene engineering. It is considered that cancer cell killing can be achieved by virus replication, because the generation of replication-incompetent viruses by UV light irradiation led to the loss of their cell-killing activity. However, the effect of

viral components on cancer cells should be analyzed further. Since synthetic RNA molecules such as polyinosinic:polycytidilic acid induces melanoma-specific killing, viral RNA introduced into the cytoplasm may have apoptotic effects on cancer cells through its recognition by retinoic acid-inducible gene I or melanoma differentiation-associated antigen 5 [10]. Based on the findings of those studies, a novel approach for cancer gene therapy can be developed.

Genes related to the survival of cancer stem cells

A recent highlight of cancer research is the finding of cancer stem cells in solid tumors [11]. It is still controversial whether cancer cells with authentic pluripotency are really present in tumor tissue, and the presence of cancer-initiating cells is currently considered to be more likely. Although the naming of the cancer stem cells may not be fully approved, it has been suggested that the resistance of cancer cells to chemotherapy or radiation therapy results from the existence of cancer stem cells, and that these cells can survive to form tumors, even under stressful conditions. Therefore, it is considered that the ultimate target for cancer therapy is to identify the molecule(s) responsible for the survival of cancer stem cells. Several candidates have been identified to confer resistance to chemotherapy or radiotherapy in cancer cells. For example, it is well known that ABC transporter genes are highly upregulated in chemoresistant cancer cells. The gene encoding clusterin is also a candidate. However, those are the peripheral effector genes, and a master gene that regulates survival must exist. A hint to identify a master gene may be present in the signaling pathways that enhance the characteristics of cancer stem cells. For example, the Notch signaling pathway [12] and CXCR12–CXCR4/CXCR7 pathway [13] appear to be involved in the survival of cancer stem cells. Gene therapy should target such master genes.

Cancer gene therapy has been the focus of approximately two-thirds of the clinical applications of human gene therapy. In spite of the development of various therapeutic approaches, no remarkable success has been reported. Cancer gene therapy has been applied only for patients without any response to the available treatments, and combination therapy has not been permitted in most of the trials. However, judging from the scientific viewpoint, cancer research is still immature. Optimal target genes have not yet been determined. The most effective strategy for immunotherapy is still controversial. These limitations to the cancer field are not restricted to gene therapy, they are generally true for all types of cancer therapy. Thus, more research is needed before it can be concluded whether gene therapy can be used to treat cancer.

Innovations in cancer immunotherapy

Even though cancers apparently disappear after treatment, tumor recurrence is frequently seen. It has been considered that the host immune system can eliminate residual cancer cells. Based on an optimistic view of immunotherapy, various approaches for cancer immunotherapy have been evaluated. However, there have been numerous failures of cancer immunotherapy, suggesting that cancer immunotherapy may

be quite different from immunotherapy against infectious diseases. It has been gradually elucidated that cancer tissues produce factors to induce immunological tolerance against cancers in tumor-bearing individuals [14]. Treatment with an anti-CTLA-4 antibody or anti-PD1 antibody resulted in the effective eradication of tumors by attenuation of suppressive signals for effector T cells [15]. These results indicate that the environment of immunosuppression should be broken in cancer patients in order to more effectively eradicate the tumor. For more effective immunotherapy, both the activation of effector lymphocytes against cancers and the suppression of immunosuppressive factors are necessary.

Aa Immunological tolerance against cancers: by producing various factors and recruiting regulatory T cells, cancers attenuate the host immune system, which results in cancer progression without immunological elimination. Immunological tolerance against cancer is the condition where cancers are recognized as 'self' by host immune system.

Tissue regeneration for injuries & acquired diseases

In the 2000s, cardiovascular gene therapy was very popular [101]. Most of the treatments have targeted peripheral arterial disease or ischemic heart disease, and it is expected that blood flow can be improved by angiogenesis induced using angiogenic genes. Although gene therapy for ischemic heart disease was difficult to perform, successful cases were reported for gene therapy used to treat peripheral arterial disease. However, the use of cardiovascular disease gene therapy has slowed. In the author's opinion, it may be difficult to demonstrate the superiority of gene therapy for the treatment of peripheral arterial disease because other therapeutic approaches such as cell therapy, low-molecular-weight drugs and surgical treatment are also effective in many cases.

