Cancer Gene Therapy by Viral and Non-viral Vectors

Edited by Malcolm K. Brenner and Mien-Chie Hung

Translational Oncology

SERIES EDITORS:
Robert C. Bast, Maurie Markman and Ernest Hawk

WILEY Blackwell

Cancer Gene Therapy by Viral and Non-viral Vectors

Translational Oncology

SERIES EDITORS

ROBERT C. BAST, MD

Vice President for Translational Research
The University of Texas MD Anderson Cancer Center
Houston, TX, USA

MAURIE MARKMAN, MD

Senior Vice President for Clinical Affairs
Cancer Treatment Centers of America

Clinical Professor of Medicine
Drexel University College of Medicine
Philadelphia, PA, USA

ERNEST HAWK, MD, MPH

Vice President, Division of OVP, Cancer Prevention and Population Sciences
The University of Texas MD Anderson Cancer Center
Houston, TX, USA

Cancer Gene Therapy by Viral and Non-viral Vectors

EDITED BY

Malcolm K. Brenner, MB, BChir, PhD

Director, Center for Cell and Gene Therapy
Baylor College of Medicine
Houston, TX, USA

Mien-Chie Hung, PhD

Chair, Department of Molecular and Cellular Oncology
The University of Texas MD Anderson Cancer Center
Houston, TX, USA

WILEY Blackwell

Published by John Wiley & Sons, Inc., Hoboken, New Jersey
Published simultaneously in Canada

Library of Congress Cataloging-in-Publication Data

Cancer gene therapy by viral and non-viral vectors / edited by Malcolm K. Brenner, Mien-Chie Hung.
p. ; cm. – (Translational oncology ; 4)
Includes bibliographical references and index.
ISBN 978-1-118-50162-7 (hardback)
I. Brenner, Malcolm K., 1951– editor of compilation. II. Hung, Mien-chie, editor of compilation.
[DNLM: 1. Neoplasms–therapy. 2. Genetic Therapy. 3. Genetic Vectors–therapeutic use. 4. Oncolytic Virotherapy. QZ 266]
RC268.4
616.99′4042–dc23

2013049552

Cover image: Left hand image – iStock #18471119 / © David_Ahn. Top right hand image – iStock #17000305 / © Pgiam. Bottom right hand image – iStock #24015041 / © monkeybusinessimages.
Cover design by Modern Alchemy

Printed in the United States of America

10 9 8 7 6 5 4 3 2 1

Contents

List of Contributors

Malcolm K. Brenner, MB, BChir, PhD
Center for Cell and Gene Therapy
Baylor College of Medicine
Houston, TX, USA

John C. Burnett, PhD
Department of Molecular and Cellular Biology
Beckman Research Institute of City of Hope
Duarte, CA, USA

George A. Calin, MD, PhD
Department of Experimental Therapeutics
The University of Texas MD Anderson Cancer Center
Houston, TX, USA

Vincenzo Cerullo, PhD
Laboratory of Immunovirotherapy
Centre for Drug Research (CDR) and Division of Pharmaceutical Bioscience
Faculty of Pharmacy
University of Helsinki
Helsinki, Finland

Laurence J.N. Cooper, MD, PhD
Division of Pediatrics
The University of Texas MD Anderson Cancer Center
Houston, TX, USA

Yue Ding, MSc
Department of Molecular and Cellular Biology
Irell and Manella Graduate School of Biological Sciences
Beckman Research Institute of City of Hope
Duarte, CA, USA

Adrian P. Gee, PhD
Center for Cell and Gene Therapy
Baylor College of Medicine
Houston, TX, USA

Bambi Grilley, RPh, RAC, CIP, CCRC, CCRP
Center for Cell and Gene Therapy
Baylor College of Medicine
Houston, TX, USA

Kilian Guse, PharmD, PhD
Cancer Gene Therapy Group
Haartman Institute
University of Helsinki
Helsinki, Finland

Akseli Hemminki MD, PhD
Cancer Gene Therapy Group
Haartman Institute
University of Helsinki
Helsinki, Finland

Jennifer L. Hsu, PhD
Department of Molecular and Cellular Oncology
The University of Texas MD Anderson Cancer Center
Houston, TX, USA

Yi-Hsin Hsu, PhD
Department of Molecular and Cellular Oncology
The University of Texas MD Anderson Cancer Center
Houston, TX, USA

Leaf Huang, PhD
Division of Molecular Pharmaceutics
Eshelman School of Pharmacy
UNC-NCSU Joint Department of Biomedical Engineering
University of North Carolina at Chapel Hill
Chapel Hill, NC, USA

Mien-Chie Hung, PhD
Department of Molecular and Cellular Oncology
The University of Texas MD Anderson Cancer Center
Houston, TX, USA

Longfei Huo, PhD
Department of Molecular and Cellular Oncology
The University of Texas MD Anderson Cancer Center
Houston, TX, USA

Chia-Wei Li, PhD
Department of Molecular and Cellular Oncology
The University of Texas MD Anderson Cancer Center
Houston, TX, USA

Hui Ling, MD, PhD
Department of Experimental Therapeutics
The University of Texas MD Anderson Cancer Center
Houston, TX, USA

Zhuyong Mei, MD
Center for Cell and Gene Therapy
Baylor College of Medicine
Houston, TX, USA

Judy S.E. Moyes, MB, BChir
Division of Pediatrics
The University of Texas MD Anderson Cancer Center
Houston, TX, USA

Amer M. Najjar, PhD
Cancer Systems Imaging
The University of Texas MD Anderson Cancer Center
Houston, TX, USA

John Rossi, PhD
Department of Molecular and Cellular Biology
Beckman Research Institute of City of Hope
Duarte, CA, USA

Andrew B. Satterlee, BS
Division of Molecular Pharmaceutics
Eshelman School of Pharmacy
UNC-NCSU Joint Department of Biomedical Engineering
University of North Carolina at Chapel Hill
Chapel Hill, NC, USA

Markus Vähä-Koskela, PhD
Cancer Gene Therapy Group
Haartman Institute
University of Helsinki
Helsinki, Finland

Yan Wang, PhD
Department of Molecular and Cellular Oncology
The University of Texas MD Anderson Cancer Center
Houston, TX, USA

Jiehua Zhou, PhD
Department of Molecular and Cellular Biology
Beckman Research Institute of City of Hope
Duarte, CA, USA

Series Foreword

While our knowledge of cancer at a cellular and molecular level has increased exponentially over the last decades, progress in the clinic has been more gradual, largely depending upon empirical trials using combinations of individually active anticancer drugs to treat the average patient. The challenge for the immediate future is to accelerate the pace of progress in clinical cancer care by enhancing the bidirectional interaction between laboratory and clinic. Our new understanding of human cancer biology and the heterogeneity of cancers at a molecular level must be used to identify novel targets for therapy, prevention, and detection focused on each individual. Barriers must be removed to facilitate the flow of targeted agents and fresh approaches from the laboratory to the clinic, while returning relevant human specimens, images, and data from the clinic to the laboratory for further analysis.

In this title we will provide a brief overview of our current understanding of human cancer biology that is driving interests in targeted therapy and personalized management. Further development of molecular diagnostics should facilitate earlier detection, more precise prognostication and prediction of response across the spectrum of cancer development. Targeted therapy has already had a dramatic impact on several forms of cancer and strategies are being developed to identify small groups of patients who would benefit from novel targeted drugs in combination with each other or with more conventional surgery, radiotherapy or chemotherapy. Development of personalized interventions – whether preventive or therapeutic in nature – will require multidisciplinary teams of investigators and the infrastructure to match patient samples and agents in real time.

To accelerate translational cancer research, greater alignment will be required between academic institutions, the US National Cancer Institute, the US Food and Drug Administration, foundations, pharma, and community oncologists. Ultimately, new approaches to prevention, detection, and therapy must be sustainable. In the long run, translational research and personalized management can reduce the cost of cancer care which has escalated in recent years. More accurate and specific identification of at-risk members and risk stratification will be helpful to minimize the risks of overdiagnosis and overtreatment, while maximizing the benefits of screening, early detection, and preventive intervention. Patients who would benefit most can be identified and funds saved by avoiding treatment in those whose cancers would not respond. Participation and education of community oncologists will be required, as will modification of practice patterns. For progress in the clinic to occur at an optimal pace, leaders of translational teams must envision a clear path to bring new concepts and new agents from the laboratory to the clinic, to complete pharmaceutical or biological development, to obtain regulatory approval, and to bring new strategies for detection, prevention, and treatment to patients in the community.

In a series of additional volumes regarding translational cancer research, several topics are explored in greater depth, including *Biomarkers*, *Targeted Therapy*, *Immunotherapy*, and this volume concerning *Cancer Gene Therapy by Viral and Non-Viral Vectors*. The purpose of these books has been not only to describe different strategies for particular forms of cancer, but also to identify some of the barriers to translation using different reagents or different strategies around common therapeutic or diagnostic modalities. Potential

barriers include not only the need for a deeper understanding of science, methods to overcome the challenge of tumor heterogeneity, the development of targeted therapies, the availability of patients with an appropriate phenotype and genotype within a research center with the investigators, research teams, and infrastructure required for clinical/translational research and the design of novel trials, but also adequate financial support, a viable connection to diagnostic and pharmaceutical development, and a strategy for regulatory approval, as well as for dissemination in the community.

Cancer Gene Therapy by Viral and Non-Viral Vectors considers many of these areas, including the strengths and limitations of the several types of viral and non-viral delivery systems, the potential importance of tumor-specific promoter systems, examples of where gene therapy has succeeded, the challenge of targeting all cancer cells, the advantages of targeting the tumor stroma and immunocytes, and the logistic barriers to preparation of materials required for clinical trials. The need for substantial antitumor activity and the importance of clinical responses in phase I–II trials are also highlighted. Overall, this volume provides substantial perspective regarding the translational potential of cancer gene therapy.

Robert C. Bast
Maurie Markman
Ernest Hawk

Preface

The idea of gene therapy was first proposed to correct errors associated with genetic disease by supplementing defective or missing genes. Advances in DNA technology and in understanding the basis of genetic diseases gave high hopes that gene therapy would be the next big breakthrough in medicine. However, the journey ahead was not without challenges and roadblocks. In 1999, a tragedy occurred when an 18-year-old gene therapy trial participant, Jessie Gelsinger, died 4 days after receiving adenoviral treatment for a genetic disorder from a massive immune response that led to multiple organ failure. This incident caused a major setback in the gene therapy field and the US Food and Drug Administration placed a hold on active gene therapy trials. Yet another event that followed brought more bad news to the field. In 2003, five patients who received CD34+ hematopoietic (bone marrow) stem cells transduced with a retrovirus carrying the interleukin-2 receptor γ chain gene to treat inherited X-linked severe combined immunodeficiency (SCID-XI) developed T-cell leukemia. One patient later died. Despite the dismissal of promising hopes of gene therapy in the early days as a result of these events, there is now optimism as more current research data have shown substantial progress in the clinical development of gene therapy after years of intense investigations to improve vector design and safety. Several successful gene therapy trials, including treatment of an inherited eye disease (Leber's congential amaurosis), Parkinson's disease, blood disorders, SCID-XI, adenosine deaminase-deficient SCID, and Siemerling–Creutzfeldt disease (X-linked adrenoleukodystrophy), have been reported in the last few years. Most recently, two studies published in July 2013 in *Science* reported clinical efficacy in lentivirial-mediated gene therapy to treat metachromatic leukodystrophy and Wiskott–Aldrich symdrome (see Chapter 2 for more details). In addition to human trials, studies conducted in canines showed that achromatopsia (an inherited form of total color blindness), diabetes, and Duchenne muscular dystrophy were successfully treated by gene therapy; these encouraging findings will undoubtedly continue to pave the way for conducting human clinical trials to develop new drugs to treat these diseases.

While no gene therapy has yet been approved in the USA, two have been approved for use in other parts of the world. The Chinese State Federal Drug Administration approved the world's first gene therapy to treat head and neck cancer using Gencidine, an adenoviral vector expressing tumor suppressor p53. However, concerns about the therapeutic efficacy have been raised [1], and there are no further reported clinical outcomes after a decade of approval. In 2012, the European Commission approved the first gene therapy product (Glybera) in the Western world to treat lipoprotein lipase deficiency, a rare inherited disease of fat metabolism. The company uniQure is currently seeking regulatory approval in the USA, Canada, and other countries.

Cancer, cardiovascular, and infectious diseases, among many others, are also targets of gene therapy. Adenovirus remains the most popular type of vector used in gene therapy clinical trials worldwide, followed by retrovirus, naked/plasmid DNA, vaccinia virus, and lipofection in the top five. For viral vectors, the important parts of the virus required for gene delivery are kept and those that are not required are deleted, and the development of self-inactivating integrating viruses such as retrovirus and lentivirus eliminates the transactivation of neighboring genes after integration. Current

investigations also continue to broaden the viral vectors' cell host range. For non-viral vectors, improvements have focused on the delivery system for therapeutic agents, including plasmid DNA, RNA interference (RNAi), and microRNAs, by increasing cellular uptake, protecting against microphage digestion, and optimizing nucleic acid payload release.

In the USA, clinical studies must be reviewed by regulatory committees such as the Institutional Review Board (IRB), Food and Drug Administration (FDA), Institutional Biosafety Committee (IBC), and Recombinant DNA Advisory Committee (RAC). Moreover, manufacture must also comply with Good Manufacturing Practice (GMP) guidelines set out by the FDA. The development of sufficient manufacturing capacity to meet the clinical demands after gene therapy attains approval is another concern.

The discovery of monoclonal antibodies brought much excitement as a new treatment modality in early 1980s. However, the lack of efficacy and the rapid clearance of murine monoclonal antibodies due to the development of human antimouse antibodies in patients led to the failure of many clinical trials. Nonetheless, perseverance allowed the development of technological improvements resulting in the eventual clinical success of monoclonal antibodies, which are now a standard approach for producing therapeutics targeting cell surface receptors. In a similar way, further improvements in gene therapy may allow this approach to follow the successful journey of monoclonal antibodies.

To ensure that gene therapy can be successfully developed into new drugs following the fate of monoclonal antibodies, there are several areas needing critical improvement, including efficient delivery, specificity, and well-designed clinical trials. In this book, we have invited experts to discuss the current updates on cancer gene therapy. The opening chapter by Cerullo et al. describes various types of viral therapy, particularly DNA viruses (adenovirus, vaccinia virus, herpes virus, parvovirus) and provides examples of their use in clinical studies. The following chapter by Zhou et al. focuses on the principal types and evolution of lentiviruses in cancer and HIV therapy with special interest in gene silencing by RNAi. The next two chapters describe non-viral delivery systems. First, in Chapter 3 Najjar et al. review various methods of plasmid DNA delivery, optimization of gene expression, and their application for therapy including cancer. Satterlee and Huang then explain in Chapter 4 the design and challenges of nanoparticles to deliver therapeutic RNAi. In the second part, starting with Chapter 5, Hsu et al. provide an introduction to the clinical applications of tissue-specific and cancer-targeting promoters in cancer gene therapy. As aberrant microRNA expression has been implicated in promoting and initiating carcinogenesis, in Chapter 6 Ling and Calin present an overview of the role of microRNAs in cancer and other diseases and discuss examples of anti-microRNA therapeutics.

The last part of the book provides some insight on the regulatory compliance of gene therapy clinical trials focusing on manufacturing regulations of viral vectors by Gee and Mei in Chapter 7 and review processes and requirements prior to obtaining FDA approval by Grilley in Chapter 8. In the closing chapter, Brenner discusses the tasks that must be accomplished to make gene therapy drugs more broadly applicable and the improvements in clinical trial design, as the development pathway of cancer gene therapy is distinct from and more complex than the traditional pharmaceutical model. It is our hope that this book can facilitate the maturation of gene therapy for its clinical application.

Malcolm K. Brenner
Mien-Chie Hung

Reference

1 Guo J, Xin H. (2006) Splicing out the West? *Science* 314: 1232–1235.

PART I

Delivery Systems

1

CHAPTER 1

Translational Cancer Research: Gene Therapy by Viral and Non-viral Vectors

Vincenzo Cerullo, Kilian Guse, Markus Vähä-Koskela, and Akseli Hemminki
University of Helsinki, Helsinki, Finland

Adenovirus

Adenovirus is among the most used vectors for gene therapy and gene transfer, and about 23% of all vector-based clinical trials have been performed with it (www.wiley.com//legacy/wileychi/genmed/clinical/). Adenovirus was first isolated in 1953 from human adenoids [1]. To date, 55 different human serotypes, subdivided into seven subgroups (A–G), have been characterized [2,3].

Adenovirus is a nonenveloped double-stranded DNA virus surrounded by an icosahedral protein capsid (Table 1.1). The capsid comprises penton and hexon proteins with knobbed fibers protruding from the vertices of the capsid [4]. Soon after its entry into the target cell viral DNA reaches the nucleus where starts its replication. *Early genes*, mainly involved in DNA replication, are transcribed first [5], followed by *late genes* mainly coding for structural proteins [4].

Adenoviruses tend to be species-specific with regard to permissivity to replication. However, there may be some exceptions to this general rule. It has been reported that adenovirus serotype 5 subgroup C (usually referred as Ad5, the most used gene therapy vector) can replicate to some degree in cotton rats [6,7], New Zealand rabbits [8], and Syrian hamsters [9]. This feature of Ad5 has been very important for scientists around the world because it has allowed them to use these animal models to develop new therapies for disease.

Historically adenovirus has been the most used vector for gene therapy and gene-transfer purposes. In 1970s F. Graham and colleagues discovered the importance of the *E1* gene, that made possible the use of adenovirus as a viral vector for gene therapy [10]. In fact, as *E1* gene products initiate the replication of the viral DNA, serotype 5 adenoviruses with *E1* deleted are incapable of replicating and remain episomal. Taking advantage of this characteristic, scientists replaced *E1* with different expression cassettes to avoid virus replication while promoting expression of the transgene inserted in place of *E1*. Later on, *E1*-deleted adenoviral vectors, also known as *first-generation* adenoviral vectors (FG-Ad), were developed into *high-capacity* adenoviral vectors or *Helper-dependent* adenoviral vectors (Hd-Ad). HD-Ad are devoid of all viral genes except the two inverted terminal repeats (ITRs) and the packaging signal (psi). They show a high cloning capacity (up to 36 kb) and reduced immunogenicity and toxicity [11] (Figure 1.1). Since then, it has been mainly used as vector for gene transfer for genetic diseases [12] or to treat cancer [13]. The immunogenicity of adenovirus may render it unsuitable for long-term

Cancer Gene Therapy by Viral and Non-viral Vectors, First Edition. Edited by Malcolm K. Brenner and Mien-Chie Hung.

Table 1.1 The main characteristics of the viruses discussed in this chapter.

	Adenovirus	*Vaccinia virus*	*Herpes simplex virus*	*Parvovirus*
				
Genome	Linear dsDNA, 36 kb	Linear dsDNA, 200 kb	Linear dsDNA, 150 kb	Linear ssDNA, 5.1 kb
Transgene capacity				
• Replication-competent vectors	≈3 kb	> 25 kb	>25 kb?	
• First-generation adenoviral vectors	FG-Ad: 7.8 kb	MVA, ΔD4R: > 50 kb	≈40 kb?	
• Helper-dependent adenoviral vectors	HD-Ad: ≈36 kb		HSV amplicon: ≈150 kb	<4.6 kb
Genetic targeting	• Deletion of genes essential for replication in normal cells (E1A, E1B) • Tumor-specific promoters • MicroRNA targets in genome	• Deletion of genes essential for replication in normal cells (VGF, TK) • MicroRNA targets in genome	• Deletion of genes essential for replication in normal cells (TK, RR, γ34.5) • Tumor-specific promoters • MicroRNA targets in genome	• Tumor-specific promoters
Particle retargeting	• scAb-binding domain in knob • Cell-specific peptides in fiber/knob • Serotype fiber/knob exchange	• scAb-binding domain in knob • Cell-specific peptides in fiber/knob • serotype fiber/knob exchange	• scAb-binding domain in knob • Cell-specific peptides in fiber/knob • Serotype fiber/knob exchange	

dsDNA, double-stranded DNA; HSV, herpes simplex viruse; MVA, modified vaccinia Ankara; ssDNA, single-stranded DNA; TK, thymidine kinase; VGF, vaccinia growth factor.

gene expression but makes it attractive for treatment of cancer. Use of a replication-deficient adenovirus as a gene delivery vehicle is the classic approach, with some exciting clinical results [14,15,16,17], but no products have been approved outside of China. This approach has been reviewed recently [18]. In the past decade, many adenoviral gene therapists have focused on use of adenovirus as a replication-competent oncolytic virus and thus this will be focus of this chapter.

Oncolytic Adenoviruses for Treatment of Cancer

Oncolytic adenoviruses are specifically modified to selectively replicate in and destroy cancer cells. This selectivity is achieved by modifications of the genes involved in viral replication so that the life cycle of the virus can occur only in cells than can transcomplement the defect, including cancer cells, while the replication of the virus is arrested in normal cells (transcriptional targeting) (Figure 1.2). An alternative approach is to use tumor-specific promoters to "drive" *E1* expression to allow selective replication of the virus in cancer cells [19] (Figure 1.2).

Historically, the first adenoviruses used in patients were wild-type viruses [20]. The concept was revived with the first adenovirus proposed to have tumor selectivity, dl1520 (today known as ONYX-015) [21]. This adenovirus bears a naturally occurring variation that results in a nonfunctional E1B-55k product. E1B-55k is one of the proteins encoded by the early gene *E1* and its normal

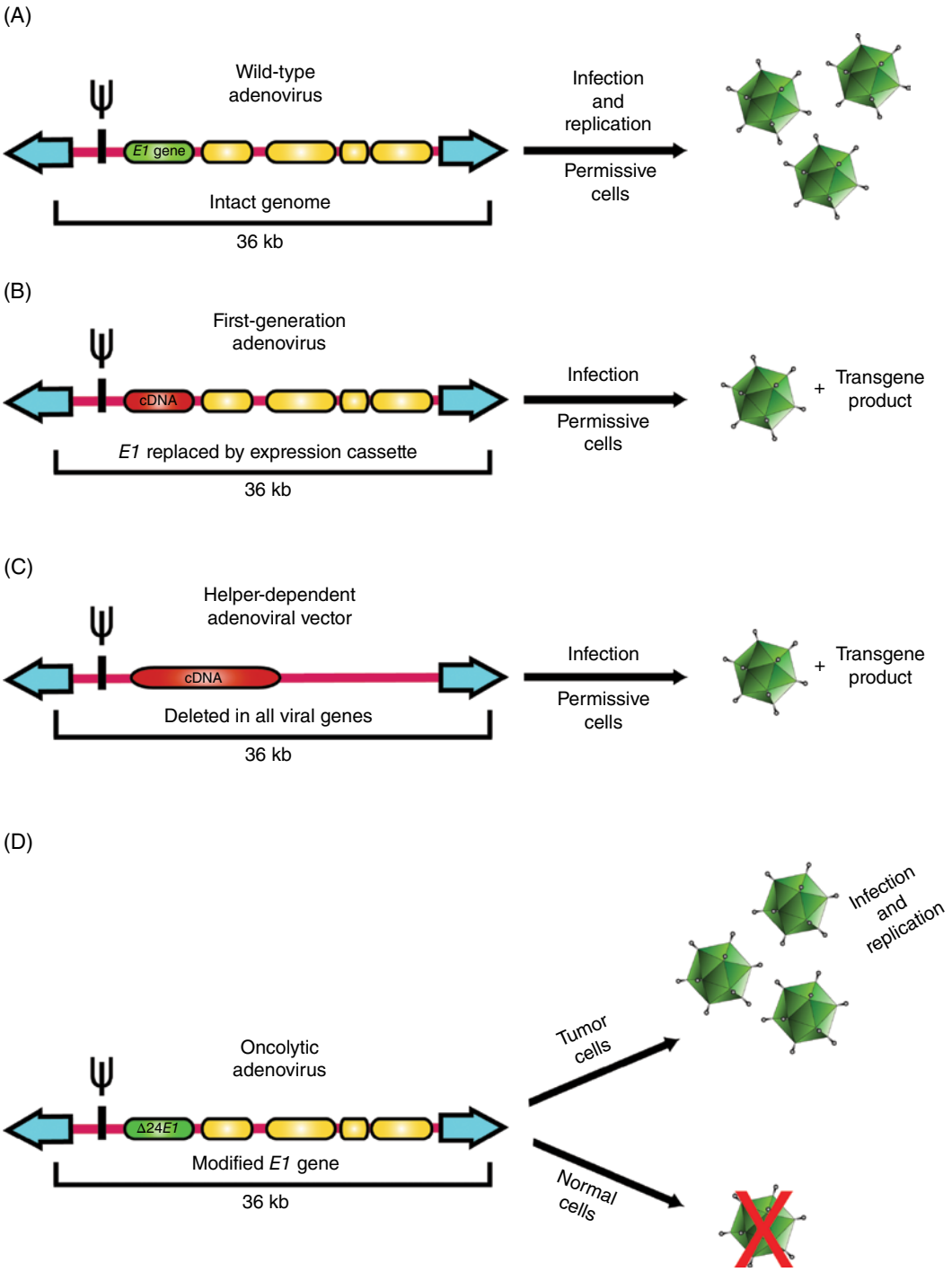


Figure 1.1 Schematic diagram representing the different kinds of adenovirus-derived vectors used for gene therapy. (A) Wild-type adenovirus is able to replicate and kill all permissive cells. (B) The *E1* gene is replaced by the expression cassette; this vector can infect all permissive cells but they cannot replicate unless *E1* is not transcomplemented by the packaging cell line. (C) All viral genes are deleted except ITRs and the packaging signal. These vectors can infect all permissive cells but they cannot replicate. (D) Oncolytic adenoviruses. These viruses have been engineered to selectively replicate in and kill cancer cells.

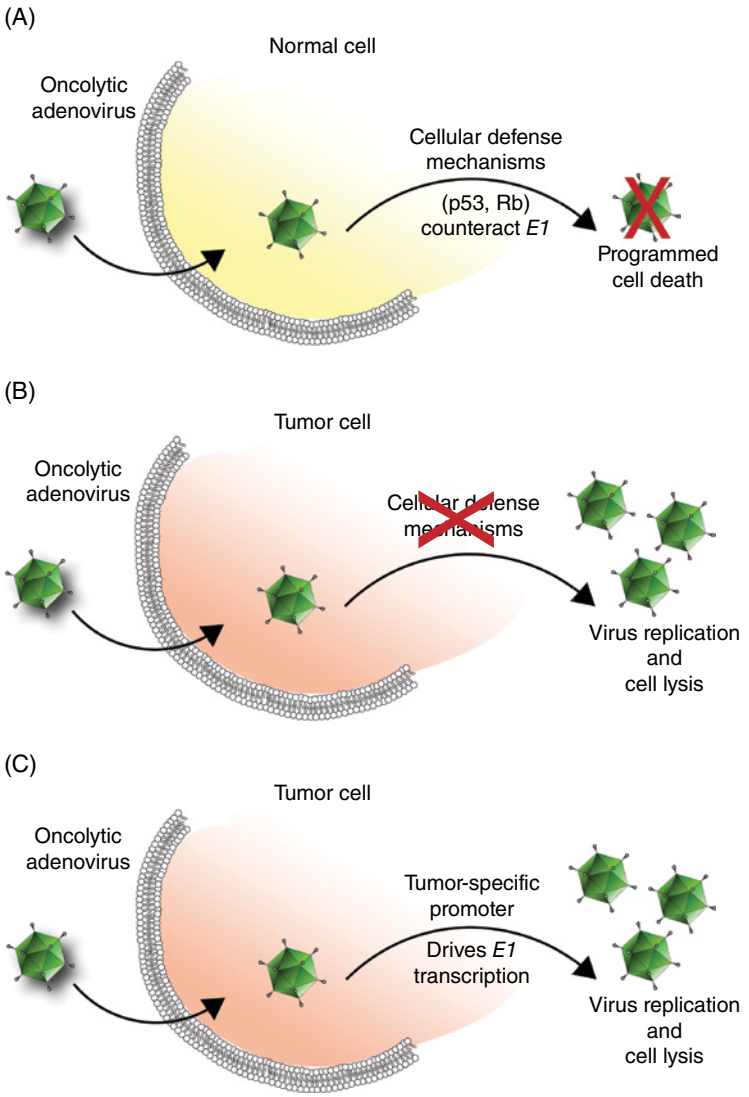


Figure 1.2 Transcriptional targeting. Simplified schematic illustrating the strategies used to achieve transcriptional targeting of tumor cells. (A) For example, a viral genome is modified to not be able to counteract the defense mechanisms that a normal cell turns on following a viral infection. (B) Tumor cells are defective of such mechanisms hence the virus can have its normal life cycle. (C) Tumor-specific promoter can initiate virus DNA replication, starting its life cycle.

function is to promote the degradation of p53 to avoid the infected cell undergoing apoptosis [22]. In infected normal cells p53 is not degraded by the mutated E1B-55k so that they can smoothly continue towards cell cycle arrest and apoptosis, which causes the arrest of the virus's life cycle; on the other hand, in cancer cells, where the p53/p14ARF pathway is universally defective, the mutation is not needed to avoid apoptosis [21]. An issue with this type of virus is that E1B-55k is needed for late mRNA transport and its absence results in ineffective oncolysis, several orders of magnitude less than with the wild-type virus [23].

An alternative strategy used to generate adenoviruses selective for cancer cells is a 24 bp deletion of the *E1A* gene [23,24,25]. This deletion results in the inability of E1A to bind to retinoblastoma tumor-suppressor protein (Rb) and to release

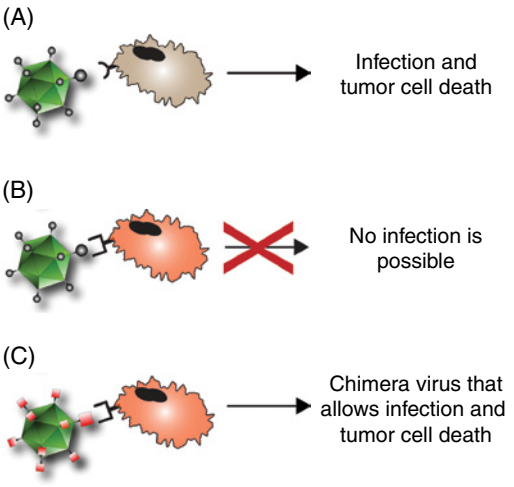


Figure 1.3 Transductional targeting. Schematic diagram representing the strategies used for transductional targeting. (A) Tumor expressing a specific receptor can be infected and killed by an adenovirus that infects through the same receptor, e.g. adenovirus serotype 5 and Coxackie adenovirus receptor-expressing tumor. (B) Some tumors do not express Coxackie adenovirus receptors hence they are not susceptible to infection by Ad5. (C) Viruses bearing the knob from a different serotype (chimera viruses) are used to overcome the lack of Coxackie adenovirus receptors.

eukaryotic initiation factor E2F, which in the case of wild-type adenovirus would result in S-phase induction in normal cells. Therefore the "delta-24" viruses are unable to induce S-phase in host cells and no viral replication follows. In contrast to normal cells, most if not all cancer cells have a defective Rb/p16 pathway, rendering the Rb-binding property of E1A dispensable [26]. An important difference to dl1520 is that these types of viruses are not attenuated in comparison to wild-type adenovirus with regard to replication in cancer cells [24].

Another strategy to restrict virus replication to tumor tissue is to drive *E1A* gene expression with a tumor-specific promoter. The first example of this type of modification was an adenovirus with prostate-specific antigen promoter driving expression of E1A [27]. Since then a multitude of different tissue-specific promoters have been used, including α-fetoprotein for hepatic cancer [28], tyrosinase for melanoma [29], and carcinoembryonic antigen (CEA) for colorectal cancer [30]. Also, tissue-specific promoters that are activated in a variety of cancer types have been employed, including cyclo-oxygenase 2 promoter [31,32,33,34], L-plastin promoter [29,35], and human telomerase reverse transcriptase promoter [36,37]. The selectivity of dl1520 and delta-24 occurs after *E1* expression, while tumor-specific promoters act prior to *E1* expression. Therefore, an appealing approach is to combine both [33].

In addition to these strategies that restrict viral replication to tumor cells (transcriptional targeting), effort has also been put into modifying the adenovirus capsid to increase transduction of cancer cells (transductional targeting) (Figure 1.3). To this purpose different serotypes or chimeras have been tested to increase tumor transduction (recently reviewed by Cerullo and colleagues [13]). Particularly noteworthy has been the adenovirus 5/3 chimera. This modified adenovirus has been generated by placing the Ad3 fiber knob into the Ad5 backbone, resulting in an Ad5/3 chimera that displays the cell-binding properties of serotype 3 [38,39]. These chimeras also exhibit enhanced gene delivery and efficacy in preclinical animal models [39,40,41]. Recently this approach was taken a step further by developing the first fully serotype 3 (Ad3)-based oncolytic adenovirus, which has shown very encouraging results in animal models and human patients [42,43]. Transcriptional targeting is fully compatible with transductional targeting and an appealing concept is to combine them, as seen in many advanced-generation viruses [33].

Importantly, oncolytic adenoviruses have also been used as delivery vehicles to produce molecules (such as antibodies, drugs and prodrugs, cytokines and chemokines, and so on) directly at the tumor site [13]. This approach has been particularly helpful because it allows, especially for molecules that have high systemic toxicity, a high local concentration associated with less systemic exposure.

For this purpose, a common way to insert foreign DNA into the adenovirus genome is by replacement of small proteins encoded by early or late genes. Transgenes can completely replace *E3* [44] or just part of this gene [26,28]. Transgenes can also be inserted in the late genes and the expression level of the transgene could depend on the insertion site [45].

A multitude of different proteins have been investigated as "arming devices" for oncolytic adenoviruses. Tumor-suppressor genes such as *p53* have been used to enhance oncolytic cell killing regardless of the *p53* status of the cancer cell line [46]. Prodrug-converting-enzyme-based systems commonly employ either cytosine deaminase for 5-fluorocytosine conversion to 5-fluorouracil [35,47], HSV-tk for ganciclovir conversion to its active metabolite [48], or both [49]. Antiangiogenic molecules have also been used for arming [50], in addition to various other molecules such as human sodium iodide symporter, which has been used to concentrate radioiodine in target cells [51]. Furthermore, immunostimulatory cytokines such as granulocyte macrophage colony-stimulating factor (GM-CSF) [52,53,54,55] used to boost antitumoral immunity have been under active investigation as transgenes.

Another interesting approach recently explored for adenovirus has been the enrichment of its genome with TLR9-specific sequences to increase TLR9 stimulation and consequently to enhance the antitumor immunity [56]. Along the same line arming the adenovirus with ligand for CD40 has also shown enhanced antitumor immunity in animal models and in cancer patients as well [57,58]. Unfortunately oncolytic viruses – which need most of their genome to replicate – have a limited cargo size capacity. A larger payload can be achieved by FG-Ad or HD-Ad but these vectors are not capable of replicating in cancer cells. In fact it would be interesting to combine the cloning capacity of HD-Ad with the killing capacity of oncolytic viruses; these approaches are under investigation. This should be feasible as oncolytic viruses have already been utilized to amplify first-generation vectors to combine the merits of each [59].

Use of Adenovirus in the Clinic

The observation that wild-type adenoviruses can kill cancer cells has been acknowledged for a long time. In fact, the first in-human use of adenovirus was in the 1950s [20], when 10 different serotypes were used to treat 30 cervical cancer patients. The treatments were quite safe, which is remarkable considering that wild-type viruses were used. With regard to efficacy, two-thirds of the patients had a "marked to moderate local tumor response" [20] with necrosis and ulceration of the tumor. Although "response" was not defined, these numbers are not so far from what is seen with modern viruses [58,60].

Since then, a multitude of different oncolytic adenoviruses have been conceived and tested in human clinical trials for different tumor types such as pancreatic cancer [61], brain tumors [62], prostate cancer [63], bladder cancer [64], and ovarian cancer [65], among others.

The first oncolytic adenovirus used in clinical trials in modern times was ONYX-015. More than 300 cancer patients with different tumor types were treated in several clinical trials from phase I to phase II, but a phase III trial was never initiated in the West. Instead, in China a similar virus, H101, was rapidly taken through all phases and approved in 2006 as Oncorine [66]. The overall results from these clinical trials were that this virus is safe and selective for cancer [67], and has antitumor efficacy, especially when combined with chemotherapy. However, preclinical data suggest that the oncolytic potency is up to 100 times lower than, for example delta-24-type viruses [25]. Also, an unarmed virus might be at a disadvantage compared to armed viruses.

Recently, scientists have realized that the use of oncolytic adenoviruses for treatment of cancer is particularly intriguing given their ability to wake up the immune system, stimulating a response to the cancer [13,68]. Although a clear mechanism on how this happens still remains to be fully clarified, we believe that even in the

tumor-immunosuppressive microenvironment adenoviral particles have the ability to (i) stimulate dendritic cells, predisposing them to *cross-priming*, (ii) promote antitumor immunity by enhancing the release of tumor-associated antigens in the presence of a danger signal [69], and (iii) break tolerance of the immunosuppresive tumor environment through interaction with pathogen-associated molecular pattern receptors [11,70,71,72,73]. These "natural" features can be further improved when adenoviruses are armed with immunostimulatory molecules.

We have been among the first laboratories to demonstrate the involvement of the immune system in human cancer patients. In our first study patients were treated with a GM-CSF-encoding serotype 5 virus (Ad5D24-GM-CSF) bearing a 24 bp deletion in *E1A* [68]. We assessed the tumor-specific immune response by ELISPOT and by flow cytometry. ELISPOT was performed on fresh peripheral blood mononuclear cells pulsed for 12 h with tumor-specific and adenovirus-specific pools of peptides. Tumor specificity was assessed using survivin as an example of a pan-carcinoma antigen commonly expressed by most tumors [74].

Similar immunological data were observed with a serotype chimera 5/3 (Ad5/3D24-GM-CSF) [60] and with an integrin-targeted virus [75]. In an interesting contrast, when an unarmed oncolytic adenovirus (Ad3hTERT) was used in humans, antiviral responses were equally emphatic but less evidence of antitumor response was seen [42]. It remains to be studied how important the immunostimulatory transgene is or if the serotype also plays a role.

Interesting results have also been reported by Li et al. [76]. They present the data of a phase I dose-escalating trial with an oncolytic adenovirus expressing the heat shock protein 70 (HSP70), emphasizing some aspects of the antitumor immune-mediated response. Specifically they observed elevation of the number of CD4+ and CD8+ T cells as well as natural killer (NK) cells in the blood of the patients after the administration of the virus [76].

Similar results were also reported in another phase I trial with an oncolytic adenovirus expressing GM-CSF [54]. Similarly an important involvement of the immune system was also demonstrated with CD40L-expressing oncolytic adenoviruses [57,58].

Vaccinia Virus

Vaccinia virus (VV) is best known for its use as an efficient vaccine against smallpox, which led to the worldwide eradication of the disease. Due to this important historical role, VV has the longest and most extensive history of use in humans of any virus and a wealth of basic, preclinical, and clinical data are now available. Different strains of VV exist, of which modified vaccinia Ankara (MVA), New York VV (NYVAC), Lister, Wyeth, and Western Reserve (WR) are the most commonly used. Some of these (MVA, NYVAC, Lister) are completely or partially replication-deficient and are therefore mostly used as gene-transfer vehicles and vaccines. Wyeth and WR instead are mostly employed as replicating viruses in experimental cancer therapies due to their strong oncolytic properties. VV has an approximately 200 kb double-stranded DNA genome, which encodes about 200 genes (Table 1.1). The large, enveloped, and brick-shaped virus particles are about 300 nm in the longest dimension. VV does not require specific cell-surface receptors for transduction of target cells. Rather, it enters cells through membrane fusion or macropinocytosis. Thus, VV is able to infect a wide range of cell types. Upon entering the cytosol, VV immediately starts transcribing a defined set of early mRNAs using the transcription enzymes that the virus brings with it. Subsequently ribosomes and other components of the host cell translation machinery are recruited into defined granular structures called virus factories. Here viral gene replication and viral protein production take place and new infectious viral particles are formed. The majority of the new particles are intracellular mature virions, which have a single lipid bilayer envelope and remain inside the cell until lysis. However, a small subset of the viral particles wrap themselves in an additional lipid bilayer derived from the trans-Golgi network before egressing from the cell as extracellular enveloped virions (EEVs). The EEV particles can spread efficiently through the system of the host and are largely shielded from the immune system; therefore, they are also known as *stealth particles*.

VV for Treatment of Cancer

VV has several unique features that make it an attractive cancer gene therapy vector. Firstly, VV has a wide host range and is able to efficiently transduce a broad range of mammalian cell types *in vitro*. However, *in vivo* after intravenous injection in mice VV exhibits a natural tropism to tumors, which has been suggested to be due to the leaky vasculature in cancer tissue that allows the large virus particles to enter. Another advantage is that VV can hold up to 25 kb of foreign DNA, allowing the insertion of large genes and/or multiple expression cassettes. Furthermore, for efficient expression of the genes of interest, a number of strong viral promoters exist. Another advantage is that VV is a highly immunogenic agent that triggers a strong antibody and T-cell response, making VV an efficient vaccine vector. Moreover, replication-competent VV strains are highly oncolytic due to their rapid and efficient replication cycle, resulting in strong antitumor efficacy. Lastly, VV does not enter the nucleus at any time during the infection; thus, there is no danger of insertional mutagenesis. These attributes make recombinant VVs attractive agents for the treatment of many diseases, especially cancer. For cancer therapy VV has mainly been used in three ways: (i) as a replication-deficient expression vector of therapeutic genes; (ii) as a replication-competent, oncolytic virus; and (iii) as a vaccine expressing cancer epitopes and/or immune-stimulatory molecules. Many VV constructs, some of which combine the above-mentioned mechanisms of action, have been generated and evaluated preclinically and some of them have entered clinical trials with exciting results.

A Lister strain VV that expresses p53 is one example of the use of VV as a gene-transfer vector. This vector showed antitumor efficacy and minimal toxicity in a murine glioma model, even when irradiated with ultraviolet light to make it completely replication-incompetent [77]. However, despite the high transduction efficiency of VV, penetration within the tumor and infection of all cancer cells is generally difficult to achieve due to intratumoral barriers. Consequently, vectors expressing therapeutic genes with a bystander effect, such as an MVA strain VV expressing *FCU1* (a secreted suicide gene) that has been evaluated in colon cancer models, have shown promising results [78]. Another strategy to improve tumor penetration and increase transgene expression inside the tumor is to use replication-competent VVs that at the same time kill cancer cells by oncolysis. There are many examples of oncolytic VVs – mostly of the Wyeth and WR strains – that have shown promising antitumor efficacy in various preclinical cancer models. To ensure tumor-selective replication, viral genes that are necessary for replication in normal cells but dispensable in cancer cells can be deleted. In line with this strategy, disruption of the viral thymidine kinase (TK) and vaccinia growth factor genes, which are both complemented in cancer cells but not in normal cells, significantly increased tumor specificity [79]. To enhance antitumor efficacy of oncolytic VVs, therapeutic genes can be incorporated into the vectors. Examples include VVs armed with suicide genes, antiangiogenic molecules, and immune-stimulatory proteins (reviewed in [80]). Vectors expressing immune-stimulatory agents might be particularly effective since induction of antitumor immunity could be the key to successful and sustained therapy. JX-594, a Wyeth strain VV deleted in TK and expressing GM-CSF, is an example of an oncolytic VV with enhanced immune-stimulatory properties that has demonstrated convincing preclinical results and is currently being evaluated in phase II clinical trials with encouraging preliminary results (reviewed in [80]). In most clinical studies so far, VV has been injected intratumorally, which is thought to be the safer route of administration. However, animal models and a clinical trial have shown that intravenously administered Wyeth and WR viruses have the potential to reach the tumor with minimal effect on normal tissue [81]. This opens up the possibility of reaching multiple tumor sites and metastases with a single virus injection, which would be highly desirable.

VV as a Cancer Vaccine

For cancer immunotherapy approaches, VV can be used to deliver nonspecific immune-modulatory molecules or tumor-specific antigens. Examples of immune-stimulatory molecules that have been delivered by VV include CD40L, GM-CSF, interleukin (IL)-2, B7-1, intracellular

cell-adhesion molecule (ICAM)-1, and lymphocyte function-associated antigen 3 (LFA-3 or CD58). These factors are cytokines and costimulatory molecules that are expected to alter the local immune-privileged tumor environment, which could lead to the host's immune system attacking the primary tumor site as well as distant sites and metastases. An MVA vector expressing the combination of B7-1, ICAM-1, and LFA-3 (designated TRICOM) has been particularly efficient in activating T cells, which are among the most efficient immune cells in cancer therapy [82]. VVs expressing tumor-associated antigens are another attractive approach in cancer immunotherapy. The rationale is that the host's immune system would be primed for antigens that are present on tumor cells but absent in normal tissue. Many different antigens have been incorporated in VVs, such as CEA, prostate-specific antigen (PSA), and the oncofetal antigen 5T4. VVs expressing these antigens showed significant antitumor efficacy in various animal models primarily due to induction of cytotoxic T cells. In clinical trials VV-CEA and VV-PSA have demonstrated antitumor efficacy especially when used in a prime-boost scheme in combination with avipox vectors. Trovax, a VV expressing 5T4, has been evaluated in phase II clinical trials in patients with metastatic colorectal cancer, metastatic renal cancer, and prostate cancer demonstrating strong 5T4 immune responses, which correlated with indicators of clinical benefit [83]. A phase IIb trial with an MVA expressing the tumor-associated antigen MUC1 and IL-2 (TG4010) in lung cancer in combination with chemotherapy as a first-line therapy suggested that the therapeutic vaccine improves clinical outcome [84]. Toxicity of VVs as cancer vaccines has generally been low in clinical trials, mostly consisting of transient fever, malaise, skin reactions, and pain at injection site.

In summary, VV seems to be well suited as a gene-therapy agent in cancer therapy in particular because of its high transduction efficiency, strong oncolytic effect, and immune-stimulatory properties. Based on the good safety profile of VV, the field will likely move towards developing more efficient cancer vaccines, combining immune-stimulatory features with oncolytic potential.

Herpes Virus

General Properties and Life Cycle

Herpes simplex viruses (HSVs) are double-stranded DNA viruses with a relatively large genome of about 150 kb encoding 100–200 genes (Table 1.1). The genome is packaged within an icosahedral protein capsid, which itself is surrounded by a host cell-derived double lipid membrane envelope. Holding the envelope in place is a layer of virus proteins called the tegument. The HSV genome consists of two sequences, UL and US, each of which is surrounded by a pair of inverted repeat sequences (IRL and IRS) [85]. A substantial portion of the genome consists of accessory genes, which may facilitate but are not necessary for completion of the virus life cycle in permissive cells. Thus, it has been estimated that replacement of up to 50 kb of accessory genes should be possible in HSV vectors without fully compromising the capacity of the virus to replicate.

HSV bears several virus-specific glycoproteins on its membrane, which mediate entry into cells through common cell-surface glycosaminoglycans, including nectin and heparan sulfate proteoglycans, giving the virus broad tropism. Infection starts by virion attachment to the cell-surface plasma membrane, followed by membrane fusion and release of the capsid into the cytoplasm. The capsid, in turn, is transported via microtubules to the nucleus, where it is disassembled and the genome is released into the nucleus through the nuclear pores. Virus replication ensues, beginning by transcription of early genes and culminating with either establishment of latency or release of new viruses by budding at the plasma membrane and, in many cases, death of the infected cell.

Cancer Targeting by Recombinant Herpes Viruses

Several members of the herpesviridae family have been found suitable for gene delivery and/or cancer targeting. The first genetically engineered herpes virus vectors were designed as vaccines against herpes and incorporated a deletion of the immediate-early gene *ICP4*. However, since *ICP4* is an essential gene, the resultant recombinant vector is replication-defective [85]. Since then, these and other replication-defective vectors, such as HSV

amplicons, in which all virus DNA save for the inverted terminal repeats has been replaced with foreign DNA, have been used for cancer targeting in preclinical settings. As another example, so-called disabled infectious single-cycle HSV (DISC HSV) vectors were shown to mediate tumor regression when engineered to express GM-CSF [86]. While such vectors are useful for gene transfer and cancer gene therapy when armed with, for example, immune-stimulating cytokines, in cancer targeting replication-competent or replication-selective vectors have been favored.

The first replication-competent HSV vector, *dls*ptk, featured a deletion of the *UL23* gene encoding TK. In a landmark study which pioneered cancer targeting by any recombinant virus (not just in the context of HSV), Martuza and colleagues used TK-deleted HSV in nude mice harboring human glioma xenografts to substantiate the concept of killing tumor cells in living hosts while sparing normal healthy tissue [87]. However, TK-deleted herpes viruses were considered unfavorable for clinical application as they were neurotoxic at high doses and lacked sensitivity to common antiherpetics (a failsafe against unwanted virus replication). Instead, new constructs were engineered. For example, *UL39*, which encodes herpes virus protein ICP6, the ribonucleotide reductase, was deleted in hrR3, based on HSV type 1 strain 1716. This agent demonstrated similar safety and efficacy in glioma targeting in experimental models as the TK-deleted vector *dls*ptk [88] and continues to be a popular vector for cancer targeting today. Around the same time, another HSV gene was deleted, *RL1*, which encodes protein ICP34.5 (more commonly known as γ34.5). γ34.5 counteracts the antiviral effects of double-stranded RNA-activated protein kinase R (PKR) by promoting dephosphorylation of eukaryotic initiation factor 2α, thereby ensuring that host cell (and virus) translation is not shut down during infection [89]. In cancer cells, PKR is typically rendered inactive or its antiviral effects are stunted, which allows even *RL1*-deleted HSV to replicate in these cells but not in normal cells. The deletion markedly attenuates herpes virus pathogenicity by rendering the virus unable to replicate productively in neurons. A recombinant vector based on HSV strain 1716 (trade name SEPREHVIR) which lacks both copies of the *RL1* gene, is being developed by Virttu Biologics (formerly Crusade Laboratories). This virus has shown excellent safety in both glioma and melanoma patients (47 and five patients, respectively; www.virttu.com). While the survival of one patient with glioma has given cause to continue clinical development of HSV1716 in glioma [90], no activity (e.g. necrosis or virus replication) was seen in biopsies of 20 patients with oral squamous cell carcinoma following three direct intratumoral injections of up to 5×10^5 plaque-forming units (PFUs) of HSV-1716 [91]. Importantly, both *UL39*- and *RL1*-deleted HSV vectors still carry the *TK* gene, which allows additional control of these viruses by TK-targeted antiherpetic drugs, such as ganciclovir.

Multimutated HSV Recombinants in Clinical Use

Because of lingering safety concerns with single-gene-deleted recombinants, two or more genetic deletions have been tested in the same virus. These second- (multimutated) and third- (multimutated + armed) generation viruses have since become the mainstay in cancer gene therapy. The first HSV-1-based recombinant to enter clinical testing was G207, which is deleted for both copies of the *RL1* gene and which has an insertion of *LacZ* in one of the *UL39* genes [92]. This virus has so far undergone two phase I/Ib studies in glioblastoma and demonstrated good safety and tolerability as well as signs of virus replication by reverse transcriptase-mediated PCR in tumor tissue in the brain [93].

Another virus in clinical development, NV1020, is based on a recombinant virus R7020 originally designed as a vaccine for HSV-2 [94]. It contains several genetic modifications, such as *TK* under the control of the ICP4 promoter as well as glycoprotein sequences from HSV-2, meant to immunize hosts against this member of the virus family without altering replicative or pathogenic properties of NV1020 compared to parental HSV-1 [95]. This virus is being tested in patients with colorectal cancer metastases in the liver following intrahepatic arterial infusion. In a phase I/II trial, 50% of patients displayed stable disease with a

median time to progression of 6.4 months at the maximum tolerated dose (1×10^8 PFU [96]). A follow-up phase II/III study is planned. The first third-generation recombinant HSV vector G47Δ features deletion of HSV gene *α47* in addition to *RL1* and *UL39*. In cells infected with wild-type HSV, *α47* eliminates MHC I from the cell surface, thereby reducing the capacity of the cell to present antigen to the immune system and allowing the infection to go unnoticed. Deletion of this gene thus increases immunogenicity of the infection, and while it increases recognition of the virus by the immune system, it also facilitates bystander immune responses against the tumor, resulting in increased overall antitumor efficacy. HSV G47Δ demonstrated excellent antitumor efficacy in several preclinical models and a phase I safety study was initiated in 2009, with results still pending publication [97].

In addition to selectively engineered gene-deletion recombinants, HF10 is a laboratory clone of a stock HSV-1 virus which displays several gene deletions and other mutations compared to the oncolytic viruses described above. While its exact mechanisms of attenuation remain unclear, HF10 has displayed excellent antitumor efficacy in several different preclinical cancer models and has now undergone at least five phase I studies in cancer patients. Lastly, Oncovex-GM-CSF, currently known as talimogene laherparepvec or T-VEC, proved safe and efficacious in phase I/II studies in advanced melanoma (28% objective response rate) and has recently completed a phase III trial, the potentially groundbreaking results of which are anticipated in the near future. The virus, originally developed by Biovex and now being developed by Amgen, is based on HSV-1 and features deletions in *RL1* and *α47* genes in addition to expressing the cytokine GM-CSF, which may increase overall therapeutic efficacy of the vector by stimulating monocyte maturation [98]. However, the discovery that high intratumoral GM-CSF expression in some cancers is associated with heavy immune-suppressor cell infiltration (including myeloid-derived suppressor cells, tumor-associated macrophages, and neutrophils) would certainly warrant closer study of GM-CSF when expressed by recombinant viruses [98].

Other Herpes Virus Family Members with Cancer-Targeting Potential

The main advantage of herpesviral vectors is their capacity to carry large transgenes, exceeding 150 kb in amplicons and up to 50 kb in replication-competent vectors, while the main drawbacks include difficult cloning (even using new BAC systems), safety issues such as neurotoxicity at high virus doses, and the risk of recombination with or without activation of endogenous herpes viruses. In order to address some of these issues, other members of the herpes virus family have been tested as gene-delivery/-therapy vehicles. In addition to the vectors described above, several new laboratory strains of HSV-1 have been generated by serial passages on human cancer cells in cell culture [99]. These recombinants typically harbor multiple mutations throughout the genome that increase replication fitness in cancer cells while reducing it in normal cells. However, the safety and potentially new molecular mechanisms of such mutants warrants characterization to avoid unexpected side effects. In addition, herpes viruses are, like most viruses, prone to Muller's ratchet and will lose fitness upon serial passages in a limited host cell repertoire, potentially reducing the usefulness of the passaged mutant viruses as broad-spectrum therapeutics [100].

Since HSV-2 is also a human pathogen with similar features to HSV-1, it has been tested for oncolytic potential. A vector called FusOn-H2 was created by deletion of the ribonucleotide reductase gene *ICP10* (corresponding to the *ICP6* gene in HSV-1), which abrogates the oncogenic potential of the vector and confers syncytium-forming properties [101]. FusOn-H2 was more efficacious in mouse tumor models than common vectors based on HSV-1. Other human herpes viruses developed for cancer targeting include Epstein–Barr virus (EBV) and human herpes viruses 6 and 7, which are noncytolytic but still able to efficiently transduce specific types tumor cells. EBV is a gammaherpesvirus which displays an inherent tropism to lymphoid cells, particularly of the B-cell lineage, which is contrast to the neurotropic simplex subfamily members on which most oncolytic viruses are built. Therefore, EBV-based vectors could potentially carry less risk of neurological complications than vectors based on HSV-1, or,

correspondingly, EBV-based vectors may be better suited to target B-cell-related cancers than HSV-1 viruses. In the case of EBV, three genes have been removed from the virus to abrogate its capacity to transform cells, also rendering it replication-defective [102]. An attenuated vaccine strain of varicella zoster virus, causative of chicken pox in children and herpes zoster in adults, also demonstrated capacity to infect and kill human glioblastoma cells *in vitro* [103]. Because the vaccine virus is safe for humans it may be possible to develop it for targeting brain (and other) tumors in the future.

As for nonhuman herpes virus family members, vectors based on equine herpes virus 1 were recently shown to possess oncolytic potential against human glioblastoma [104]. Also, both bovine herpes virus 1 and 4 are being developed as oncolytics [105,106], with the latter demonstrating oncolytic potential in glioblastoma in mice. Further, vectors based on pig pseudorabies and monkey saimiri virus (prototype γ-2 herpes virus) show targeting potential in human cancer cells, although these viruses are generally not cytolytic on their own and require additional modification (i.e. "arming") to be useful as therapeutics. In addition, while, for example, saimiri virus can transduce human T cells, lingering concern about oncogenic potential may limit translation of these nonhuman herpes viruses into the clinic [107]. This would likely be a concern for vectors based on EBV and other potentially oncogenic viruses as well.

Herpes Virus Retargeting

Several different strategies have been developed to increase virus specificity. For example, a single-chain antibody-binding domain was successfully incorporated into a variable loop of glycoprotein G, allowing for coating of the virion by a single-chain antibody of choice. By this approach, the natural broad cellular tropism of HSV could be diminished and the virus retargeted to cells expressing antibody target receptors, such as human epidermal growth factor receptor 2 (HER-2). Such HER-2-targeted HSV vectors showed robust antitumor activity in a target-dependent fashion [108]. A more straightforward approach has been to restrict virus gene expression to desired cell types by placing the genes under control of cell-specific promoters. For instance, as an alternative to gene deletion, which reduces virus replication even in cancer cells, the main neurovirulence gene *RL1* (encoding γ34.5) was placed under control of tumor-restricted promoters, which allowed the virus to replicate with nearly undiminished efficacy in several types of cancer cells [109]. Indeed, several other HSV genes, essential or not, have been tested under a variety of tissue-specific promoters, demonstrating the feasibility of directing HSV replication to only desired cell types/tissues and in some cases, due to relative promoter strengths, even increasing the replicative efficacy of the tissue-specific vectors compared to gene-deleted viruses [110]. Finally, HSV replication may be regulated through manipulation of virus gene transcripts by microRNA targets through which the cellular RNA-silencing mechanism may be harnessed to reduce translation of the targeted genes. It is possible to combine more than one targeting approach to maximize tissue specificity, as exemplified by a liver-targeted oncolytic HSV recombinant in which virus replication was on one hand restricted to the liver by a liver-specific promoter driving the essential virus glycoprotein gene H and on the other hand abrogated in normal liver cells through microRNA targets in gene H which are sensitive to RNA silencing in normal liver cells but not liver cancer cells [111].

Parvovirus

Parvoviruses (PVs) are small (from Latin *parvus* meaning small; about 25 nm in diameter) icosahedral particles containing a single-stranded DNA of about 5000 nucleotides [112] (Table 1.1). Upon infection of permissive cells PVs undergo viral replication and release of virus progenies. Infection by some but not all PVs results in cell death. In order for the cell lysis to occur, the cell has to be of the right animal species, it has to be proliferating, and it has be rather undifferentiated [113]. Many of these characteristics can be found in tumor cells, and in fact some PVs can be used as oncolytic viruses, in which case we will talk of oncolysis. On the other hand, other PVs are better suited for gene delivery, as they do not kill the infected cells. Most PVs are not known to cause any diseases in humans and thus they are often called "viruses in

search of diseases." Some PVs such as adenoassociated virus are low in pathogenicity and immunogenicity and are popular vehicles for treatment of many types of genetic and metabolic diseases [112].

The most used and studied PV for treatment of cancer is the rat PV, called H-1PV, which is attractive for its capability of infecting and replicating in humans (including human cancer cells) without causing significant clinical signs due to death of normal cells [112].

The oncotropism of PVs is not due to better virus uptake by transformed cells, but to a more efficient replication in these cells. Specifically, the conversion of the viral single-stranded DNA to double-stranded replication forms, and the transcription of these duplex forms, depend on factors that are often dysregulated in cancer cells [cyclin A, E2F, and cAMP-response-element-binding/activating transcription factors (CREB/ATF) among others], allowing the virus to preferentially replicate (and kill) these cells rather than normal cells. The killing of cancer cells is mainly due to the accumulation of a cytotoxic protein called NS1. Interestingly, it has been discovered that not only are cancer cells more susceptible to NS1 but also that in these cells the concentration of this protein is often significantly higher than in normal cells; the mechanism for this is still unknown and under investigation. These characteristics make PV a selective anticancer oncolytic virus [112].

Given its efficacy and selectivity for tumor tissue, H-1PV has been particularly useful for treatment of brain tumors and non-Hodgkin lymphoma. Together with its oncolytic activity it has also been recently observed that PVs are able to influence the immune system to recognize the tumor [114].

As with other oncolytic viruses, the possibility of arming these platforms to generate more potent and more immunogenic devices makes them even more appealing for treatment of cancer [115]. Like adenovirus, PVs have also been enriched with TLR9-specific sequences to enhance antitumor immunity [116].

Recently a phase I/IIa trial has started in Germany for patients with recurrent glioblastoma multiforme (www.clinicaltrials.gov, identifier NCT01301430). This will be the first clinical trial with H-1PV in Germany.

Final Remarks

In conclusion, while gene delivery with nonreplicating viruses may have its uses in the treatment of cancer, replication-competent oncolytic viruses have become popular in the last decade. Although several RNA and DNA viruses are being studied, the latter are furthest along in clinical use due to their stability, predictability, ease of construction, and production. Predictable pharmaceutical properties are key regulatory aspects of clinical translation of "advanced therapy medicinal products." The most popular oncolytic DNA viruses are adenoviruses, VVs, and herpes simplex viruses, all of which have entered randomized clinical trials; the first product approvals are expected by 2015. One virus, Oncorine, has already been approved in China. These viruses feature different characteristics and all of them are active in many different tumor types. Although a "magic bullet" to eradicate cancer as global disease will never be identified, it seems likely that viruses will enter the oncologist's arsenal to design tumor-specific and patient-specific therapies. Immunotherapy is a missing sector in the pie chart of antitumor approaches and oncolytic viruses could contribute to filling the void. For this to be achieved it is of utmost importance to profoundly understand the biology of oncolytic viruses at the interface between the virus and host so that we might rationally design combination therapies that can attack tumors from different angles.

References

1 Rowe WP, Huebner RJ, Gilmore LK, Parrott RH, Ward TG. (1953) Isolation of a cytopathogenic agent from human adenoids undergoing spontaneous degeneration in tissue culture. *Proceedings of the Society for Experimental Biology and Medicine* 84: 570–573.

2 Khare R, et al. (2011) Generation of a Kupffer cell-evading adenovirus for systemic and liver-directed gene transfer. *Molecular Therapy* 19: 1254–1262.

3 Khare R, Chen CY, Weaver EA, Barry MA. (2011) Advances and future challenges in adenoviral vector pharmacology and targeting. *Current Gene Therapy* 11: 241–258.

4 Berk JA. (2007) Adenoviridae: the viruses and their replication. *Field's Virology*, 5th edn, DA Knipe and PM Howley (eds), pp. 2355–2394. Philadelphia, PA: Lippincott Williams & Wilkins.

5 Russell WC. (2000) Update on adenovirus and its vectors. *Journal of General Virology* 81: 2573–2604.

6 Pacini DL, Dubovi EJ, Clyde WA Jr (1984) A new animal model for human respiratory tract disease due to adenovirus. *Journal of Infectious Disease* 150: 92–97.

7 Toth K, Spencer JF, Tollefson AE, Kuppuswamy M, Doronin K, Lichtenstein DL, et al. (2005) Cotton rat tumor model for the evaluation of oncolytic adenoviruses. *Human Gene Therapy* 16: 139–146.

8 Gordon YJ, Romanowski E, Araullo-Cruz T. (1992) An ocular model of adenovirus type 5 infection in the NZ rabbit. *Investigative Ophthalmology & Visual Science* 33: 574–580.

9 Thomas MA, Spencer JF, La Regina MC, Dhar D, Tollefson AE, Toth K, et al. (2006) Syrian hamster as a permissive immunocompetent animal model for the study of oncolytic adenovirus vectors. *Cancer Research* 66: 1270–1276.

10 Graham FL, Smiley J, Russell WC, Nairn R. (1977) Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *Journal of General Virology* 36: 59–74.

11 Seiler MP, Cerullo V, Lee B. (2007) Immune response to helper dependent adenoviral mediated liver gene therapy: challenges and prospects. *Current Gene Therapy* 7: 297–305.

12 Brunetti-Pierri N, Ng P. (2008) Progress and prospects: gene therapy for genetic diseases with helper-dependent adenoviral vectors. *Gene Therapy* 15: 553–560.

13 Cerullo V, Koski A, Vaha-Koskela M, Hemminki A. (2012) Oncolytic adenoviruses for cancer therapy: data from mice, hamsters and humans. *Advances in Cancer Research* 115: 265–318.

14 Hecht JR, Farrell JJ, Senzer N, Nemunaitis J, Rosemurgy A, Chung T, et al. (2012) 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. *Gastrointestinal Endoscopy* 75: 332–338.

15 Kircheis R, Wagner E. (2003) Technology evaluation: TNFerade GenVec. *Current Opinion in Molecular Therapeutics* 5: 437–447.

16 van Putten EH, Dirven CM, van den Bent MJ, Lamfers ML. (2010) Sitimagene ceradenovec: a gene-based drug for the treatment of operable high-grade glioma. *Future Oncology* 6: 1691–1710.

17 Raty JK, Pikkarainen JT, Wirth T, Yla-Herttuala S. (2008) Gene therapy: the first approved gene-based medicines, molecular mechanisms and clinical indications. *Current Molecular Pharmacology* 1: 13–23.

18 Nielsen LL, Gurnani M, Syed J, Dell J, Hartman B, Cartwright M, et al. (1998) Recombinant E1-deleted adenovirus-mediated gene therapy for cancer: efficacy studies with p53 tumor suppressor gene and liver histology in tumor xenograft models. *Human Gene Therapy* 9: 681–694.

19 Guse K, Hemminki A. (2009) Cancer gene therapy with oncolytic adenoviruses. *Journal of BUON* 14 Suppl 1: S7–S15.

20 Smith RR, Huebner RJ, Rowe WP, Schatten WE, Thomas LB (1956) Studies on the use of viruses in the treatment of carcinoma of the cervix. *Cancer* 9: 1211–1218.

21 Bischoff JR, Kirn DH, Williams A, Heise C, Horn S, Muna M, et al. (1996) An adenovirus mutant that replicates selectively in p53-deficient human tumor cells. *Science* 274: 373–376.

22 Hollstein M, Sidransky D, Vogelstein B, Harris CC. (1991) p53 mutations in human cancers. *Science* 253: 49–53.

23 Heise C, Hermiston T, Johnson L, Brooks G, Sampson-Johannes A, Williams A, et al. (2000) An adenovirus E1A mutant that demonstrates potent and selective systemic anti-tumoral efficacy. *Nature Medicine* 6: 1134–1139.

24 Fueyo J, Gomez-Manzano C, Alemany R, Lee PS, McDonnell TJ, Mitlianga P, et al. (2000) A mutant oncolytic adenovirus targeting the Rb pathway produces anti-glioma effect in vivo. *Oncogene* 19: 2–12.

25 Heise C, Lemmon M, Kirn D. (2000) Efficacy with a replication-selective adenovirus plus cisplatin-based chemotherapy: dependence on sequencing but not p53 functional status or route of administration. *Clinical Cancer Research* 6(12): 4908–4914.

26 Cody JJ, Douglas JT. (2009) Armed replicating adenoviruses for cancer virotherapy. *Cancer Gene Therapy* 16: 473–488.

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

28 Kim J, Lee B, Kim JS, Yun CO, Kim JH, Lee YJ, et al. (2002) Antitumoral effects of recombinant adenovirus YKL-1001, conditionally replicating in alpha-fetoprotein-producing human liver cancer cells. *Cancer Letters* 180: 23–32.

29 Zhang L, Akbulut H, Tang Y, Peng X, Pizzorno G, Sapi E, et al. (2002) Adenoviral vectors with E1A regulated by tumor-specific promoters are selectively cytolytic for breast cancer and melanoma. *Molecular Therapy* 6: 386–393.

30 Li Y, Chen Y, Dilley J, Arroyo T, Ko D, Working P, et al. (2003) Carcinoembryonic antigen-producing cell-specific oncolytic adenovirus, OV798, for colorectal cancer therapy. *Molecular Cancer Therapeutics* 2: 1003–1009.

31 Ono HA, Davydova JG, Adachi Y, Takayama K, Barker SD, Reynolds PN, et al. (2005) Promoter-controlled infectivity-enhanced conditionally replicative adenoviral

vectors for the treatment of gastric cancer. *Journal of Gastroenterology* 40: 31–42.

32 Kanerva A, Bauerschmitz GJ, Yamamoto M, Lam JT, Alvarez RD, Siegal GP, et al. (2004) A cyclooxygenase-2 promoter-based conditionally replicating adenovirus with enhanced infectivity for treatment of ovarian adenocarcinoma. *Gene Therapy* 11: 552–559.

33 Bauerschmitz GJ, Guse K, Kanerva A, Menzel A, Herrmann I, Desmond RA, et al. (2006) Triple-targeted oncolytic adenoviruses featuring the cox2 promoter, E1A transcomplementation, serotype chimerism for enhanced selectivity for ovarian cancer cells. *Molecular Therapy* 14: 164–174.

34 Pesonen S, Cerullo V, Escutenaire S, Raki M, Kangasniemi L, Nokisalmi P, et al. (2010) Treatment of patients with advanced and refractory solid tumors with oncolytic adenoviruses Ad5-D24-RGD and Ad5-RGD-D24-GMCSF. *Molecular Therapy* 18: 1874–1884.

35 Akbulut H, Zhang L, Tang Y, Deisseroth A. (2003) Cytotoxic effect of replication-competent adenoviral vectors carrying L-plastin promoter regulated E1A and cytosine deaminase genes in cancers of the breast, ovary and colon. *Cancer Gene Therapy* 10: 388–395.

36 Wirth T, Kuhnel F, Kubicka S. (2005) Telomerase-dependent gene therapy. *Current Molecular Medicine* 5: 243–251.

37 Hemminki O, Bauerschmitz G, Hemmi S, Lavilla-Alonso S, Diaconu I, Guse K, et al. (2011) Oncolytic adenovirus based on serotype 3. *Cancer Gene Therapy* 18: 288–296.

38 Krasnykh VN, Mikheeva GV, Douglas JT, Curiel DT. (1996) Generation of recombinant adenovirus vectors with modified fibers for altering viral tropism. *Journal of Virology* 70: 6839–6846.

39 Kanerva A, Mikheeva GV, Krasnykh V, Coolidge CJ, Lam JT, Mahasreshti PJ, et al. (2002) Targeting adenovirus to the serotype 3 receptor increases gene transfer efficiency to ovarian cancer cells. *Clinical Cancer Research* 8: 275–280.

40 Kanerva A, Zinn KR, Chaudhuri TR, Lam JT, Suzuki K, Uil TG, et al. (2003) Enhanced therapeutic efficacy for ovarian cancer with a serotype 3 receptor-targeted oncolytic adenovirus. *Molecular Therapy* 8: 449–458.

41 Volk AL, Rivera AA, Kanerva A, Bauerschmitz G, Dmitriev I, Nettelbeck DM, et al. (2003) Enhanced adenovirus infection of melanoma cells by fiber-modification: incorporation of RGD peptide or Ad5/3 chimerism. *Cancer Biology & Therapy* 2: 511–515.

42 Hemminki O, Diaconu I, Cerullo V, Pesonen SK, Kanerva A, Joensuu T, et al. (2012) Ad3-hTERT-E1A, a fully serotype 3 oncolytic adenovirus, in patients with chemotherapy refractory cancer. *Molecular Therapy* 20(9): 1821–1830.

43 Hemminki O, Bauerschmitz G, Hemmi S, Lavilla-Alonso S, Diaconu I, Desmond RA, et al. (2009) Fully non-serotype 5 oncolytic adenovirus based on serotype 3. *Molecular Therapy* 17: S186.

44 Zhang JF, Hu C, Geng Y, Selm J, Klein SB, Orazi A, et al. (1996) Treatment of a human breast cancer xenograft with an adenovirus vector containing an interferon gene results in rapid regression due to viral oncolysis and gene therapy. *Proceedings of the National Academy of Sciences USA* 93: 4513–4518.

45 Robinson M, Ge Y, Ko D, Yendluri S, Laflamme G, Hawkins L, et al. (2008) Comparison of the E3 and L3 regions for arming oncolytic adenoviruses to achieve a high level of tumor-specific transgene expression. *Cancer Gene Therapy* 15: 9–17.

46 van Beusechem VW, van den Doel PB, Grill J, Pinedo HM, Gerritsen WR. (2002) Conditionally replicative adenovirus expressing p53 exhibits enhanced oncolytic potency. *Cancer Research* 62: 6165–6171.

47 Zhan J, Gao Y, Wang W, Shen A, Aspelund A, Young M, et al. (2005) Tumor-specific intravenous gene delivery using oncolytic adenoviruses. *Cancer Gene Therapy* 12: 19–25.

48 Zhang JF, Wei F, Wang HP, Li HM, Qiu W, Ren PK, et al. (2010) Potent anti-tumor activity of telomerase-dependent and HSV-TK armed oncolytic adenovirus for non-small cell lung cancer in vitro and in vivo. *Journal of Experimental & Clinical Cancer Research* 29: 52.

49 Freytag SO, Rogulski KR, Paielli DL, Gilbert JD, Kim JH. (1998) A novel three-pronged approach to kill cancer cells selectively: concomitant viral, double suicide gene, radiotherapy. *Human Gene Therapy* 9: 1323–1333.

50 Guse K, Diaconu I, Rajecki M, Sloniecka M, Hakkarainen T, Ristimaki A, et al. (2009) Ad5/3-9HIF-Delta24-VEGFR-1-Ig, an infectivity enhanced, dual-targeted and antiangiogenic oncolytic adenovirus for kidney cancer treatment. *Gene Therapy* 16: 1009–1020.

51 Hakkarainen T, Rajecki M, Sarparanta M, Tenhunen M, Airaksinen AJ, Desmond RA, et al. (2009) Targeted radiotherapy for prostate cancer with an oncolytic adenovirus coding for human sodium iodide symporter. *Clinical Cancer Research* 15: 5396–5403.

52 Lei N, Shen FB, Chang JH, Wang L, Li H, Yang C, et al. (2009) An oncolytic adenovirus expressing granulocyte macrophage colony-stimulating factor shows improved specificity and efficacy for treating human solid tumors. *Cancer Gene Therapy* 16: 33–43.

53 Ramesh N, Ge Y, Ennist DL, Zhu M, Mina M, Ganesh S, et al. (2006) CG0070, a conditionally replicating granulocyte macrophage colony-stimulating factor-armed oncolytic adenovirus for the treatment of bladder cancer. *Clinical Cancer Research* 12: 305–313.

54 Chang J, Zhao X, Wu X, Guo Y, Guo H, Cao J, et al. (2009) A Phase I study of KH901, a conditionally replicating granulocyte-macrophage colony-stimulating factor: armed oncolytic adenovirus for the treatment of head and neck cancers. *Cancer Biology & Therapy* 8: 676–682.

55 Ahonen MT, Diaconu I, Pesonen S, Kanerva A, Baumann M, Parviainen ST, et al. (2010) Calcium gluconate in phosphate buffered saline increases gene delivery with adenovirus type **5**. *PLoS One* 5(9).

56 Cerullo V, Diaconu I, Romano V, Hirvinen M, Ugolini M, Escutenaire S, et al. (2012) An oncolytic adenovirus enhanced for Toll-like receptor 9 stimulation increases antitumor immune responses and tumor clearance. *Molecular Therapy* 20(11): 2076–2086.

57 Diaconu I, Cerullo V, Hirvinen ML, Escutenaire S, Ugolini M, Pesonen SK, et al. (2012) Immune response is an important aspect of the antitumor effect produced by a CD40L-encoding oncolytic adenovirus. *Cancer Research* 72: 2327–2338.

58 Pesonen S, Diaconu I, Kangasniemi L, Ranki T, Kanerva A, Pesonen SK, et al. (2012) Oncolytic immunotherapy of advanced solid tumors with a CD40L-expressing replicating adenovirus: assessment of safety and immunologic responses in patients. *Cancer Research* 72: 1621–1631.

59 Hemminki A, Dmitriev I, Liu B, Desmond RA, Alemany R, Curiel DT. (2001) Targeting oncolytic adenoviral agents to the epidermal growth factor pathway with a secretory fusion molecule. *Cancer Research* 61: 6377–6381.

60 Koski A, Kangasniemi L, Escutenaire S, Pesonen S, Cerullo V, Diaconu I, et al. (2010) Treatment of cancer patients with a serotype 5/3 chimeric oncolytic adenovirus expressing GMCSF. *Molecular Therapy* 18: 1874–1884.

61 Wennier S, Li S, McFadden G. (2011) Oncolytic virotherapy for pancreatic cancer. *Expert Reviews in Molecular Medicine* 13: e18.

62 Gomez-Manzano C, Fueyo J. (2010) Oncolytic adenoviruses for the treatment of brain tumors. *Current Opinion in Molecular Therapeutics* 12: 530–537.

63 Ekblad M, Hallden G. (2010) Adenovirus-based therapy for prostate cancer. *Current Opinion in Molecular Therapeutics* 12: 421–431.

64 Burke J. (2010) Virus therapy for bladder cancer. *Cytokine and Growth Factor Reviews* 21: 99–102.

65 Young A, McNeish IA. (2009) Oncolytic adenoviral gene therapy in ovarian cancer: why we are not wasting our time. *Future Oncology* 5: 339–357.

66 Guo J, Xin H. (2006) Chinese gene therapy. splicing out the West? *Science* 314: 1232–1235.

67 Kirn DH, Thorne SH. (2009) Targeted and armed oncolytic poxviruses: a novel multi-mechanistic therapeutic class for cancer. *Nature Reviews Cancer* 9: 64–71.

68 Cerullo V, Diaconu I, Romano V, Hirvinen M, Ugolini M, Escutenaire S, et al. (2010) Oncolytic adenovirus coding for granulocyte macrophage colony-stimulating factor induces antitumoral immunity in cancer patients. *Cancer Research* 70: 4297–4309.

69 Cerullo V, Koski A, Vaha-Koskela M, Hemminki A. (2012) Oncolytic adenoviruses for cancer immunotherapy: data from mice, hamsters, humans. *Advances in Cancer Research* 115: 265–318.

70 Suzuki M, Cela R, Bertin TK, Sule GJ, Cerullo V, Rodgers JR, et al. (2011) NOD2 signaling contributes to the innate immune response against helper-dependent adenovirus vectors independently of MyD88 in vivo. *Human Gene Therapy* 22(9): 1071–1082.

71 Suzuki M, Cerullo, Bertin T, Cela, Clarke, Guenther M, et al. (2010) MyD88-dependent silencing of transgene expression during the innate and adaptive immune response to Helper-dependent adenovirus. *Human Gene Therapy* 21(3): 325–336.

72 Cerullo V, Seiler MP, Mane V, Brunetti-Pierri N, Clarke C, Bertin TK, et al. (2007) Toll-like receptor 9 triggers an innate immune response to helper-dependent adenoviral vectors. *Molecular Therapy* 15: 378–385.

73 Cerullo V, McCormack W, Seiler M, Mane V, Cela R, Clarke C, et al. (2007) Antigen-specific tolerance of human alpha1-antitrypsin induced by helper-dependent adenovirus. *Human Gene Therapy* 18: 1215–1224.

74 Lladser A, Sanhueza C, Kiessling R, Quest AF. (2011) Is survivin the potential Achilles' heel of cancer? *Advances in Cancer Research* 111: 1–37.

75 Pesonen S, Diaconu I, Cerullo V, Escutenaire S, Raki M, Kangasniemi L, et al. (2011) Integrin targeted oncolytic adenoviruses Ad5-D24-RGD and Ad5-RGD-D24-GMCSF for treatment of patients with advanced chemotherapy refractory solid tumors. *International Journal of Cancer* 130: 1937–1947.

76 Li JL, Liu HL, Zhang XR, Xu JP, Hu WK, Liang M, et al. (2009) A phase I trial of intratumoral administration of recombinant oncolytic adenovirus overexpressing HSP70 in advanced solid tumor patients. *Gene Therapy* 16: 376–382.

77 Timiryasova TM, Chen B, Fodor I. (2001) Replication-deficient vaccinia virus gene therpay vector: evaluation of exogenous gene expression mediated by PUV-inactivated virus in glioma cells. *Journal of Gene Medicine* 3: 468–477.

78 Erbs P, Findeli A, Kintz J, Cordier P, Hoffmann C, Geist M, et al. (2008) Modified vaccinia virus Ankara as a vector for suicide gene therapy. *Cancer Gene Therapy* 15: 18–28.

79 McCart JA, Ward JM, Lee J, Hu Y, Alexander HR, Libutti SK, et al. (2001) Systemic cancer therapy with a tumor-selective vaccinia virus mutant lacking thymidine kinase and vaccinia growth factor genes. *Cancer Research* 61: 8751–8757.

80 Guse K, Cerullo V, Hemminki A. (2011) Oncolytic vaccinia virus for the treatment of cancer. *Expert Opinion on Biological Therapy* 11: 595–608.

81 Breitbach CJ, Burke J, Jonker D, Stephenson J, Haas AR, Chow LQ, et al. (2011) Intravenous delivery of a multi-mechanistic cancer-targeted oncolytic poxvirus in humans. *Nature* 477: 99–x.

82 Hodge JW, Sabzevari H, Yafal AG, Gritz L, Lorenz MG, Schlom J. (1999) A triad of costimulatory molecules synergize to amplify T-cell activation. *Cancer Research* 59: 5800–5807.

83 Amato RJ. (2010) 5T4-modified vaccinia Ankara: progress in tumor-associated antigen-based immunotherapy. *Expert Opinion on Biological Therapy* 10: 281–287.

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

85 Marconi P, Argnani R, Berto E, Epstein AL, Manservigi R. (2008) HSV as a vector in vaccine development and gene therapy. *Human Vaccines* 4(2): 91–105.

86 Assudani DP, Ahmad M, Li G, Rees RC, Ali SA. (2006) Immunotherapeutic potential of DISC-HSV and OX40L in cancer. *Cancer Immunology Immunotherapy* 55: 104–111.

87 Martuza RL, Malick A, Markert JM, Ruffner KL, Coen DM. (1991) Experimental therapy of human glioma by means of a genetically engineered virus mutant. *Science* 252: 854–856.

88 Markert JM, Malick A, Coen DM, Martuza RL, Kornblith PL, Hinton DR. (1993) Reduction and elimination of encephalitis in an experimental glioma therapy model with attenuated herpes simplex mutants that retain susceptibility to acyclovir. *Neurosurgery* 32(4): 597–603.

89 He B, Gross M, Roizman B. (1997) The gamma(1)34.5 protein of herpes simplex virus 1 complexes with protein phosphatase 1alpha to dephosphorylate the alpha subunit of the eukaryotic translation initiation factor 2 and preclude the shutoff of protein synthesis by double-stranded RNA-activated protein kinase. *Proceedings of the National Academy of Sciences USA* 94(3): 843–848.

90 Harrow S, Papanastassiou V, Harland J, Mabbs R, Petty R, Fraser M, et al. (2004) HSV1716 injection into the brain adjacent to tumour following surgical resection of high-grade glioma: safety data and long-term survival. *Gene Therapy* 11: 1648–1658.

91 Mace AT, Ganly I, Soutar DS, Brown SM. (2008) Potential for efficacy of the oncolytic Herpes simplex virus 1716 in patients with oral squamous cell carcinoma. *Head Neck* 30: 1045–1051.

92 Markert JM, Medlock MD, Rabkin SD, Gillespie GY, Todo T, Hunter WD, et al. (2000) Conditionally replicating herpes simplex virus mutant, G207 for the treatment of malignant glioma: results of a phase I trial. *Gene Therapy* 7: 867–874.

93 Markert JM, Liechty PG, Wang W, Gaston S, Braz E, Karrasch M, et al.. (2009) Phase Ib trial of mutant herpes simplex virus G207 inoculated pre-and post-tumor resection for recurrent GBM. *Molecular Therapy* 17(1): 199–207.

94 Meignier B, Longnecker R, Roizman B. (1988) In vivo behavior of genetically engineered herpes simplex viruses R7017 and R7020: construction and evaluation in rodents. *Journal of Infectious Disease* 158: 602–614.

95 Kelly KJ, Wong J, Fong Y. (2008) Herpes simplex virus NV1020 as a novel and promising therapy for hepatic malignancy. *Expert Opinion on Investigational Drugs* 17(7): 1105–1113.

96 Geevarghese SK, Geller DA, de Haan HA, Hörer M, Knoll AE, Mescheder A, et al. (2010) Phase I/II study of oncolytic herpes simplex virus NV1020 in patients with extensively pretreated refractory colorectal cancer metastatic to the liver. *Human Gene Therapy* 21(9): 1119–1128.

97 Todo T. (2012) Active immunotherapy: oncolytic virus therapy using HSV-1. *Advances in Experimental and Medical Biology* 746: 178–186.