Therefore, the author is pessimistic about gene therapy for cardiovascular disease, but the concept of *in vivo* angiogenesis provides us with an attractive basis for tissue regenerative medicine. Embryonic stem cells have been considered to be excellent tools to regenerate damaged tissues. The development of induced pluripotent stem cells accelerated the movement of tissue regeneration medicine [16]. However, those cells are still associated with problems that need to be solved, including their tumorigenecity, homogeneity and genome alterations. Even if these problems are solved, such strategies are based on cell or tissue transplantation. However, angiogenesis gene therapy can lead to *in vivo*-induced tissue regeneration. Therefore, additional investigations on the potency and mechanism(s) of regeneration of damaged tissues are needed. In particular, the stem cells that differentiate in the damaged tissue should be identified, and the factors that recruit these stem cells and promote their differentiation should be elucidated. Generally,

Aa Mesenchymal stem cells: cells that have the potential to differentiate into cells of various tissue types, such as osteoblasts, chondrocytes, adipocytes and keratinocytes. Mesenchymal stem cells are present in the bone marrow, adipose tissue, muscle, dental pulp, among others.

mesenchymal stem cells (MSCs) appear to have a potential for tissue regeneration. The cells are not homogeneous, and therefore have varying potentials. Previously, stage-specific embryonic antigen-3-positive human MSCs were shown to be nontumorigenic stem cells with the ability to differentiate into many cell types. If these stem cells are recruited to damaged tissue, tissue regeneration may occur without transplantation of *ex vivo*-treated stem cells [17]. It has been reported that high mobility group B1 can recruit MSCs to damaged tissues from bone marrow [18]. SDF-1 is also involved in the recruitment of MSCs to injured sites. Although injured tissue may produce those factors, gene transfer of factors for stem cell recruitment will probably enhance tissue regeneration.

From the viewpoint of practical medicine

Gene therapy application at earlier stages of disease

At the tumor initiation stage, cancer cells can be more easily eliminated by the host immune system. When the activities of the immune system are attenuated by factors such as senescence, stress and immunosuppressive drugs, cancers can escape from immune surveillance. Finally, a large tumor mass can overwhelm the immune system. At that time, it is too late to eliminate cancer cells using immunotherapy. Therefore, immunotherapy should be started at an early stage of cancer.

Gene therapy is considered to have unknown risks. Therefore, it has been permitted only for patients at the terminal stage of disease or without any other available treatments. Although most new therapies are initially evaluated in the patients, in the case of cancer gene therapy, late-stage patients are still the only ones evaluated, even after Phase I and II trials. During the 20 years since the initiation of gene therapy, the safety of several gene therapy strategies has been proven. It should therefore be made available to patients with various stages of cancers. Recently, gene therapy for adrenoleukodystrophy using a lentiviral vector was applied for patients before neurological degeneration was detected [19]. This may be the breakthrough in gene therapy. Similar approaches should therefore be conducted regarding other diseases that can be targeted by gene therapy.

Multimodal gene therapy for cancer

As described above, cancer tissues produce factors that induce immunological tolerance in tumor-bearing individuals. Although it is

difficult to conclude precisely when immunotherapy should be started, postoperative immunotherapy may be beneficial for prolonging the patient survival rate. Therefore, immunotherapy should be initiated after as much of the tumor mass has been removed as possible. Therefore, immunotherapy should be combined with surgical treatment or radiotherapy.

Multimodal therapy is not merely a combination of different treatments. In cancer gene therapy, a vector with anti-tumor activity may be beneficial for the eradication of cancers. The delivery of therapeutic molecules to cancer cells using a vector with anti-tumor activity may have synergistic anticancer effects. A typical example is an armed-type oncolytic virus. The oncolytic viruses used for cancer therapy include natural mutants, such as the E1B-55K deletion adenovirus and the Edmonston strain of the measles virus, and artificial mutants developed by viral gene engineering, such as telomerase-controlled adenovirus, ICP6/γ34.5-deleted herpes virus and thymidine kinase-deleted vaccinia virus (JX-594) [20]. These viruses are live viruses that are expected to replicate selectively in cancer cells. An armed-type oncolytic virus is an oncolytic virus carrying a therapeutic gene that augments its anti-tumor effects. A cytosine deaminase/thymidine kinase gene-loaded E1B-55K deletion adenovirus and *GM-CSF* gene-loaded JX-594 are representative examples of such viruses [20]. These viral vectors may be promising therapeutic tools in the future.

Gene therapy for preventive medicine

Gene expression is easily obtained by intramuscular, intradermal or subcutaneous injection of naked DNA. The immune response against the product encoded by transgenes is also detected. This suggests the utility of gene vaccination against infectious diseases and cancers. Humans are at the risk of being exposed to new infectious diseases by mutant viruses, as illustrated by recent H5N1 mutant influenza virus and severe acute respiratory syndrome virus pandemics. The advances in genome detection and analysis technologies now enable the rapid construction of expression vectors for viral proteins. Using such clinical-grade vectors, the prevention of a pandemic infection can be achieved by gene vaccination of healthy individuals. Naked DNA injection is very common in human gene therapy [101], and the safety of the approach has already been evaluated, mainly for cardiovascular gene therapy. Based on the evidence, gene vaccination should be more thoroughly evaluated as a method for preventing serious infectious diseases.