98 Hamilton JA, Anderson GP. (2004) GM-CSF biology. *Growth Factors* 22: 225–231.

99 Taneja S, MacGregor J, Markus S, Ha S, Mohr I. (2001) Enhanced antitumor efficacy of a herpes simplex virus mutant isolated by genetic selection in cancer cells. *Proceedings of the National Academy of Sciences USA* 98: 8804–8808.

100 Jaramillo N, Domingo E, Munoz-Egea MC, Tabares E, Gadea I. (2013) Evidence of Muller's ratchet in herpes simplex virus type 1. *Journal of General Virology* 94: 366–375.

101 Fu X, Tao L, Cai R, Prigge J, Zhang X. (2006) A mutant type 2 herpes simplex virus deleted for the protein kinase domain of the ICP10 gene is a potent oncolytic virus. *Molecular Therapy* 13: 882–890.

102 Hellebrand E, Mautner J, Reisbach G, Nimmerjahn F, Hallek M, Mocikat R, et al. (2006) Epstein-Barr virus vector-mediated gene transfer into human B cells:

potential for antitumor vaccination. *Gene Therapy* 13: 150–162.

103 Leske H, Haase R, Restle F, Schichor C, Albrecht V, Vizoso Pinto MG, et al. (2012) Varicella zoster virus infection of malignant glioma cell cultures: a new candidate for oncolytic virotherapy? *Anticancer Research* 32(4): 1137–1144.

104 Courchesne MJ, White MC, Stanfield BA, Frampton AR. (2012) Equine herpesvirus type 1-mediated oncolysis of human glioblastoma multiforme cells. Journal of Virology 86(5): 2882–2886.

105 Redaelli M, Franceschi V, Capocefalo A, D'Avella D, Denaro L, Cavirani S, et al.. (2012) Herpes simplex virus type 1 thymidine kinase-armed bovine herpesvirus type 4-based vector displays enhanced oncolytic properties in immunocompetent orthotopic syngenic mouse and rat glioma models. *Neuro Oncology* 14(3): 288–301.

106 Rodrigues R, Cuddington B, Mossman K. (2010) Bovine herpesvirus type 1 as a novel oncolytic virus. *Cancer Gene Therapy* 17(5): 344–355

107 Cabanillas JA, Cambronero R, Pacheco-Castro A, Garcia-Rodriguez MC, Martin-Fernandez JM, Fontan G, et al. (2002) Characterization of Herpesvirus saimiri-transformed T lymphocytes from common variable immunodeficiency patients. *Clinical & Experimental Immunology* 127: 366–373.

108 Menotti L, Nicoletti G, Gatta V, Croci S, Landuzzi L, De Giovanni C, et al. (2009) Inhibition of human tumor growth in mice by an oncolytic herpes simplex virus designed to target solely HER-2-positive cells. *Proceedings of the National Academy of Sciences USA* 106: 9039–9044.

109 Mullen JT, Kasuya H, Yoon SS, Carroll NM, Pawlik TM, Chandrasekhar S, et al.. (2002) Regulation of herpes simplex virus 1 replication using tumor-associated promoters. *Annals of Surgery* 236(4): 502–513.

110 Carson J, Haddad D, Bressman M, Fong Y. (2010) Oncolytic herpes simplex virus 1 (HSV-1) vectors: increasing treatment efficacy and range through strategic virus design. *Drugs of the Future* 35(3): 183–195.

111 Fu X, Rivera A, Tao L, De Geest B, Zhang X. (2012) Construction of an oncolytic herpes simplex virus that precisely targets hepatocellular carcinoma cells. *Molecular Therapy* 20: 339–346.

112 Rommelaere J, Geletneky K, Angelova AL, Daeffler L, Dinsart C, Kiprianova I, et al. (2010) Oncolytic parvoviruses as cancer therapeutics. *Cytokine and Growth Factor Reviews* 21: 185–195.

113 Cotmore SF, Tattersall P. (2007) Parvoviral host range and cell entry mechanisms. *Advances in Virus Research* 70: 183–232.

114 Morales O, Richard A, Martin N, Mrizak D, Senechal M, Miroux C, et al. (2012) Activation of a helper and not regulatory human CD4+ T cell response by oncolytic H-1 parvovirus. *PLoS One* 7: e32197.

115 Dempe S, Lavie M, Struyf S, Bhat R, Verbeke H, Paschek S, et al. (2012) Antitumoral activity of parvovirus-mediated IL-2 and MCP-3/CCL7 delivery into human pancreatic cancer: implication of leucocyte recruitment. *Cancer Immunology Immunotherapy* 61(11): 2113–2123.

116 Raykov Z, Grekova S, Leuchs B, Aprahamian M, Rommelaere J. (2008) Arming parvoviruses with CpG motifs to improve their oncosuppressive capacity. *International Journal of Cancer* 122: 2880–2884.

2

CHAPTER 2

Retroviruses for Cancer Therapy

Jiehua Zhou, Yue Ding, John C. Burnett, and John Rossi
Beckman Research Institute of City of Hope, Duarte, CA, USA

Introduction

Retroviruses are single-stranded, positive, sense RNA viruses that carry a diploid RNA genome. They are characterized by their use of viral reverse transcriptase (RT) and integrase (IN) to stably insert a DNA copy of their own genetic information into the host genome [1]. By taking advantage of this ability, vectors derived from retroviruses can be used to permanently integrate DNA into the genomes of infected host cells (transduction), thereby leading to stable and prolonged expression of the introduced genetic information. Retrovirus-based vectors have been used in human gene-transfer experiments that are designed to introduce therapeutic molecules into cells to combat human disease. The use of retroviruses as vectors to deliver non-oncogenic genes into cells dates back to the 1980s [2,3]. Retroviral vectors based on gammaretroviruses, such as the murine leukemia viruses (MLVs), were some of the first vectors to be used in human gene-transfer experiments [4]. Over the last three decades, various retroviral vectors based on different retroviral genera have been developed, optimized, and used both for basic biological studies and gene therapy. According to clinical data provided by the *Journal of Gene Medicine*, viral vectors were used in approximately 75% of clinical trials involving gene therapy from 1989 to 2012 worldwide (www.wiley.co.uk/genmed/clinical). Because they have been used in more than 360 initial clinical phase I/II gene therapy trials, retroviral vectors are the second most commonly used gene-delivery vehicle after adenovirus-derived vectors [5].

The first clinical trials of human gene transfer using retroviral-based technology were for the treatment of infants and children with hereditary severe combined immune deficiency (SCID), a disease that results from adenosine deaminase (ADA) deficiency. These trials used MLV-based gammaretroviral vectors that expressed ADA in autologous T lymphocytes [6], autologous umbilical cord blood CD34+ hematopoietic stem and progenitor cells [7], or the combination of autologous peripheral blood lymphocytes and bone marrow progenitors [8]. Since then, about one-third of the clinical trials involving viral vectors have used retroviral-based vectors. In comparison to non-viral vectors, viral vectors can achieve a higher transfection/transduction efficiency and long-term effect because the designed transgenes are integrated into the target cell genome and are transmitted to progeny cells, which continue to express the transgene. However, there were incidents of leukemia that developed in X-linked SCID patients treated with the MLV-based vectors, which raised biosafety concerns and so limits on the use of retroviral vectors were introduced. Although the transduced cells established and corrected the immunologic deficiency in 19 of 20 patients, five of the treated patients ultimately developed T-cell leukemia [9]. This is because the pattern of retroviral integration is more like a random event and not completely decoded or yet controlled. Integration of the retroviral vector close to oncogenes can activate them, so normal cells can transform into tumor cells [10]. A second safety

Cancer Gene Therapy by Viral and Non-viral Vectors, First Edition. Edited by Malcolm K. Brenner and Mien-Chie Hung.

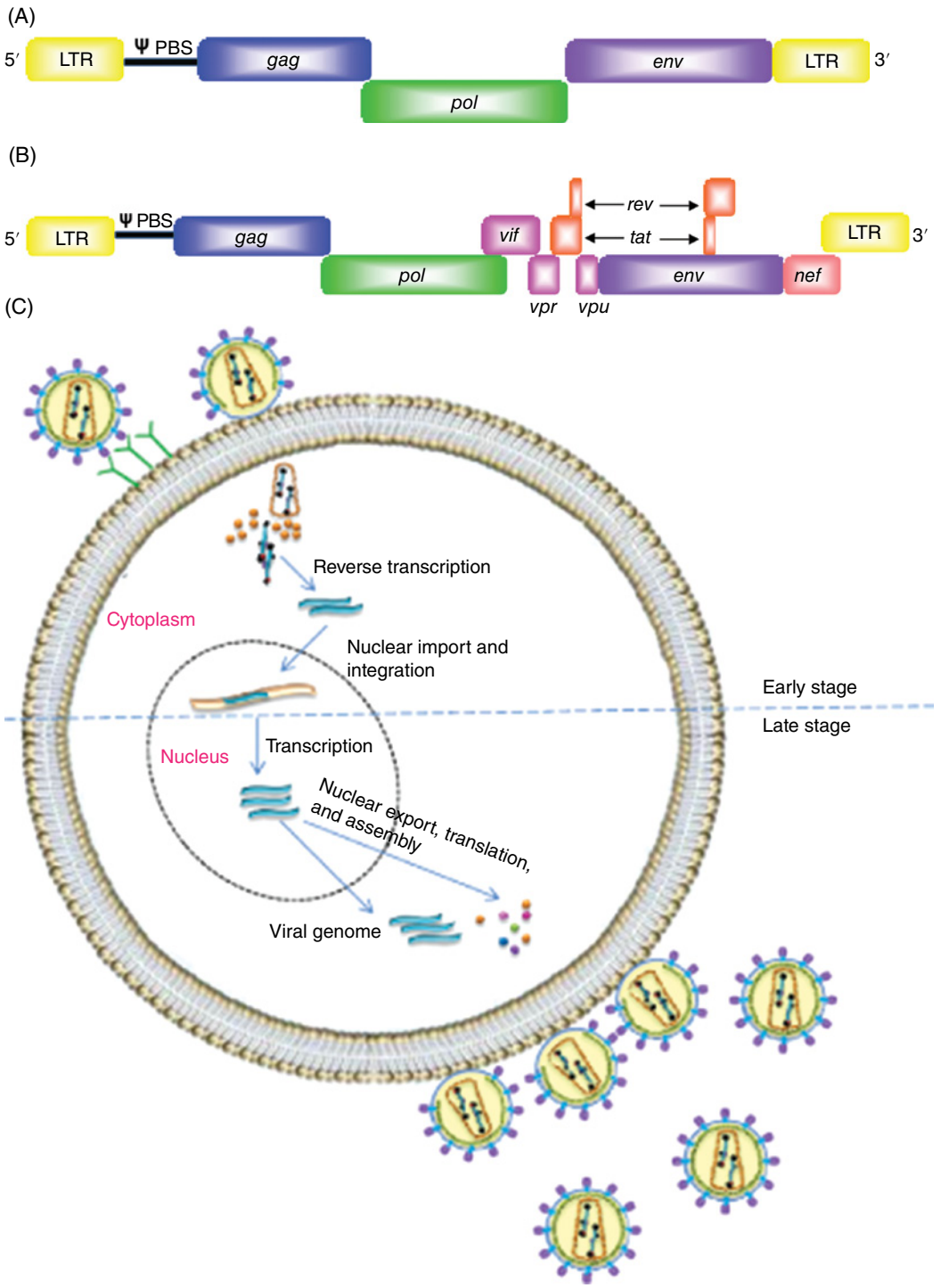


Figure 2.1 Schematic representations of retrovirus genomes and HIV-1 life cycle. (A) A simple retrovirus genomic RNA. (B) HIV-1 genomic RNA. In addition to three indispensable retroviral genes (*gag*, *pol*, and *env*), the HIV-1 genome also contains six accessory genes (*tat*, *rev*, *vpr*, *vif*, *vpu*, and *nef*). PBS, primer-binding site. (C) Life cycle of HIV-1. Early stage: the HIV-1 envelope protein recognizes specific receptors on the surface of target cells to mediate entry and release of HIV-1 virion into the cytoplasm. After entry, the viral RNA is reverse transcribed into cDNA, imported into the host cell nucleus, and integrated into the host genome to become a provirus. Late stage: using the host cell transcription and translation machinery, the provirus is transcribed and the transcripts are exported to cytoplasm where they are translated into viral packaging proteins or used to generate the viral genome of next-generation virus. These viral components are then assembled into mature virions that exit the host cell through budding, to become next generation viruses.

concern with retroviral vectors is the possibility of generating replication-competent retroviruses, although this can be addressed by the construction of attenuated vectors that lack most viral genes. Moreover, MLV vectors exhibit tumor selectivity due to their inability to transduce quiescent cells. Therefore, the road toward retroviral vector modification and optimization has included efforts to improve both biosafety and the transduction efficiency of the retroviral vector.

One common modification, such as that found in the self-inactivating (SIN) vector, is to remove the enhancer sequences from the U3 region of the long terminal repeats (LTRs) (Figure 2.1) [11,12]. Due to abolishment of promoter activity from the LTR, the attenuated SIN LTRs lack the ability to activate nearby proto-oncogenes. Moreover, although both of retroviral and lentiviral vectors favor integration into coding rather than noncoding regions, they show different integration-site preferences. Moloney MLV tends to integrate near the transcription start site, while human immunodeficiency virus (HIV) shows a stronger preference for integrating within introns of expressed genes [13]. HIV-1-derived lentiviral vectors appear to be safer and therefore they are currently of considerable interest. Additionally, lentiviral vectors have the advantage of being able to transduce nondividing cells. At present, HIV-1-derived lentiviral vectors are the vectors most frequently used in translational studies and clinical trials. In this chapter we will discuss the principles of retrovirology and advances in retroviral vector technology. The basics and progress of lentiviral vectors systems are included. We also address the development of RNA interference (RNAi) therapies that employ HIV-1-derived lentiviral systems, and we highlight several representative examples.

Overview of Retroviral Vectors

Basics of Retrovirus

Retroviral capsids are 80–100 nm in diameter and contain linear, nonsegmented, single-stranded RNA (ssRNA) genomes that vary from 7 to 12 kb in length. According to the International Committee on Taxonomy of Viruses, the retroviridae family consists of the orthoretroviridae and spumaretroviridae subfamilies. Six genera (alpharetrovirus, betaretrovirus, gammaretrovirus, deltaretrovirus, epsilonretrovirus, and lentivirus) belong to orthoretroviridae subfamily, whereas the spumaretroviridae only has one member: spumavirus (foamy virus). Each genus further consists of different types of subspecies. Both lentivirus and spumavirus have relatively complex structures compared to the other five retroviral genera. In addition to the universally existing retroviral promoter/enhancers in the LTRs, spumavirus has a unique functional intragenic promoter/enhancer located at the 3′ end of the envelope gene (*env*) that mediates the transcription of two genes located between the *env* gene and the 3′ LTR [14].

The life cycle of retroviruses is divided into early and late stages. Virus entry, reverse transcription of the ssRNA genome, and integration of the cDNA into host genome all occur during the early stage. Retroviruses enter targeted host cells through the binding of the envelope protein to specific membrane-bound receptors on the host cell surface. After entry, the ssRNA genome is released from the virion particle and is reverse transcribed into a DNA intermediate (provirus) in the cytoplasm. The DNA provirus then binds to a series of stable nucleoprotein complexes to form a preintegration complex (PIC) [15,16]. Entry of the PIC into the nucleus is blocked by nuclear membranes so the integration of the provirus into the host genome usually occurs only during mitosis, when the nuclear membranes are fragmented [17]. In the late stage, the integrated provirus is transcribed and translated into proteins required for virion assembly and budding, and then assembled to become the next generation of viruses.

A schematic of a simple retrovirus genomic RNA is shown in Figure 2.1A. All replication-competent retroviruses contain the *gag*-, *pol*-, and *env*-coding sequences in their RNA genomes regardless of species, origin, or pathogenic potential [18]. These genes encode the retroviral core proteins, enzymatic proteins (reverse transcriptase/RNase H/integrase), and envelope glycoproteins, all of which are necessary for virion assembly and viral budding. The noncoding domains in retroviral RNA genomes are located in the 5′ and 3′ terminal regions. The two identical LTRs play important roles in viral gene expression and polyadenylation of mRNA transcripts [18]. Reverse transcription is primed by specific tRNAs that

bind to a primer-binding site outside of the LTRs and the retroviral life cycle is then initiated [19]. The retroviral packaging signal and genome dimerization signal, psi, is located between the 5′ LTR and *gag*.

Principles of Retroviral Vectors

In brief, retroviral vectors are generated by recombinant DNA manipulation of plasmids that contain a retroviral provirus. To derive retroviral vectors that are defective in replication, all of the *trans*-acting elements of the genome, including the *gag*, *pol*, and *env* genes, must be removed and can be substituted with DNA encoding the gene of interest. The final product retains only the viral LTRs, psi element for packaging/encapsidation, and the sites essential for viral gene expression. This design reduces the risk of forming a replication-competent retrovirus. So far, many retroviral vectors have been developed. The split-packaging, tropism-expansion, and SIN designs are three most important methods for the development and optimization of retroviral vectors.

Split-packaging design

To turn infectious retroviruses into gene vectors and avoid generating replication-competent viruses, researchers have split the retroviral gene sequences that are essential for maintaining retroviral packaging ability into three plasmid constructs and deleted all the other unnecessary sequences [20]. One essential packaging plasmid contains the retroviral genes *gag* and *pol*, which are required for integration, while another has the *env* or a substitute envelope gene, such as the envelope glycoprotein of vesicular stomatitis virus (VSV-G), required for retroviral entry. Each of these plasmids encodes heterologous promoters to initiate gene transcription. The third plasmid is the transgene-transfer vector that contains the gene to be transferred (i.e. the transgene), flanking LTRs, and the Ψ retroviral packaging signal. Vector replication can only occur in packaging cell lines that have been cotransfected with the two packaging plasmids and the third plasmid containing the transgene. The transfected packaging cells can then be used to infect target cells where reverse transcription and integration of the vector take place, followed by host cell expression of the transgene.

Tropism expansion

Retrovirus entry is mediated by the Env protein, which binds to specific cellular receptors that determine viral tropism. Specific tropism greatly limits the broad use of retroviral vectors. In order to expand retroviral tropism and develop retroviral vectors that can be used to transduce a broader range of cells, different viral envelope proteins can be substituted for the natural Env protein, according to infection requirements. This replacement is called pseudotyping. For example, a glycoprotein of a retroviral vector derived from T-tropic HIV-1 can only infect CD4+ T cells through binding to the CD4 receptor on the T-cell surface. In contrast, VSV-G binds to phosphatidylserine, which is presented on cell membranes of all kinds of mammalian cells, allowing VSV-G to infect a broad range of cell types and tissues. By using the VSV-G envelope protein for pseudotyping, HIV-1-derived vectors have achieved high infection efficiencies for cell types as different as HeLa cells, rat fibroblasts, human primary macrophages, nondividing airway epithelial cells *in vitro*, and terminally differentiated neurons *in vivo* [21,22,23]. Moreover, due to the high stability of the VSV-G envelope protein, viral particles can be concentrated and purified by high-speed ultracentrifugation [24]. In this regard, VSV-G has been widely used to pseudotype a variety of viral vectors.

SIN vector

A key safety feature of retroviral vectors is the engineered SIN vectors, which inactivate the retroviral promoter within the LTR U3 regions [12]. LTRs flanking the transgene in the transfer vector construct contain promoter/enhancer sequences in their U3 subregions. The U3 region of the 3′ LTR is the template for generating both U3 sequences in the LTRs in the integrated provirus. In SIN vectors a substantial portion of the U3 promoter is deleted, which abrogates the promoter/enhancer activity of the 3′ LTR and eliminates transcription of the integrated provirus in infected cells [11]. Instead, a heterologous promoter [such as a cytomegalovirus (CMV) promoter] ensures transcription of the transgene mRNA. This design contributes to minimizing insertional oncogenesis in infected cells and excludes the chance recreation of replication-competent, wild-type-like viruses.

Gammaretroviral and Alpharetroviral Vectors

Gamamretroviral and alpharetroviral vectors are two types of retroviral vectors that are derived from the two simple retroviruses, gammaretroviruses and alpharetroviruses, respectively. Simpler than lentiviral vectors, accessory gene constructs are not required for gammaretroviral vector (γ-RV) and alpharetroviral vector (α-RV) systems. Transduction by α-RVs and γ-RVs is dependent on cell cycling [25]. γ-RVs are one of the four main clinically applicable and studied viral vectors (the other three are adenoviral vectors, lentiviral vectors, and adeno-associated viral vectors) [26]. The most frequently used and studied γ-RVs are MLV-derived [6,7,8,27]. γ-RVs can be produced in large scale and at clinical grades, and transduce and integrate into genomes of dividing cells stably and efficiently [28,29,30,31]. All these characteristics have made γ-RVs a promising candidate in gene therapy. γ-RVs have been used to treat many diseases such as Gaucher disease [32], hypercholesterolemia [33], and chronic granulomatous disease [34]. However, γ-RVs can only be used *ex vivo*, as the target cells must be transduced and then re-infused back into the patient because of the sensitivity of these viruses to mammalian complement. Other significant limitations of γ-RVs include the high frequency of integration in or near proto-oncogenes and the inability to infect nondividing cells. Recently α-RVs have gained in interest as an alternative retroviral vector. Compared to γ-RVs and LVs, α-RVs have relatively neutral and favorable integration patterns [13]. Suerth et al. have successfully constructed SIN α-RVs that can transduce murine and human CD34+ hematopoietic cells with low multiplicity of infection [35]. Their follow-up study showed that SIN α-RVs can mediate long-term gene expression within the progeny of transplanted CD34+ hematopoietic cells and have low risk of insertional mutagenesis [36]. Kaufmann et al. further evaluated SIN α-RVs in an X-linked granulomatous disease model, which supports the value of SIN α-RVs for gene therapy [37].

Foamyviral (Spumaviral) Vectors

Foamyviral vectors (FVs) are derived from foamy viruses (spumaviruses), which have a low prevalence in humans. Although there are reports of human infection after interaction with non-human primates [38,39,40], foamy viruses are not pathogenic and do not spread horizontally in humans [41,42,43]. This characteristic makes foamy viruses a relatively safe and promising candidate for gene delivery. Foamy viruses are complex retroviruses and contain accessory genes in viral genomes. However, no accessory gene constructs are required in an FV system for gene delivery [44,45,46]. FVs complete reverse transcription mostly in virion-producing cells rather than in target cells [47,48]. As a result, the transducing viral genomes are cDNA. This would explain how FVs persist as a stable transduction intermediate when transducing quiescent cells and only become effective when cells enter their replicative cycle [49]. Preclinical studies [50,51,52] have demonstrated the potential of FVs to mediate gene transfer in hematopoietic stem cells (HSCs).

HIV-1-Derived Lentiviral Vectors

Basics of Lentiviruses

Lentivirus, a genus of retroviridae family known as complex retroviruses, infects and inserts its genome into host cells in the same manner as other retroviruses, but possesses some unique characteristics. The lentiviral capsid shares a structure with the simple retroviruses and the lentiviral genome also contains a ssRNA genome of 7–12 kb in length. Similar to the simple retroviruses, the HIV-1 genome contains the *gag*, *pol*, and *env* genes. However the genome also contains six other genes, giving it a total of nine open reading frames that can encode nine proteins. All these additional proteins play important roles in HIV infection and determine HIV pathogenic properties. Because the viral accessory genes (*vif*, *vpr*, *vpu*, *nef*, *env*, and *tat*) are not necessary for vector production and functionality, they can either be deleted from the vector or replaced with another retroviral gene, such as the case for *env*. In consequence, the HIV-1-derived lentiviral vectors retain only the HIV-1 *gag*, *pol*, and *rev* genes that are necessary for viral packaging. Schematics of HIV-1 genomic RNA and the HIV-1 life cycle are shown in Figures 2.1B and C.

Gag, Pol, and Env

The Gag protein is the viral core protein that is synthesized in the cytoplasm of infected cells and orchestrates the assembly and release of HIV-1 particles. It is both necessary and sufficient for the formation of non-infectious virus-like particles. It is responsive to both assembly and virion maturation signals after particle release and the entry and postentry steps during virus replication. The Pol protein is a set of enzymatic proteins including a protease, reverse transcriptase, and integrase. HIV-1 synthesizes these proteins products using a translational frameshift mechanism. The core and enzymatic proteins are derived from a Gag-Pol precursor protein. In this way, HIV-1 can regulate the relative amounts of Gag and Pol protein that are translated to ensure normal viral replication [53]. The Env precursor glycoprotein gp160 is cleaved into the mature viral surface gp120 and transmembrane gp41 proteins. Env proteins are necessary to initiate viral infection; they bind to receptors and coreceptors that they recognize on target cell surfaces [54].

Tat and Rev

Tat is a transactivator that interacts with the transactivation response element (TAR) that is located between nucleotides −17 and +80 in the LTR. Tat, which is only found in the complex retroviruses, promotes elongation of HIV-1 transcription. It affects viral replication [55,56] and is essential for the replication of HIV-1. The HIV-1 Rev, a virally encoded, sequence-specific RNA-binding protein, plays a critical role in the nuclear export of HIV-1 RNA that contains introns. Rev is translated from fully spliced viral mRNA, and mediates the transport unspliced viral mRNAs from nucleus to cytoplasm through an interaction with a Rev response element (RRE) that is located in *env* region [57]. By increasing transport of viral mRNA to cytoplasm, the half-life of viral mRNAs and the production of viral particles are enhanced [57,58].

Nef, Vpr, Vpu, and Vif

Nef, Vpr, Vpu, and Vif are four accessory proteins essential for efficient HIV-1 propagation/virulence in primary cells and in models *in vivo*. Nef is a myristoylated protein that quickly accumulates to detectable levels following HIV-1 infection [59]. Nef affects viral replication and pathogenesis by modulating cell-surface receptors, such as CD4, CXCR4, and MHC I, on the infected cell so it escapes the immune response [60,61,62,63]. Vpr can facilitate the nuclear import of viral PIC. Unlike other retroviruses, lentiviruses such as HIV-1 can infect both dividing and nondividing cells. A nuclear localization sequence in the Gag matrix (MA) protein of HIV-1 induces the conjugation of MA to integrase, and Vpr to form PIC, then mediates active transport of PIC into nucleus through nucleopores during interphase [64,65,66,67]. Although the integrase protein alone is sufficient to mediate the nuclear import of HIV-1 PIC in cell lines [67], Vpr is required for HIV-1 infection and replication in primary cells (macrophages) [68,69]. Similar to Nef, Vpu can also modulate CD4 receptor expression. However, as a protein that is expressed and accumulates early, Nef mainly acts on mature CD4 molecules presenting at the cell surface [70]. In contrast, Vpu is expressed late in the viral life cycle and sequesters newly synthesized CD4 molecules in the endoplasmic reticulum [71]. Vpu can also antagonize BST-2/Tetherin to stimulate the release of virions [72,73,74]. Vif promotes HIV-1 infection and replication in primary lymphocytes and macrophages by suppressing an inhibitory host cell factor apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G (APOBEC3G) [75,76,77,78].

LTRs

The HIV-1 LTRs are approximately 640 bp in length, and contain a number of *cis*-acting elements that are necessary for proviral DNA synthesis, integration of proviral DNA into the host genome, transcription of the viral genes, and regulation of viral gene expression. The nine coding genes in the HIV-1 genome, as well as integrated provirus, are flanked by LTRs at the 5′ and 3′ ends. From 5′ to 3′, the LTR has three subregions: U3, R, and U5. The U3 subregion contains many different transcription factor-binding sites that function in viral gene expression and integrated provirus transcription. The *nef* gene overlaps U3 upstream of promoter/enhancer sequences. The critical subregions in the U3 region are the TATAA box (promoter), GC-rich binding sites of SpI, and the binding sites of nuclear factor κB (NF-κB)-related factors (enhancers). In the integrated provirus, the 5′ LTR serves as a viral promoter for transcription and the 3′ LTR mediates the polyadenylation of nascent viral transcripts. Thus, LTRs directly influence many aspects of the virus lifecycle from infection to pathogenesis.

Principles of Lentiviral Vectors

Retroviral vectors developed from lentiviruses are called lentiviral vectors. Lentivirus-mediated gene-transfer systems share many features of retrovirus systems. The viral genome integrates into host chromosomes, and then the inserted genes are permanently maintained in the cells. Unlike simple retroviruses such as MLV, lentiviruses tend to integrate in introns, distal to promoters, there by potentially limiting their overall oncogenicity and immune response [13,79]. In addition, lentiviral vectors are capable of transducing dividing cells and nondividing cells, including HSCs and neurons, and they can specifically target the nucleus. Therefore, they have been applied in a broader range of cell lines and tissues, and are used for many local applications as well as for *ex vivo* gene therapy. Moreover, lentiviruses can permanently integrate into the genome of host cells, and are able to maintain long-term expression. They also have a larger packaging capacity (an overall transgene insert size of 8–10 kb) so either a transgene of interest or construct that will produce RNAi to suppress expression of a target gene can be packaged.

In 1991, Sodroski and coworkers described the first lentiviral vector that was developed from HIV using the same design strategy as for retroviral vectors [80]. All viral coding sequences were removed, but the viral structural and enzymatic proteins in trans, together with an envelope gene, were provided in a separate expression vector. Lentiviruses from different species, such as human (HIV-1 and HIV-2), monkey (simian immunodeficiency virus, SIV), cat (feline immunodeficiency virus, FIV), horse (equine infectious anemia virus, EIAV), cow (bovine immunodeficiency virus, BIV), goat, and sheep (caprine arthritis and encephalitis virus, CAEV), show distinct structural differences in their genomes, as well as receptor usage and pathogenicity. Because the physiopathology associated with interspecies transmission of nonhuman lentiviruses is highly unpredictable, the restriction mechanisms might reduce the transduction efficiency of human cells by these vectors. Consequently, HIV-1-derived lentiviral vectors are the most frequently used in translational studies and clinical trials. Next, we therefore focus on HIV-1-derived lentiviral vectors.

Evolution of HIV-1-Derived Lentiviral Vectors

Genetically modified HIV has been used for gene delivery for over 20 years. The earliest lentiviral vectors were replication-competent viruses that carried a transgene. Considering safety issues, virus elements in the first prototypes were separated onto two plasmids. One plasmid encoded the HIV-1 proviral DNA with a deletion of the *env* gene, and another one expressed Env. The resulting vectors produced viruses that could undergo only a single round of infection. After that, more sophisticated lentiviral vectors have been developed with improved biosafety and efficiency.

Continuous improvements have been made to subsequent "generations" of HIV-derived lentiviral vectors. The difference between these generations is mainly in the number of plasmids used for packaging. The use of multiple plasmids allows the *cis*- and *trans*-elements to be split among them and improves the safety profile of virus preparation. As accepted through common practice, the "generation" of lentiviral vectors does not include the early replication-competent prototypes of HIV vectors described above. Three major generations of HIV-1 lentiviral vectors and their advanced derivatives are described here and illustrated in Figure 2.2.

The First-Generation HIV-1-Derived Lentiviral Vectors

The first-generation replication-deficient, recombinant HIV-1 vectors were achieved by splitting genomic components onto three separate plasmids to increase safety [21] (Figure 2.2A). One plasmid expresses packaging construct genes to generate HIV-1 viral proteins Gag, Pol, and the regulatory and accessory proteins. A second plasmid expresses a viral Env glycoprotein of an unrelated virus VSV-G. These two different packaging vectors express their viral proteins from heterologous promoters. The third construct, a transfer vector construct, is a plasmid that contains the transgene and the packaging, reverse transcription, and integration signal elements (Ψ, LTRs, etc.). This three-plasmid system allows delivery of the gene of interest, but does not allow the expression of viral proteins in target cells. Although the use of VSV-G Env reduces risk of recombination, it does not eliminate the risk

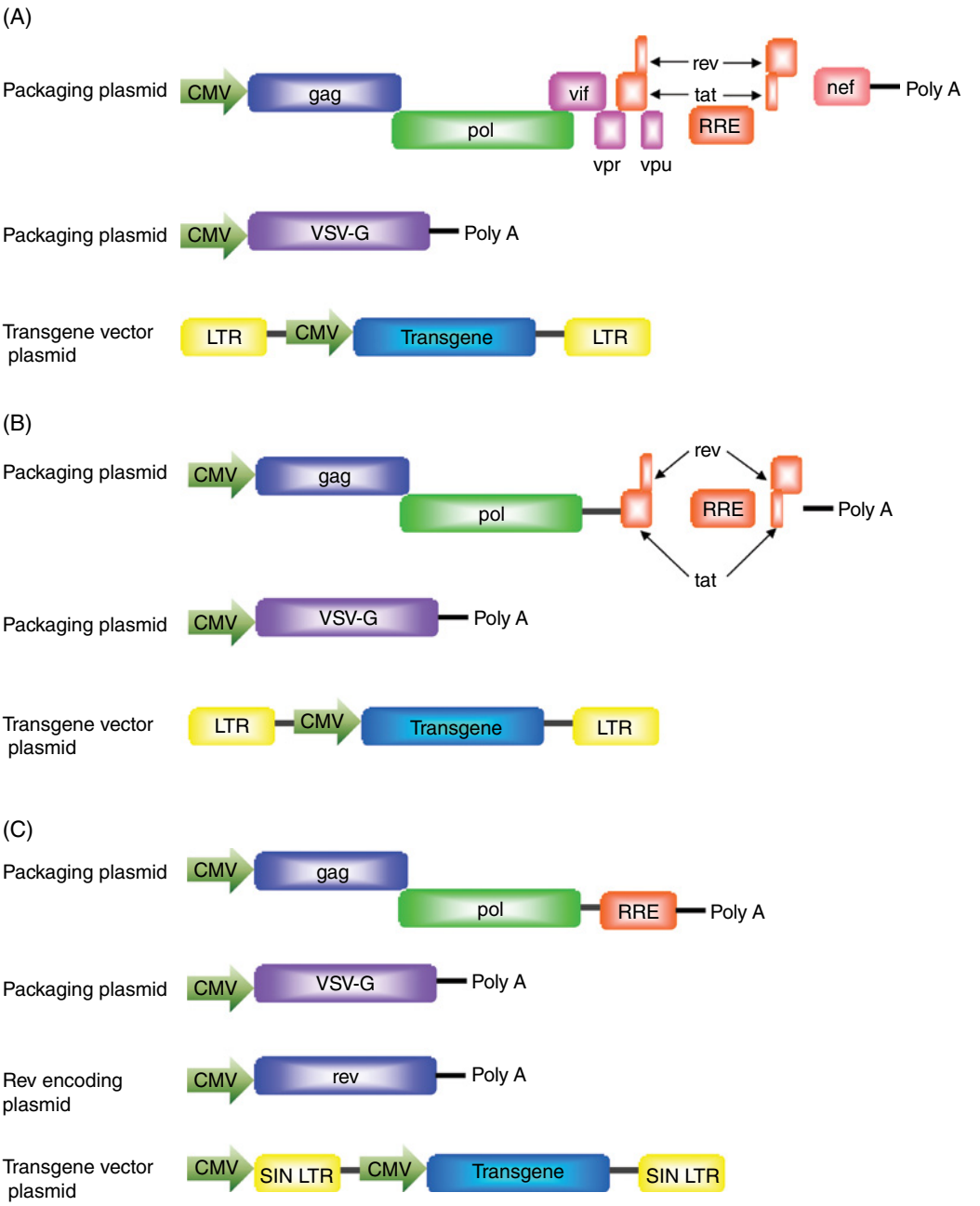


Figure 2.2 Scheme of lentiviral vectors derived from HIV-1. (A) The first-generation vector consists of three plasmid constructs. HIV-1 envelope glycoprotein is substituted by VSV-G. (B) On the basis of the first-generation vectors, the second-generation vectors exclude the four accessory genes (*vpr*, *vif*, *vpu*, and *nef*) of HIV-1. (C) The third-generation vector further deletes the HIV-1 accessory gene *tat* and is composed of four plasmid constructs. Rev is expressed by a separate plasmid construct and SIN LTRs are included in transgene plasmid construct.

of acquiring a new type of virus. Therefore, it is highly desirable to identify the minimal requirements for viral genes and elements in order to obtain an efficient transduction vector.

The Second-Generation HIV-1-Derived Lentiviral Vectors

In second-generation lentiviral vectors (Figure 2.2B) most viral accessory genes (*vif*, *vpu*, *vpr*, *nef*) were eliminated and only *tat* and *rev* were retained. As described above, HIV-1 viral accessory genes are associated with HIV-1 infection and replication ability in primary cells and *in vivo*. For example, Fouchier et al. reported that Vif-deficient HIV-1 cannot replicate in lymphocytes and a limited number of immortalized T-lymphoid lines (non-permissive cells), and does not influence the expression of any other virion components [81]. Aldrovandi et al. showed that deletion of *vpu*, *vip*, or *nef* significantly attenuates virus infectivity and pathogenicity in a SCID human mouse model of HIV-1 infection [82]. Therefore, the presence of these genes could cause serious safety issues in gene therapy applications. However, they can be deleted without affecting viral replication in certain human lymphoid cell lines. Therefore, by deleting these viral accessory genes, the second-generation lentiviral vectors, which possess only four of the nine HIV-1 genes, *gag*, *pol*, *tat*, and *rev*, have been developed to increase biosafety. Fortunately, deletion of these four accessory genes does not reduce the transduction efficiency of the corresponding lentivirus when packaged.

Kim et al. studied the minimal requirements for an efficient HIV-1-based vector. When all four accessory genes and the regulatory gene *tat* were excluded, and they replaced the U3 of 5′ LTR with a human CMV promoter [83], the transduction efficiency of their lentiviral vector in both proliferating and nondividing cells was not affected. At the same time, Zufferey et al. also demonstrated that multiple mutations in the four accessory genes did not affect transduction efficiency both *in vitro* and *in vivo* [84]. Soon after, Dull et al. confirmed the study of Kim et al. and proposed a design for a third-generation lentiviral vector [85].

The Third-Generation HIV-1-Derived Lentiviral Vector

According to the studies of Kim et al. and Dull et al. [83,85], among the six auxiliary systems (*tat*/TAR, *rev*/RRE, *nef*, *vpr*, *vpu*, and *vif*), only *rev*/RRE is required for constructing an applicable lentiviral vector based on HIV-1. On the basis of the second-generation vectors, the third-generation system (Figure 2.2C) uses four different plasmids and excludes the *tat* gene, whose gene product contributes to multiple AIDS-associated diseases such as AIDS-Kaposi's sarcoma, AIDS-associated cholangiopathy, and human papillomavirus-related oral squamous cell carcinoma in HIV-infected patients [86]. The four constructs of the third-generation vectors are (i) a packaging construct containing HIV-1 *gag* and *pol*, (ii) a packaging construct expressing VSV-G *env*, (iii) a plasmid expressing *Rev*, and (iv) a transgene vector construct containing transgene, enhancer/promoter activity-eliminated HIV-1 3′ LTR, and chimeric HIV-1 5′ LTR. Normally *Tat* is essential for HIV-1 replication but eliminating the need for *tat* by replacing the U3 promoter region of the 5′ LTR further increases safety of the third-generation vectors. Kim et al. [83] indicated that this simplified vector can be concentrated easily by ultracentrifugation, with high recovery rate.

Another change in the third-generation vectors was the introduction of the SIN vector, which also improves biosafety. Developed by Miyoshi et al. [87], 133 bp of the U3 region of the HIV-1 3′ LTR in the transfer vector construct was removed. The sequences deleted included the TATAA box and the binding sites for transcription factors Sp1 and NF-κB. Miyoshi et al. showed that the titer of the SIN vector virus particles was not significantly decreased and that they could mediate efficient gene delivery into neurons in rat brain. Zufferey et al. [11] have also described a SIN vector that has a 400 bp deletion in the 3′ LTR, thereby eliminating its promoter activity. Compared to a vector with a full-length LTR, their SIN vector did not affect virus titer, transgene expression *in vitro*, or transduction efficiency *in vivo*. The deletion in the 3′ LTR together with chimeric 5′ LTR reduced the risks of integrated vector mobilization, oncogenesis by insertional activation of oncogene,

and recombinational generation of replication-competent viruses while maintaining viral titers.

Advanced Lentiviral Vectors

cPPT- and WPRE-containing lentiviral vector

Further modifications have been made to the transgene vector construct to increase transduction efficiency and transgene expression. The central polypurine tract (cPPT) is a *cis* element within the HIV-1 *pol* gene that can promote nuclear translocation of the viral PIC. To enhance transgene expression further, the WPRE (a post-transcriptional regulatory element of woodchuck hepatitis virus, WHV) enhancer can be inserted to function as a *cis*-acting element to increase the accumulation of unspliced RNAs in the nucleus and cytoplasm [88]. Sense orientation and site-specific insertion are two requirements for these two elements to function. Inserting cPPT at the 5′ end between the promoter and transgene and WPRE at the 3′ end between the transgene and the SIN 3′ LTR can increase transduction efficiency in several types of human primary cells, including HSCs. This advanced vector showed superior efficiency to the third-generation vectors when used to transduce growth-arrested cells, such as cardiomyocytes, both *in vitro* and *in vivo* [89].

Targeting lentiviral vector

Pseudotyping with VSV-G has already greatly expanded the transduction range of lentiviral vectors. The enthusiasm for targeted gene therapy inspired researchers to further modify lentiviral vectors to achieve cell-type-specific targeting. To this end, targeting ligands or antibodies that specifically recognize cell-surface receptors or antigens are fused with the lentiviral vector Env glycoprotein. For example, Yang et al. successfully incorporated anti-CD20 antibody into an Env lentiviral vector and obtained a virus particles that effectively targeted human B cells [90]. However, it was noted that the fusion of ligands or antibodies to Env may change vector entry pattern. In Yang et al.'s study [90], the modified targeting vectors entered the Be cells through endocytosis. Therefore, Yang's group fused another pH-responsive protein to the vector to assist in its release from the endosome. Most recently, Liang et al. [91] created a so-called "targeting nanovirus." They shielded original lentiviral vectors outside with a thin polymer shell and then introduced binding-specific peptides to the polymer shell to realize cell targeting. The nanovirus has a transfection efficiency that is comparable to those of the original vectors and uses the same entry path to target cells, but with increased stability in the presence of human serum.

Non-integrating lentiviral vector

Biosafety issues, such as oncogenesis induced by nonspecific integration and the potential to generate replication-competent viruses through the recombination of viral vectors in the host genome, are always concerns when deciding to use viral vectors for gene therapy. Non-integrating lentiviral vectors are constructed by introducing class I mutations into the integrase protein to induce integration defects. As a result, double-stranded episomal DNA circles with one LTR (homologous recombination) or two LTRs (nonhomologous end joining) accumulate in the host nucleus. Non-integrating lentiviral vectors can mediate stable transduction and efficient transgene expression both *in vitro* and *in vivo* [92].

Retroviral Vector-Mediated RNAi Therapy: From Basic Science to Clinical Trial

MLV RNAi Delivery

RNAi is a powerful tool for functional analysis of genes and developing a potential therapeutic strategy for disease. A number of publications have reported using retroviral vectors to deliver a short hairpin RNA (shRNA) library expressing transgenes for high-throughput screening. For example, using a retroviral vector based on MLV as a transfer vehicle, Paddison et al. designed a large-scale library of RNAi-inducing shRNA-expression cassettes [93]. The resulting MLV-based shRNA vector was capable of targeting approximately a third of all human genes, which could be used for both genetic selection and screening. Similarly, in 2004 Bern et al. reported the construction of a set of retroviral vectors (MLV) encoding 23 742 distinct shRNAs, which was used to identify one known and five new modulators of p53-dependent proliferation arrest in mammalian cells [94].

Additionally, it was reported that a retrovirally delivered shRNA induced long-term silencing of HIV-1 transcription [95]. In this case, the authors used MLV-based vectors to express shRNAs, which target the NF-κB-binding motif in the U3 region of the HIV-1 LTR. Following retroviral RNAi delivery, shRNA-expressing CD4(+) T cells (Molt-4) were established, where HIV-1 gene expression was profoundly suppressed for 1 year.

Lentiviral RNAi Delivery

HIV-derived lentiviral vectors are extensively used in both basic biology and clinical trials to overexpress transgenes, immunize, generate transgenic animals, persistently silence genes, or engineer and modify stem cells. Lentiviral-vector-mediated RNAi technology offers great therapeutic potential against a variety of human diseases [96], including cancer, HIV-1, ocular disease, cardiovascular disease, and neurodegenerative diseases. One application for HIV-1-derived lentiviral vectors was *ex vivo* gene transfer into human HSCs to treat adrenoleukodystrophy, and clinical trials aiming to treat primary immune deficiencies could follow soon. A number of in-depth reviews of and seminal research into RNAi and lentiviral vectors are available in various fields [97,98]. In this section, we draw from our own experience to concentrate on the development of RNAi therapy mediated by lentiviral vectors for HIV-1 research and cancer treatment.

Production of RNAi triggers from a lentiviral system

RNA polymerase III-driven expression cassettes in lentiviral vectors are typically used to generate the double-stranded RNA triggers for RNAi, which are known as small interfering RNAs (siRNAs), or precursors of siRNAs, known as shRNAs. Construction of these cassettes involves cloning the oligonucleotides containing the siRNA sequence into viral vectors that will endogenously express an siRNA or shRNA. In one system, the guide and passenger strands of the siRNA molecule are expressed from two separate promoters. In another system, the passenger, loop, and guide sequence are generated from one promoter, thereby producing a shRNA, a different version of an RNAi trigger that is subsequently processed in the cytoplasm into siRNAs. Currently, shRNA expression cassettes are primarily in use.

Briefly, to produce lentiviral particles, the transfer vector encoding the siRNA or shRNA is cotransfected into a packaging cell line with Gag-Pol and VSV-G packaging plasmids (Figure 2.3). RNAi triggers (siRNA or shRNA) from a lentiviral-based vector system may be expressed from a constitutive promoter or an inducible promoter when it is desired to control the "on/off" expression of the RNAi triggers during the course of the experiment.

Generally, the recombinant lentiviruses are prepared by transient transfection into 293 T/17 packaging cells using a calcium phosphate solution. The 293 T/17 cells are a highly transfectable derivative of

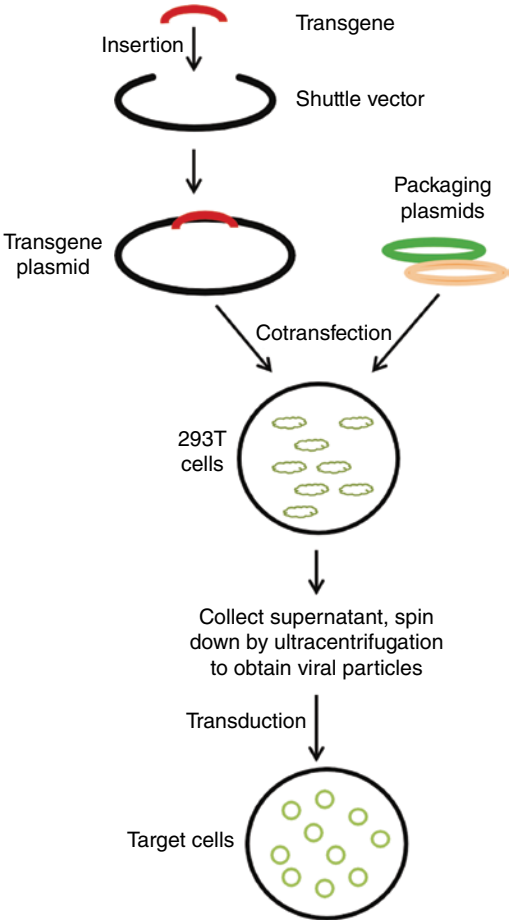


Figure 2.3 Schematic representation of lentiviral vector production and transduction. Transgene plasmid construct and packaging plasmids are cotransfected into 293 T cells for viral production. Then viral particles are collected by ultracentrifugation, resuspended, and titrated to determine the needed amount for target cell infection.

293 human fetal kidney cell line that can be used as packaging cells. After 24 or 48 h of transfection, the *trans*- and *cis*-elements combine together to produce the lentiviral vector particles. The virus can then be harvested from the supernatant, concentrated, and titrated for use in future experiments.

Anticancer therapy

Lung, breast, colorectal, stomach, and prostate cancers have high incidences and account for most cancer-related mortality worldwide. Effective cures for cancer are unavailable despite the many efforts that have been made towards developing useful therapies. Carcinogenesis is a complex process in which the normal cells are transformed to a state where they proliferate without limit and metastasize to invade other tissues. During carcinogenesis many proto-oncogenes are activated to become oncogenes, or tumor-suppressor genes are deactivated, thereby losing tumor-suppressing effects. The crosstalk between signaling pathways that involve these tumor-related gene products determines many aspects of the tumors, such as cell growth rate, metastasis, and angiogenesis. Therefore, those activated tumor-related genes are considered as potential therapeutic targets for cancer treatment. SiRNAs have been demonstrated to posttranscriptionally suppress target gene expression without affecting other genes [99]. Given the high transduction efficiency and long-term expression of transgenes, lentiviral-vector-mediated RNAi triggers have better gene-silencing capability than synthetic siRNAs. Many preclinical studies have been completed or are in progress to explore the most feasible therapeutics for various cancers. In the USA about one in eight women will develop invasive breast cancer during their lifetime (www.breastcancer.org) and about one in five breast cancer survivors will probably suffer a recurrence within 10 years after treatment [100]. Currently, chemotherapies are the best treatments available. As our understanding of breast cancer continues to expand, more attention may be directed towards the regulation of oncogenic factors/pathways to control and suppress breast tumors at the gene level. Studies have shown that lentiviral-vector-mediated RNAi triggers are promising tools for gene regulation and manipulation of gene expression. Leptin/leptin receptor (ObR) signaling and its crosstalk with other signaling pathways, including phosphoinositide 3-kinase/Akt, STAT3, and estrogen receptor (ER) signaling, are critical for how breast cancer and cancer stem cells determine their growth, migration, invasion, angiogenesis, and metastasis [101]. Therefore, the leptin/ObR pathway could be an effective therapeutic target for breast cancer treatment. Intratumoral injection of lentiviral RNAi triggers targeting ObR can significantly downregulate ObR expression and other signaling pathway factors [ERα and vascular endothelial growth factor (VEGF)] to suppress established tumor growth in xenograft nude mice [102]. Moreover, lentiviral-vector-mediated RNAi triggers have been also used to permanently silence some "undruggable" targets such as the *Hsp25*/*Hsp27* gene to induce the regression of established breast tumors in untreated mice [103]. Cervical cancer is another common type of cancer in women. Lentiviral vector-based RNAi triggers that target the *E6* and *E7* oncogenes of papillomavirus can inhibit cervical tumor growth both *in vitro* and *in vivo* [104]. Lentivirus-based RNAi triggers have also been applied to treat small-cell lung cancer and showed good effect in inhibiting cancer cell growth and bone metastasis [105]. Despite these inspiring results, *in vivo* systemic delivery of viral vectors remains an unresolved issue. Viral vectors may induce host immune responses and most of the available lentiviral vectors lack cell specificity, although targeting lentiviral vectors are under study. Moreover, tumor metastasis, which may result in off-target/nontarget delivery and low-titer viral vectors, also poses difficulty for *in vivo* application of viral vectors.

Anti-HIV therapy

Despite the marked success of highly active antiretroviral therapy (HAART), it is likely never to be curative, and drug resistance and toxicity issues still remain a great concern. Given the well-recognized limitation of current therapeutic approaches, nucleic acid-based therapeutics have been considered as an alternative to or adjuvant with HAART. RNAi can function as a gene-specific therapeutic option for controlling HIV-1 replication. Intensive efforts have been made to use lentiviral vectors for shRNA delivery against HIV infection. Analogous to HAART, in which several small-molecule active drugs are used against HIV-1,

several shRNAs and different inhibitory agents have been combined to combat the virus, minimize viral escape, and prevent the emergence of resistance. For example, ter Brake et al. designed and tested a multiple shRNA expression strategy using different promoters (*U6*, *H1*, *7SK*, and *U1* promoters) in a lentiviral vector [106]. In an *in vitro* cell culture system they demonstrated that the combination of the four shRNA expression cassettes prevented HIV-1 escape, which is often observed in a single shRNA expression system.

From our own efforts using RNAi against HIV infection [107], we successfully combined three different inhibitory genes into a single-lentiviral-vector backbone, thereby simultaneously expressing (i) an shRNA that targets the HIV-1 *tat*/*rev* mRNA, (ii) a nucleolus-localizing decoy that binds and sequesters the HIV Tat protein, and (iii) a ribozyme that cleaves and downregulates the CCR5 chemokine receptor used by HIV for cellular entry. The resulting triple combination (lentiviral vector rHIV7-shI-TAR-CCR5RZ) effectively suppressed HIV-1 replication in primary hematopoietic cells [108]. Moreover, it demonstrated increased suppression of HIV-1 over 42 days when compared to a single anti-*tat*/*rev* shRNA or double combinations of shRNA/ribozyme or decoy.

To reduce the need for continuous and lifelong treatment in HAART there is growing interest in developing a lifelong cure. It has been proposed that long-lived, self-renewing, multilineage HSCs could be engineered such that they and their progeny are able to resist HIV infection. The engraftment of these engineered HSCs in the patient will produce an HIV-resistant immune system, thus creating a curative approach. Recently, the aforementioned lentiviral vector has been used for *ex vivo* gene delivery to HSCs in a human clinical trial [109] (ClinicalTrials.gov identifier NCT00569985). In this case, CD34+ hematopoietic progenitor stem cells were mobilized and collected from the patients, and then transduced in tissue culture with the vector. After *ex vivo* expansion, up to 10^8–10^9 transduced stem cells were isolated and re-infused into the patients. After introduction of these modified cells it was expected that the host could be repopulated with HIV-resistant HSCs. The results demonstrated that transfected cells were successfully engrafted in all four infusion patients by day 11 and no unexpected infusion-related toxicities were observed. In a follow-up experiment, persistent expression of the anti-*tat*/*rev* shRNA and CCR5 ribozyme was detectable in the peripheral blood for up to 36 months after infusion [109,110]. Due to the low frequency of modified cells in this study (less than 0.2% of circulating peripheral blood mononuclear cells), no clinical benefit was observed; however, the modified cells showed good tolerance and long-term persistence for at least 36 months. Further development to improve the transduction process and optimize the transplant procedures will be expected to result in a higher percentage of gene-modified cells, which therefore probably satisfies the requirement for clinical application.

Lentiviral-vector-mediated inherited disorder therapy

Most recently, two impressive studies published in *Science* showed clinical efficacy from lentiviral-vector-mediated inherited disorder therapy. Metachromatic leukodystrophy (MLD) is a genetic disorder caused by deficiency of the enzyme arylsulphatase A (ARSA). The late-infantile form that is the most common form of MLD (50–60%) affects children's walking within the first year of life and other symptoms follow within the second year of life. Untreated, most children with this form of MLD die by age 5, often much sooner. Currently there is no cure for MLD. Biffi and colleagues [111] used a lentiviral vector to introduce a functional ARSA gene into HSCs *ex vivo*. The resulting transduced HSCs were transplanted into three children with ARSA deficiency and mutations associated with early-onset MLD. Clinical and objective evaluations were conducted up to 24 months after treatment. All the patients showed a high-level stable engraftment of the transduced HSCs in bone marrow and peripheral bloods at all times tested without apparent vector genotoxicity, and 45–80% of bone marrow-derived hematopoietic colonies contained the lentiviral vector. Markedly, ARSA activity was reconstituted to above normal values in the hematopoietic lineages and in the cerebrospinal fluid. In another similar study, Aiuti et al. [112] reported to use a lentiviral vector to introduce a functional *WAS* gene into autologous HSCs *ex vivo* for the treatment of Wiskott–Aldrich

syndrome (WAS), which is a rare X-linked recessive disease characterized by eczema, thrombocytopenia, and immune deficiency. In a phase I/II clinical trial, autologous CD34+ cells were transduced with a lentiviral *WAS* vector. The resulting gene-corrected HSCs were re-infused into three patients with WAS. After transplantation, high, stable levels (25–50%) of blood cells expressing the lentiviral vectors were observed in the bone marrow of all three patients. Most importantly, all three patients showed improved platelet counts, protection from bleeding and severe infections, and resolution of eczema. Compared to gammaretroviral gene therapy, their lentiviral-vector-mediated therapy did not trigger *in vivo* selection of clones carrying an integration near oncogenes. Both studies suggested that lentiviral-vector-mediated gene transfer would be a feasible means to engineer human HSCs.

Concluding Remarks and Challenges

A number of features of retroviruses make them unique as gene-delivery vehicles. Retroviral gene-transfer systems have demonstrated stable integration into the host genome and subsequent long-term ectopic expression of a therapeutic transgene in several clinical trials and lead to sustained long-term correction of genetic defects. For example, the Moloney MLV gammaretrovirus was the first vector employed in a human gene-transfer trial. However, gammaretroviruses tend to integrate in introns, potentially producing oncogenicity. Moreover, in contrast to lentiviral vectors, γ-RVs are reported to be restricted in their ability to transduce growth-arrested cells. Lentiviral vectors possess obvious advantages over other retroviral vectors in that they can efficiently deliver the gene or therapeutic molecule of interest into both dividing and nondividing cells. Through extensive improvements, safer and more effective lentiviral vectors have been established. They are now widely used in biological research and gene therapy applications. The highly specific mechanism of RNAi that inhibits the expression of particular genes is extremely attractive for biotechnology and pharmaceutical companies to develop gene therapies that treat a wide range of human diseases. Combining RNAi with viral gene therapy vectors is a powerful approach that can introduce therapeutic RNAi molecules into cells and induce persistent gene silencing. In particular, there is growing enthusiasm for creating programmable HSCs through the use of lentiviral-vector RNAi systems. However, safety and efficiency issues are still critical challenges for the clinical translation of lentiviral-vector-based gene therapy. The goal in designing a vector is to minimize the potential risk that may be observed long term, but to also maximize the efficacy of gene silencing by the gene therapy system. Such viral vectors should be carefully engineered. More comprehensive and mechanistic understanding of retroviral integration patterns will be helpful for generating safer and more effective vector platforms.

Acknowledgments

We thank Dr Margaret Morgan for critical reading and editing the manuscript. This work was funded by National Institutes of Health grants AI42552 and HL07470 awarded to JR. Jiehua Zhou and Yue Ding made equal contribution on this book chapter.

References

1 Coffin JM. (1979) Structure, replication, and recombination of retrovirus genomes: some unifying hypotheses. *Journal of General Virology* 42(1): 1–26.

2 Shimotohno K, Temin HM. (1981) Formation of infectious progeny virus after insertion of herpes simplex thymidine kinase gene into DNA of an avian retrovirus. *Cell* 26(1 Pt 1): 67–77.

3 Wei CM, Gibson M, Spear PG, Scolnick EM. (1981) Construction and isolation of a transmissible retrovirus containing the src gene of Harvey murine sarcoma virus and the thymidine kinase gene of herpes simplex virus type 1. *Journal of Virology* 39(3): 935–944.

4 Mann R, Mulligan RC, Baltimore D. (1983) Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. *Cell* 33(1): 153–159.

5 Edelstein ML, Abedi MR, Wixon J. (2007) Gene therapy clinical trials worldwide to 2007–an update. *Journal of Gene Medicine* 9(10): 833–842.

6 Blaese RM, Culver KW, Miller AD, Carter CS, Fleisher T, Clerici M, et al. (1995) T lymphocyte-directed gene therapy for ADA-SCID: initial trial results after 4 years. *Science* 270(5235): 475–480.

7 Kohn DB, Weinberg KI, Nolta JA, Heiss LN, Lenarsky C, Crooks GM, et al. (1995) Engraftment of gene-modified

umbilical cord blood cells in neonates with adenosine deaminase deficiency. *Nature Medicine* 1(10): 1017–1023.

8 Bordignon C, Notarangelo LD, Nobili N, Ferrari G, Casorati G, Panina P, et al. (1995) Gene therapy in peripheral blood lymphocytes and bone marrow for ADA-immunodeficient patients. *Science* 270(5235): 470–475.

9 Fischer A, Hacein-Bey-Abina S, Cavazzana-Calvo M. (2010) 20 years of gene therapy for SCID. *Nature Immunology* 11(6): 457–460.

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

11 Zufferey R, Dull T, Mandel RJ, Bukovsky A, Quiroz D, Naldini L, et al. (1998) Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. *Journal of Virology* 72(12): 9873–9880.

12 Yu SF, von Ruden T, Kantoff PW, Garber C, Seiberg M, Ruther U, et al. (1986) Self-inactivating retroviral vectors designed for transfer of whole genes into mammalian cells. *Proceedings of the National Academy of Sciences USA* 83(10): 3194–3198.

13 Mitchell RS, Beitzel BF, Schroder AR, Shinn P, Chen H, Berry CC, et al. (2004) Retroviral DNA integration: ASLV, HIV, and MLV show distinct target site preferences. *PLoS Biology* 2(8): E234.

14 Lochelt M, Muranyi W, Flugel RM. (1993) Human foamy virus genome possesses an internal, Bel-1-dependent and functional promoter. *Proceedings of the National Academy of Sciences USA* 90(15): 7317–7321.

15 Bowerman B, Brown PO, Bishop JM, Varmus HE. (1989) A nucleoprotein complex mediates the integration of retroviral DNA. *Genes and Development* 3(4): 469–478.

16 Li M, Mizuuchi M, Burke TR, Jr., Craigie R. (2006) Retroviral DNA integration: reaction pathway and critical intermediates. *EMBO Journal* 25(6): 1295–1304.

17 Roe T, Reynolds TC, Yu G, Brown PO. (1993) Integration of murine leukemia virus DNA depends on mitosis. *EMBO Journal* 12(5): 2099–2108.

18 Varmus HE. (1982) Form and function of retroviral proviruses. *Science* 216(4548): 812–820.

19 Marquet R, Isel C, Ehresmann C, Ehresmann B. (1995) tRNAs as primer of reverse transcriptases. *Biochimie* 77(1–2): 113–124.

20 Soneoka Y, Cannon PM, Ramsdale EE, Griffiths JC, Romano G, Kingsman SM, et al. (1995) A transient three-plasmid expression system for the production of high titer retroviral vectors. *Nucleic Acids Research* 23(4): 628–633.

21 Naldini L, Blomer U, Gallay P, Ory D, Mulligan R, Gage FH, et al. (1996) In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* 272(5259): 263–267.

22 Goldman MJ, Lee PS, Yang JS, Wilson JM. (1997) Lentiviral vectors for gene therapy of cystic fibrosis. *Human Gene Therapy* 8(18): 2261–2268.

23 Naldini L, Blomer U, Gage FH, Trono D, Verma IM. (1996) Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. *Proceedings of the National Academy of Sciences USA* 93(21): 11382–11388.

24 Yee JK, Friedmann T, Burns JC. (1994) Generation of high-titer pseudotyped retroviral vectors with very broad host range. *Methods in Cell Biology* 43(A): 99–112.

25 Katz RA, Greger JG, Skalka AM. (2005) Effects of cell cycle status on early events in retroviral replication. *Journal of Cellular Biochemistry* 94(5): 880–889.

26 Flotte TR. (2007) Gene therapy: the first two decades and the current state-of-the-art. *Journal of Cellular Physiology* 213(2): 301–305.

27 Rosen CA, Sodroski JG, Campbell K, Haseltine WA. (1986) Construction of recombinant murine retroviruses that express the human T-cell leukemia virus type II and human T-cell lymphotropic virus type III trans activator genes. *Journal of Virology* 57(1): 379–384.

28 Andreadis ST, Roth CM, Le Doux JM, Morgan JR, Yarmush ML. (1999) Large-scale processing of recombinant retroviruses for gene therapy. *Biotechnology Progress* 15(1): 1–11.

29 Palu G, Parolin C, Takeuchi Y, Pizzato M. (2000) Progress with retroviral gene vectors. *Reviews in Medical Virology* 10(3): 185–202.

30 McTaggart S, Al-Rubeai M. (2002) Retroviral vectors for human gene delivery. *Biotechnology Advances* 20(1): 1–31.

31 Feldman SA, Goff SL, Xu H, Black MA, Kochenderfer JN, Johnson LA, et al. (2011) Rapid production of clinical-grade gammaretroviral vectors in expanded surface roller bottles using a "modified" step-filtration process for clearance of packaging cells. *Human Gene Therapy* 22(1): 107–115.

32 Dunbar CE, Kohn DB, Schiffmann R, Barton NW, Nolta JA, Esplin JA, et al. (1998) Retroviral transfer of the glucocerebrosidase gene into CD34+ cells from patients with Gaucher disease: in vivo detection of transduced cells without myeloablation. *Human Gene Therapy* 9(17): 2629–2640.

33 Gough PJ, Raines EW. (2003) Gene therapy of apolipoprotein E-deficient mice using a novel macrophage-specific retroviral vector. *Blood* 101(2): 485–491.

34 Ott MG, Schmidt M, Schwarzwaelder K, Stein S, Siler U, Koehl U, et al. (2006) Correction of X-linked chronic granulomatous disease by gene therapy, augmented by insertional activation of MDS1-EVI1, PRDM16 or SETBP1. *Nature Medicine* 12(4): 401–409.

35 Suerth JD, Maetzig T, Galla M, Baum C, Schambach A. (2010) Self-inactivating alpharetroviral vectors with a split-packaging design. *Journal of Virology* 84(13): 6626–6635.

36 Suerth JD, Maetzig T, Brugman MH, Heinz N, Appelt JU, Kaufmann KB, et al. (2012) Alpharetroviral self-inactivating

vectors: long-term transgene expression in murine hematopoietic cells and low genotoxicity. *Molecular Therapy* 20(5): 1022–1032.

37 Kaufmann KB, Brendel C, Suerth JD, Mueller-Kuller U, Chen-Wichmann L, Schwable J, et al. (2013) Alpharetroviral vector-mediated gene therapy for X-CGD: functional correction and lack of aberrant splicing. *Molecular Therapy* 21(3): 648–661.

38 Schweizer M, Turek R, Hahn H, Schliephake A, Netzer KO, Eder G, et al. (1995) Markers of foamy virus infections in monkeys, apes, and accidentally infected humans: appropriate testing fails to confirm suspected foamy virus prevalence in humans. *AIDS Research and Human Retroviruses* 11(1): 161–170.

39 Heneine W, Switzer WM, Sandstrom P, Brown J, Vedapuri S, Schable CA, et al. (1998) Identification of a human population infected with simian foamy viruses. *Nature Medicine* 4(4): 403–407.

40 Switzer WM, Bhullar V, Shanmugam V, Cong ME, Parekh B, Lerche NW, et al. (2004) Frequent simian foamy virus infection in persons occupationally exposed to nonhuman primates. *Journal of Virology* 78(6): 2780–2789.

41 Linial M. (2000) Why aren't foamy viruses pathogenic? *Trends in Microbiology* 8(6): 284–289.

42 Boneva RS, Switzer WM, Spira TJ, Bhullar VB, Shanmugam V, Cong ME, et al. (2007) Clinical and virological characterization of persistent human infection with simian foamy viruses. *AIDS Research and Human Retroviruses* 23(11): 1330–1337.

43 Boneva RS, Grindon AJ, Orton SL, Switzer WM, Shanmugam V, Hussain AI, et al. (2002) Simian foamy virus infection in a blood donor. *Transfusion* 42(7): 886–891.

44 Trobridge GD, Russell DW. (1998) Helper-free foamy virus vectors. *Human Gene Therapy* 9(17): 2517–2525.

45 Bock M, Heinkelein M, Lindemann D, Rethwilm A. (1998) Cells expressing the human foamy virus (HFV) accessory Bet protein are resistant to productive HFV superinfection. *Virology* 250(1): 194–204.

46 Heinkelein M, Dressler M, Jarmy G, Rammling M, Imrich H, Thurow J, et al. (2002) Improved primate foamy virus vectors and packaging constructs. *Journal of Virology* 76(8): 3774–3783.

47 Zamborlini A, Renault N, Saib A, Delelis O. (2010) Early reverse transcription is essential for productive foamy virus infection. *PLoS One* 5(6): e11023.

48 Yu SF, Sullivan MD, Linial ML. (1999) Evidence that the human foamy virus genome is DNA. *Journal of Virology* 73(2): 1565–1572.

49 Trobridge G, Russell DW. (2004) Cell cycle requirements for transduction by foamy virus vectors compared to those of oncovirus and lentivirus vectors. *Journal of Virology* 78(5): 2327–2335.

50 Zucali JR, Ciccarone T, Kelley V, Park J, Johnson CM, Mergia A. (2002) Transduction of umbilical cord blood CD34+ NOD/SCID-repopulating cells by simian foamy virus type 1 (SFV-1) vector. *Virology* 302(2): 229–235.

51 Josephson NC, Trobridge G, Russell DW. (2004) Transduction of long-term and mobilized peripheral blood-derived NOD/SCID repopulating cells by foamy virus vectors. *Human Gene Therapy* 15(1): 87–92.

52 Leurs C, Jansen M, Pollok KE, Heinkelein M, Schmidt M, Wissler M, et al. (2003) Comparison of three retroviral vector systems for transduction of nonobese diabetic/severe combined immunodeficiency mice repopulating human CD34+ cord blood cells. *Human Gene Therapy* 14(6): 509–519.

53 Karacostas V, Wolffe EJ, Nagashima K, Gonda MA, Moss B. (1993) Overexpression of the HIV-1 gag-pol polyprotein results in intracellular activation of HIV-1 protease and inhibition of assembly and budding of virus-like particles. *Virology* 193(2): 661–671.

54 Checkley MA, Luttge BG, Freed EO. (2011) HIV-1 envelope glycoprotein biosynthesis, trafficking, and incorporation. *Journal of Molecular Biology* 410(4): 582–608.

55 Ruben S, Perkins A, Purcell R, Joung K, Sia R, Burghoff R, et al. (1989) Structural and functional characterization of human immunodeficiency virus tat protein. *Journal of Virology* 63(1): 1–8.

56 Feinberg MB, Baltimore D, Frankel AD. (1991) The role of Tat in the human immunodeficiency virus life cycle indicates a primary effect on transcriptional elongation. *Proceedings of the National Academy of Sciences USA* 88(9): 4045–4049.

57 Hadzopoulou-Cladaras M, Felber BK, Cladaras C, Athanassopoulos A, Tse A, Pavlakis GN. (1989) The rev (trs/art) protein of human immunodeficiency virus type 1 affects viral mRNA and protein expression via a cis-acting sequence in the env region. *Journal of Virology* 63(3): 1265–1274.

58 Felber BK, Hadzopoulou-Cladaras M, Cladaras C, Copeland T, Pavlakis GN. (1989) rev protein of human immunodeficiency virus type 1 affects the stability and transport of the viral mRNA. *Proceedings of the National Academy of Sciences USA* 86(5): 1495–1499.

59 Kim SY, Byrn R, Groopman J, Baltimore D. (1989) Temporal aspects of DNA and RNA synthesis during human immunodeficiency virus infection: evidence for differential gene expression. *Journal of Virology* 63(9): 3708–3713.

60 Garcia JV, Miller AD. (1992) Downregulation of cell surface CD4 by nef. *Research in Virology* 143(1): 52–55.

61 Levesque K, Finzi A, Binette J, Cohen EA. (2004) Role of CD4 receptor down-regulation during HIV-1 infection. *Current HIV Research* 2(1): 51–59.

62 Hrecka K, Swigut T, Schindler M, Kirchhoff F, Skowronski J. (2005) Nef proteins from diverse groups of primate lentiviruses downmodulate CXCR4 to inhibit migration to the chemokine stromal derived factor 1. *Journal of Virology* 79(16): 10650–10659.

63 Schwartz O, Marechal V, Le Gall S, Lemonnier F, Heard JM. (1996) Endocytosis of major histocompatibility complex class I molecules is induced by the HIV-1 Nef protein. *Nature Medicine* 2(3): 338–342.

64 Bukrinsky MI, Haggerty S, Dempsey MP, Sharova N, Adzhubel A, Spitz L, et al. (1993) A nuclear localization signal within HIV-1 matrix protein that governs infection of nondividing cells. *Nature* 365(6447): 666–669.

65 Heinzinger NK, Bukinsky MI, Haggerty SA, Ragland AM, Kewalramani V, Lee MA, et al. (1994) The Vpr protein of human immunodeficiency virus type 1 influences nuclear localization of viral nucleic acids in nondividing host cells. *Proceedings of the National Academy of Sciences USA* 91(15): 7311–7315.

66 Gallay P, Swingler S, Song J, Bushman F, Trono D. (1995) HIV nuclear import is governed by the phosphotyrosine-mediated binding of matrix to the core domain of integrase. *Cell* 83(4): 569–576.

67 Gallay P, Hope T, Chin D, Trono D. (1997) HIV-1 infection of nondividing cells through the recognition of integrase by the importin/karyopherin pathway. *Proceedings of the National Academy of Sciences USA* 94(18): 9825–9830.

68 Connor RI, Chen BK, Choe S, Landau NR. (1995) Vpr is required for efficient replication of human immunodeficiency virus type-1 in mononuclear phagocytes. *Virology* 206(2): 935–944.

69 Nitahara-Kasahara Y, Kamata M, Yamamoto T, Zhang X, Miyamoto Y, Muneta K, et al. (2007) Novel nuclear import of Vpr promoted by importin alpha is crucial for human immunodeficiency virus type 1 replication in macrophages. *Journal of Virology* 81(10): 5284–5293.

70 Rhee SS, Marsh JW. (1994) Human immunodeficiency virus type 1 Nef-induced down-modulation of CD4 is due to rapid internalization and degradation of surface CD4. *Journal of Virology* 68(8): 5156–5163.

71 Willey RL, Maldarelli F, Martin MA, Strebel K. (1992) Human immunodeficiency virus type 1 Vpu protein regulates the formation of intracellular gp160-CD4 complexes. *Journal of Virology* 66(1): 226–234.

72 Klimkait T, Strebel K, Hoggan MD, Martin MA, Orenstein JM. (1990) The human immunodeficiency virus type 1-specific protein vpu is required for efficient virus maturation and release. *Journal of Virology* 64(2): 621–629.

73 Neil SJ, Zang T, Bieniasz PD. (2008) Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu. *Nature* 451(7177): 425–430.

74 Douglas JL, Viswanathan K, McCarroll MN, Gustin JK, Fruh K, Moses AV. (2009) Vpu directs the degradation of the human immunodeficiency virus restriction factor BST-2/Tetherin via a {beta}TrCP-dependent mechanism. *Journal of Virology* 83(16): 7931–7947.

75 Gabuzda DH, Lawrence K, Langhoff E, Terwilliger E, Dorfman T, Haseltine WA, et al. (1992) Role of vif in replication of human immunodeficiency virus type 1 in CD4+ T lymphocytes. *Journal of Virology* 66(11): 6489–6495.

76 von Schwedler U, Song J, Aiken C, Trono D. (1993) Vif is crucial for human immunodeficiency virus type 1 proviral DNA synthesis in infected cells. *Journal of Virology* 67(8): 4945–4955.

77 Madani N, Kabat D. (1998) An endogenous inhibitor of human immunodeficiency virus in human lymphocytes is overcome by the viral Vif protein. *Journal of Virology* 72(12): 10251–10255.

78 Sheehy AM, Gaddis NC, Choi JD, Malim MH. (2002) Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. *Nature* 418(6898): 646–650.

79 Schroder AR, Shinn P, Chen H, Berry C, Ecker JR, Bushman F. (2002) HIV-1 integration in the human genome favors active genes and local hotspots. *Cell* 110(4): 521–529.

80 Poznansky M, Lever A, Bergeron L, Haseltine W, Sodroski J. (1991) Gene transfer into human lymphocytes by a defective human immunodeficiency virus type 1 vector. *Journal of Virology* 65(1): 532–536.

81 Fouchier RA, Simon JH, Jaffe AB, Malim MH. (1996) Human immunodeficiency virus type 1 Vif does not influence expression or virion incorporation of gag-, pol-, and env-encoded proteins. *Journal of Virology* 70(12): 8263–8269.

82 Aldrovandi GM, Zack JA. (1996) Replication and pathogenicity of human immunodeficiency virus type 1 accessory gene mutants in SCID-hu mice. *Journal of Virology* 70(3): 1505–1511.

83 Kim VN, Mitrophanous K, Kingsman SM, Kingsman AJ. (1998) Minimal requirement for a lentivirus vector based on human immunodeficiency virus type 1. *Journal of Virology* 72(1): 811–816.

84 Zufferey R, Nagy D, Mandel RJ, Naldini L, Trono D. (1997) Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. *Nature Biotechnology* 15(9): 871–875.

85 Dull T, Zufferey R, Kelly M, Mandel RJ, Nguyen M, Trono D, et al. (1998) A third-generation lentivirus vector with a conditional packaging system. *Journal of Virology* 72(11): 8463–8471.

86 Li JC, Yim HC, Lau AS. (2010) Role of HIV-1 Tat in AIDS pathogenesis: its effects on cytokine dysregulation and contributions to the pathogenesis of opportunistic infection. *AIDS* 24(11): 1609–1623.

87 Miyoshi H, Blomer U, Takahashi M, Gage FH, Verma IM. (1998) Development of a self-inactivating lentivirus vector. *Journal of Virology* 72(10): 8150–8157.

88 Zufferey R, Donello JE, Trono D, Hope TJ. (1999) Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors. *Journal of Virology* 73(4): 2886–2892.

89 Bonci D, Cittadini A, Latronico MV, Borello U, Aycock JK, Drusco A, et al. (2003) 'Advanced' generation lentiviruses as efficient vectors for cardiomyocyte gene transduction in vitro and in vivo. *Gene Therapy* 10(8): 630–636.

90 Yang L, Bailey L, Baltimore D, Wang P. (2006) Targeting lentiviral vectors to specific cell types in vivo. *Proceedings of the National Academy of Sciences USA* 103(31): 11479 –11484.

91 Liang M, Yan M, Lu Y, Chen IS. (2013) Retargeting vesicular stomatitis virus glycoprotein pseudotyped lentiviral vectors with enhanced stability by in situ synthesized polymer shell. *Human Gene Therapy Methods* 24(1): 11–18.

92 Yanez-Munoz RJ, Balaggan KS, MacNeil A, Howe SJ, Schmidt M, Smith AJ, et al. (2006) Effective gene therapy with nonintegrating lentiviral vectors. *Nature Medicine* 12(3): 348–353.

93 Paddison PJ, Silva JM, Conklin DS, Schlabach M, Li M, Aruleba S, et al. (2004) A resource for large-scale RNA-interference-based screens in mammals. *Nature* 428(6981): 427–431.

94 Berns K, Hijmans EM, Mullenders J, Brummelkamp TR, Velds A, Heimerikx M, et al. (2004) A large-scale RNAi screen in human cells identifies new components of the p53 pathway. *Nature* 428(6981): 431–437.

95 Yamagishi M, Ishida T, Miyake A, Cooper DA, Kelleher AD, Suzuki K, et al. (2009) Retroviral delivery of promoter-targeted shRNA induces long-term silencing of HIV-1 transcription. *Microbes and Infection/Institut Pasteur* 11(4): 500–508.

96 D'Costa J, Mansfield SG, Humeau LM (2009) Lentiviral vectors in clinical trials: current status. *Current Opinion in Molecular Therapeutics* 11(5): 554–564.

97 Sliva K, Schnierle BS. (2010) Selective gene silencing by viral delivery of short hairpin RNA. *Virology Journal* 7: 248.

98 Manjunath N, Wu H, Subramanya S, Shankar P. (2009) Lentiviral delivery of short hairpin RNAs. *Advanced Drug Delivery Reviews* 61(9): 732–745.

99 Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 411(6836): 494–498.

100 Brewster AM, Hortobagyi GN, Broglio KR, Kau SW, Santa-Maria CA, Arun B, et al. (2008) Residual risk of breast cancer recurrence 5 years after adjuvant therapy. *Journal of the National Cancer Institute* 100(16): 1179–1183.

101 Guo S, Liu M, Wang G, Torroella-Kouri M, Gonzalez-Perez RR. (2012) Oncogenic role and therapeutic target of leptin signaling in breast cancer and cancer stem cells. *Biochimica et Biophysica Acta* 1825(2): 207–222.

102 Xue RQ, Gu JC, Du ST, Yu W, Wang Y, Zhang ZT, et al. (2012) Lentivirus-mediated RNA interference targeting the ObR gene in human breast cancer MCF-7 cells in a nude mouse xenograft model. *Chinese Medical Journal (English Edition)* 125(9): 1563–1570.

103 Kaur P, Nagaraja GM, Asea A. (2011) Combined lentiviral and RNAi technologies for the delivery and permanent silencing of the hsp25 gene. *Methods in Molecular Biology* 787: 121–136.

104 Gu W, Putral L, Hengst K, Minto K, Saunders NA, Leggatt G, et al. (2006) Inhibition of cervical cancer cell growth in vitro and in vivo with lentiviral-vector delivered short hairpin RNA targeting human papillomavirus E6 and E7 oncogenes. *Cancer Gene Therapy* 13(11): 1023–1032.

105 Ma NQ, Liu LL, Min J, Wang JW, Jiang WF, Liu Y, et al. (2011) The effect of down regulation of calcineurin Aalpha by lentiviral vector-mediated RNAi on the biological behavior of small-cell lung cancer and its bone metastasis. *Clinical & Experimental Metastasis* 28(8): 765–778.

106 ter Brake O, t Hooft K, Liu YP, Centlivre M, von Eije KJ, Berkhout B. (2008) Lentiviral vector design for multiple shRNA expression and durable HIV-1 inhibition. *Molecular Therapy* 16(3): 557–564.

107 Li M, Rossi JJ. (2005) Lentiviral vector delivery of siRNA and shRNA encoding genes into cultured and primary hematopoietic cells. *Methods in Molecular Biology* 309: 261–272.

108 Li MJ, Kim J, Li S, Zaia J, Yee JK, Anderson J, et al. (2005) Long-term inhibition of HIV-1 infection in primary hematopoietic cells by lentiviral vector delivery of a triple combination of anti-HIV shRNA, anti-CCR5 ribozyme, and a nucleolar-localizing TAR decoy. *Molecular Therapy* 12(5): 900–909.

109 DiGiusto DL, Krishnan A, Li L, Li H, Li S, Rao A, et al. (2010) RNA-based gene therapy for HIV with lentiviral vector-modified CD34(+) cells in patients undergoing transplantation for AIDS-related lymphoma. *Science Translational Medicine* 2(36): 36ra43.

110 Burnett JC, Zaia JA, Rossi JJ. (2012) Creating genetic resistance to HIV. *Current Opinion in Immunology* 24(5): 625–632.

111 Biffi A, Montini E, Lorioli L, Cesani M, Fumagalli F, Plati T, et al. (2013) Lentiviral hematopoietic stem cell gene therapy benefits metachromatic leukodystrophy. *Science* 341(6148): 853–858.

112 Aiuti A, Biasco L, Scaramuzza S, Ferrua F, Cicalese MP, Baricordi C, et al. (2013) Lentiviral hematopoietic stem cell gene therapy in patients with Wiskott-Aldrich syndrome. *Science* 341(6148): 12331–12351.

3

CHAPTER 3

DNA Plasmids for Non-viral Gene Therapy of Cancer

Amer M. Najjar, Judy S.E. Moyes, and Laurence J.N. Cooper
The University of Texas MD Anderson Cancer Center, Houston, TX, USA

Delivery

There are approximately 1500 clinical trials that employ DNA currently open to accrual on ClinicalTrials.gov [1]. The majority of these use electroporation and hydrodynamic injection to achieve rapid and direct delivery of non-viral payloads. These methods of plasmid DNA delivery are optimized to address specific treatment needs with the clinical outcome being largely dictated by the nature of the disease and the therapeutic modality employed. Tissue-specific expression of systemically delivered plasmid DNA, such as by hydrodynamic injection, can be achieved by promoters engineered to drive transgene expression in desired cell populations. Alternatively, direct intravital electrotransfer of plasmid DNA may be utilized to limit expression to specific organs or *ex vivo* electrotransfer of DNA into defined cell types.

Electroporation

Electroporation is the application of an external electric field to cell suspensions or tissues to induce temporary plasma membrane permeablization that enables intracellular transfer of genetic material or drug molecules. Studies of electric current-induced cell membrane permeability in the 1960s helped establish the application of electric fields to facilitate nucleic acid uptake via transient formation of membrane pores [2]. One of the first successful demonstrations of gene transfer into eukaryotic cells by electroporation was performed by Neumann et al. [3]. The application of this gene-transfer technique has since been standardized and extended to genetically modify a wide variety of plant and animal cells. Direct electrotransfer of plasmid DNA and transgene expression in animal tissue was first demonstrated in mouse skeletal muscle over two decades ago [4]. Since then, the methodology has been expanded to many cell types and organs including cardiac myocytes, whole heart, tumor cells, and skin [5,6]. Skeletal muscle electroporation remains the most widely used and efficient plasmid-transfer technique that can yield high and sustained levels of gene expression [7]. This approach can be harnessed to systemically express therapeutic proteins into circulation, for example, by converting muscle into endocrine tissue [8]. Electrotransfer of DNA into the skin has appeal due to accessibility and its less invasive nature. However, the physical barrier formed by the *stratum corneum* significantly reduces transfection efficiency and rapid regeneration of the epidermal layer contributes to the gradual decline of transgene expression [9]. These optimized DNA electrotransfer techniques have spawned a diverse array of therapeutic applications, which can be grouped into three general categories: restoration of deficient or defective gene products, vaccination achieved from (transient) expression of antigens and intratumoral delivery

Cancer Gene Therapy by Viral and Non-viral Vectors, First Edition. Edited by Malcolm K. Brenner and Mien-Chie Hung.

and expression of transgenes (coding for antiproliferative, cytokine, and tumor-targeting products to induce and/or augment immunity against tumor-associated antigens).