In addition to infectious diseases, cancer prevention is also an important issue. It is well known that human papillomavirus causes cervical cancers,

and that human papillomavirus vaccination of young females is permitted to prevent cervical cancers. The vaccine is basically derived from a viral envelope without the viral genome. A DNA vaccine using DNA encoding viral envelope proteins is also available. Gene vaccines against other tumor viruses such as human T-cell leukemia virus-1, hepatitis B virus, hepatitis C virus and Epstein–Barr virus would also be easy to prepare. Although these viruses cause cancers, they are categorized as infectious diseases.

Tumor vaccines derived from tumor antigens may also be effective. Melanoma-associated antigens have been extensively investigated and numerous HLA-restricted antigens have been identified. Although these antigens may be available for vaccination, it is unlikely that cancer prevention can be achieved by this approach. Tumors can change their biological characteristics to survive under stressful conditions, which results in tumor recurrence. Therefore, gene vaccination using tumor antigen genes will probably be unsuccessful for the prevention of cancers.

From the viewpoint of administration

Since administratives differ from nation to nation, the author understands that his view does not necessarily fit into the situation of all countries. However, further improvement of administration is a problem that all countries face. The author hopes that his opininon here will stimulate readers to pay more attention to the administration of gene therapy.

Education of officers specializing in gene therapy

The guidelines related to human gene therapy and the approval system for protocols for clinical trials are different in each country. In the USA and EU, these systems are more advanced and well organized than other countries such as Japan. Even though the degree of preparation in these systems is varied, it is obviously necessary to increase the number of officers to control the administrative tasks related to gene therapy. For this purpose, the tasks of academia are to educate students about gene therapy, and to help them obtain government jobs as regulatory officers. These officers may come back to academia in the future to support translational research. The author proposes the establishment of an exchange program to educate specialists for translational research, including gene therapy, who would alternate between the administrative and academic sectors.

Funding for gene therapy

It is generally difficult to obtain funding specialized for gene therapy in every country. However, from a scientific viewpoint, special funding for

gene therapy is not necessary. The seeds of gene therapy are produced from basic research. Scientists should keep in mind that gene therapy should be based on basic science. At this point, the acquisition of funding is not a problem, as it still depends on the ability of the scientists. However, at the next step (for preclinical studies and for Phase I and II studies), it is difficult to get funding. While there may be some grants for that purpose, it is almost impossible for clinicians to promote Phase III trials by themselves without the support of a large pharmaceutical company. Unfortunately, if a treatment is not very promising during early Phase I and II clinical trials, no companies take over the trial.

Another source of funding is donations from various companies. In the EU, nonprofit organizations collect donations from many companies to enable the use of gene therapy for hereditary diseases. As a result, a lot of money is available for human gene therapy or rare diseases. This is because there is a tax exemption for big companies for such donations. The author's proposal is to establish such a nonprofit organization in other countries to collect donations from companies for gene therapy. It may be necessary to change the relevant laws for this purpose.

Central concept for the production of gene-based drugs

As described above, a large amount of money is needed to promote translational research, including gene therapy, toward drug development. Therefore, it is necessary to avoid waste, especially dispersion of money. A new branch of the NIH in the USA, the National Center for Advancing Translational Research, has started to speed up the production of drugs and other therapies [21]. Seven programs will be created in the National Center for Advancing Translational Research, and funding for translational research is going to be concentrated. Recently, in Japan, the Ministry of Health, Labor and Welfare selected five centers to achieve advances in translational research toward clinical trials and drug production. They will support those centers financially for 5 years. This direction of centralization may be inevitable for the success of translational research in countries worldwide. However, this centralization concept should be applied only for the realization of translational research, not for the support of basic science. Academic sectors outside the centers will use the centers to accomplish the translational research. Therefore, it is very important for each center to have successful systems in place, such as good manufacturing practice facilities, data centers to maintain patient records, special research hospitals for patients and prepared staff members with specialized training for clinical trials.

Financial & competing interests disclosure

The author has no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

Summary

- Gene correction therapy can be utilized for hereditary diseases based on homologous recombination after the development of improved specificity of the target sites.
- Genes that induce cancer cell-specific apoptosis can be isolated through the analysis of epigenetic regulation of gene expression. Cancer immunotherapy should be performed by both enhancing the effector cells and attenuating the immune system suppression.
- An *in vivo* tissue regeneration strategy will be useful for the treatment of acquired diseases. More attention should be paid to the potential of mesenchymal stem cells.
- Gene therapy applications should be performed for earlier stages of disease, and used in combination with other therapeutics.
- An administrative system that can promote the education of gene therapy specialists as officers of the government, collect funds and establish centers for clinical trials should be considered and improved by the government of each country.