Mechanism of electroporation

As illustrated in Figure 3.1, intracellular electrotransfer of nucleic acids is a multistep process that involves interaction of DNA molecules with the destabilized membrane, passage across transient pores, and transport to the nucleus for subsequent expression [10]. Prior to electrical induction, nucleic acids must be present in close proximity to, or in direct contact with, cell membranes. In the following step, electric field pulses destabilize cell membranes and induce the transient formation of nanopores through which nucleic acids flow. Cells temporarily exposed to electric fields mimic capacitors as their nonconductive bipolar lipid membranes behave as dielectric insulators separating two oppositely charged extracellular and intracellular ionic environments. Mathematical models suggest that membrane pores form when electric field lines induce cellular

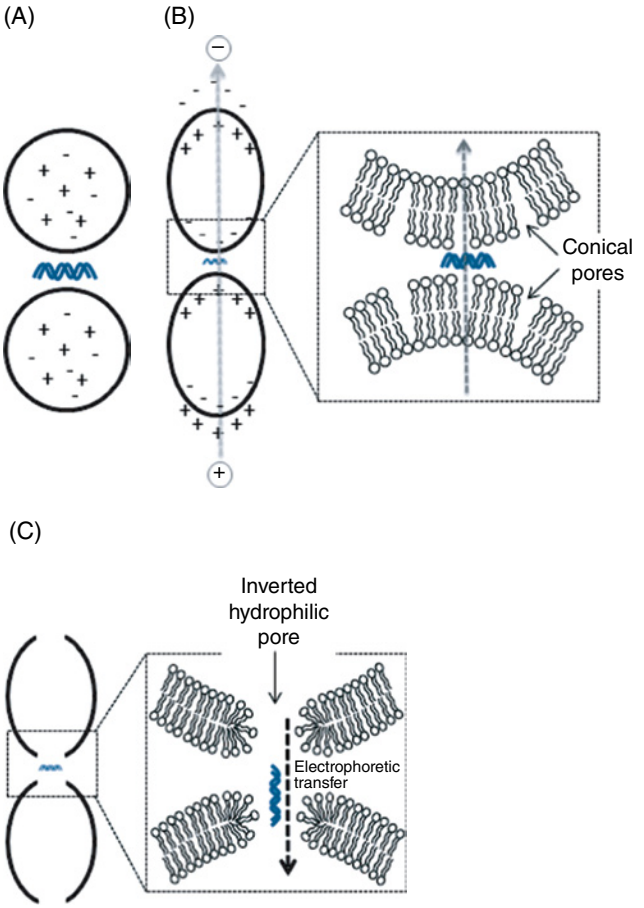


Figure 3.1 Model of electroporation leading to channel formation and DNA transfer. (A) DNA is present in the proximity or in direct contact with cell membranes prior to the application of an electric field. (B) Upon application of an electric current, the cells elongate along electric field lines. Consequently, the curvature radii at the apices of the aligned cells increase and the internal and external charges redistribute to form a transmembrane potential. Subsequent discharge of electric potential across the membrane leads to the formation of conical pores that permit ion flux. (C) Under the continuous influence of electric field currents, the pores coalesce to form larger inverted hydrophilic channels that facilitate the passage of nucleic acid. Intracellular transport of nucleic acid may be passive or may be facilitated by the application of electrophoretic currents.

alignment and membrane elongation resulting in reduced curvature radii at the apices. When the dielectric field strength of the membrane is exceeded, allowing the electric potential to permeate the membrane, small conical pores form and eventually coalesce to form larger inverted hydrophilic pores that remain intact for minutes following electric field induction. These pores seal when the membranes revert to ground state [11]. Exposure of cells to electric fields may potentially result in an undesired side effect of electrofusion, where oppositely charged proximal ends of aligned polarized cells fuse, leading to loss of cell viability [12]. Following membrane permeablization, nucleic acids passively enter cells based on the concentration gradient. Low-voltage pulses can also promote intracellular translocation of DNA molecules into the permeabilized cells by electrophoretic force transfer [13]. The transient pores close, entrapping the nucleic acids in the cytosol. The final step requires movement of the introduced DNA across the nuclear envelope for the expression of transgenes. Nuclear translocation of plasmids can be further advanced by electrophoretic currents produced by continued application of an electric field [14] and on the microtubule cytoskeletal network, forming complexes with importin α1 and β1, for trafficking and subsequent nuclear import [15].

An *ex vivo* electrotransfer technique termed nucleofection has been developed to enhance nuclear transport and subsequent expression of electroporated nucleic acids in postmitotic or quiescent cells. Nucleofection combines cell-specific reagents and typically short high-voltage pulses that enlarge or create transient nuclear pores to improve the efficiency of non-viral gene transfer through nuclear electrophoretic transfer while maintaining cell viability [16]. Buffering solutions mixed with the DNA protect cells from damaging free radicals, further enhancing cell survival [17]. The process of nucleofection does not appear to alter the functional and differentiation potential of transfected T cells or stem cells [18]. Indeed, we have adapted the nucleofector technology for human application and are currently undertaking four clinical trials (ClinicalTrials.gov identifiers NCT00968760, NCT01497184, NCT01362452, and NCT01653717) at the MD Anderson Cancer Center in Houston, TX, USA, based on this approach to non-viral gene transfer (Table 3.1) [19,20].

The efficiency of electroporation-mediated gene transfer can be improved by varying the pulse length, number of pulses, and field magnitude. Cell type, size, number, and health, as well as *ex vivo* culture conditions and the *in vivo* extracellular milieu, govern the successful application of electrotransfer. Given the variability of conditions and environments, empirical determination of electrotransfer parameters is typically required to achieve the desired levels of transgene expression while minimizing cell damage. For example, tissues that contain tight cell junctions effectively

Table 3.1 Ongoing clinical trials targeting B-cell malignancies infusing T cells (i) genetically modified *ex vivo* using the *Sleeping Beauty* (SB) system to express a CD19-specific chimeric antigen receptor and (ii) propagated on engineered artificial antigen-presenting cells.

ClinicalTrials.gov identifier	*Goal*
NCT00968760	Determine the persistence and maximally tolerated dose of patient-derived genetically modified T cells after autologous HSCT.
NCT01497184	Determine the persistence and maximally tolerated dose of donor-derived genetically modified T cells after allogeneic HSCT from related and unrelated donors (including haploidentical donors).
NCT01362452	Determine the persistence and maximally tolerated dose of donor-derived genetically modified T cells after allogeneic umbilical cord blood transplantation.
NCT01653717	Determine the persistence and maximally tolerated dose of autologous genetically modified T cells after chemotherapy.

HSCT, hematopoietic stem cell transplantation.

behave as single-cell units capable of continuous electrical conductivity and consequently require a lower electric field threshold to induce permeability. Nonhomogeneous and variable extracellular milieu can complicate the empirical determination of suitable electroporation conditions. In contrast to *in vitro* electroporation parameters which can be controlled and standardized, the complex *in vivo* architecture is often difficult to model and thus parameters cannot be readily cross-applied. Electrotransfer of DNA into organs and tissues is further challenged by their predominant composition of quiescent cells, which are less amenable to gene transfer than proliferating cells. To help predict the optimal electroporation parameters required for *in vivo* gene transfer, three-dimensional spheroid models have been used to mimic *in vivo* tissue environments [21]. Plasmid size and structure also contribute to the efficiency of DNA electrotransfer. Small plasmids tend to be more readily transferred than larger DNA species [22]. Thus, tightly knotted minicircle DNA molecules, produced by the action of type II DNA topoisomerase, yield higher transfection rates [23].

Routes of DNA electrotransfer

As mentioned, the electrotransfer of plasmid DNA into the skin is feasible and convenient due to its accessibility. Most applications require only short-term expression of transgenes, such as the transient expression of cytokines, stimulatory hormones, or vaccine antigens. The relatively large distances between electrodes in traditional caliper or plate devices can result in significant muscle stimulation and associated pain. The spacing also compromises control and direction of the applied field direction, curtailing the efficiency of transfection. As a result, multielectrode array devices have been developed to improve the efficiency of plasmid DNA delivery and thus expression of encoded transgenes in skin. These noninvasive devices are clinically appealing as they minimize skin damage and reduce unpleasant muscle twitching [24]. Multielectrode array devices consist of multiple electrode pairs positioned at fixed distances for intradermal plasmid delivery. Increasing the magnitude and duration of the applied voltage may enhance the level of transgene expression and can expand gene delivery to larger surface areas [25]. Therapeutic levels of transgene expression have been achieved with dermal electrotransfer of DNA plasmids coding for erythropoietin (EPO). EPO expression maximized at 2 weeks and returned to baseline at 8 weeks with a correlated elevation in mouse hemoglobin levels for more than 3 months [26].

Muscle is an attractive target tissue for the long-term expression of transgenes due to its accessibility and high efficiency of electrotransfer. Tight cell junctions enable continuous electrical conductivity requiring a lower electric field to induce permeability for efficient electrotransfer of genes [27]. Pretreatment of muscle with hyaluronidase can further enhance permeability to DNA and increase the efficiency of electrotransfer [28]. In preclinical studies, electroporated murine muscle has been exploited to function as an endocrine organ secreting CT-1 to correct hereditary progressive motor neuronopathy [29] or EPO to increase hematocrit [30]. Electrotransfer of DNA into muscle has also been used as gene-replacement therapy such as for Duchenne muscular dystrophy, where recombinant dystrophin was expressed to restore muscle function in dystrophic *mdx* mice [31]. Catheter-mediated electrotransfer of DNA into swine cardiac muscle has been successfully applied to deliver therapeutic transgenes without resulting in notable procedure-related cardiac abnormalities [32].

Some clinical scenarios require expression of desired gene products within target organs. Intravital DNA electrotransfer and expression in liver has been demonstrated in a rat model of dimethylnitrosamine-induced cirrhosis. Apoptosis of hepatocytes was inhibited by direct delivery of a DNA plasmid encoding Fas-Fc [33]. As indicated by the presence of hemagglutinin (HA)-specific IgA antibody in circulation, intranasal delivery of vaccine DNA encoding HA from influenza A H5N1 or H1N1 2009 immunized mice against influenza. This direct tissue transfection was enhanced by inclusion of polyethylenimine as a DNA carrier [34].

Hydrodynamic Injection

Hydrodynamic DNA delivery is based on the rapid application of an osmotic pressure gradient induced by high-volume injection of DNA solution to disrupt endothelial cell junctions in microcapillaries, thereby exposing and permeablizing parenchymal cells. A DNA solution is swiftly injected causing elevated hydrostatic pressure to force cellular permeablization and subsequent intracellular delivery of the genetic material. It has been applied for delivery of transgene resulting in systemic levels of the encoded protein.

This technique was first demonstrated by Budker et al. when it was employed to transfer naked DNA into the skeletal muscles of rats [35]. Systemic administration and expression of plasmid DNA was subsequently demonstrated in mice and its application has since been widened to a variety of animal disease models [36]. Indeed, promising preclinical data have been obtained in large animals, using a dog model of hemophilia [37].

Hydrodynamic injection has been used to deliver transgenes to a variety of tissues and organs including liver and skeletal muscle [38,39]. The longevity and levels of gene expression of hydrodynamic injections have been improved using these models. This has resulted in the preclinical application of hydrodynamic gene delivery for gene therapy of genetic deficiencies such as Fabry disease and phenylketonuria [40,41], and also non-genetic abnormalities such as diabetes and liver degeneration [42,43].

Experimentally, hydrodynamic injection via the tail vein offers the most convenient method of transgene expression in the liver for specific liver-targeting applications or for systemic gene product expression, where the liver serves in the capacity of an endocrine organ. This protocol entails a rapid (5–7 s) bolus injection of a large volume of DNA solution in physiological buffer, equivalent to about 10% of the body weight, via the tail vein of rodents. The introduced volume bolus travels via the inferior vena cava to the heart where it causes cardiac congestion that forces the injected volume retrograde to the liver via the hepatic vein. As illustrated in Figure 3.2, the rapid increase in blood volume disrupts the endothelial cell junctions and inflates the hepatocytes, inducing the formation of transient membrane pores (hydroporation) through which naked DNA enters into the cytosol. Other models suggest entry of DNA into the cytosol via vesicles created by macropinocytosis [44]. This mechanism of hydrodynamically mediated DNA delivery to the liver is supported by the predominant expression of transgenes within the hepatic pericentral region [44] and

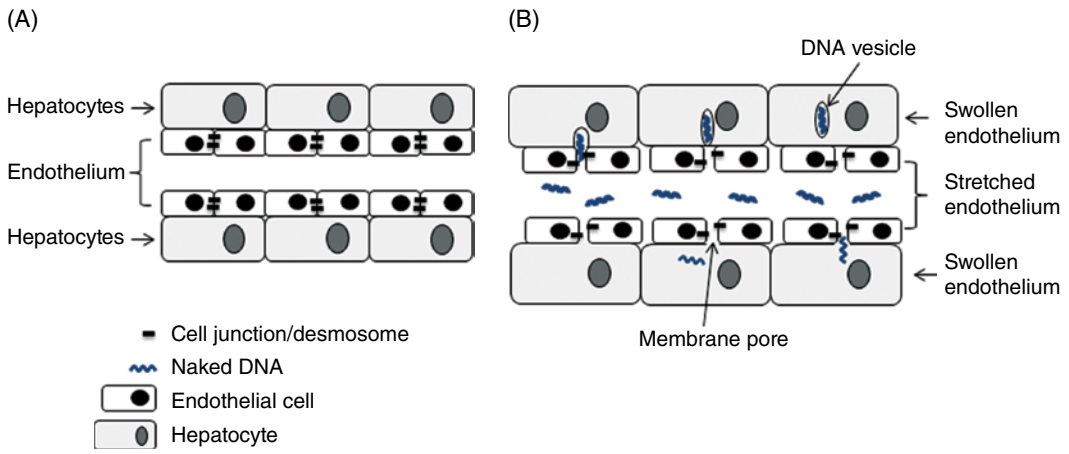


Figure 3.2 Mechanism of hydrodynamic gene delivery. (A) Tight junctions between endothelial cells form the vascular wall surrounded by hepatocytes in undisrupted liver tissue. (B) Upon hydrodynamic injection of naked DNA solution, the rapid increase in vascular volume disrupts the tight endothelial cell junctions, placing the hepatocytes in direct contact with the circulation. Transient pores form in the cell membranes allowing naked DNA in circulation to enter the cytosol. Other models suggest entry of DNA into the cytosol via vesicles created by macropinocytosis.

the immediate increase in the concentration of liver enzymes in circulation, which indicates cytosolic leakage of the hepatocytes [45].

The abnormal osmotic pressure environment created by hydrodynamic injection may contribute to acute adverse effects. Hydrodynamic delivery is associated with transient heart function irregularity, elevated venous pressure, liver fenestrae enlargement, and damage to hepatocytes [46]. These irregularities, however, are usually transient in nature and resolve within 72 h of injection [45]. Although testing in large porcine and canine models has been undertaken as a step towards clinically implementing hydrodynamic delivery of DNA plasmids, safety concerns over the potentially lethal acute side effects in a clinical setting have thus far limited its application to experimental disease models.

Liposomes

Liposome-mediated delivery of DNA offers several advantages over viral and other non-viral transfer methods. Cationic liposome-DNA complexes are safe and efficient vehicles for transferring DNA into cells *in vitro* and *in vivo*. The positively charged lipids and the negatively charged DNA spontaneously form precise complexes with defined morphologies upon mixing. The DNA–liposome complex morphologies are largely dependent on the lipid formulation used. DNA–liposome complexes are nonimmunogenic, enabling repetitive systemic delivery of therapeutic genes. Furthermore, the nucleic acid size cargo capacity is unlimited, allowing molecules ranging from single nucleotides to artificial chromosomes to be delivered. The relative low cost of production and efficient scalability are additional advantages that promote the utilization of liposome-based DNA delivery [47].

Nucleic acid loads are delivered in liposomes into target cells by either endocytosis or fusion with the cell membrane. Endocytosis-mediated delivery involves several steps, starting with binding of the DNA–liposome complex to the cell membrane followed by internalization into endosomes that fuse with intracellular lysosomes. The DNA–liposome complexes are broken down by lytic enzymes in the lysosomes and the freed DNA then enters the nucleus through the nuclear pores or during cell division as the nuclear envelope dissolves and reforms [48].

Although *in vitro* transfection of cultured cells with cationic DNA–liposome complexes can be efficient, it does not necessarily correlate with the efficacy of *in vivo* gene delivery following systemic administration. In circulation, DNA–liposome complexes are subjected to pharmacokinetic and metabolic forces that affect their stability, biodistribution, and ultimately potential for expression in target tissues. In addition, the capacity of this approach to deliver DNA to deep tissue regions by traversing tight cell junctions is limited. These limitations have been addressed with modified lipid formulations that prolong stability and improve tissue extravasation. Addition of polyethylene glycol, for example, extends the half-life of liposomes from minutes to hours in circulation. Such complexes, however, may impede gene delivery to cells by hindering the ionic interactions between the DNA–liposome and the cell membrane. Some modified lipid formulations can enhance deep tissue penetration as DNA-encapsulating bilamellar invaginated vesicles formed with 1,2-bis (oleoyloxy)-3-(trimethylammonio)propane (DOTAP) and cholesterol formulations exhibit enhanced tissue extravasation properties [49].

Sonoporation

Ultrasound-mediated DNA transfer using piezoelectric transducers to transiently disrupt cellular structures allows naked DNA to transport across cellular membranes. This method relies on cell exposure to ultrasound resonance (e.g. 980kHz) for up to a few seconds to enable gene transfer [50]. Sonoporation has found application in targeting tissues/organs for gene transfer that are difficult to modify with electroporation or hydrodynamic methods. The transfection efficiency of sonoporation is further enhanced by inclusion of (clinical-grade) microbubble solutions (such as Optison and Levovist), which increase ultrasound-mediated uptake of DNA by 10-fold [51]. These echo-contrast agents enhance the acoustic cavitation effect leading to significant improvements in the transfection of target tissue without any indications of toxicity [52].

Sonoporation has been utilized to genetically modify the central nervous system through its capacity to disrupt the blood–brain barrier [53].

DNA can be transferred to specific regions of the spinal cord by intrathecal injection into specific regions followed by ultrasound treatment of the exposed region to facilitate transfer [54]. This capacity to genetically modify the spinal cord is augmented by the inclusion of an echo-contrast agent [55]. Another application is vascular transfection using intravascular ultrasound catheters to deliver a therapeutic gene, such as to prevent restenosis following balloon angioplasty of porcine coronary arteries [56]. Other preclinical applications of sonoporation include direct DNA delivery into tumors to induce immunity [57], into mouse Achilles tendons to promote tissue regeneration [58], and into rat kidney to transfect the glomeruli, tubules, and interstitial area for the potential treatment of renal diseases [59].

Physical Disruption

High-throughput mechanical deformation to induce pore formation in cell membranes is being developed as an *ex vivo* approach to introduce genetic material. This is an emerging technology that overcomes the limitations of other approaches involving direct manipulation and instrumentation such as micro-injection. These microfluidic devices temporarily distort cells through constricted channels that are 30–80% narrower than the cell diameter. Compression and shear forces induce the formation of transient membrane pores through which DNA, proteins, or nanoparticles in the surrounding buffer pass into the cells. Over 80% of transfected cells remain viable upon return to physiological growth conditions [60,61]. This technology is particularly useful for the genetic modification of cells that are typically difficult to transfect using conventional methods. These microfluidic devices have appeal as they can be fabricated and operated to genetically modify defined cell types and are disposable, precluding cross-contamination between experiments.

Expression of Transgenes from DNA Plasmid

Improving Transgene Expression

Transient and stable expression of transgenes encoded by introduced plasmid DNA *in vivo* is dependent on transfection efficiency, cell viability, and integration [copy number, site(s) of insertion into genome, epigenetic modification]. Features of plasmid design can improve transgene expression to achieve desired therapeutic effect. In general, smaller plasmids (such as those lacking antibiotic resistance genes) tend to yield higher transfection efficiencies [62]. These minimized plasmid species have reduced bacterial sequence content, which is known to induce innate immune responses that may gradually silence transgene expression. To avoid the use of bacterial drug-resistance genes in plasmids, amber mutation suppressor tRNA genes have been used to selectively clone plasmids in specialized *Escherichia coli* containing an amber mutation in the *thyA* gene [63].

Long-term expression of integrated DNA is also dependent on the types of promoters and enhancers used to drive expression of the transgene. For example, the cytomegalovirus (CMV) promoter may initially yield high levels of expression for a few days following hydrodynamic delivery. However, this expression declines rapidly, lasting only up to 3 weeks [64]. Other promoters are capable of sustaining the long-term expression of transgenes. For example, we and others use human elongation factor 1 hybrid promoter to express transgenes in T cells for clinical application [65]. Inclusion of simian virus 40 (SV40) enhancer sequences can also prolong transgene expression [66]. Another viral enhancer, from CMV, appended to the 5′ end of the ubiquitin B UBB promoter (CMV-Ub), increased persistence of transgene expression [67]. Insertion of a poly-A tail downstream of transgenes augmented the abundance of transcribed mRNA by increasing the half-life of the messenger product [22]. Furthermore, downstream incorporation of a human T-lymphotropic virus type I (HTLV-I) R region has been shown to increase efficiency of mRNA translation [68]. DNA plasmids containing a scaffold/matrix-attached region (S/MAR) exhibit sustained episomal replication, prolonged transgene expression, and resistance to silencing in dividing cells *in vitro* and following hydrodynamic delivery into mice [69].

Codon optimization can increase the longevity of transgene expression and enhance the level of protein produced. This manipulation has improved the efficacy of DNA vaccines by elevating expressed antigen levels and by reducing the required dose of

DNA needed to achieve therapeutic outcome. The augmented production of recombinant antigen improves T-cell responses, as demonstrated with codon-optimized DNA vaccines in mouse models of tetanus infection and malaria [70,71]. Codon optimization has also been used to improve DNA vaccines against viral infections, as low levels of expressed antigen limits their therapeutic potential. This is evident with human immunodeficiency virus (HIV) DNA vaccines, which often fail to induce an adequate immune response in clinical trials. Such optimization may potentially improve immunogenicity to HIV antigens by increasing their levels in circulation following DNA delivery as illustrated with codon-optimized HIV Tat and Env gp160 antigens [72,73]. The immunogenicity of other poorly expressed viral antigens has also been improved by codon optimization. Codon optimization of the respiratory syncytial virus *F* gene led to an increase in therapeutic antibody levels in mice [74]. Similarly, optimized L1 protein of the human papillomavirus type 11 was expressed to a greater level in mice compared to the native sequence [75]. Gene-replacement therapy also benefits from codon optimization to increase production of a desired protein. In a mouse model of Duchenne muscular dystrophy, intramuscular and systemic administration of codon-optimized microdystrophin-encoding plasmids significantly increased levels of microdystrophin mRNA, restoring muscle function and substantially protecting against contraction-induced injury [31]. Electroporation of a mammalian codon-optimized *EPO* transgene into murine skeletal muscle resulted in increased circulatory levels of this hormone due to enhanced transcription that was sustained for over a year [76]. Codon optimization has also been used to improve the expression of transgenes from DNA plasmids introduced into human cells *ex vivo* for *in vivo* clinical application. For example, the transgene coding for a chimeric antigen receptor (CAR) used to redirect the specificity of T cells has been codon-optimized and is used in clinical trials to treat B-cell malignancies [77].

Inactivation of Transgene Expression

Loss or rapid decline of transgene expression may be due to many factors including promoter inactivation via methylation or clearance of transfected cells by innate or antigen-specific pathways. The loss of transgene expression under control of a CMV promoter may be due to methylation of the plasmid DNA [64]. Inclusion of *cis*-acting elements derived from the native genomic DNA may limit silencing. For example, a 19 kb genomic fragment containing the α1-antitrypsin (AAT) sequence can help maintain expression [78].

Noncoding sequences in plasmids can induce an undesired immune response or inflammatory reactions that may lead to the clearance of transfected cells. Immune responses have been associated with cytosine-guanine (CpG)-rich sequences in DNA plasmids [79]. Inflammatory responses following intramuscular or hydrodynamic administration of plasmid DNA are directly correlated with the number of CpG motifs in DNA plasmids [80]. The low abundance of CpG motifs in the human genome, compared to prokaryotic chromosomes, may be a factor contributing to the recognition of plasmid DNA as a foreign substance by mammalian immune systems [81]. This immune response appears to be specific to unmethylated CpG motifs in plasmid DNA as methylation of these dinucleotides prior to administration reduces inflammation and extends the presence of plasmids in target tissue [79,82]. However, methylation of CpG motifs may also lead to transgene silencing if the methylation loci exist within regulatory elements of the plasmid [83]. Enhanced and persistent transgene expression was observed with minicircle DNA engineered to be devoid of CpG motifs and minimized to only contain the transgene expression cassettes [84]. Thus, CpG-reduced plasmid vectors are employed to enhance long-term transgene expression required to achieve a desired therapeutic outcome.

Safety

Safety is an advantage provided by non-viral plasmid DNA-based gene therapy as it avoids complications associated with use of virus-based vectors to achieve gene transfer. Nevertheless, several precautionary factors should be taken into consideration to minimize associated health risks while maximizing therapeutic outcomes.

Plasmid DNA Purity and Immunogenicity

Impurities in plasmid DNA preparations used for direct administration may cause necrosis and inflammation in target tissue that may compromise efficacy or pose a health risk to recipients. Therefore, additional purification may be necessary to produce pharmaceutical DNA by removing any contaminants in the final DNA preparation that may cause toxicity and compromise efficacy. Lipopolysaccharide (endotoxin) is a common inflammatory contaminant derived from *E. coli* that can be removed using well-established chromatography and extraction protocols [85]. Another contaminant, colonic acid, may be removed by colonic acid-degrading enzyme [86]. Size-exclusion chromatography can remove extraction detergent additives and animal-derived enzymes [87]. Use of activated charcoal in DNA-purification protocols reduces the presence of interfering nucleotide fragments that may compromise the transfection efficiency and the efficacy of plasmid gene therapy [88]. Contamination of plasmid preparations with bacterial genomic DNA, which has been shown to cause necrosis in muscle tissue following hydrodynamic limb vein injection, can be removed by enzymatic digestion [89]. As mentioned, CpG motifs in plasmid DNA may also cause undesired immune responses, which can be minimized by using plasmids devoid of CpG-rich bacterial backbones.

Delivery Mechanisms

Delivery mechanisms also pose potential health risks arising from physical and physiological responses. These physical stresses should be considered when administering DNA-based therapies. As mentioned, hydrodynamic injection induces transient changes in osmotic pressure that may cause transient physical and mechanical stress on the circulatory system leading to acute tissue damage. Tissue inflammation and necrosis can also occur at sites of intramuscular injection and electroporation of DNA. Epidermal electrotransfer of DNA may result in necrosis [90], but reduction of applied electric currents may lower tissue damage and inflammation [91]. The degree to which these factors hinder clinical outcome is highly dependent on the disease mode and therapeutic goal. With these factors controlled and risks minimized, plasmid DNA-based therapies emerge as an attractive approach to gene therapy *in vitro* and *in vivo*.

Much safety and feasibility data related to plasmid-based gene therapy stem from phase I clinical trials of DNA-based vaccines targeting tumors and opportunistic infections. These trials provide data attesting to the overall safety and feasibility of plasmid gene therapy. In general, DNA plasmid vaccines are well tolerated and cause no clinically significant adverse effects. DNA vaccination with plasmids coding for HIV gag co-administered with plasmids encoding cytokine adjuvants were well tolerated [92]. DNA plasmid vaccines against human CMV can effectively induce humoral and innate immunity and are well tolerated, inducing only mild side effects of malaise, myalgia, and pain at sites of injection [93]. DNA vaccines against the H5N1 subtype of avian influenza virus were also shown to be safe and effective [94]. These representative clinical trials attest to the safety of utilizing naked plasmid DNA in gene therapy applications.

Preclinical and clinical gene therapy trials of ischemia and muscular disorders have also provided significant safety data pertaining to the intramuscular delivery of DNA. Intramuscular electrotransfer of plasmid DNA is generally well tolerated in patients. Plasmids encoding human hepatocyte growth factor (HGF) or vascular endothelial growth factor (VEGF) have been used in attempts to alleviate critical limb ischemia (CLI) by restoring circulation. Intramuscular and intravascular (hydrodynamic) injections in limb veins to deliver angiogenic genes for therapy of muscle disorders in rats and nonhuman primates were found to be well tolerated [95]. A phase I study administering DNA encoding HGF by intramuscular injection of patients with CLI established that plasmid delivery was well tolerated and safe in human subjects [96]. Clinical trials to treat Duchenne muscular dystrophy by restoring the expression of recombinant dystrophin by intra-arterial delivery to skeletal muscle [97] or by intramuscular administration of plasmids [98] have also demonstrated the safety of intramuscular DNA delivery. Direct electrotransfer of DNA plasmids into the myocardium has also been shown to be well tolerated [99]. Indeed, intramyocardial injections of DNA encoding VEGF were safely performed in patients with refractory angina [100].

Genomic Integration

DNA plasmid expression systems have been developed to achieve stable transgene integration for sustained gene expression. These systems, *piggyBac* (PB), *Sleeping Beauty* (SB), and phiC31 integrase-based transposon/transposase (Figure 3.3), are based on cotransfection of transgene- and integrase-encoding plasmids that facilitate transgene integration into genomic homology sites. The PB transposase system can express a large cargoload and has been used to genetically modify many cell types, including T cells and stem cells [101,102]. We have adapted the SB transposon/transposase system for human application and opened four clinical trials using this non-viral approach to genetically modify T cells (Table 3.1). The SB-derived integrase uses a cut-and-paste mechanism to insert the transgene from the SB transposon into TA dinucleotide repeats scattered throughout the genome [103,104]. As may occur with genetic modification using integrating virus or alternative transposon/transposase systems, insertional mutagenesis is a potential risk factor associated with genomic integration of plasmid DNA. Clinical-grade T cells genetically modified using the SB system typically exhibit an average of one to two genomic insertions of the transgene per cell [105]. This has implications for the improved safety of SB systems used in human trials, as a decrease in the number of insertions presumably reduces the chance of inadvertent genomic integration events that may potentially be oncogenic. This risk may be further reduced if the insertion site can be targeted to a "safe harbor" using artificial genome-editing enzymes such as zinc-finger nucleases (ZFNs) [106], transcription activator-like (TAL) effector nucleases (TALENs) [107], or clustered regularly interspaced short palindromic repeat (CRISPR)-associated (Cas) systems [108]. Inclusion of p5 integration efficiency elements have also been used to direct genomic integration into the adenovirus-associated virus (AAV) integration site [109]. The phiC31 integrase coexpression system has been used to restrict transgene integration into specific loci based on att sequence homology [110]. However, this integrase leads to a DNA damage response and chromosomal aberrations which preclude the human application of this system [111].

As an alternative to genomic integration, DNA-based episomal-expression systems have also been developed. Sustained expression from episomal plasmids is largely restricted to quiescent cells. However, inclusion of SV40 107/402-T sequences enables introduced plasmids to replicate as chromosomes yielding thousands of copies and thereby enhancing and sustaining transgene expression [112]. The application of nonintegrating episomal plasmids in humans has not yet been achieved.

Therapeutic Applications

DNA Vaccines

DNA plasmids are well suited for vaccination as immunity does not necessitate long-term sustained expression and can be established from short-term expression of antigen. Plasmid-based vaccines provide multiple advantages which include ease of production, rapid scalability to express multiple antigen epitopes, and configurability to target specific antigens, bypassing the need to use potentially harmful attenuated pathogens as immunogens.

The capacity to induce immunity against plasmid-encoded antigens has been demonstrated with a variety of different viral proteins. DNA vaccines encoding the hepatitis C virus nonstructural 5A protein induced T-cell immunity [113]. The intradermal electrotransfer of DNA plasmids encoding influenza virus H5 hemagglutinin and nucleoprotein of influenza H1N1 elicited and sustained humoral and cellular immune responses [91]. Intramuscular electroporation of plasmids encoding neutralizing hemagglutinin-specific monoclonal antibodies were detected up to 130 days following administration in mice and provided adequate protection against influenza virus challenge [114]. DNA vaccines against two flaviviruses, Japanese encephalitis virus (JEV) and West Nile virus (WNV), were developed based on the structural domain III (DIII) of E protein, which is known to induce neutralizing antibodies. Delivery of WNV DIII-encoding plasmids induced humoral immunity and coimmunization with interleukin (IL)-15 further enhanced the response [115]. Intradermal electroporation of mice with a plasmid encoding HIV-1

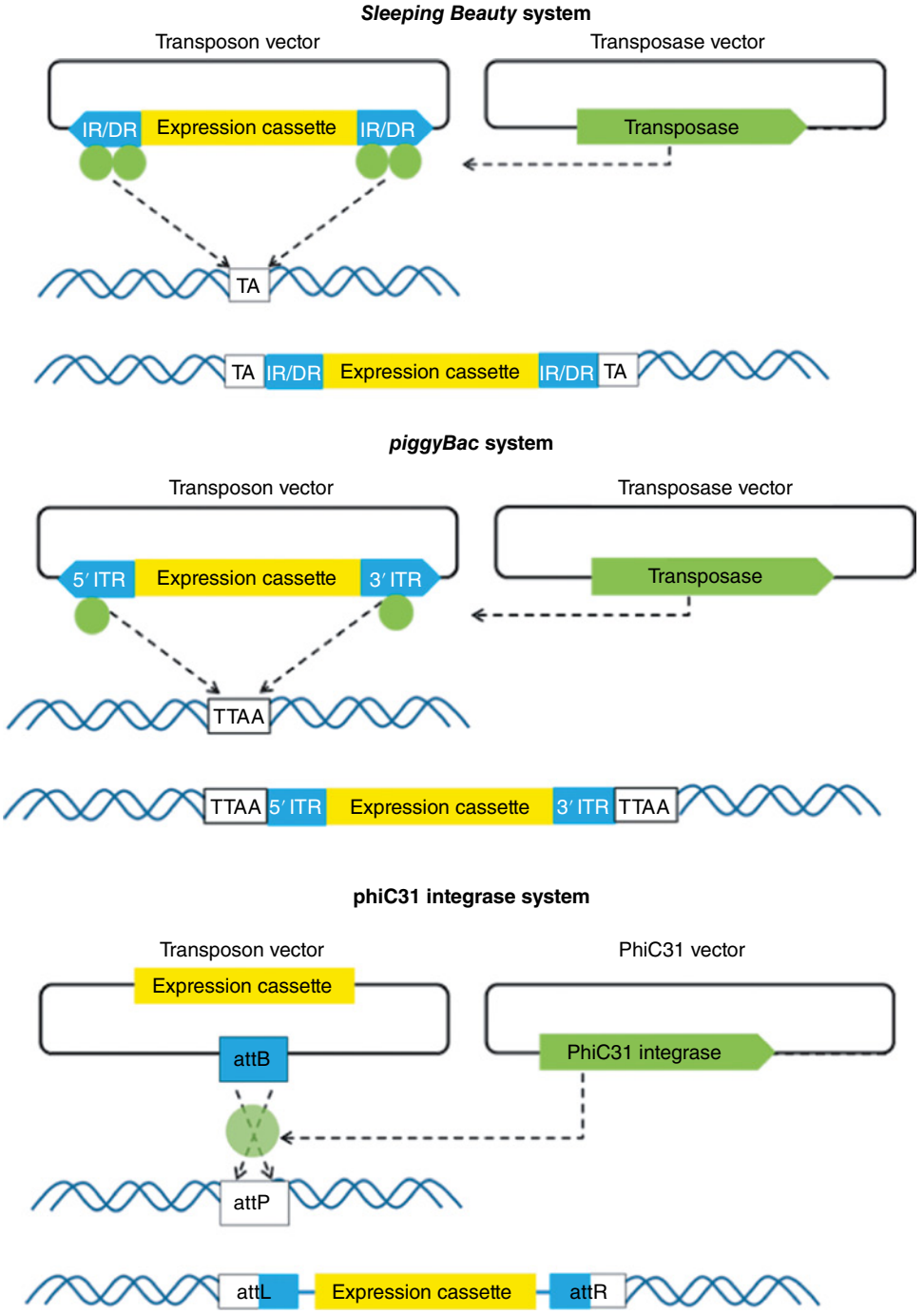


Figure 3.3 Non-viral genomic integration systems for stable expression of therapeutic genes. These systems are based on the cotransfection of expression cassettes and transposase genes to facilitate genomic integration. These genes may be on separate vectors, as shown, or encoded on the same plasmid. In the *Sleeping Beauty* system, the expression cassette is flanked by inverted terminal repeat/direct repeat (IR/DR) sequences. Transposase binds to the IR/DR elements forming a synaptic complex that is transposed into genomic TA dinucleotide sites. The *piggyBac* expression cassette is flanked by inverted terminal repeat (ITR) elements that target TTAA sequences in the target cell genome. The phiC31 integrase system is based on recombination between two attachment (att) sites: attB on the vector and so-called pseudo-attP genomic sites that are similar to native bacterial attP sequences. The stably integrated expression cassette is flanked by the newly recombined att sites (attR and attL).

Gag induced strong cellular immune response against the antigen. Intramuscular injection, however, was not as effective in inducing a Gag-specific response [116].

Synchronous co-administration of multiple DNA plasmids coding for multiple proteins may generate a broad immune response. This multi-antigen expression strategy may be desirable in inducing adequate immunity against highly evasive targets such as HIV. Mice immunized with combinations of plasmids containing the avian influenza virus antigens HA, neuraminidase, nucleoprotein (NP), matrix protein 1, and matrix protein 2 genes exhibited resistance to challenge with virus [117]. HA and NP proteins coexpressed after intramuscular electroporation resulted in protective immunity against multiple influenza antigens [118].

Electroporation-based DNA vaccines have been tested in large animals such as cows and monkeys [119]. Immunization of macaques with multiple DNA plasmids encoding immunogens from simian immunodeficiency virus (SIV) elicited T cell and humoral immune responses against a broad range of viral antigens [120,121]. SIV-infected macaques immunized by electrotransfer of a DNA plasmid encoding HIV Gag into the muscle developed cellular immune responses which correlated with IL-15 expression and expansion of antigen-specific T cells [122].

In some applications, integral CpG motifs, which are inherently immunogenic, can provide an added benefit as adjuvants to DNA vaccines. These motifs are recognized by Toll-like receptor (TLR) 9 on dendritic and B cells and thus promote cytokine release [123]. The cellular and humoral responses to hepatitis C virus E1 and E2 antigens encoded by DNA are enhanced by the presence of CpG-rich regions contained within these plasmids [124]. Inclusion of CpG motifs into DNA vaccines against HIV has improved the immune response against the HIV envelope [125]. Co-administration of short CpG oligodeoxynucleotides has also been used as a strategy to enhance the immunogenicity of DNA vaccines against hepatitis C virus to induce release of transforming growth factor (TGF)-β and IL-1α [126].

Adoptive T-cell Therapy

DNA plasmids have been used to genetically modify T cells, in compliance with current good manufacturing practices, that have then been administered in human trials. Electroporation has been employed to stably express DNA coding for CD19- and CD20-specific CARs [127,128]. These initial trials established the safety and feasibility of the electrotransfer of DNA expression vectors. However, despite the use of CAR molecules with an advanced design (third-generation technology activating T cells via three signaling motifs) the clinical data demonstrated minimal therapeutic impact. This was likely due to the lengthy culture period needed to retrieve clinically sufficient numbers of T cells following the low rates of integration of electrotransferred naked DNA during which time the biologic product may enter replicative senescence. Furthermore, the inclusion of genes expressed for *in vitro* selection using cytocidal concentration of antibiotics or to render the cells susceptible to conditional ablation *in vivo* may lead to a host immune response targeting infused T cells expressing the bacterial or viral immunogenic proteins. This has been overcome by the human application of a transposon/transposase from the SB system to improve the efficiency of integration following electrotransfer of DNA plasmids and selective propagation of CAR^+ T cells on designer artificial antigen-presenting cells [129]. The successful first-in-human application of the SB system provides a foundation for DNA plasmids to be tested in other clinical settings, such as infusing CAR^+ T cells with specificity other than CD19 and using this approach to non-viral gene transfer to genetically modify cells other than T cells.

Cytokine Therapy

DNA plasmids delivered by hydrodynamic injection or electroporation into muscle have been used to express recombinant cytokines to enhance humoral and cytotoxic immune responses against tumors or infectious agents. Hydrodynamic delivery and expression of IL-2 in liver has been shown to inhibit hepatocarcinoma growth [130]. IL-15 electroporation slows the growth of colon and bladder tumors by enhancing the infiltration of cytotoxic T cells [131,132]. Expression of an

IL-15–apolipoprotein A-I conjugate (ApoAI-IL15) extends the half-life of the cytokine in circulation by incorporating it into high-density lipoprotein. ApoAI-IL15 stimulates natural killer (NK) and T-cell production and accumulation in liver, spleen, and blood and exhibits therapeutic potential against metastatic lung cancer [133].

Direct intratumoral expression of IL-12 elicits an antitumor effect by enhancing NK and T-cell infiltration into tumors and by inhibiting angiogenesis [134]. The antitumor potential of plasmid-encoded IL-12 has also been demonstrated in several large animal models and clinical trials of melanoma patients and has been shown to be effective, safe, and well tolerated [135,136]. The antitumor activity of IL-12 may be further enhanced by sequential therapy with IL-27 as demonstrated in small animal models [137]. Coexpression of cytokines *in vivo* also serves as an adjuvant to DNA vaccine therapy. Plasmids encoding IL-12 and tumor necrosis factor (TNF) have been used as adjuvants to enhance cellular immunity to anti-HIV DNA vaccines by stimulating CD8+ T-cell response [138,139]. DNA vaccines against bacterial infections also benefit from adjuvant cytokine expression [140].

Antiangiogenesis and Antiproliferative Cancer Therapy

An approach to plasmid-based therapy of tumors is demonstrated through the expression of recombinant protein products with antiangiogenic or antiproliferative properties. The intratumoral electrotransfer of three plasmids encoding the antiangiogenic genes of ADAM-15, thrombospondin 1, and the soluble VEGF receptor 1 (sFlt-1) into B16F10 melanoma tumors doubled the survival time of mice and decreased the metastatic potential of the tumors [141]. Survivin-specific T cells were generated following intradermal electroporation of survivin-encoding DNA plasmid which inhibited angiogenesis in B16 melanoma tumors [142]. Intratumoral electrotransfer of plasmids encoding angiostatin and endostatin enhanced survival of mice expressing melanoma tumors [143]. *Ex vivo* electroporation of retinal or iris pigment epithelial cells stably expressing pigment epithelium-derived factor (PEDF), a potent antiangiogenic and neuroprotective factor, followed by subretinal transplantation has been proposed as a cell-based therapy of macular degeneration. The *PEDF* gene was stably integrated and sustained expression was maintained for over 6 months [144].

Autoimmune Diseases and Allograft Tolerance

DNA plasmids can be used to alleviate autoimmune diseases and improve allograft tolerance. The *ex vivo* electroporation of IL-10 into dendritic cells was used to suppress the development of experimental murine autoimmune optic neuritis and encephalitis [145]. Tolerance to allografts can be enhanced by the expression of recombinant IL-10 and HGF encoded by DNA plasmid [146].

Cardiac Disorders

DNA plasmid-based approaches have focused on atrial fibrillation, congenital abnormalities, and ischemic disorders. Pharmacologic and ablative therapies for atrial fibrillation have yielded suboptimal efficacy. A new candidate approach for atrial fibrillation is the targeting of parasympathetic signaling with plasmids expressing $G\alpha_i$ C-terminal peptide ($G\alpha_i$ctp) either alone or in combination with $G\alpha_o$ctp to control vagal-induced atrial fibrillation in the left atrium [147]. The feasibility of gene delivery into heart muscle has also facilitated the treatment of ischemic cardiomyopathy based on the induction of angiogenesis. Retrograde delivery of plasmid DNA into transiently occluded coronary sinus followed by electric pulse resulted in gene expression without immediate signs of toxicity [99]. Delivery and expression of angiogenic factors such as stromal cell-derived factor-1 in heart tissue have shown evidence of alleviating ischemic cardiomyopathy [148]. Large-animal electroporation-mediated delivery of VEGF into porcine heart has also been shown to increase cardiac expression of the angiogenic factor [149].

Ischemia

Alleviation of CLI is one of the most successful applications of DNA plasmid-based gene therapy and has been proven to be safe and feasible in numerous clinical trials. A randomized placebo-controlled

multicenter trial assessed the safety and efficacy of delivery of HGF transgene in patients with CLI. The intramuscular delivery of DNA plasmid was safe and demonstrated a favorable therapeutic impact [150]. Also, the expression of HGF from DNA plasmid injected into muscles of patients with CLI was shown to be safe, well tolerated, and resulted in improved prognosis [151,152]. Intramuscular delivery of VEGF- and HGF-encoding plasmids has shown increased perfusion [153]. Intramuscular administration of DNA coding for fibroblast growth factor 1 reduced the risk of amputation and death in patients [154], and intradermal electroporation of DNA coding for fibroblast growth factor-2 (FGF-2) led to sustained expression of this transgene, improved perfusion, and increased ischemic limb blood flow [155].

Endocrine

Plasmids have been used for the experimental treatment of diabetic neuropathy by enforcing the expression of neurotrophic placental growth factor-2 (PlGF-2) from muscle electroporated with DNA expression vector [156]. Plasmids have also been used to produce human growth hormone. A single electroporation of DNA yielded comparable results to repeated injections of growth hormone [157].

Summary

DNA plasmids can be used *in vitro* and *in vivo* to achieve short- and long-term gene expression. They are simple to generate and test and thus provide a nimble platform for assessing the therapeutic potential of transgenes. They can be readily produced at pharmaceutical grade by academic facilities and for-profit entities. The manufacture of clinical-grade DNA plasmids is a fraction of the cost of producing recombinant virus for human application. Given the safety track record of DNA plasmids, there is an established pathway and regulatory infrastructure for translating preclinical observations into gene therapy. Already, there have been notable successes achieved with plasmid-based gene therapy trials, most significantly demonstrating the safety and feasibility of DNA vaccines and the effective treatment of ischemic disorders. The emerging application of the SB system as a cost-effective and nimble approach to *ex vivo* genetic manipulation of clinical-grade T cells offers investigators a clear line of sight between immunology at the bench and immunotherapy at the bedside. The inherent advantages of scalability, customization, improved frequency of integration, and clinical feasibility will further expand the application of plasmid-based gene therapy in the treatment of human malignancy.

References

1 ClinicalTrials.gov. (2013) Bethesda: National Institutes of Health. http://clinicaltrials.gov/ct2/results?term=DNA&recr=Open.

2 Coster HG. (1965) A quantitative analysis of the voltage–current relationships of fixed charge membranes and the associated property of "punch-through". *Biophysics Journal* 5(5): 669–686.

3 Neumann E, Schaefer-Ridder M, Wang Y, Hofschneider PH. (1982) Gene transfer into mouse lyoma cells by electroporation in high electric fields. *EMBO Journal* 1(7): 841–845.

4 Wolff JA, Malone RW, Williams P, Chong W, Acsadi G, Jani A, et al. (1990) Direct gene transfer into mouse muscle in vivo. *Science* 247(4949 Pt 1): 1465–1468.

5 Lin H, Parmacek MS, Morle G, Bolling S, Leiden JM. (1990) Expression of recombinant genes in myocardium in vivo after direct injection of DNA. *Circulation* 82(6): 2217–2221.

6 Yang JP, Huang L. (1996) Direct gene transfer to mouse melanoma by intratumor injection of free DNA. *Gene Therapy* 3(6): 542–548.

7 Jaroszeski MJ, Gilbert R, Nicolau C, Heller R. (1999) In vivo gene delivery by electroporation. *Advanced Drug Delivery Reviews* 35(1): 131–137.

8 Goldspink G. (2003) Skeletal muscle as an artificial endocrine tissue. *Best Practice & Research Clinical Endocrinology & Metabolism* 17(2): 211–222.

9 Zhang L, Li L, Hoffmann GA, Hoffman RM. (1996) Depth-targeted efficient gene delivery and expression in the skin by pulsed electric fields: an approach to gene therapy of skin aging and other diseases. *Biochemical and Biophysical Research Communications* 220(3): 633–636.

10 Rols MP. (2008) Mechanism by which electroporation mediates DNA migration and entry into cells and targeted tissues. *Methods in Molecular Biology* 423: 19–33.

11 Teissie J, Rols MP. (eds) (1992) *Time Course of Electropermeabilization*. Boston: Birkhauser Press.

12 Jaroszeski MJ, Gilbert R, Fallon PG, Heller R. (1994) Mechanically facilitated cell-cell electrofusion. *Biophysics Journal* 67(4): 1574–1581.
13 Pavlin M, Flisar K, Kanduser M. (2010) The role of electrophoresis in gene electrotransfer. *Journal of Membrane Biology* 236(1): 75–79.
14 Neumann E, Kakorin S, Toensing K. (1999) Fundamentals of electroporative delivery of drugs and genes. *Bioelectrochemistry and Bioenergetics* 48(1): 3–16.
15 Badding MA, Lapek JD, Friedman AE, Dean DA. (2013) Proteomic and functional analyses of protein-DNA complexes during gene transfer. *Molecular Therapy* 21(4): 775–785.
16 Gresch O, Engel FB, Nesic D, Tran TT, England HM, Hickman ES, et al. (2004) New non-viral method for gene transfer into primary cells. *Methods* 33(2): 151–163.
17 Zeitelhofer M, Vessey JP, Xie Y, Tubing F, Thomas S, Kiebler M, et al. (2007) High-efficiency transfection of mammalian neurons via nucleofection. *Nature Protocols* 2(7): 1692–1704.
18 Singh H, Manuri PR, Olivares S, Dara N, Dawson MJ, Huls H, et al. (2008) Redirecting specificity of T-cell populations for CD19 using the Sleeping Beauty system. *Cancer Research* 68(8): 2961–2971.
19 Singh H, Figliola MJ, Dawson MJ, Olivares S, Zhang L, Yang G, et al. (2013) Manufacture of clinical-grade CD19-specific T cells stably expressing chimeric antigen receptor using sleeping beauty system and artificial antigen presenting cells. *PLoS One* 8(5): e64138.
20 Huls MH, Figliola MJ, Dawson MJ, Olivares S, Kebriaei P, Shpall EJ, et al. (2013) Clinical application of Sleeping Beauty and artificial antigen presenting cells to genetically modify T cells from peripheral and umbilical cord blood. *Journal of Visualized Experiments* 72: e50070.
21 Chopinet L, Wasungu L, Rols MP. (2012) First explanations for differences in electrotransfection efficiency in vitro and in vivo using spheroid model. *International Journal of Pharmacy* 423(1): 7–15.
22 Ribeiro S, Mairhofer J, Madeira C, Diogo MM, Lobato da Silva C, Monteiro G, et al. (2012) Plasmid DNA size does affect nonviral gene delivery efficiency in stem cells. *Cellular Reprogramming* 14(2): 130–137.
23 Tolmachov OE. (2010) Tightly-wound miniknot vectors for gene therapy: a potential improvement over supercoiled minicircle DNA. *Medical Hypotheses* 74(4): 702–704.
24 Heller R, Cruz Y, Heller LC, Gilbert RA, Jaroszeski MJ. (2010) Electrically mediated delivery of plasmid DNA to the skin, using a multielectrode array. *Human Gene Therapy* 21(3): 357–362.
25 Ferraro B, Heller LC, Cruz YL, Guo S, Donate A, Heller R. (2011) Evaluation of delivery conditions for cutaneous plasmid electrotransfer using a multielectrode array. *Gene Therapy* 18(5): 496–500.
26 Gothelf A, Hojman P, Gehl J. (2010) Therapeutic levels of erythropoietin (EPO) achieved after gene electrotransfer to skin in mice. *Gene Therapy* 17(9): 1077–1084.
27 Fear EC, Stuchly MA. (1998) Biological cells with gap junctions in low-frequency electric fields. *IEEE Transactions on Biomedical Engineering* 45(7): 856–866.
28 Molnar MJ, Gilbert R, Lu Y, Liu AB, Guo A, Larochelle N, et al. (2004) Factors influencing the efficacy, longevity, and safety of electroporation-assisted plasmid-based gene transfer into mouse muscles. *Molecular Therapy* 10(3): 447–455.
29 Lesbordes JC, Bordet T, Haase G, Castelnau-Ptakhine L, Rouhani S, Gilgenkrantz H, et al. (2002) In vivo electrotransfer of the cardiotrophin-1 gene into skeletal muscle slows down progression of motor neuron degeneration in pmn mice. *Human Molecular Genetics* 11(14): 1615–1625.
30 Terada Y, Tanaka H, Okado T, Inoshita S, Kuwahara M, Akiba T, et al. (2001) Efficient and ligand-dependent regulated erythropoietin production by naked dna injection and in vivo electroporation. *American Journal of Kidney Diseases* 38(4 Suppl 1): S50–S53.
31 Foster H, Sharp PS, Athanasopoulos T, Trollet C, Graham IR, Foster K, et al. (2008) Codon and mRNA sequence optimization of microdystrophin transgenes improves expression and physiological outcome in dystrophic mdx mice following AAV2/8 gene transfer. *Molecular Therapy* 16(11): 1825–1832.
32 Gwon HC, Jeong JO, Kim HJ, Park SW, Lee SH, Park SJ, et al. (2001) The feasibility and safety of fluoroscopy-guided percutaneous intramyocardial gene injection in porcine heart. *International Journal of Cardiology* 79(1): 77–88.
33 Miyahara T, Umeda Y, Yoshikawa S, Matsuno Y, Iwata H, Takemura H. (2012) Selective blockade of apoptosis by in vivo electroporation-mediated gene transfer combined with portal infusion of plasmid DNA attenuates liver cirrhosis. *Minerva Chirurgica* 67(3): 249–255.
34 Torrieri-Dramard L, Lambrecht B, Ferreira HL, Van den Berg T, Klatzmann D, Bellier B. (2011) Intranasal DNA vaccination induces potent mucosal and systemic immune responses and cross-protective immunity against influenza viruses. *Molecular Therapy* 19(3): 602–611.
35 Budker V, Zhang G, Danko I, Williams P, Wolff J. (1998) The efficient expression of intravascularly delivered DNA in rat muscle. *Gene Therapy* 5(2): 272–276.
36 Liu F, Song Y, Liu D. (1999) Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. *Gene Therapy* 6(7): 1258–1266.
37 Hackett PB Jr, Aronovich EL, Hunter D, Urness M, Bell JB, Kass SJ, et al. (2011) Efficacy and safety of Sleeping Beauty transposon-mediated gene transfer

in preclinical animal studies. *Current Gene Therapy* 11(5): 341–349.

38 Eastman SJ, Baskin KM, Hodges BL, Chu Q, Gates A, Dreusicke R, et al. (2002) Development of catheter-based procedures for transducing the isolated rabbit liver with plasmid DNA. *Human Gene Therapy* 13(17): 2065–2077.

39 Zhang G, Ludtke JJ, Thioudellet C, Kleinpeter P, Antoniou M, Herweijer H, et al. (2004) Intraarterial delivery of naked plasmid DNA expressing full-length mouse dystrophin in the mdx mouse model of duchenne muscular dystrophy. *Human Gene Therapy* 15(8): 770–782.

40 Chu Q, Joseph M, Przybylska M, Yew NS, Scheule RK. (2005) Transient siRNA-mediated attenuation of liver expression from an alpha-galactosidase A plasmid reduces subsequent humoral immune responses to the transgene product in mice. *Molecular Therapy* 12(2): 264–273.

41 Chen L, Woo SL. (2005) Complete and persistent phenotypic correction of phenylketonuria in mice by site-specific genome integration of murine phenylalanine hydroxylase cDNA. *Proceedings of the National Academy of Sciences USA* 102(43): 15581–15586.

42 He CX, Shi D, Wu WJ, Ding YF, Feng DM, Lu B, et al. (2004) Insulin expression in livers of diabetic mice mediated by hydrodynamics-based administration. *World Journal of Gastroenterology* 10(4): 567–572.

43 Nejak-Bowen KN, Thompson MD, Singh S, Bowen WC Jr, Dar MJ, Khillan J, et al. (2010) Accelerated liver regeneration and hepatocarcinogenesis in mice overexpressing serine-45 mutant beta-catenin. *Hepatology* 51(5): 1603–1613.

44 Crespo A, Peydro A, Dasi F, Benet M, Calvete JJ, Revert F, et al. (2005) Hydrodynamic liver gene transfer mechanism involves transient sinusoidal blood stasis and massive hepatocyte endocytic vesicles. *Gene Therapy* 12(11): 927–935.

45 Kobayashi N, Nishikawa M, Hirata K, Takakura Y. (2004) Hydrodynamics-based procedure involves transient hyperpermeability in the hepatic cellular membrane: implication of a nonspecific process in efficient intracellular gene delivery. *The Journal of Gene Medicine* 6(5): 584–592.

46 Zhang G, Budker VG, Ludtke JJ, Wolff JA. (2004) Naked DNA gene transfer in mammalian cells. *Methods in Molecular Biology* 245: 251–264.

47 Smyth Templeton N. (2002) Liposomal delivery of nucleic acids in vivo. *DNA and Cell Biology* 21(12): 857–867.

48 Luo D, Saltzman WM. (2000) Synthetic DNA delivery systems. *Nature Biotechnology* 18(1): 33–37.

49 Templeton NS, Lasic DD, Frederik PM, Strey HH, Roberts DD, Pavlakis GN. (1997) Improved DNA: liposome complexes for increased systemic delivery and gene expression. *Nature Biotechnology* 15(7): 647–652.

50 Rodamporn S, Harris NR, Beeby SP, Boltryk RJ, Sanchez-Elsner T. (2011) HeLa cell transfection using a novel sonoporation system. *IEEE Transactions on Biomedical Engineering* 58(4): 927–934.

51 Taniyama Y, Tachibana K, Hiraoka K, Aoki M, Yamamoto S, Matsumoto K, et al. (2002) Development of safe and efficient novel nonviral gene transfer using ultrasound: enhancement of transfection efficiency of naked plasmid DNA in skeletal muscle. *Gene Therapy* 9(6): 372–380.

52 Lawrie A, Brisken AF, Francis SE, Cumberland DC, Crossman DC, Newman CM. (2000) Microbubble-enhanced ultrasound for vascular gene delivery. *Gene Therapy* 7(23): 2023–2027.

53 Huang Q, Deng J, Xie Z, Wang F, Chen S, Lei B, et al. (2012) Effective gene transfer into central nervous system following ultrasound-microbubbles-induced opening of the blood–brain barrier. *Ultrasound in Medicine and Biology* 38(7): 1234–1243.

54 Shimamura M, Sato N, Taniyama Y, Kurinami H, Tanaka H, Takami T, et al. (2005) Gene transfer into adult rat spinal cord using naked plasmid DNA and ultrasound microbubbles. *The Journal of Gene Medicine* 7(11): 1468–1474.

55 Manome Y, Nakayama N, Nakayama K, Furuhata H. (2005) Insonation facilitates plasmid DNA transfection into the central nervous system and microbubbles enhance the effect. *Ultrasound in Medicine and Biology* 31(5): 693–702.

56 Phillips LC, Klibanov AL, Bowles DK, Ragosta M, Hossack JA, Wamhoff BR. (2010) Focused in vivo delivery of plasmid DNA to the porcine vascular wall via intravascular ultrasound destruction of microbubbles. *Journal of Vascular Research* 47(3): 270–274.

57 Casey G, Cashman JP, Morrissey D, Whelan MC, Larkin JO, Soden DM, et al. (2010) Sonoporation mediated immunogene therapy of solid tumors. *Ultrasound in Medicine and Biology* 36(3): 430–440.

58 Delalande A, Bouakaz A, Renault G, Tabareau F, Kotopoulis S, Midoux P, et al. (2011) Ultrasound and microbubble-assisted gene delivery in Achilles tendons: long lasting gene expression and restoration of fibromodulin KO phenotype. *Journal of Controlled Release* 156(2): 223–230.

59 Koike H, Tomita N, Azuma H, Taniyama Y, Yamasaki K, Kunugiza Y, et al. (2005) An efficient gene transfer method mediated by ultrasound and microbubbles into the kidney. *The Journal of Gene Medicine* 7(1): 108–116.

60 Sharei A, Zoldan J, Adamo A, Sim WY, Cho N, Jackson E, et al. (2013) A vector-free microfluidic platform for intracellular delivery. *Proceedings of the National Academy of Sciences USA* 110(6): 2082–2087.

61 Lee J, Sharei A, Sim WY, Adamo A, Langer R, Jensen KF, et al. (2012) Nonendocytic delivery of functional engineered nanoparticles into the cytoplasm of live cells using a novel, high-throughput microfluidic device. *Nano Letters* 12(12): 6322–6327.

62 Peng J, Zhao Y, Mai J, Guo W, Xu Y. Short noncoding DNA fragment improve efficiencies of in vivo electroporation-mediated gene transfer. *Journal of Gene Medicine*. 2012 Sep-Oct;14(9–10): 563–569.

63 Marie C, Vandermeulen G, Quiviger M, Richard M, Preat V, Scherman D. (2010) pFARs, plasmids free of antibiotic resistance markers, display high-level transgene expression in muscle, skin and tumour cells. *The Journal of Gene Medicine* 12(4): 323–332.

64 Herweijer H, Zhang G, Subbotin VM, Budker V, Williams P, Wolff JA. (2001) Time course of gene expression after plasmid DNA gene transfer to the liver. *The Journal of Gene Medicine* 3(3): 280–291.

65 Davies JK, Singh H, Huls H, Yuk D, Lee DA, Kebriaei P, et al. (2010) Combining CD19 redirection and alloanergization to generate tumor-specific human T cells for allogeneic cell therapy of B-cell malignancies. *Cancer Research* 70(10): 3915–3924.

66 Blomberg P, Eskandarpour M, Xia S, Sylven C, Islam KB. (2002) Electroporation in combination with a plasmid vector containing SV40 enhancer elements results in increased and persistent gene expression in mouse muscle. *Biochemical and Biophysical Research Communications* 298(4): 505–510.

67 Gazdhar A, Bilici M, Pierog J, Ayuni EL, Gugger M, Wetterwald A, et al. (2006) In vivo electroporation and ubiquitin promoter–a protocol for sustained gene expression in the lung. *The Journal of Gene Medicine* 8(7): 910–918.

68 Luke JM, Vincent JM, Du SX, Gerdemann U, Leen AM, Whalen RG, et al. (2011) Improved antibiotic-free plasmid vector design by incorporation of transient expression enhancers. *Gene Therapy* 18(4): 334–343.

69 Hagedorn C, Wong SP, Harbottle R, Lipps HJ. (2011) Scaffold/matrix attached region-based nonviral episomal vectors. *Human Gene Therapy* 22(8): 915–923.

70 Narum DL, Kumar S, Rogers WO, Fuhrmann SR, Liang H, Oakley M, et al. (2001) Codon optimization of gene fragments encoding Plasmodium falciparum merzoite proteins enhances DNA vaccine protein expression and immunogenicity in mice. *Infection & Immunity* 69(12): 7250–7253.

71 Stratford R, Douce G, Zhang-Barber L, Fairweather N, Eskola J, Dougan G. (2000) Influence of codon usage on the immunogenicity of a DNA vaccine against tetanus. *Vaccine* 19(7–8): 810–815.

72 Ramakrishna L, Anand KK, Mohankumar KM, Ranga U. (2004) Codon optimization of the tat antigen of human immunodeficiency virus type 1 generates strong immune responses in mice following genetic immunization. *Journal of Virology* 78(17): 9174–9189.

73 Megati S, Garcia-Hand D, Cappello S, Roopchand V, Masood A, Xu R, et al. (2008) Modifying the HIV-1 env gp160 gene to improve pDNA vaccine-elicited cell-mediated immune responses. *Vaccine* 26(40): 5083–5094.

74 Ternette N, Tippler B, Uberla K, Grunwald T. (2007) Immunogenicity and efficacy of codon optimized DNA vaccines encoding the F-protein of respiratory syncytial virus. *Vaccine* 25(41): 7271–7279.

75 Mossadegh N, Gissmann L, Muller M, Zentgraf H, Alonso A, Tomakidi P. (2004) Codon optimization of the human papillomavirus 11 (HPV 11) L1 gene leads to increased gene expression and formation of virus-like particles in mammalian epithelial cells. *Virology* 326(1): 57–66.

76 Kosovac D, Wild J, Ludwig C, Meissner S, Bauer AP, Wagner R. (2011) Minimal doses of a sequence-optimized transgene mediate high-level and long-term EPO expression in vivo: challenging CpG-free gene design. *Gene Therapy* 18(2): 189–198.

77 Cooper LJ, Topp MS, Serrano LM, Gonzalez S, Chang WC, Naranjo A, et al. (2003) T-cell clones can be rendered specific for CD19: toward the selective augmentation of the graft-versus-B-lineage leukemia effect. *Blood* 101(4): 1637–1644.

78 Stoll SM, Sclimenti CR, Baba EJ, Meuse L, Kay MA, Calos MP. (2001) Epstein-Barr virus/human vector provides high-level, long-term expression of alpha1-antitrypsin in mice. *Molecular Therapy* 4(2): 122–129.

79 Sato Y, Roman M, Tighe H, Lee D, Corr M, Nguyen MD, et al. (1996) Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. *Science* 273(5273): 352–354.

80 Stan AC, Casares S, Brumeanu TD, Klinman DM, Bona CA. (2001) CpG motifs of DNA vaccines induce the expression of chemokines and MHC class II molecules on myocytes. *European Journal of Immunology* 31(1): 301–310.

81 Bird AP. (1987) Cpg islands as gene markers in the vertebrate nucleus. *Trends in Genetics* 3(12): 342–347.

82 Reyes-Sandoval A, Ertl HC. (2004) CpG methylation of a plasmid vector results in extended transgene product expression by circumventing induction of immune responses. *Molecular Therapy* 9(2): 249–261.

83 Mitsui M, Nishikawa M, Zang L, Ando M, Hattori K, Takahashi Y, et al. (2009) Effect of the content of unmethylated CpG dinucleotides in plasmid DNA on the sustainability of transgene expression. *The Journal of Gene Medicine* 11(5): 435–443.

84 Chen ZY, He CY, Ehrhardt A, Kay MA. (2003) Minicircle DNA vectors devoid of bacterial DNA result in persistent and high-level transgene expression in vivo. *Molecular Therapy* 8(3): 495–500.

85 Wicks IP, Howell ML, Hancock T, Kohsaka H, Olee T, Carson DA. (1995) Bacterial lipopolysaccharide copurifies with plasmid DNA: implications for animal models and human gene therapy. *Human Gene Therapy* 6(3): 317–323.

86 Firozi P, Zhang W, Chen L, Quiocho FA, Worley KC, Templeton NS. (2010) Identification and removal of colanic acid from plasmid DNA preparations: implications for gene therapy. *Gene Therapy* 17(12): 1484–1499.

87 Horn NA, Meek JA, Budahazi G, Marquet M. (1995) Cancer gene therapy using plasmid DNA: purification of DNA for human clinical trials. *Human Gene Therapy* 6(5): 565–573.

88 Kim JY, Cho C, Cho BN. (2010) Plasmid DNA of high quality purified by activated charcoal. *Journal of Bioscience and Bioengineering* 110(5): 608–613.

89 Wooddell CI, Subbotin VM, Sebestyen MG, Griffin JB, Zhang G, Schleef M, et al. (2011) Muscle damage after delivery of naked plasmid DNA into skeletal muscles is batch dependent. *Human Gene Therapy* 22(2): 225–235.

90 Dincer Z, Jones S, Haworth R. (2006) Preclinical safety assessment of a DNA vaccine using particle-mediated epidermal delivery in domestic pig, minipig and mouse. *Experimental and Toxicologic Pathology* 57(5–6): 351–357.

91 Hutnick NA, Myles DJ, Ferraro B, Lucke C, Lin F, Yan J, et al. (2012) Intradermal DNA vaccination enhanced by low-current electroporation improves antigen expression and induces robust cellular and humoral immune responses. *Human Gene Therapy* 23(9): 943–950.

92 Kalams SA, Parker S, Jin X, Elizaga M, Metch B, Wang M, et al. (2012) Safety and immunogenicity of an HIV-1 gag DNA vaccine with or without IL-12 and/or IL-15 plasmid cytokine adjuvant in healthy, HIV-1 uninfected adults. *PLoS One* 7(1): e29231.

93 Wloch MK, Smith LR, Boutsaboualoy S, Reyes L, Han C, Kehler J, et al. (2008) Safety and immunogenicity of a bivalent cytomegalovirus DNA vaccine in healthy adult subjects. *Journal of Infectious Disease* 197(12): 1634–1642.

94 Smith LR, Wloch MK, Ye M, Reyes LR, Boutsaboualoy S, Dunne CE, et al. (2010) Phase 1 clinical trials of the safety and immunogenicity of adjuvanted plasmid DNA vaccines encoding influenza A virus H5 hemagglutinin. *Vaccine* 28(13): 2565–2572.

95 Hegge JO, Wooddell CI, Zhang G, Hagstrom JE, Braun S, Huss T, et al. (2010) Evaluation of hydrodynamic limb vein injections in nonhuman primates. *Human Gene Therapy* 21(7): 829–842.

96 Henry TD, Hirsch AT, Goldman J, Wang YL, Lips DL, McMillan WD, et al. (2011) Safety of a non-viral plasmid-encoding dual isoforms of hepatocyte growth factor in critical limb ischemia patients: a phase I study. *Gene Therapy* 18(8): 788–794.

97 Thioudellet C, Blot S, Squiban P, Fardeau M, Braun S. (2002) Current protocol of a research phase I clinical trial of full-length dystrophin plasmid DNA in Duchenne/Becker muscular dystrophies. Part I: rationale. *Neuromuscular Disorders* 12(Suppl 1):S49–S51.

98 Romero NB, Benveniste O, Payan C, Braun S, Squiban P, Herson S, et al. (2002) Current protocol of a research phase I clinical trial of full-length dystrophin plasmid DNA in Duchenne/Becker muscular dystrophies. Part II: clinical protocol. *Neuromuscular Disorders* 12(Suppl 1):S45–S48.

99 Ayuni EL, Gazdhar A, Giraud MN, Kadner A, Gugger M, Cecchini M, et al. (2010) In vivo electroporation mediated gene delivery to the beating heart. *PLoS One* 5(12): e14467.

100 Kalil RA, Salles FB, Giusti II, Rodrigues CG, Han SW, Sant'Anna RT, et al. (2010) VEGF gene therapy for angiogenesis in refractory angina: phase I/II clinical trial. *Revista Brasileira de Cirurgia Cardiovascular* 25(3): 311–321.

101 Manuri PV, Wilson MH, Maiti SN, Mi T, Singh H, Olivares S, et al. (2010) piggyBac transposon/transposase system to generate CD19-specific T cells for the treatment of B-lineage malignancies. *Human Gene Therapy* 21(4): 427–437.

102 Li X, Burnight ER, Cooney AL, Malani N, Brady T, Sander JD, et al. (2013) piggyBac transposase tools for genome engineering. *Proceedings of the National Academy of Sciences USA* 110(25): E2279–E2287.

103 Hackett PB, Largaespada DA, Switzer KC, Cooper LJ. (2013) Evaluating risks of insertional mutagenesis by DNA transposons in gene therapy. *Translational Research* 161(4): 265–283.

104 Hackett PB, Largaespada DA, Cooper LJ. (2010) A transposon and transposase system for human application. *Molecular Therapy* 18(4): 674–683.

105 Jin Z, Maiti S, Huls H, Singh H, Olivares S, Mates L, et al. (2011) The hyperactive Sleeping Beauty transposase SB100X improves the genetic modification of T cells to express a chimeric antigen receptor. *Gene Therapy* 18(9): 849–856.

106 Durai S, Mani M, Kandavelou K, Wu J, Porteus MH, Chandrasegaran S. (2005) Zinc finger nucleases: custom-designed molecular scissors for genome engineering of plant and mammalian cells. *Nucleic Acids Research* 33(18): 5978–5990.

107 Carlson DF, Fahrenkrug SC, Hackett PB. (2012) Targeting DNA with fingers and TALENs. *Molecular Therapy Nucleic Acids* 1: e3.

108 Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, et al. (2013) Multiplex genome engineering using CRISPR/Cas systems. *Science* 339(6121): 819–823.

109 Huang S, Kawabe Y, Ito A, Kamihira M. (2012) Adeno-associated virus Rep-mediated targeting of integrase-defective retroviral vector DNA circles into human chromosome 19. *Biochemical and Biophysical Research Communications* 417(1): 78–83.

110 Ginsburg DS, Calos MP. (2005) Site-specific integration with phiC31 integrase for prolonged expression of therapeutic genes. *Advances in Genetics* 54: 179–187.

111 Liu J, Skjorringe T, Gjetting T, Jensen TG. (2009) PhiC31 integrase induces a DNA damage response and chromosomal rearrangements in human adult fibroblasts. *BMC Biotechnology* 9: 31.

112 Cooper MJ, Lippa M, Payne JM, Hatzivassiliou G, Reifenberg E, Fayazi B, et al. (1997) Safety-modified episomal vectors for human gene therapy. *Proceedings of the National Academy of Sciences USA* 94(12): 6450–6455.

113 Holmstrom F, Pasetto A, Nahr V, Brass A, Kriegs M, Hildt E, et al. (2013) A synthetic codon-optimized hepatitis C virus nonstructural 5A DNA vaccine primes polyfunctional CD8+ T Cell responses in wild-type and NS5A-transgenic mice. *Journal of Immunology* 190(3): 1113–1124.

114 Yamazaki T, Nagashima M, Ninomiya D, Arai Y, Teshima Y, Fujimoto A, et al. (2011) Passive immune-prophylaxis against influenza virus infection by the expression of neutralizing anti-hemagglutinin monoclonal antibodies from plasmids. *Japanese Journal of Infectious Disease* 64(1): 40–49.

115 Ramanathan MP, Kutzler MA, Kuo YC, Yan J, Liu H, Shah V, et al. (2009) Coimmunization with an optimized IL15 plasmid adjuvant enhances humoral immunity via stimulating B cells induced by genetically engineered DNA vaccines expressing consensus JEV and WNV E DIII. *Vaccine* 27(32): 4370–4380.

116 Hallengard D, Haller BK, Maltais AK, Gelius E, Nihlmark K, Wahren B, et al. (2011) Comparison of plasmid vaccine immunization schedules using intradermal in vivo electroporation. *Clinical and Vaccine Immunology* 18(9): 1577–1581.

117 Chen Q, Kuang H, Wang H, Fang F, Yang Z, Zhang Z, et al. (2009) Comparing the ability of a series of viral protein-expressing plasmid DNAs to protect against H5N1 influenza virus. *Virus Genes* 38(1): 30–38.

118 Xu K, Ling ZY, Sun L, Xu Y, Bian C, He Y, et al. (2011) Broad humoral and cellular immunity elicited by a bivalent DNA vaccine encoding HA and NP genes from an H5N1 virus. *Viral Immunology* 24(1): 45–56.

119 van Drunen Littel-van den Hurk S, Luxembourg A, Ellefsen B, Wilson D, Ubach A, Hannaman D, et al. (2008) Electroporation-based DNA transfer enhances gene expression and immune responses to DNA vaccines in cattle. *Vaccine* 26(43): 5503–5509.

120 Yin J, Dai A, Lecureux J, Arango T, Kutzler MA, Yan J, et al. (2011) High antibody and cellular responses induced to HIV-1 clade C envelope following DNA vaccines delivered by electroporation. *Vaccine* 29(39): 6763–6770.

121 Valentin A, von Gegerfelt A, Rosati M, Miteloudis G, Alicea C, Bergamaschi C, et al. (2010) Repeated DNA therapeutic vaccination of chronically SIV-infected macaques provides additional virological benefit. *Vaccine* 28(8): 1962–1974.

122 Rosati M, Valentin A, Jalah R, Patel V, von Gegerfelt A, Bergamaschi C, et al. (2008) Increased immune responses in rhesus macaques by DNA vaccination combined with electroporation. *Vaccine* 26(40): 5223–5229.

123 Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, et al. (2000) A Toll-like receptor recognizes bacterial DNA. *Nature* 408(6813): 740–745.

124 Ma X, Forns X, Gutierrez R, Mushahwar IK, Wu T, Payette PJ, et al. (2002) DNA-based vaccination against hepatitis C virus (HCV): effect of expressing different forms of HCV E2 protein and use of CpG-optimized vectors in mice. *Vaccine* 20(27–28): 3263–3271.

125 Kojima Y, Xin KQ, Ooki T, Hamajima K, Oikawa T, Shinoda K, et al. (2002) Adjuvant effect of multi-CpG motifs on an HIV-1 DNA vaccine. *Vaccine* 20(23–24): 2857–2865.

126 Verfaillie T, Cox E, Goddeeris BM. (2005) Immunostimulatory capacity of DNA vaccine vectors in porcine PBMC: a specific role for CpG-motifs? *Veterinary Immunology and Immunopathology* 103(1–2): 141–151.

127 Till BG, Jensen MC, Wang J, Chen EY, Wood BL, Greisman HA, et al. (2008) Adoptive immunotherapy for indolent non-Hodgkin lymphoma and mantle cell lymphoma using genetically modified autologous CD20-specific T cells. *Blood* 112(6): 2261–2271.

128 Jensen MC, Popplewell L, Cooper LJ, DiGiusto D, Kalos M, Ostberg JR, et al. (2010) Antitransgene rejection responses contribute to attenuated persistence of adoptively transferred CD20/CD19-specific chimeric antigen receptor redirected T cells in humans. *Biology of Blood and Marrow Transplantation* 16(9): 1245–1256.

129 Kebriaei P, Huls H, Jena B, Munsell M, Jackson R, Lee DA, et al. (2012) Infusing CD19-directed T cells to augment disease control in patients undergoing autologous hematopoietic stem-cell transplantation for advanced B-lymphoid malignancies. *Human Gene Therapy* 23(5): 444–450.

130 Cheng W, Miao L, Zhang H, Yang O, Ge H, Li Y, et al. (2013) Induction of interleukin 2 expression in the liver for the treatment of H22 hepatoma in mice. *Digestive and Liver Disease* 45(1): 50–57.

131 He X, Li W, Wang Y, Hou L, Zhu L. (2012) Inhibition of colon tumor growth by IL-15 immunogene therapy. *Molecular Medicine Reports* 5(1): 96–102.

132 Matsumoto K, Kikuchi E, Horinaga M, Takeda T, Miyajima A, Nakagawa K, et al. (2011) Intravesical interleukin-15 gene therapy in an orthotopic bladder cancer model. *Human Gene Therapy* 22(11): 1423–1432.

133 Ochoa MC, Fioravanti J, Rodriguez I, Hervas-Stubbs S, Azpilikueta A, Mazzolini G, et al. (2013) Antitumor immunotherapeutic and toxic properties of an HDL-conjugated chimeric IL-15 fusion protein. *Cancer Research* 73(1): 139–149.

134 Yin X, Yan X, Yang Q, Cao H, Liang H. (2010) Antitumor mechanism of recombinant murine interleukin-12 vaccine. *Cancer Biotherapy & Radiopharmaceuticals* 25(3): 263–268.

135 Pavlin D, Cemazar M, Sersa G, Tozon N. (2012) IL-12 based gene therapy in veterinary medicine. *J Translational Medicine* 10: 234.

136 Cemazar M, Jarm T, Sersa G. (2010) Cancer electrogene therapy with interleukin-12. *Current Gene Therapy* 10(4): 300–311.

137 Zhu S, Lee DA, Li S. (2010) IL-12 and IL-27 sequential gene therapy via intramuscular electroporation delivery for eliminating distal aggressive tumors. *Journal of Immunology* 184(5): 2348–2354.

138 Rodriguez AM, Pascutti MF, Maeto C, Falivene J, Holgado MP, Turk G, et al. (2012) IL-12 and GM-CSF in DNA/MVA immunizations against HIV-1 CRF12_BF Nef induced T-cell responses with an enhanced magnitude, breadth and quality. *PLoS One* 7(5): e37801.

139 Li S, Qi X, Gao Y, Hao Y, Cui L, Ruan L, et al. (2010) IL-15 increases the frequency of effector memory CD8+ T cells in rhesus monkeys immunized with HIV vaccine. *Cellular and Molecular Immunology* 7(6): 491–494.

140 Yoon WS, Choi HJ, Park YK. (2011) Salmonella typhimurium harboring plasmid expressing interleukin-12 induced attenuation of infection and protective immune responses. *Journal of General and Applied Microbiology* 57(2): 115–122.

141 Daugimont L, Vandermeulen G, Defresne F, Bouzin C, Mir LM, Bouquet C, et al. (2011) Antitumoral and antimetastatic effect of antiangiogenic plasmids in B16 melanoma: Higher efficiency of the recombinant disintegrin domain of ADAM 15. *European Journal of Pharmaceutics and Biopharmaceutics* 78(3): 314–319.

142 Lladser A, Ljungberg K, Tufvesson H, Tazzari M, Roos AK, Quest AF, et al. (2010) Intradermal DNA electroporation induces survivin-specific CTLs, suppresses angiogenesis and confers protection against mouse melanoma. *Cancer Immunology Immunotherapy* 59(1): 81–92.

143 Chan RC, Gutierrez B, Ichim TE, Lin F. (2009) Enhancement of DNA cancer vaccine efficacy by combination with anti-angiogenesis in regression of established subcutaneous B16 melanoma. *Oncology Reports* 22(5): 1197–1203.

144 Johnen S, Izsvak Z, Stocker M, Harmening N, Salz AK, Walter P, et al. (2012) Sleeping Beauty transposon-mediated transfection of retinal and iris pigment epithelial cells. *Investigative Ophthalmology & Visual Science* 53(8): 4787–4796.

145 Matsuda R, Kezuka T, Nishiyama C, Usui Y, Matsunaga Y, Okunuki Y, et al. (2012) Interleukin-10 gene-transfected mature dendritic cells suppress murine experimental autoimmune optic neuritis. *Investigative Ophthalmology & Visual Science* 53(11): 7235–7245.

146 Fakin R, Hamacher J, Gugger M, Gazdhar A, Moser H, Schmid RA. (2011) Prolonged amelioration of acute lung allograft rejection by sequential overexpression of human interleukin-10 and hepatocyte growth factor in rats. *Experimental Lung Research* 37(9): 555–562.

147 Aistrup GL, Cokic I, Ng J, Gordon D, Koduri H, Browne S, et al. (2011) Targeted nonviral gene-based inhibition of Galpha(i/o)-mediated vagal signaling in the posterior left atrium decreases vagal-induced atrial fibrillation. *Heart Rhythm* 8(11): 1722–1729.

148 Penn MS, Mendelsohn FO, Schaer GL, Sherman W, Farr M, Pastore J, et al. (2013) An open-label dose escalation study to evaluate the safety of administration of non-viral stromal cell-derived factor-1 plasmid to treat symptomatic ischemic heart failure. *Circulation Research* 112(5): 816–825.

149 Marshall WG Jr, Boone BA, Burgos JD, Gografe SI, Baldwin MK, Danielson ML, et al. (2010) Electroporation-mediated delivery of a naked DNA plasmid expressing VEGF to the porcine heart enhances protein expression. *Gene Therapy* 17(3): 419–423.

150 Powell RJ, Goodney P, Mendelsohn FO, Moen EK, Annex BH. (2010) Safety and efficacy of patient specific intramuscular injection of HGF plasmid gene therapy on limb perfusion and wound healing in patients with ischemic lower extremity ulceration: results of the HGF-0205 trial. *Journal of Vascular Surgery* 52(6): 1525–1530.

151 Shigematsu H, Yasuda K, Iwai T, Sasajima T, Ishimaru S, Ohashi Y, et al. (2010) Randomized, double-blind, placebo-controlled clinical trial of hepatocyte growth factor plasmid for critical limb ischemia. *Gene Therapy* 17(9): 1152–1161.

152 Morishita R, Aoki M, Hashiya N, Makino H, Yamasaki K, Azuma J, et al. (2004) Safety evaluation of clinical gene therapy using hepatocyte growth factor to treat peripheral arterial disease. *Hypertension* 44(2): 203–209.