References

1 Delelis O, Zamborlini A, Thierry S *et al*. Chromosomal tethering and proviral integration. *Biochim. Biophys. Acta* 1799(3–4), 207–216 (2010).

2 Nienhuis AW, Dunbar CE, Sorrentino BP. Genotoxicity of retroviral integration in hematopoietic cells. *Mol. Ther.* 13(6), 1031–1049 (2006).

3 Urnov FD, Miller JC, Lee YL *et al*. Highly efficient endogenous human gene correction using designed zinc-finger nucleases. *Nature* 435(7042), 646–651 (2005).

4 Li H, Haurigot V, Doyon Y *et al*. *In vivo* genome editing restores haemostasis in mouse model of hemophilia. *Nature* 475(7355), 217–221 (2011).

5 Olsen PA, Gelazauskaite M, Randol M *et al*. Analysis of illegitimate genomic integration mediated by zinc-finger nucleases: implications for specificity of targeted gene correction. *BMC Mol. Biol.* 11, 35 (2010).

6 Fiscer PB. Is mda-7/IL-24 a magic bullet for cancer? *Cancer Res*. 65(22), 10128–10138 (2005).

7 Abarzua F, Sakaguchi M, Takaishi M *et al*. Adenovirus-mediated overexpression of REIC/Dkk-3 selectively induces apoptosis in human prostate cancer cells through activation of c-Jun-NH2-kinase. *Cancer Res.* 65(21), 9617–9622 (2005).

8 Kelly E, Russell SJ. History of oncolytic viruses: genesis to genetic engineering. *Mol. Ther.* 15(4), 651–659 (2007).

9 Davis JJ, Fang B. Oncolytic virotherapy for cancer treatment; challenges and solutions. *J. Gene Med*. 7(11), 1380–1389 (2005).

10 Besch R, Poeck H, Hohenauer T *et al*. Proapoptotic signaling induced by RIG-I and MDA-5 results in type I interferon-independent apoptosis in human melanoma cells. *J.Clin. Invest.* 119(8), 2399–2411 (2009).

11 O'Brien CA, Kreso A, Jamieson CH. Cancer stem cells and self-renewal. *Clin. Cancer Res.* 16(12), 3113–3120 (2010).

12 Duda DG, Kozin SV, Kirkpatrick ND *et al*. CXCL12(SDF1α)–CXCR4/CXCR7 pathway inhibition; an emerging sensitizer for anticancer therapeutics? *Clin. Cancer Res.* 17(8), 2074–2080 (2011).

13 Pannuti A, Foreman K, Rizzo P *et al*. Targeting notch to target cancer stem cells. *Clin. Cancer Res.* 16(12), 3141–3152 (2010).

14 Mellman I, Coukos G, Dranoff G. Cancer immunotherapy comes of age. *Nature* 480(7378), 480–489 (2011).

15 Alexandrescu DT, Ichim TE, Marincola FM *et al*. Immunotherapy for melanoma; current status and perspectives. *J. Immunother.* 33(6), 570–590 (2010).

16 Yamanaka S. Strategies and new developments in the generation of patient-specific pluripotent stem cells. *Cell Stem Cell* 1(1), 39–49 (2007).

17 Kuroda Y, Kitada M, Wakao S *et al*. Unique multipotent cells in adult human mesenchymal cell populations. *Proc. Natl Acad. Sci. USA* 107(19), 8639–8643 (2010).

18 Tamai K, Yamazaki T, Chino T *et al*. PDGFR-α-positive cells in bone marrow are mobilized by HMGB1 to regenerate injured epithelia. *Proc. Natl Acad. Sci. USA* 108(16), 6609–6614 (2011).

19 Cartier N, Hacein-Bey-Abina S, Bartholomae CC. Hematopoietic stem cell gene therapy with a lentiviral vector in X-linked adrenoleukodystrophy. *Science* 326(5954), 818–823 (2009).

20 Kirn DH, Thorne SH. Targeted and armed oncolytic poxviruses, a novel multi-mechanistic therapeutic class for cancer. *Nat. Rev. Cancer* 9(1) 64–71, (2009).

21 Wadman M. US translational-science centre gets under way. *Nature* 481(7380), 128 (2012).

Website

101 Gene therapy clinical trials worldwide. www.abedia.com/wiley/index.html

Index