153 Makarevich P, Tsokolaeva Z, Shevelev A, Rybalkin I, Shevchenko E, Beloglazova I, et al. (2012) Combined transfer of human VEGF165 and HGF genes renders potent angiogenic effect in ischemic skeletal muscle. *PLoS One* 7(6): e38776.

154 Nikol S, Baumgartner I, Van Belle E, Diehm C, Visona A, Capogrossi MC, et al. (2008) Therapeutic angiogenesis with intramuscular NV1FGF improves amputation-free survival in patients with critical limb ischemia. *Molecular Therapy* 16(5): 972–978.

155 Ferraro B, Cruz YL, Baldwin M, Coppola D, Heller R. (2010) Increased perfusion and angiogenesis in a hindlimb ischemia model with plasmid FGF-2 delivered by noninvasive electroporation. *Gene Therapy* 17(6): 763–769.

156 Murakami T, Imada Y, Kawamura M, Takahashi T, Fujita Y, Sato E, et al. (2011) Placental growth factor-2 gene transfer by electroporation restores diabetic sensory neuropathy in mice. *Experimental Neurology* 227(1): 195–202.

157 Higuti E, Cecchi CR, Oliveira NA, Vieira DP, Jensen TG, Jorge AA, et al. (2012) Growth responses following a single intra-muscular hGH plasmid administration compared to daily injections of hGH in dwarf mice. *Current Gene Therapy* 12(6): 437–443.

4

CHAPTER 4

Cancer Therapy with RNAi Delivered by Non-viral Membrane/Core Nanoparticles

Andrew B. Satterlee and Leaf Huang
University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

Introduction

Since the 1970s, researchers and clinicians have been attempting to deliver DNA to cells as a therapy for many diseases in which a gene no longer functions correctly or when delivery of a gene can have a therapeutic effect (therapy by gain of function) [1]. Since then, significant progress has been made against diseases such as hemophilia [2], neurodegenerative disease [3], and cancer [4,5,6]. Although many diseases can potentially be treated this way, others require the downregulation of an undesired gene by silencing it through RNA interference (RNAi) (therapy through loss of function).

In 1998, it was discovered that cells inherently have the machinery to prevent expression of specific proteins [7]. Since that time, the details of endogenous RNAi have been further elucidated. After transcription, double-stranded RNA (dsRNA) translocates from the nucleus to the cytoplasm and binds with the enzyme Dicer [8]. Dicer has a dsRNA-binding domain (dsRBD) and two RNase III domains that it uses to cleave the dsRNA into an oligonucleotide that is approximately 21 bp long with a two-nucleotide 3′ overhang [9] (Figure 4.1). In *Drosophila*, Dicer and another dsRBD-containing protein, R2D2, help load the newly formed small dsRNA, now called small interfering RNA (siRNA), onto another protein called Argonaute-2 (Ago2) [10,11]. Ago2 unwinds the RNA duplex and discards the "passenger" strand while retaining the "guide" strand. The guide strand is chosen based on the thermodynamic asymmetry of the siRNA's 5′ ends [12]. Before loading onto Ago2, R2D2 binds to the 5′ end that is more stable, leaving the less stable 5′ end for Dicer to bind. Upon Ago2 loading, the dsRNA is oriented so that the guide strand is the one with the less stable 5′ end. Once the passenger strand is discarded, the RNA-induced silencing complex (RISC) is mature and ready to function. In humans, the protein TRBP carries out similar functions to R2D2, and together with Dicer, Ago2, and the siRNA guide strand, can compose one of many functional RISC complexes [13].

RISC silences gene expression through endonucleolytic cleavage or "slicing" of mRNA, thereby prohibiting translation [14,15]. The siRNA guide strand binds to a target mRNA through Watson–Crick base pairing [16], and displays high specificity in gene silencing because the target sequence generally must show full complementarity with the siRNA. mRNA cleavage occurs between the nucleotides complementary to nucleotides 10 and 11 of the guide strand and, once cleaved, the two mRNA

Cancer Gene Therapy by Viral and Non-viral Vectors, First Edition. Edited by Malcolm K. Brenner and Mien-Chie Hung.

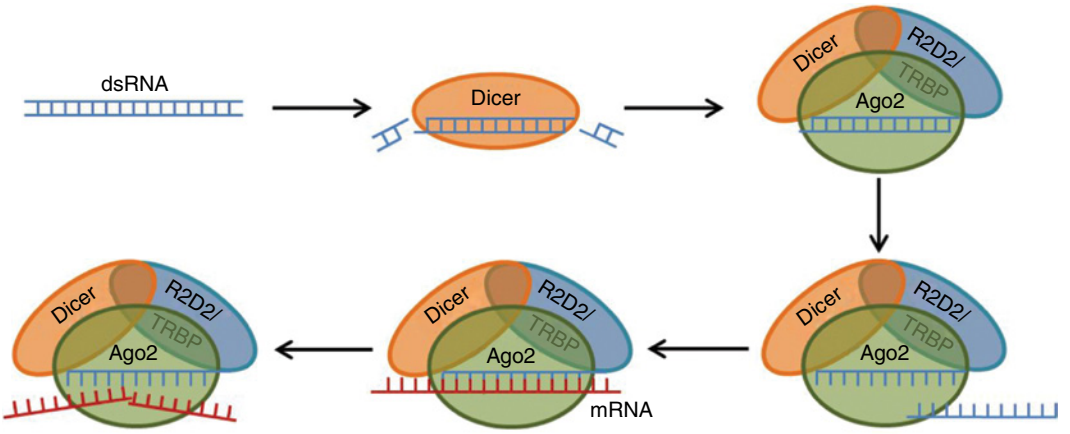


Figure 4.1 Cytoplasmic double-stranded DNA binds to Dicer, which cleaves it into a short oligonucleotide. Upon binding with R2D2/TRBP and Ago2, one RNA strand is discarded and the other is used as the template strand that is complementary to the target mRNA. mRNA then binds to this guide strand and is cleaved and released, while the siRNA guide strand remains a part of the RNA-induced silencing complex (RISC) and is able to bind and cleave additional mRNA.

fragments are degraded by ribonucleases [15]. The siRNA guide strand remains intact after mRNA cleavage, and RISC can subsequently move on to find other complementary mRNAs to cleave [11]. In this way, the RNAi mechanism through RISC can inhibit the translation of certain proteins. siRNA is not the only oligonucleotide responsible for endogenous RNAi; in Chapter 6 in this volume, the roles of microRNA (miRNA), which are not limited RNAi, are discussed in depth.

The discovery of endogenous RNAi initiated a desire to deliver and use siRNA and miRNA therapeutically. siRNA has emerged as the most popular oligonucleotide for exogenous RNAi, and over the past decade synthetic siRNA libraries have been compiled and commercialized for easy access to siRNA against many genes. Designing effective siRNA sequences, however, is not a trivial matter. Not all complementary siRNAs can cause silencing in their target mRNA [17]. Furthermore, partial complementarity of an siRNA sequence with an off-target mRNA can lead to nonspecific binding of the siRNA, lowering therapeutic efficiency by causing a loss of available siRNA while at times also resulting in degradation the off-target mRNA [18]. Therefore, an effective siRNA sequence is one that shows minimal complementarity with all sequences in a transcriptome, except in the gene of interest. Many algorithms have been developed to optimize the sensitivity and specificity of siRNA, including using the Basic Local Alignment Search Tool (BLAST) to return possible secondary siRNA targets, but there are many other features that must be considered and prioritized during optimization, such as secondary structures and thermodynamic properties [17,18,19]. The most effective algorithms blend many established algorithms together in an attempt to design efficient siRNAs that present minimal off-target effects.

RNAi is especially effective against cancer, as many cancers develop through oncogenic mutations that increase the expression or activity of certain proteins that promote cell growth or proliferation, or inhibit apoptosis. Some of these oncogenic proteins, such as the mutant B-raf^{V600E} protein (mutation arising from a substitution of glutamic acid for valine at amino acid position 600), already have US Food and Drug Administration (FDA)-approved drugs against them (in this case, the drug vemurafenib). Other proteins, the so-called "undruggable" targets, do not currently have a drug that can inhibit their activity, making RNAi the preferred therapeutic approach. Examples of such targets can be found in the last section of this chapter. Despite the great therapeutic potential of siRNA, there are few RNAi-based therapies currently favored over traditional chemotherapy in the clinic; however, the next generation of stimulus-responsive and theranostic delivery systems may facilitate a change in this paradigm.

Barriers

Several chemical, physical, and biological barriers impede systemic siRNA delivery to tumor cells (Table 4.1 [6,20,21,22,23,24,25,26,27]). If unmodified and naked (i.e. without a vector) siRNA is delivered systemically, it is quickly degraded by nucleases and cleared by the kidney and the mononuclear phagocyte system (MPS; previously referred to as the reticuloendothelial system). siRNA can be modified to improve circulation half-life and reduce nuclease degradation, but these modifications do not alter siRNA's negative charge or nonspecific interaction with serum proteins, which also impede transfection efficiency and gene silencing [28,29]. To overcome these and other barriers, shown in Table 4.1, many viral and non-viral delivery systems have been developed, and a general membrane/core design, in which siRNA is protected in the core of a lipid and/or polymer-based nanostructure, has been particularly successful. Cationic lipids, first used in 1987 to encapsulate DNA [30], are composed of a positively charged hydrophilic headgroup and a hydrophobic chain that are connected by a linker. The lipids arrange in bilayers

Table 4.1 Barriers that hinder delivery efficiency and necessitate strategies to overcome them.

Barrier	*Description*
Complexation	Efficient siRNA complexation into a NP requires a different strategy than that for DNA. The smaller size and increased stiffness of dsRNA decreases its avidity to cationic structures that easily condense DNA and can result in larger, more diffuse particles [20].
MPS recognition	Cationic structures used to complex nucleic acids attract serum proteins and other anionic compounds *in vivo*. These opsonins are recognized by Kupffer cells in the liver and macrophages in the spleen and the bone marrow which take up the particles and remove them from circulation [21,22]. Glycosaminoglycans, present in the extracellular matrix, can also act as opsonins or directly disrupt a cationic membrane [23].
Extravasation	NPs must be a certain size to effectively deliver their cargo to the tumor site. They must be large enough to bypass fensetrae in the liver (≈100 nm) [24] but small enough to extravasate from the tumor vasculature into the interstitial space. Capillary pore sizes in tumor masses are generally much larger than in other tissues, but pore sizes vary with tumor location and are spatially and temporally heterogeneous in a single tumor [25]. Thus, a generally acceptable upper bound for effective tumor extravasation is a NP diameter of ≈200 nm [26].
Targeting and internalization	The enhanced permeability of tumor vasculature coupled with low lymphatic drainage and increased retention of NPs (the EPR effect) allow for NP accumulation in the tumor mass, but this alone is not sufficient for NP internalization into cells. Targeting tumor cells through receptor-mediated endocytosis is a more effective cellular internalization process [27].
Endosome escape	NPs internalized via endocytosis are transported through the cytoplasm in a vesicle called an endosome. NPs must be able to escape the endosome in order to access the intracellular environment, and must do so before endosomes return to the cell surface or fuse with lysosomes, which degrades the siRNA payload [6].
Release	If a NP is robust enough to enter the cytoplasm of a tumor cell and still contain its siRNA, it must then be able to efficiently release that siRNA to have any therapeutic effect.

MPS, mononuclear phagocyte system; NP, nanoparticle.

to form liposomes, hollow spherical structures with an internal aqueous phase that can encapsulate siRNA. Cationic polymers, which are generally not amphipathic and have a much larger molecular weight than lipids, can readily complex and condense DNA through electrostatic interaction. Diblock copolymers have also been designed to form micelles that electrostatically bind to and entrap siRNA [31]. Polymer–DNA/–RNA complexes can be further functionalized or loaded into liposomes for *in vivo* delivery. It is important to note that the inherent toxicity of cationic lipids and polymers limits their tolerable dose [32], and is in part why delivery efficiency is so crucial.

Overcoming Delivery Barriers

Complexation

Polycationic polymers such as protamine and polyethyleneimine (PEI) are readily able to complex with and condense polyanionic DNA for delivery largely because of their high avidity (multiple charge–charge interactions). However, the small size of an siRNA molecule lowers its avidity with cationic molecules and increases its rigidity, hindering its ability to be condensed [20]. Several strategies have been developed to overcome this barrier. To allow better complexation, siRNA can be modified in ways that allow covalent attachment to polymers, such as by adding a thiol group to the siRNA backbone for a disulfide bond [33], or by conjugating the siRNA to PEI through amino-ketal branches [34]. Other strategies include entrapping siRNA in an amorphous calcium phosphate precipitate core [26,35] or adding a large, nonfunctional, polyanionic molecule such as calf thymus DNA or hyaluronic acid to the complex to trap the siRNA in a high-avidity interaction [36,37,38].

MPS Recognition and the Role of Polyethylene Glycol

Opsonization, which is the act of "tagging" a cell, particle, or protein for phagocytosis, and subsequent MPS uptake of nanoparticles (NPs) in systemic circulation is a major cause of loss of injected dose. The zeta potential, or surface charge, of a NP is often measured to estimate the rate of opsonization that may occur, as more cationic NPs are more quickly bound by serum proteins [39]. In the early 1990s, three different and independent groups discovered that when polyethylene glycol (PEG) is incorporated onto the outer surface of liposomes, the blood circulation half-life of the liposomes is greatly increased [40,41,42]. Since that time, the consequences of PEGylation have been extensively researched and PEG is now conjugated to many types of particles to improve circulation half-life. NP PEGylation masks the particle's surface charge and sterically hinders opsonization [43] as well as cationic NP aggregation [44]. PEG's hydrophilicity also allows the binding of a tight layer of water molecules via hydrogen bonding, which further deters opsonization [45]. For PEGylation to be fully effective, however, the PEG must completely cover the NP surface. Dilute PEG concentrations on the NP membrane generate a diffuse "mushroom" conformation, while higher PEG concentrations begin to form the "brush" conformation (Figure 4.2A and B), where the PEG is sterically forced to extend away from the NP surface. The planar brush conformation is defined as being when the distance between two PEG molecules at the NP surface is less than twice the PEG's Flory radius (r) [46], and holds true with little error in NPs with low curvature. While the mushroom conformation generally arises at PEG concentrations of up to 8 mol% [43], this number is also dependent on NP size, as smaller particles with greater curvature increase the distance between the distal ends of PEG molecules (Figure 4.2C) and require a higher PEG density to reach the brush conformation. Because a brush conformation favorably protects NPs from opsonization, widespread focus has been placed on maximizing PEG density for *in vivo* delivery, but new data suggest that too much PEG can hinder receptor-mediated NP endocytosis and that a moderate amount of PEG (<10%) is optimal for NP uptake [47]. While this data underlines the drawbacks of overPEGylation in regard to target cell uptake, it does not consider any potential advantages that higher PEG densities present when avoiding MPS uptake. An optimal PEG density is likely one that balances MPS evasion with uptake by the target cell.

Unpublished data from this laboratory by Liu et al. have shown recently that on some lipid bilayers the brush conformation may not exist at high PEG densities. Data show that when coating a NP with a

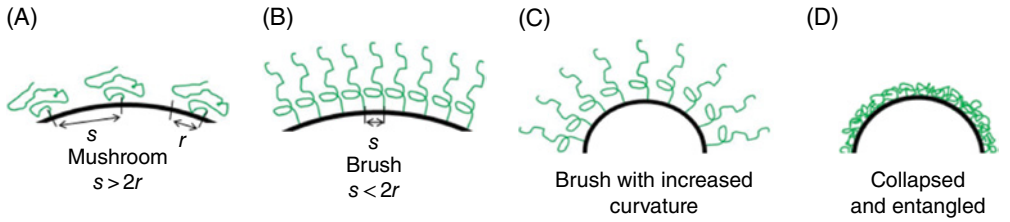


Figure 4.2 Depictions of mushroom, brush, and collapsed conformations. (A) When $s > 2r$, PEG molecules do not sterically hinder each other and are free to spread out close to the NP surface. (B) When $s < 2r$, the PEG molecules are closer together and their interaction causes them to extend out from the NP. (C) When the NP curvature is increased, the same PEG density on the NP surface provides less shielding because the distal ends of each PEG molecule are now farther from each other. (D) High-density PEG may collapse on the surface of NPs.

highly curved lipid bilayer, such as the lipid-calcium phosphate (LCP) NP, with 20 mol% PEG, the PEG molecules may actually interact with each other and form a collapsed and entangled layer on the surface of the NP (Figure 4.2D). If true, this could help explain why NP uptake is hindered at high PEG densities, as receptor-ligand binding for endocytosis would be obstructed. However, the data also show that LCP NPs formulated with 20 mol% PEG effectively evade the MPS and still efficiently internalize in their target cells; that is, hepatocytes. It is not clear whether this occurs in larger bilayer-coated NPs with less curvature, or if the collapsed PEG layer may protect from the MPS as effectively as a conventional brush. The driving force behind this collapsed conformation is also unclear, although it may be related to the PEG dehydrating as water molecules increasingly prefer hydrogen bonding with each other.

Although tightly packed PEG offers better protection from the MPS, the detergent-like properties of PEG–lipid conjugates will destabilize a polar NP membrane in high concentrations. Several different approaches have therefore been utilized to either covalently attach PEG to the NP or support the NP membrane to allow for denser PEGylation. Thiol groups have been used to crosslink and strengthen PEG–cationic polymer conjugates with disulfide bonds [31], while PEG has also been conjugated to PEI through an acetal linker to improve stability [48]. A calcium phosphate core used to complex siRNA has also been used to complex the phosphate headgroups of the inner leaflet lipids of its membrane, increasing lipid bilayer stability [26]. Simple electrostatic interaction between an anionic core and cationic membrane can also stabilize the membrane enough to tolerate higher PEG densities [21].

Further research regarding PEG has revealed that PEG can induce its own immune response upon repeated dosing. Although the precise mechanism of induction is unclear, there is evidence that splenic B cells produce anti-PEG IgM in response to an initial dose of PEGylated lipid, and that this response is intensified if the lipid is encapsulating nucleic acid [49,50]. The response causes subsequent doses of PEGylated lipid up to 4 weeks after the initial injection to be opsonized and rapidly cleared mostly by the liver in a phenomenon known as "accelerated blood clearance" [49,51]. In addition, the immune response to PEG can cause hypersensitivity and result in antibody-mediated acute toxicity upon repeated dosing. The immune response can be lessened by shortening PEG's alkyl chain, which allows faster passive diffusion of PEG from the NP [52]; however, the issue of PEG's immunostimulatory effects remains mostly unresolved.

Although PEG is the most widely used stealth compound in NPs, there are others currently being researched that attempt to improve on some of PEG's weaknesses, which also include susceptibility to oxidative or thermal damage [53]. A recently developed polymer with a similar structure to PEG, poly(2-methyl-2 oxazoline) (PMOXA), is bioinert and possesses thermal stability and resistance to oxidative stress [54]. Several zwitterionic molecules have also been used instead of PEG to decrease opsonization, as their electrostatic interaction with water is stronger than the hydrogen bonding exhibited by PEG [45]. Polymers from the zwitterions phosphobetane,

sulfobetane, and carboxybetane are all biomemetic and can reduce nonspecific protein adsorption. The carboxylate-anion groups in each monomer of poly(carboxybetane) make this polymer particularly functionalizable and valuable as an alternative to PEG [45]. As with PEG, high surface packing density of zwitterions is required for effective stealth properties. Poly(amino acids), polyglycerols, and polysaccharides have also been used as alternatives to PEG [53].

Extravasation, Targeting, and Internalization

The longer circulation time afforded to NPs by PEG allows greater extravasation into the tumor extracellular environment, which is further aided by the EPR effect (the enhanced permeability of tumor vasculature coupled with low lymphatic drainage and increased retention of NPs; see Table 4.1). At this stage, PEG becomes a hindrance to delivery efficiency. The cationic surface of NPs can associate to the anionic surface of cancer cells and facilitate endocytosis, but just as PEG masks the NP surface to prevent opsonization, PEG also prevents the association between the NP and the cell surface. This "PEG dilemma" has called for strategies to expose the NP's cationic surface by shedding PEG in response to physiological conditions in the extracellular tumor environment. The pH in this environment is more acidic than in systemic circulation (≈6.0 compared to ≈ 7.3), and PEG that is conjugated to the NP with acid-cleavable acetal [48] or ketal [55] linkages will be cleaved and expose the cationic core. PEG can also be linked to the NP using reducible bonds, such as disulfide bonds [56], as many cell surfaces also express proteins such as protein disulfide isomerase and NADH oxidase that are able to reduce these bonds. A PEG–peptide–lipid conjugate has also been designed in which the peptide is cleavable by matrix metalloproteinase, an enzyme that is specifically expressed in cancer cells and subsequently secreted into their extracellular environment [57]. Notice that in many cases the same bonds used to stabilize PEG on the NP are synthesized to be degradable in response to an internal stimulus.

1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE)-PEG that is inserted into liposomal NPs can also be shed at a quantifiable rate by passive diffusion instead of an active, tumor-specific mechanism [43,58]. DSPE-PEG tends to gradually diffuse from the lipid membrane at body temperature, and the kinetics of this diffusion are based on the length and degree of saturation of the DSPE-PEG alkyl chain. This approach allows initial shielding from NP opsonization while allowing efficient cellular uptake and endosomal escape in the tumor microenvironment at later time points [59]. Diffusible PEG also lessens the immune recognition of PEG described previously.

While the association between cationic NPs and the tumor cell surface can facilitate endocytosis, a much more efficient strategy is to increase NP uptake through receptor-mediated endocytosis by attaching a targeting ligand to the NP. Ligands that are conjugated to the NP membrane will require that any PEG be shed before they are able to target their receptor, and recent data show that even ligands attached to the distal end of PEG molecules can be hindered at high PEG densities [47]. Many effective targeting ligands have been identified, including the anisamide ligand for the sigma receptor [26], transferrin for the transferrin receptor [60], folic acid for the folate receptor [61], and others [62,63]. These receptors are overexpressed in cancer cells, which allows for selective tumor targeting. A mathematical model depicting endocytosis has shown that the first receptor-ligand binding event produces a local reduction of free energy, which causes nearby receptors to diffuse toward the NP and bind to other exposed ligands [64]. The receptor concentration gradient caused by this diffusion facilitates further diffusion of receptors to the NP. The cell membrane eventually completely wraps around the NP and internalizes it in an endosomal compartment. Smaller particles are therefore more quickly and efficiently endocytosed, although a NP's cationic surface charge can also positively affect endocytosis [64].

Endosome Escape

The endosomal trafficking system transports NPs around the cytoplasm as the endosome gradually decreases in pH by importing H^+ through proton pumps in the endosomal membrane. As an early endosome progresses to a late endosome and finally merges with a lysosome,

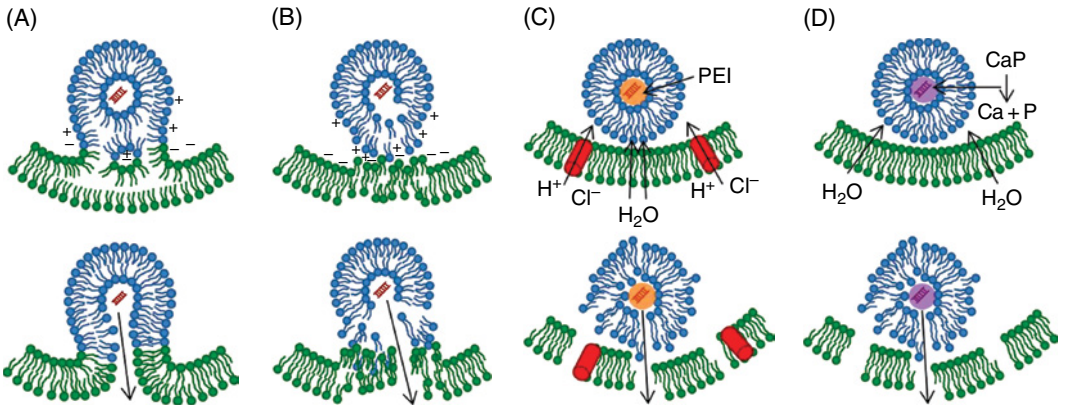


Figure 4.3 Mechanisms of endosomal escape. (A) Ion-pair formation. Cationic lipids in the NP form ion pairs with anionic lipids in the endosome, leading to destabilization and fusion of both membranes. (B) Electrostatic interaction. Highly charged cationic lipids (or other particles) in the NP disrupt the anionic endosome. (C) The proton sponge effect. A proton sponge molecule (e.g. PEI) acts as a buffer while proton pumps in the endosomal membrane actively pump in H^+ and Cl^-. Therefore, the pH in the endosome does not drop, and more H^+ and Cl^- are pumped in, followed by water moving with its osmotic gradient. The increased pressure in the endosome ruptures it and allows the release of siRNA. (D) Osmotic rupture. A solid precipitate (e.g. calcium phosphate, shown by CaP) dissolves in the acidic endosome, increasing the osmotic pressure in the endosome, and ushering in water moving with its osmotic gradient. The increased pressure in the endosome ruptures it and allows the release of siRNA.

the pH drops to around 4.5, low enough to degrade the NP and its encapsulated siRNA [65]. It is therefore imperative that the siRNA is quickly released from the endosomal compartment. There are currently several proposed models describing endosome escape, including ion-pair formation, electrostatic destabilization, the proton sponge effect, and non-proton sponge osmotic rupture [26,58]. Ion-pair formation and electrostatic destabilization both require interaction between the cationic NP membrane and the anionic endosomal membrane, thus any PEG that was not shed outside the cell must be shed in the endosome before efficient destabilization occurs. Because the endosomal environment is very acidic, acid-cleavable bonds can again facilitate PEG shedding (Figure 4.3).

In the ion-pair model, the NP and endosomal membranes interact directly through ion pairing, which creates an unstable inverted micellar H_{II} phase, fusing the membranes and exposing the core of the NP to the cytoplasm [43,66]. NP surface modification with a peptide such as GALA can help induce membrane fusion, as protonation of GALA at an acidic pH causes a conformational change that allows selective membrane fusion [67]. Electrostatic destabilization occurs slightly differently, as NP cations interact with and destabilize the endosomal membrane without causing the inverted H_{II} phase and membrane fusion [58].

The proton sponge effect is a hypothesis used to describe why highly branched polyamines such as PEI [68] or polyamidoamine dendrimers [69] facilitate such high transfection efficiency. Endosomes drop in pH by pumping in H^+ chaperoned by Cl^-, but, according to the theory, if PEI is present in the endosome, the amine groups in PEI will become protonated, decreasing the free hydrogen ion concentration in the endosome and preventing the pH from dropping. As a result, more H^+ and Cl^- will be pumped in, further increasing the concentration of Cl^- ions in the endosome. Water will subsequently follow the osmotic gradient and the increased pressure will burst the endosome, releasing its contents into the cytoplasm. Although this model has been understood as fact in many instances, there is currently insufficient proof that polyamines facilitate endosome escape in this manner. More research must be directed toward elucidating the exact mechanisms surrounding proton absorption

by polyamines and if the resultant increase in osmotic pressure is alone sufficient to burst the endosome [70]. There is experimental evidence for the proton sponge effect which shows that, when used in polyplexes with DNA, titratable polyamines facilitate more endosome swelling and lysis than non-titratable polyamines [69], but data have also shown that once complexed with nucleic acids, proton sponges like PEI lose much of their buffering capacity, requiring either a high nitrogen/phosphate complexation ratio or delivery with free PEI for efficient transfection [71]. Recent hypotheses propose that this free PEI instead drives endosome lysis by electrostatically interacting with and destabilizing the endosomal membrane, and also by preventing lysosomes from fusing with endosomes, thus increasing the available time for endosome escape [72]. These data, taken together, suggest that while more data must be collected to prove the existence of the proton sponge effect, titratable polyamines such as PEI are still effective transfection agents, albeit possibly under a different mechanism. To be sure, osmotic endosomal rupture may still be an effective model as separate from the proton sponge effect, as the dissolution of precipitated particles, such as an acid-responsive calcium phosphate core, in an endosome may facilitate release in this manner [26].

Release

For siRNA to pass from systemic circulation into the cellular cytoplasm, it must remain encapsulated across many barriers, but be immediately free and functional once it arrives at its target. siRNA has often been complexed with the cationic polymer protamine, which encapsulates very efficiently, but has no active mechanism for release [21]. In a case such as this, siRNA is not efficiently released and must rely on indirect processes such as competition with cytoplasmic proteins for protamine's positive charge. Many models have been developed to facilitate efficient siRNA release, and several again utilize acid [73] and redox-cleavable [31,74] bonds that can detach siRNA from its carrier in the endosome or cytoplasm, as the cytoplasm contains a high concentration of reducing agents such as glutathione [31].

Putting it All Together: Model Examples of Innovative and Successful NPs

To date, around 20 RNAi clinical trials have been initiated against a number of diseases, including cancer [75]. These products currently represent the most proven, effective formulations, but because the road from bench to bedside is long and difficult, many NPs in clinical trials are not nearly as advanced as those that have recently been developed. New approaches for NP delivery and function include the novel field of theranostics, in which NPs offer diagnostic imaging applications as well as their conventional therapies, and the utilization of naturally occurring vesicles, called exosomes, to better deliver siRNA across biological barriers. The following sections will provide a brief description of several diverse NPs – of which, Atu027, ALN-VSP, and CALAA-01 are currently in clinical trials – to demonstrate the varied methods of delivery that have overcome many, if not all, of the barriers described above (see Table 4.2).

Atu027

The siRNA-lipoplex Atu027 is a product of Silence Therapeutics and is currently in phase I clinical trials for patients with advanced solid tumors [76,77,78]. The trials are currently ongoing and dose-escalation studies have shown that Atu027 is well tolerated in humans. Atu027 delivers siRNA against protein kinase N3 (PKN3), a downstream effector of the phosphoinositide 3-kinase (PI3K) signaling pathway with pro-angiogenic function. The formulation for Atu027 was first developed in 2006 [79]. The multilamellar (multiple concentric bilayer) lipoplex contains a custom-synthesized lipid with a highly charged head group called AtuFECT01, the neutral fusogenic helper lipid DphyPE to aid cell and endosome membrane association and subsequent endosomal release, and just 1 mol% DSPE-PEG. A low PEG density was chosen because the unsupported bilayer in this design was destabilized upon addition of just 5 mol% PEG, but this low density also provided less binding inhibition of the lipoplex to the cell surface. PKN3 siRNA was modified with 2′-O-methyl sugar moieties to

Table 4.2 NP characteristics.

	Properties			*Barriers*				
NP	*Size (nm)*	*Zeta (mV)*	*Imaging?*	*Complexation*	*MPS*	*Target/internalize*	*Endosome escape*	*Release*
Atu027	120	46	No	Electrostatic	PEG	Passive	Ion pair	Fusogenic lipid-mediated
ALN-VSP	80–100	< 6	No	Ethanol destabilization	PEG	Passive	Ion pair	Fusogenic lipid mediated
CALAA-01	70	–25 to 15	No	Cyclodextrin	PEG	Transferrin	Proton sponge	Acid-degradable bonds
LPH	115–170	10–25	No	Electrostatic	PEG	Anisamide, GC4 scFv	Ion pair	No active mechanism
LCP	42–50	25	No	Calcium phosphate	PEG	Anisamide, galactose	Osmotic rupture/ion pair	CaP dissolution at low pH
Mesoporous silica	165	–32 to 12	Capable	Electrostatic	PEG	SP94	Ion pair	Acid-mediated destabilization
AuNP	194, 115	17.2	Yes	Amino-ketal bonds	PEG	Cationic surface exposure	Proton sponge	Amino-ketal bonds
Iron oxide NP	75	–30	Yes	Disulfide bonds	PEG	RGD peptide	Charge–charge destabilization	Reducible bonds
Exosome	40–100	N/A	No	Electroporation	NA	RVG peptide	NA	Direct cytosol delivery

For definitions of NPs, see text; NA, not applicable.

increase resistance to serum nuclease, and was efficiently electrostatically bound to the highly charged AtuFECT01 lipids between bilayers or on the outside of the lipoplex. Simple mixing of the liposomal dispersion with siRNA was enough to efficiently synthesize the siRNA-lipoplex and provide ≈ 120 nm particles with a zeta potential of +46 mV. *In vitro*, Atu027 can completely suppress PKN3 levels and provide an IC_{50} value of between 5 and 10 nmol/L. *In vivo*, tail-vein-administered doses of 2.8 mg/kg siRNA showed significant pulmonary metastasis inhibition, but did not affect primary tumor growth, likely because of the disparity in tumor-growth-related angiogenesis between metastases and the primary tumor. The lung metastasis data for Atu027 as shown in [77] is robust and suggests that Atu027 may be clinically applicable against highly metastatic tumors but there are many improvements that could increase its effectiveness and broaden its usefulness, including supporting the outer bilayer to allow an increase in PEG concentration, and perhaps encapsulating a second siRNA molecule which targets a non-angiogenic oncogenic pathway.

ALN-VSP

ALN-VSP, a lipid NP by Alnylam Pharmaceuticals that is formulated based on the small nucleic-acid-lipid particle (SNALP) design [80,81] has recently completed its phase I clinical trial in patients with liver cancer [82]. The 41-subject dose-escalation trial, which evaluated toxicity, pharmacokinetics, proof-of-mechanism for RNAi, and tumor response, showed relative safety and efficacy, with multiple patients responding to treatment, including one complete response, a rarity in phase I trials. ALV-VSP encapsulates siRNA against vascular endothelial growth factor (VEGF) and kinesin spindle protein (KSP) inside a lipid membrane made up of the ionizable cationic lipid DSPC, the fusogenic lipid DLinDMA, cholesterol, and a low concentration of PEG-C-DMA. Encapsulation occurs by simply destabilizing the lipid in 40% ethanol and then incubating it with the siRNA [83]. The final particle is 80–100 nm in diameter and has a low zeta potential of less than +6 mV at physiological pH. The cationic lipids facilitate passive cellular uptake, while the fusogenic lipids expedite fusion with the cellular and endosomal membrane, accelerating release of siRNA. Although simple, ALN-VSP continues to produce data in favor of use in the clinic.

CALAA-01

CALAA-01 is a sophisticated NP by Calando Pharmaceuticals that is also currently in phase I clinical trials for patients with solid tumors [60,84]. An ongoing dose-escalation trial will determine the maximum tolerated dose of CALAA-01, and evaluate the pharmacokinetics, immune response, and tumor response to this NP. Published data from three subjects in this study show heterogeneous distribution of NPs in tumor biopsies after systemic NP injection. This was the first time that a systemically delivered targeted NP was shown to have dose-dependent tumor accumulation in humans. The siRNA used in this trial is against the M2 subunit of ribonucleotide reductase, RRM2. There are four components to this delivery system, including the delivered siRNA. The first of the three delivery components is a cyclodextrin-containing cationic polymer (CDP) with five or six repeating cyclodextrin units and several cationic charge centers to complex the siRNA. The other two components are adamantane (AD)–PEG and AD–PEG–transferrin (Tf) conjugates. AD has a very high association constant with cyclodextrin and will form strong non-covalent bonds in solution, while PEG's stealth properties are well documented, and Tf is a well-known targeting ligand for several types of cancer cells. Upon simple mixing of siRNA with the three delivery components, ≈70 nm NPs can be reproducibly synthesized in high concentrations. CALAA-01 can handle a high PEG density, with an average CDP/PEG ratio of 2.5, allowing for effective shielding, although only a small percentage of PEG can be conjugated with Tf before Tf–Tf-induced particle aggregation occurs. Desired proportions of the AD–PEG conjugate can also be modified to AD–anionic charge–PEG to tune the zeta potential from +15 to −25 mV. Delivery efficiency can be further increased by adding the imidazole functional group to the termini of the CDP, which acts as a pH buffer in the endosome and may facilitate escape through the proton sponge effect. Around a pH of 6 the CDP dissociates to release the siRNA and break into components small enough to be routinely cleared

by the kidney. The design of this NP accounts for all major barriers to efficient delivery.

Lipid–Polycation–Hyaluronic Acid

Lipid–polycation–hyaluronic acid (LPH) was developed as an improved version of the Lipid–polycation–DNA (LPD) lipoplex [37,38]. The core of LPD is formed by electrostatic interaction of polyanionic siRNA (low avidity) and polyanionic calf thymus DNA (high avidity) with polycationic protamine. The cationic 1,2-bis(oleoyloxy)-3-(trimethylammonio)propane (DOTAP)/cholesterol (1:1) lipid bilayer is supported by the anionic core by electrostatic interaction and thus can withstand a larger amount of PEG (>10 mol%) than an unsupported bilayer [21]. LPH is formed by replacing calf thymus DNA with the high-avidity polyanion hyaluronic acid, a low immunostimulatory, FDA-approved polysaccharide that maintains a high siRNA encapsulation efficiency of 90% [37,38]. This improves the biocompatability of the formulation, as calf thymus DNA may present unexpected toxicity and immune response due to its high density of CpG motifs, and increases the therapeutic window of the treatment by 350%. Formulations of LPH generally have sizes ≈ 115 to 170 nm and zeta potentials of 10–25 mV. LPH-bound DSPE-PEG has been functionalized with targeting ligands including anisamide and GC4 scFv, allowing LPH to significantly inhibit lung metastasis nodules while presenting no significant toxicity at 0.45 mg siRNA/kg. LPH can also simultaneously deliver both siRNA and miRNA, and has successfully delivered c-Myc siRNA, MDM2 siRNA, VEGF siRNA, and miR-34a miRNA in a 1:1:1:3 weight proportion *in vivo*, decreasing the lung metastasis tumor load to ≈ 20% of the untreated control. Both LPD and LPH escape the endosome through ion-pair formation, but contain no active mechanism for siRNA release.

Lipid-Calcium Phosphate

This liposomal NP (Figure 4.4) encapsulates siRNA in an amorphous calcium phosphate core [26]. The inner leaflet of the bilayer is the anionic phospolipid dioleoylphosphatidic acid (DOPA) whose phosphate headgroup is incorporated into the calcium phosphate core. This supports the inner leaflet and allows a much higher incorporation of PEG with cationic DOTAP/cholesterol in the outer leaflet (up to 23 mol% DSPE-PEG). PEG can be further functionalized with a targeting ligand, such as the anisamide ligand for the sigma receptor or galactose to target the well-established hepatocyte galactose receptor [85,86]. With PEG and anisamide added, the lipid-calcium phosphate (LCP) NP has a size of 42–50 nm and a zeta potential of +25 mV. This size is much smaller than the size of liver fenestrae (≈100 nm), but the very small size of the LCP may also allow exit back out of the fenestrae for tumor-targeted delivery. Passage through the fenestrae can also be exploited by facilitating highly efficient hepatocyte-targeted delivery. After receptor-mediated uptake into the cellular endosome, it is proposed that the acid-responsive dissolution of the calcium phosphate core triggers osmotic rupture of the endosome and release of the cargo. Published data show that the *in vitro* IC_{50} of siRNA delivered in LCP was just 5 nM, and *in vivo* biodistribution showed preferential tumor accumulation, while the ED_{50} *in vivo* was 0.6 mg siRNA/kg. Intravenous injection of LCP has been shown to inhibit non-small-cell lung

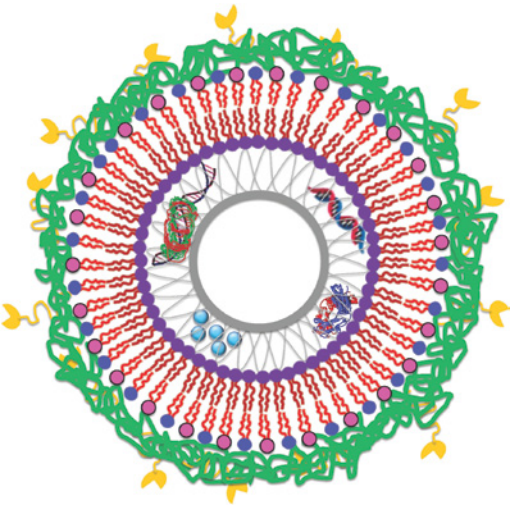


Figure 4.4 Hypothetical diagram of LCP with a collapsed and entangled PEG layer. A hollow calcium phosphate core (middle) can encapsulate siRNA, DNA, drug, or peptide. An asymmetric lipid bilayer, where the lipids in the inner leaflet differ from those in the outer leaflet, surrounds the core and is functionalized with PEG (green) and targeting ligands (yellow). Ligands may also be entangled, allowing only a fraction to be available for receptor binding. Source: B. DiPrete, unpublished diagram.

cancer xenograft tumor growth when delivering a pooled therapeutic combination of siRNA (HDM2/c-Myc/VEGF, 1:1:1) [87], and has also been shown to inhibit murine melanoma tumor growth in a lung metastasis model [88].

Mesoporous Silica

Mesoporous silica particles have recently been developed as efficient siRNA delivery vehicles because of their large available surface area and pore volume for siRNA encapsulation. Mesoporous silica can be used for sustained siRNA delivery [89], and can be externally layered with PEI [90] or a lipid bilayer [91] for further functionalization. Not until recently has siRNA been successfully loaded into the silica pores, as silica and siRNA are both negatively charged. One strategy to overcome this obstacle is to modify the silica with amine-containing silane, increasing the particle's zeta potential from −32 to +12 mV and increasing its siRNA-loading capacity. Coating the silica with cationic DOTAP also facilitated siRNA entry into the pores. These approaches led to a 10-fold increase in siRNA encapsulation per volume of NP compared to DOTAP NPs without mesoporous silica [91]. The lipid-layered mesoporous silica particles, termed "protocells," had an average diameter of 165 nm and could retain their siRNA cargo until membrane destabilization occurred under mildly acidic conditions. The protocells were further functionalized with the SP94 targeting ligand against hepatocellular carcinoma, and the acid-responsive, endosomolytic peptide H5WYG [92] was conjugated to the protocells to aid escape of the endo-/lysosome. When loaded with an equimolar mixture of cyclin A2, cyclin B1, cyclin D1, and cyclin E siRNA at a total concentration of 125 pM this protocell formulation was able to achieve ≈ 90% repression of each protein after continuous exposure to hepatocellular carcinoma for 72 h. It has been suggested that future iterations of this formulation could additionally include imaging or diagnostic agents. *In vivo* testing of this protocell has yet to be published.

Theranostic Gold NP

Optical coherence tomography (OCT) is a noninvasive imaging modality that can measure optical reflections in biological tissues [93]. Theranostic gold NPs (AuNPs) are good contrast agents for imaging using OCT and can easily be functionalized and incorporated into larger nanostructures. This specific nanostructure [55] not only utilizes the optical properties of AuNP aggregrates in a single particle, but also takes advantage of the optical shift that occurs when AuNPs dissipate away from each other. Linear PEI functionalized with acid-degradable amino-ketal branches is used to complex siRNA in the core of the particle at physiological conditions. Amino-ketal linkages facing the opposite direction also conjugate AuNPs that coat the PEI, and PEG is further conjugated on the AuNPs, facing outward, to protect the particle from opsonization (Figure 4.5A). Upon arrival in the tumor microenvironment, the outer acid-degradable linkages begin to degrade more quickly than the inner linkages, shedding the PEG–AuNPs and exposing the cationic surface of the PEI–siRNA (Figure 4.5B). This dissipation of AuNPs causes a drop in scattering intensity, as well as a shift in the maximum ultraviolet absorbance peak, which allows detection of arrival and successful acid response at the tumor site. Endocytosis is aided by the newly exposed cationic core and smaller particle diameter, and the increased endosomal acidity quickens ketal bond degradation, freeing the siRNA (Figure 4.5C). In the endosome, the PEI also acts as a proton sponge, destabilizing the endosome and releasing the siRNA into the cytoplasm. The siRNA used in this publication was against green fluorescent protein to show proof of principle. The initial, fully functionalized NP is ≈ 194 nm in size with 15 nm AuNPs and a zeta potential of +17.2 mV, and after acid hydrolysis, the cationic core is ≈ 115 nm. A therapeutic model has not yet been reported for this particle, but early data show promise for success.

Theranostic Magnetic Iron Oxide NP

Magnetic resonance imaging (MRI), which uses electromagnetic radiation to measure the nuclear magnetic resonance of atoms, is another imaging modality that theranostic NPs can utilize. Magnetic iron oxide NPs are excellent contrast agents for MRI, and boast many properties that enhance delivery effciency, such as their biocompatibility and ability to be readily functionalized. In their 2009 publication, Lee et al. [62] describe

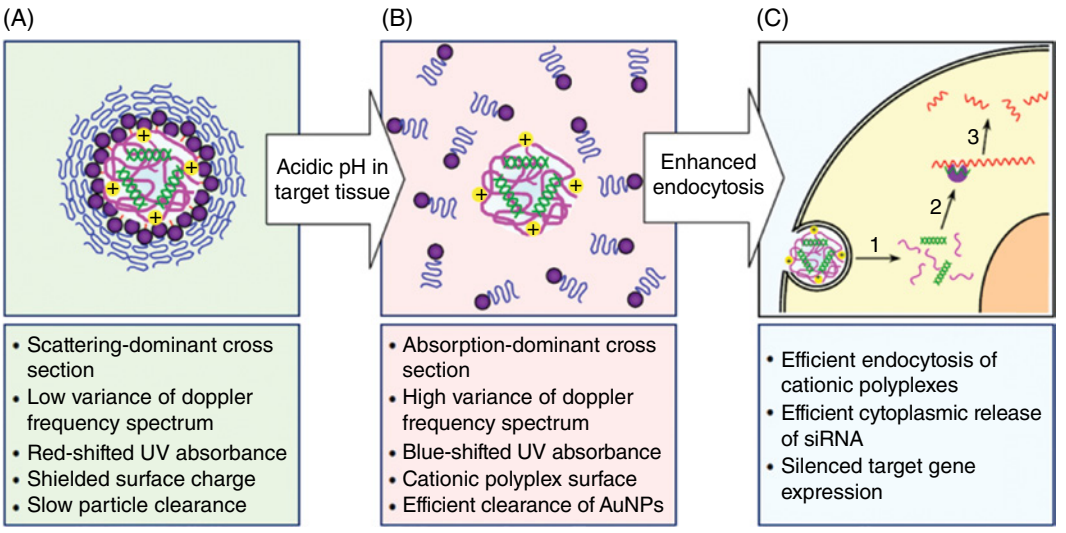


Figure 4.5 Schematic diagram of an AuNP complex. (A) The fully functionalized NP in systemic circulation. (B) Degradation of ketal linkages causes individual PEG-functionalized AuNPs to release and dissipate. (C) Exposed cationic core facilitates enhanced endocytosis and proton sponge effect, and is further degraded in the endosome to release siRNA. Source: Adapted from Shim 2010 [55]. Copyright 2012 American Chemical Society.

the production of their multifunctional magnetic NP. The core of this NP was made from 15 nm manganese-doped magnetic iron oxide NPs in which the manganese further enhanced MRI signals. The core was coated with cationized bovine serum albumin (BSA) with an isoelectric point of 6.1, allowing it to become cationic only in the tumor microenvironment. The BSA was further modified to allow disulfide functionalization, and thiolated PEG with the RGD targeting peptide attached to its distal end was conjugated to the BSA. The RGD peptide specifically binds to $\alpha_v\beta_3$ integrin, which is overexpressed in metastatic tumor cells. Thiolated siRNA against green fluorescent protein was also bound to the BSA, creating two different functional groups attached to the BSA that could be cleaved in the reducing intracellular environment. Each NP was estimated to contain ≈ 40 siRNA molecules. In this publication, Cy5 (red) dye was conjugated to the siRNA to allow intracellular imaging of successful delivery and internalization. MRI and fluorescence confocal microscopy were then used simultaneously to determine NP effectiveness. After complete conjugation, the NP had a size of ≈ 75 nm and a zeta potential of −30 mV, owing to the negatively charged siRNA at its surface. This NP has a distinct toxicological advantage over many others because under physiological conditions, it contains no cationic components; it is only at the tumor site that the BSA becomes cationic to disrupt the endosome.

Exosomes

Scientists have spent decades synthesizing complex delivery systems for nucleic acids, but the best delivery vehicle could be one that occurs naturally. Alvarez-Erviti et al. [94] was the first group to successfully use exosomes as a delivery vehicle for siRNA. Exosomes are ≈ 40–100 nm vesicles that form by the *inward* budding of endosomal membranes inside cells [95]. Exosomes can express proteins derived from their parent cell [96] and have also been found to naturally carry nucleic acids [97]. Alvarez-Erviti's group functionalized exosomes with targeting ligands by transfecting immature dendritic cells with the desired ligand constructs and then harvesting ligand-bearing exosomes from the dendritic cells that now expressed those ligands. Electroporation was then successfully used to load the exosomes with GAPDH siRNA. The most impressive data resulted when using the rabies viral glycoprotein (RVG) peptide, the ligand for the acetylcholine receptor found in

neurons. This led to downregulation of GAPDH in the brain in response to delivered siRNA, and indicated that the exosomes had successfully crossed the blood–brain barrier, the most formidable physical barrier to drug delivery due to its extremely tight junctions and astrocyte/pericyte regulation. This delivery also occurred without any immune response to the exosomes, as the delivery vehicles were purified from cells from the same murine strain they were used on. This may be the most impressive aspect of exosomal delivery; there is no worry about toxicity, opsonization, or MPS recognition because the delivery system could potentially be drawn from a patient's own cells. There is also no need to overcome the endosomal barrier, as exosomal cargo is directly delivered to the cellular cytosol after fusion with the cell membrane [98]. Of course, much is still unknown about exosomes, and even if they are eventually fully characterized it would be difficult to scale up production for clinical use. If nothing else, this breakthrough has shown that a little creativity can go a long way in the field of nanotherapeutics.

Conclusions

The ability of RNAi to silence expression at the pretranslational level sets it apart from other drugs, such as the developing field of small-molecule kinase inhibitors. It has been clear for some time that non-viral siRNA delivery has significant potential, but formulating effective delivery systems has proven more difficult than anticipated. The barriers to efficient delivery are complex, prevalent, and difficult to hurdle, but, even so, solutions have been developed for each and have been successfully implemented in NPs. Thousands of articles have been published on non-viral RNAi, and the field is reaching a biological understanding of the barriers to delivery along with a chemical and physical understanding of how to overcome them. To maximize chances for success, newly designed NPs should attempt to overcome all of these barriers, a practice that may seem obvious in principle, but is difficult to carry out. Many laboratories are working with NPs for RNAi, and most have their own unique formulation that they have been working with for years. Each has its own equipment, training, and strengths to improve upon its own design. Realistically, there is little impetus for an individual laboratory to make drastic changes if they are successfully publishing papers. In the field of cancer therapy, however, this cannot be the goal, and because non-viral delivery requires so much complexity and so many levels of design, interlaboratory collaboration is essential to accelerate discovery and expedite this therapy to patients.

Acknowledgments

The original work performed in the authors' laboratory has been supported by US National Institutes of Health grants CA129835, CA129421, CA149363, CA151652, and CA151455. ABS also acknowledges support from the NSF GRFP Fellowship.

References

1 Neufeld EF, Sweeley, CC. (1972) Gene therapy for human genetic disease? *Science* 178(4061): 648–649.
2 Fletcher BS. (2011) Advancements in non viral gene therapy for hemophilia. *Journal of Genetic Syndromes & Gene Therapy* S1:002. doi:10.4172/2157-7412.S1-002.
3 Rogers ML, Rush RA. (2012) Non-viral gene therapy for neurological diseases, with an emphasis on targeted gene delivery. *Journal of Controlled Release* 157(2): 183–189.
4 Li S, Huang L. (2000) Nonviral gene therapy: promises and challenges. *Gene Therapy* 7(1): 31–34.
5 Morille M, Passirani C, Vonarbourg A, Clavreul A, Benoit JP. (2008) Progress in developing cationic vectors for non-viral systemic gene therapy against cancer. *Biomaterials* 29(24–25): 3477–3496.
6 Wiethoff CM, Middaugh CR. (2003) Barriers to nonviral gene delivery. *Journal of Pharmaceutical Sciences* 92(2): 203–217.
7 Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391(6669): 806–811.
8 Bernstein E, Caudy AA, Hammond SM, Hannon GJ. (2001) Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* 409(6818): 363–366.
9 Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 411(6836): 494–498.
10 Kawamata T, Tomari Y. (2010) Making RISC. *Trends in Biochemical Sciences* 35(7): 368–376.
11 Filipowicz W. (2005) RNAi: the nuts and bolts of the RISC machine. *Cell* 122(1): 17–20.

12 Schwarz DS, Hutvagner G, Du T, Xu Z, Aronin N, Zamore PD. (2003) Asymmetry in the assembly of the RNAi enzyme complex. *Cell* 115(2): 199–208.
13 MacRae IJ, Ma E, Zhou M, Robinson CV, Doudna JA. (2008) In vitro reconstitution of the human RISC-loading complex. *Proceedings of the National Academy of Sciences USA* 105(2): 512–517.
14 Tuschl T, Zamore PD, Lehmann R, Bartel DP, Sharp PA. (1999) Targeted mRNA degradation by double-stranded RNA in vitro. *Genes & Development* 13(24): 3191–3197.
15 Valencia-Sanchez MA, Liu J, Hannon GJ, Parker R. (2006) Control of translation and mRNA degradation by miRNAs and siRNAs. *Genes & Development* 20(5): 515–524.
16 Rand TA, Petersen S, Du F, Wang X. (2005) Argonaute2 cleaves the anti-guide strand of siRNA during RISC activation. *Cell* 123(4): 621–629.
17 Ebalunode JO, Jagun C, Zheng W. (2011) Informatics approach to the rational design of siRNA libraries. *Methods in Molecular Biology* 672: 341–358.
18 Baranova A, Bode J, Manyam G, Emelianenko M. (2011) An efficient algorithm for systematic analysis of nucleotide strings suitable for siRNA design. *BMC Research Notes* 4: 168.
19 Gupta S, Akhoon BA, Srivastava M, Gupta SK. (2010) A novel algorithm to design an efficient siRNA by combining the pre proposed rules of siRNA designing. *Journal of Computer Science & Systems Biology* 3(1): 005–009.
20 Monaghan M, Pandit A. (2011) RNA interference therapy via functionalized scaffolds. *Advanced Drug Delivery Reviews* 63(4–5): 197–208.
21 Li SD, Huang L. (2009) Nanoparticles evading the reticuloendothelial system: role of the supported bilayer. *Biochimica et Biophysica Acta* 1788(10): 2259–2266.
22 Nolan JP. (1981) Endotoxin, reticuloendothelial function, and liver injury. *Hepatology* 1(5): 458–465.
23 Ruponen M, Yla-Herttuala S, Urtti A. (1999) Interactions of polymeric and liposomal gene delivery systems with extracellular glycosaminoglycans: physicochemical and transfection studies. *Biochimica et Biophysica Acta* 1415(2): 331–341.
24 Wisse E, Jacobs F, Topal B, Frederik P, De Geest B. (2008) The size of endothelial fenestrae in human liver sinusoids: implications for hepatocyte-directed gene transfer. *Gene Therapy* 15(17): 1193–1199.
25 Monsky WL, Fukumura D, Gohongi T, Ancukiewcz M, Weich HA, Torchilin VP, et al. (1999) Augmentation of transvascular transport of macromolecules and nanoparticles in tumors using vascular endothelial growth factor. *Cancer Research* 59(16): 4129–4135.
26 Li J, Yang Y, Huang L. (2012) Calcium phosphate nanoparticles with an asymmetric lipid bilayer coating for siRNA delivery to the tumor. *Journal of Controlled Release* 158(1): 108–114.
27 Li SD, Chen YC, Hackett MJ, Huang L. (2008) Tumor-targeted delivery of siRNA by self-assembled nanoparticles. *Molecular Therapy* 16(1): 163–169.
28 Layzer JM, McCaffrey AP, Tanner AK, Huang Z, Kay MA, Sullenger BA. (2004) In vivo activity of nuclease-resistant siRNAs. *RNA* 10(5): 766–771.
29 Pan X, Thompson R, Meng X, Wu D, Xu L. (2011) Tumor-targeted RNA-interference: functional non-viral nanovectors. *American Journal of Cancer Research* 1(1): 25–42.
30 Felgner PL, Gadek TR, Holm M, Roman R, Chan HW, Wenz M, et al. (1987) Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proceedings of the National Academy of Sciences USA* 84(21): 7413–7417.
31 Matsumoto S, Christie RJ, Nishiyama N, Miyata K, Ishii A, Oba M, et al. (2009) Environment-responsive block copolymer micelles with a disulfide cross-linked core for enhanced siRNA delivery. *Biomacromolecules* 10(1): 119–127.
32 Lv H, Zhang S, Wang B, Cui S, Yan J. (2006) Toxicity of cationic lipids and cationic polymers in gene delivery. *Journal of Controlled Release* 114(1): 100–109.
33 Meyer M, Dohmen C, Philipp A, Kiener D, Maiwald G, Scheu C, et al. (2009) Synthesis and biological evaluation of a bioresponsive and endosomolytic siRNA-polymer conjugate. *Molecular Pharmaceutics* 6(3): 752–762.
34 Shim MS, Kwon YJ. (2009) Acid-responsive linear polyethylenimine for efficient, specific, and biocompatible siRNA delivery. *Bioconjugate Chemistry* 20(3): 488–499.
35 Pittella F, Zhang M, Lee Y, Kim HJ, Tockary T, Osada K, et al. (2011) Enhanced endosomal escape of siRNA-incorporating hybrid nanoparticles from calcium phosphate and PEG-block charge-conversional polymer for efficient gene knockdown with negligible cytotoxicity. *Biomaterials* 32(11): 3106–3114.
36 Li SD, Huang L. (2006) Targeted delivery of antisense oligodeoxynucleotide and small interference RNA into lung cancer cells. *Molecular Pharmaceutics* 3(5): 579–588.
37 Chono S, Li SD, Conwell CC, Huang L. (2008) An efficient and low immunostimulatory nanoparticle formulation for systemic siRNA delivery to the tumor. *Journal of Controlled Release* 131(1): 64–69.
38 Chen Y, Zhu X, Zhang X, Liu B, Huang L. (2010) Nanoparticles modified with tumor-targeting scFv deliver siRNA and miRNA for cancer therapy. *Molecular Therapy* 18(9): 1650–1656.
39 Mahato RI, Rolland A, Tomlinson E. (1997) Cationic lipid-based gene delivery systems: pharmaceutical perspectives. *Pharmaceutical Research* 14(7): 853–859.
40 Klibanov AL, Maruyama K, Torchilin VP, Huang L. (1990) Amphipathic polyethyleneglycols effectively

prolong the circulation time of liposomes. *FEBS Letters* 268(1): 235–237.

41 Blume G, Cevc G. (1990) Liposomes for the sustained drug release in vivo. *Biochimica et Biophysica Acta* 1029(1): 91–97.

42 Woodle MC, Matthay KK, Newman MS, Hidayat JE, Collins LR, Redemann C, et al. (1992) Versatility in lipid compositions showing prolonged circulation with sterically stabilized liposomes. *Biochimica et Biophysica Acta* 1105(2): 193–200.

43 Li SD, Huang L. (2010) Stealth nanoparticles: high density but sheddable PEG is a key for tumor targeting. *Journal of Controlled Release* 145(3): 178–181.

44 Davis ME. (2002) Non-viral gene delivery systems. *Current Opinion in Biotechnology* 13(2): 128–131.

45 Jiang S, Cao Z. (2010) Ultralow-fouling, functionalizable, and hydrolyzable zwitterionic materials and their derivatives for biological applications. *Advanced Materials* 22(9): 920–932.

46 Backmann N, Kappeler N, Braun T, Huber F, Lang HP, Gerber C, et al. (2010) Sensing surface PEGylation with microcantilevers. *Beilstein Journal of Nanotechnology* 1: 3–13.

47 Hak S, Helgesen E, Hektoen HH, Huuse EM, Jarzyna PA, Mulder WJ, et al. (2012) The effect of nanoparticle polyethylene glycol surface density on ligand-directed tumor targeting studied in vivo by dual modality imaging. *ACS Nano* 6(6): 5648–5658.

48 Knorr V, Allmendinger L, Walker GF, Paintner FF, Wagner E. (2007) An acetal-based PEGylation reagent for pH-sensitive shielding of DNA polyplexes. *Bioconjugate Chemistry* 18(4): 1218–1225.

49 Tagami T, Nakamura K, Shimizu T, Ishida T, Kiwada H. (2009) Effect of siRNA in PEG-coated siRNA-lipoplex on anti-PEG IgM production. *Journal of Controlled Release* 137(3): 234–240.

50 Ishida T, Wang X, Shimizu T, Nawata K, Kiwada H. (2007) PEGylated liposomes elicit an anti-PEG IgM response in a T cell-independent manner. *Journal of Controlled Release* 122(3): 349–355.

51 Semple SC, Harasym TO, Clow KA, Ansell SM, Klimuk SK, Hope MJ. (2005) Immunogenicity and rapid blood clearance of liposomes containing polyethylene glycol-lipid conjugates and nucleic acid. *Journal of Pharmacology and Experimental Therapeutics* 312(3): 1020–1026.

52 Judge A, McClintock K, Phelps JR, Maclachlan I. (2006) Hypersensitivity and loss of disease site targeting caused by antibody responses to PEGylated liposomes. *Molecular Therapy* 13(2): 328–337.

53 Amoozgar Z, Yeo Y. (2012) Recent advances in stealth coating of nanoparticle drug delivery systems. *Wiley Interdisciplinary Reviews Nanomedicine and Nanobiotechnology* 4(2): 219–233.

54 von Erlach T, Zwicker S, Pidhatika B, Konradi R, Textor M, Hall H, et al. (2011) Formation and characterization of DNA-polymer-condensates based on poly(2-methyl-2-oxazoline) grafted poly(L-lysine) for non-viral delivery of therapeutic DNA. *Biomaterials* 32(22): 5291–5303.

55 Shim MS, Kim CS, Ahn YC, Chen Z, Kwon YJ. (2010) Combined multimodal optical imaging and targeted gene silencing using stimuli-transforming nanotheragnostics. *Journal of the American Chemical Society* 132(24): 8316–8324.

56 Takae S, Miyata K, Oba M, Ishii T, Nishiyama N, Itaka K, et al. (2008) PEG-detachable polyplex micelles based on disulfide-linked block catiomers as bioresponsive nonviral gene vectors. *Journal of the American Chemical Society* 130(18): 6001–6009.

57 Hatakeyama H, Akita H, Kogure K, Oishi M, Nagasaki Y, Kihira Y, et al. (2007) Development of a novel systemic gene delivery system for cancer therapy with a tumor-specific cleavable PEG-lipid. *Gene Therapy* 14(1): 68–77.

58 Huang L, Liu Y. (2011) In vivo delivery of RNAi with lipid-based nanoparticles. *Annual Review of Biomedical Engineering* 13: 507–530.

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

60 Davis ME. (2009) The first targeted delivery of siRNA in humans via a self-assembling, cyclodextrin polymer-based nanoparticle: from concept to clinic. *Molecular Pharmaceutics* 6(3): 659–668.

61 Dohmen C, Frohlich T, Lachelt U, Rohl I, Vornlocher HP, Hadwiger P, Wagner E. (2012) Defined folate-PEG-siRNA conjugates for receptor-specific gene silencing. *Molecular Therapy Nucleic Acids* e7(1): 1–6.

62 Lee JH, Lee K, Moon SH, Lee Y, Park TG, Cheon J. (2009) All-in-one target-cell-specific magnetic nanoparticles for simultaneous molecular imaging and siRNA delivery. *Angewandte Chemie International Edition in English* 48(23): 4174–4179.

63 Mok H, Veiseh O, Fang C, Kievit FM, Wang FY, Park JO, et al. (2010) pH-Sensitive siRNA nanovector for targeted gene silencing and cytotoxic effect in cancer cells. *Molecular Pharmaceutics* 7(6): 1930–1939.

64 Gao H, Shi W, Freund LB. Mechanics of receptor-mediated endocytosis. *Proceedings of the National Academy of Sciences USA* 2005;102(27): 9469–9474.

65 Kulkarni RP, Mishra S, Fraser SE, Davis ME. (2005) Single cell kinetics of intracellular, nonviral, nucleic acid delivery vehicle acidification and trafficking. *Bioconjugate Chemistry* 16(4): 986–994.

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

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

68 Sonawane ND, Szoka FC Jr, Verkman AS. (2003) Chloride accumulation and swelling in endosomes enhances DNA transfer by polyamine-DNA polyplexes. *Journal of Biological Chemistry* 278(45): 44826–31.

69 Patil ML, Zhang M, Taratula O, Garbuzenko OB, He H, Minko T. (2009) Internally cationic polyamidoamine PAMAM-OH dendrimers for siRNA delivery: effect of the degree of quaternization and cancer targeting. *Biomacromolecules* 10(2): 258–266.

70 Won YY, Sharma R, Konieczny SF. (2009) Missing pieces in understanding the intracellular trafficking of polycation/DNA complexes. *Journal of Controlled Release* 139(2): 88–93.

71 Boeckle S, von Gersdorff K, van der Piepen S, Culmsee C, Wagner E, Ogris M. (2004) Purification of polyethylenimine polyplexes highlights the role of free polycations in gene transfer. *Journal of Gene Medicine* 6(10): 1102–1111.

72 Yue Y, Jin F, Deng R, Cai J, Dai Z, Lin MC, et al. (2011) Revisit complexation between DNA and polyethylenimine--effect of length of free polycationic chains on gene transfection. *Journal of Controlled Release* 152(1): 143–151.

73 Lin YL, Jiang G, Birrell LK, El-Sayed ME. (2010) Degradable, pH-sensitive, membrane-destabilizing, comb-like polymers for intracellular delivery of nucleic acids. *Biomaterials* 31(27): 7150–7166.

74 Beloor J, Choi CS, Nam HY, Park M, Kim SH, Jackson A, et al. (2012) Arginine-engrafted biodegradable polymer for the systemic delivery of therapeutic siRNA. *Biomaterials* 33(5): 1640–1650.

75 Shim MS WS, Kwon YJ. (2012) siRNA as a conventional drug in the clinic? Challenges and current technologies. *Drug Discovery Today: Technologies* 9(2): e167–e173.

76 Strumberg D, Schultheis B, Traugott U, Vank C, Santel A, Keil O, et al. (2012) Phase I clinical development of Atu027, a siRNA formulation targeting PKN3 in patients with advanced solid tumors. *International Journal of Clinical Pharmacology and Therapeutics* 50(1): 76–78.

77 Santel A, Aleku M, Roder N, Mopert K, Durieux B, Janke O, et al. (2010) Atu027 prevents pulmonary metastasis in experimental and spontaneous mouse metastasis models. *Clinical Cancer Research* 16(22): 5469–5480.

78 Aleku M, Schulz P, Keil O, Santel A, Schaeper U, Dieckhoff B, et al. (2008) Atu027, a liposomal small interfering RNA formulation targeting protein kinase N3, inhibits cancer progression. *Cancer Research* 68(23): 9788–9798.

79 Santel A, Aleku M, Keil O, Endruschat J, Esche V, Fisch G, et al. (2006) A novel siRNA-lipoplex technology for RNA interference in the mouse vascular endothelium. *Gene Therapy* 13(16): 1222–1234.

80 Morrissey DV, Lockridge JA, Shaw L, Blanchard K, Jensen K, Breen W, et al. (2005) Potent and persistent in vivo anti-HBV activity of chemically modified siRNAs. *Nature Biotechnology* 23(8): 1002–1007.

81 Zimmermann TS, Lee AC, Akinc A, Bramlage B, Bumcrot D, Fedoruk MN, et al. (2006) RNAi-mediated gene silencing in non-human primates. *Nature* 441(7089): 111–114.

82 Tabernero J, Shapiro GI, Lorusso PM, Cervantes A, Schwartz GK, Weiss GJ, et al. (2013) First-in-humans trial of an RNA interference therapeutic targeting VEGF and KSP in cancer patients with liver involvement. *Cancer Discovery* 3(4): 406–417.

83 Jeffs LB, Palmer LR, Ambegia EG, Giesbrecht C, Ewanick S, MacLachlan I. (2005) A scalable, extrusion-free method for efficient liposomal encapsulation of plasmid DNA. *Pharmaceutical Research* 22(3): 362–372.

84 Davis ME, Zuckerman JE, Choi CH, Seligson D, Tolcher A, Alabi CA, et al. (2010) Evidence of RNAi in humans from systemically administered siRNA via targeted nanoparticles. *Nature* 464(7291): 1067–1070.

85 Seymour LW, Ulbrich K, Wedge SR, Hume IC, Strohalm J, Duncan R. (1991) N-(2-hydroxypropyl)methacrylamide copolymers targeted to the hepatocyte galactose-receptor: pharmacokinetics in DBA2 mice. *British Journal of Cancer* 63(6): 859–866.

86 Hu Y, Haynes M, Wang Y, Liu F, Huang L. (2013) A highly efficient synthetic vector: non-hydrodynamic delivery of DNA to hepatocyte nuclei *in vivo*. *ACS Nano* 7(6): 5376–5384.

87 Yang Y, Hu Y, Wang Y, Li J, Liu F, Huang L. (2012) Nanoparticle delivery of pooled siRNA for effective treatment of non-small cell lung cancer. *Molecular Pharmaceutics* 9(8): 2280–2289.

88 Yang Y, Li J, Liu F, Huang L. (2012) Systemic delivery of siRNA via LCP nanoparticle efficiently inhibits lung metastasis. *Molecular Therapy* 20(3): 609–615.

89 Tanaka T, Mangala LS, Vivas-Mejia PE, Nieves-Alicea R, Mann AP, Mora E, et al. (2010) Sustained small interfering RNA delivery by mesoporous silicon particles. *Cancer Research* 70(9): 3687–3696.

90 Li X, Xie QR, Zhang J, Xia W, Gu H. (2011) The packaging of siRNA within the mesoporous structure of silica nanoparticles. *Biomaterials* 32(35): 9546–9556.

91 Ashley CE, Carnes EC, Epler KE, Padilla DP, Phillips GK, Castillo RE, et al. (2012) Delivery of small interfering RNA by peptide-targeted mesoporous silica nanoparticle-supported lipid bilayers. *ACS Nano* 6(3): 2174–2188.

92 Midoux P, Kichler A, Boutin V, Maurizot JC, Monsigny M. (1998) Membrane permeabilization and efficient gene transfer by a peptide containing several histidines. *Bioconjugate Chemistry* 9(2): 260–267.

93 Huang D, Swanson EA, Lin CP, Schuman JS, Stinson WG, Chang W, et al. (1991) Optical coherence tomography. *Science* 254(5035): 1178–1181.

94 Alvarez-Erviti L, Seow Y, Yin H, Betts C, Lakhal S, Wood MJ. (2011) Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. *Nature Biotechnology* 29(4): 341–345.

95 Lakhal S, Wood MJ. (2011) Exosome nanotechnology: an emerging paradigm shift in drug delivery: exploitation of exosome nanovesicles for systemic in vivo delivery of RNAi heralds new horizons for drug delivery across biological barriers. *BioEssays* 33(10): 737–741.

96 Simpson RJ, Jensen SS, Lim JW. (2008) Proteomic profiling of exosomes: current perspectives. *Proteomics* 8(19): 4083–4099.

97 Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO. (2007) Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nature Cell Biology* 9(6): 654–659.

98 van den Boorn JG, Schlee M, Coch C, Hartmann G. (2011) SiRNA delivery with exosome nanoparticles. *Nature Biotechnology* 29(4): 325–326.

PART II

Targeted Expression

5

CHAPTER 5

Cancer Gene Therapy by Tissue-specific and Cancer-targeting Promoters

Jennifer L. Hsu, Longfei Huo, Chia-Wei Li, Yi-Hsin Hsu, Yan Wang, and Mien-Chie Hung

The University of Texas MD Anderson Cancer Center, Houston, TX, USA

Introduction

Cancer gene therapy has been extensively investigated as an alternative therapeutic option to traditional treatment modalities such as surgery, chemotherapy, and radiation therapy by introducing therapeutic genes in cancer cells. Similar to recently developed targeted therapy, one of the major goals of cancer gene therapy is to limit expression of therapeutic genes only in cancer cells to selectively suppress cancer cell growth and prevent potential toxicities caused by unwanted expression of the therapeutic gene in normal tissues. To reach this goal, investigators have explored the promoters (upstream elements required to initiate gene transcription) of genes that are highly expressed in cancer cells and developed promoters/enhancers that primarily express therapeutic genes in targeted cells. Unfortunately, many of the so-called tumor-specific promoters are relatively weaker than commonly used strong promoters such as cytomegalovirus (CMV), even in the tumor cells. Cancer-targeting expression of therapeutic genes that express proapoptotic proteins or suicide genes that produce enzymes to convert prodrugs into cytotoxic agents have demonstrated antitumor effect by inducing cell death.

One commonly used approach to deliver the therapeutic genes is through viral vectors, such as adenovirus. Currently, adenovirus vector accounts for about 23% of gene therapy clinical trials worldwide [1]. Some of the advantages of using adenovirus include its high levels of gene-transfer capability, low pathogenicity in humans, and ability to transduce nonproliferating cells [2]. Early adenoviral vectors induced strong immune response mostly due to the expression of viral genes such as *E1*, *E2*, and *E4* [2]. Moreover, it also produced undesirable toxicities as it targeted not only cancer cells but also normal tissues under powerful non-specific ubiquitous promoters such as CMV or Rous sarcoma virus (RSV) [3]. Today, modification of the adenoviral vector has substantially reduced toxicity and improved efficacy (see also Chapter 1 in this volume) [4]. Contrary to viral vectors, plasmid DNA-based non-viral vectors have been considered to be much safer due to less immunogenicity but come with poor transfection efficiency, hindering their therapeutic efficacy. Improvements are currently under intensive research [5,6] (see also Chapter 3).

In both approaches, the reduction of toxicity and enhancement of therapeutic efficacy can be

Cancer Gene Therapy by Viral and Non-viral Vectors, First Edition. Edited by Malcolm K. Brenner and Mien-Chie Hung.

achieved by restricting the expression of essential viral genes or therapeutic genes only in cancer cells through cancer-specific or cancer-targeting promoters and expression vectors. Here we describe several cancer types for which specific promoters have been developed and extensively studied to direct cancer-targeting gene expression. In addition, we also describe expression platforms, two-step transcriptional amplification (TSTA) and VP16-Gal4-WPRE integrated systemic amplifier (VISA), which can amplify the activities of promoters in cancer cells and enhance the expression of the gene of interest.

Cancer-Specific Promoters

Prostate Cancer

Prostate cancer is the most frequently diagnosed malignancy and the second-leading cause of cancer deaths in men [7]. In the USA, approximately 238,590 new cases of prostate cancer and 29,720 prostate cancer-related deaths are expected for 2013 [8]. Despite advances in the early detection and treatment of locally advanced prostate cancer, the prognosis for patients with advanced prostate cancer is poor. Most patients with advanced prostate cancer respond initially to androgen-ablation therapy, but most patients will eventually progress to castration-resistant prostate cancer, which usually results in widespread metastasis with fatal outcome [9].

Gene therapy utilizing prostate cancer-specific promoters has been widely investigated for prostate cancer treatment as a targeted approach that would limit unwanted side effects in normal tissues. The best-studied prostate-specific promoter is derived from the prostate-specific antigen (*PSA* or *hK3*) gene, which encodes an androgen-regulated serine protease that is expressed in both prostate epithelial cells and prostate cancer [10], and its expression has served as an important biomarker for the diagnosis and management of prostate cancer [11]. The upstream regulatory region of the *PSA* gene contains several components that are critical for prostate-specific gene expression, including the proximal promoter, which contains two androgen-responsive elements for androgen receptor binding, and the upstream enhancer regions. In addition, a small core enhancer region was found to be essential for strong activity and prostate specificity [12]. Studies to improve the inherently weak activity of the native PSA promoter and enhancer included the design of a chimeric PSA promoter (PSE) by shortening the intervening sequences between the proximal promoter and enhancer and duplicating the core enhancer [13]. These studies also found that duplication of the core enhancer within the chimeric PSE promoter increased the activity of the promoter by nearly 20-fold while retaining prostate specificity and androgen inducibility. Similar to *PSA*, human glandular kallikrein 2 (*hK2*) is also a prostate-restricted gene, and its promoter shares high sequence homology in gene-regulatory elements with the PSA promoter [14,15]. Another androgen-regulated promoter that maintains prostate-specific gene expression is the AAR_2PB promoter, comprising a minimal rat probasin (PB) promoter and an additional copy of the androgen-response region (ARR) containing two androgen-response elements (AREs) inserted upstream of this minimal promoter [16]. A later study by Furuhata et al. showed that modification of the PB promoter by substituting the retinoic acid-response element (RARE) for an ARE can drive transgene expression in an androgen-independent fashion [17].

Contrary to androgen-regulated PSA, hK2, and ARR_2PB promoters, the osteocalcin (OC) and prostate-specific membrane antigen (PSMA) promoters possess prostate specificity independently of androgen [18,19]. OC is a bone matrix protein that is exclusively produced by osteoblasts but its promoter has demonstrated activity in primary and metastatic prostate cancer cells [20]. The *PSMA* gene encodes a type II membrane protein with folate hydrolase activity that is expressed in primary and lymph node metastatic prostate cancer [21]. Interestingly, *PSMA* gene expression is upregulated under androgen-deprivation conditions [22]. A chimeric prostate-specific enhancing sequence (PSES) composed of critical regulatory elements of the *PSA* and *PSMA* gene mediated high levels of gene expression in *PSA*- and *PSMA*-positive prostate cancer cells with or without androgen yet remained silent in *PSA*- and *PSMA*-negative prostate and non-prostate cancer cells [23]. A review by Figueiredo et al. provides excellent information of past preclinical studies of adenoviral vector-based

gene therapy that utilized prostate-specific promoters and enhancers to restrict the expression of therapeutic genes in prostate cancer cells or to limit expression of key viral lifecycle regulators E1A and E1B for prostate-specific adenovirus replication [24]. Several preclinical animal studies since then have demonstrated successful use of the prostate-specific promoters, including PSMA- and OC-driven inducible nitric oxide synthase expression that significantly reduced tumor growth without any detrimental side effects [25], AAR_2PB-driven *Escherichia coli* purine nucleoside phosphorylase (PNP) expression in gene-directed enzyme prodrug therapy (GDEPT) that suppressed tumor growth [26], and a multimodal therapy consisting of AAR_2PB promoter-based cytolytic virotherapy and radioiodine treatment that effectively increased survival [27]. Currently, a clinical trial is ongoing for a GDEPT expressing the *E. coli* PNP under a prostate-directed promoter in ovine atadenovirus (ClinicalTrials.gov identifier NCT00625430) in which localized expression of PNP converts systemically administered fludarabine (prodrug) into 2-fluoroadenine (active agent) to provide tissue-specific chemotherapy, limiting the systemic side effects associated with traditional chemotherapy and the risks of damaging the surrounding normal tissues. This serves as an example of an ideal system that is capable of producing high levels of prostate cell-specific expression with very low expression in non-prostate cells. The results from this trial to test assess safety and tolerability will be highly anticipated.

Liver Cancer

Hepatocellular carcinoma (HCC) accounts for more than 80% of liver cancer cases. While most cases of HCC are reported in the developing countries of Asia and Africa, there has been an alarming increase in HCC cases in Western Europe as well as in the USA. The number of liver cancer deaths in the USA has been estimated to be 21,670 (6780 women, 14,890 men) for 2013, and patients with liver cancer have an overall 5-year relative survival rate of 14% [7,8]. Since HCC is a highly aggressive and metastatic disease, a systemic treatment is required for achieving an effective therapeutic outcome. Currently, liver transplantation as well as surgical resection are the potential curative treatments for HCC but are not generally applicable to most HCC patients with larger coexisting liver cirrhosis or metastatic liver [28]. These therapeutic limitations make HCC a disease with no effective treatment and poor prognosis and thus there exists an urgent need to investigate and evaluate possible alternative systemic therapeutic strategies.

The use of tumor-specific promoter to direct the expression of therapeutic genes provides alternative strategies for liver cancer therapy. Human α-fetoprotein (AFP) is highly expressed during early development in fetus; however, in normal adult tissues its expression decreases to a low or undetected level [29]. In liver neoplastic transformation AFP expression is reactivated, and nearly 70% of HCCs overexpress AFP, making it an important biomarker for diagnosis [30] and its promoter an ideal candidate for driving liver-specific gene expression. The AFP promoter has been extensively studied, and its enhancer and silencer regions located upstream of the *AFP* gene have been shown to play a critical role in HCC-selective AFP expression [31,32]. Both viral and non-viral therapeutic strategies using variations of the AFP promoter and enhancer to drive the expression of therapeutic genes or restrict the replication of virus specifically in HCC cells have been reported, including non-viral DNA–liposome nanoparticles that induce apoptosis [33], hypoxia-dependent oncolytic virotherapy that incorporates hypoxia-response elements into the AFP promoter to restrict viral replication in tumor microenvironment [34], oncolytic radiovirotherapy that also expresses sodium iodine symporter for radioiodine (^{131}I) uptake [35], gene-virotherapy that upregulates apoptotic signaling through the expression of interleukin (IL)-24 [36], and tumor-targeted horseradish peroxidase/indole-3-acetic acid enzyme prodrug virotherapy [37]. While the specificity of the AFP promoter in liver cancer to restrict gene expression/viral replication is a highly desirable characteristic, it has yet to be determined whether any of the above-mentioned preclinical studies would work well in the clinical setting.

Lung Cancer

Lung cancer accounts for 14% of cancer diagnoses and for more death in both men and women than any other cancer [7]. Gene therapy has been widely

explored as an alternative cancer treatment options, and the promoters of genes encoding the surfactant protein B, gastrin-releasing peptide, cyclooxygenase-2, and vasopressin have been utilized to drive lung cancer-specific transgene expression [38]. In addition to these previously described promoters, several others have been reported to have lung cancer specificity. The promoter of the insulinoma-associated 1 (*INSM1*) gene has demonstrated lung cancer specificity. While the *INSM1* gene was found to be highly expressed in neuroendocrine tumors, analysis of the INSM1 promoter identified a 1.7 kb region that drove high reporter-gene expression in a majority of small-cell lung cancer (SCLC) cell lines tested but not in other tumor or fibroblast cell lines [39]. A later study from the same laboratory further demonstrated that INSM1 promoter-coupled suicide platform (INSM-YCD-YUPRT/5-FC) induced high cytotoxicity in a range of SCLC cell lines and delayed tumor growth in mice by using the non-viral 1,2-bis(oleoyloxy)-3-(trimethylammonio)propane (DOTAP)/cholesterol delivery approach [40]. The TTS [*TTF1* gene under the control of human telomerase reverse transcriptase (hTERT)/human surfactant protein A1 (hSPA1) promoter] dual system has also been shown to specifically target pulmonary adenocarcinoma cells [41]. Incorporating the proapoptotic BID expression into the TTS system (Ad-TTS/Bid) synergistically enhances cisplatin and dexamethasone treatments of advanced lung cancer [42]. Besides the aforementioned promoter usage, the *cis*-acting enhancer sequence also provides plausible strategy for lung cancer therapy. For instance, the antioxidant-response element of Nrf2, which is constitutively expressed in lung cancer cells, has been shown to regulate thymidine kinase (TK)/ganciclovir-directed suicidal therapy [43]. Although a clinical trial for lung cancer gene therapy was recently completed using a liposome-mediated (DOTAP/cholesterol) approach to deliver a non-viral vector expressing a tumor-suppressor gene [44], a trial with lung cancer-specific expression vector has yet to be seen.

Breast Cancer

Breast cancer is the most commonly diagnosed cancer among American women. Although the development of targeted therapies has led to a decline in mortality, the death rate remains the second highest among women with approximately 39,620 women in the USA estimated to die from breast cancer in 2013 [7]. The first gene therapy for breast cancer consisted of the adenovirus type 5 *E1A* gene encapsulated by a cationic liposome containing 3-β-[*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl]cholesterol (DC-Chol) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine [45]. Although this trial did not specifically target the breast tissues, later studies focused on developing breast cancer-specific promoters for targeted expression of therapeutic genes.

Serial analyses of gene expression indicated that the human glyceroldehyde-3-phosphate dehydrogenase (GAPDH) promoter is selectively expressed in breast cancer cells [46]. Incorporating the GAPDH promoter with the CMV enhancer showed tissue specificity in breast cancer cells [47]. Fatty acid synthase, an enzyme that functions in palmitate synthesis, is overexpressed in a variety of cancer types including breast cancer and was shown to drive therapeutic gene expression in a wide range of breast cancer cell lines without any expression in normal fibroblast cells [48]. A composite promoter, CT90, containing a minimal promoter from topoisomerase IIa linked to the CMV immediate-early gene promoter has also been shown to selectively kill breast cancer cells *in vitro* and suppress mammary tumor development in animal model [49].

Tumor-initiating cells (TICs), also termed cancer stem cells, are a small subpopulation of cancer cells within tumors with characteristics of resistance to cancer treatments and regrowth of new tumors, and are currently considered as a major obstacle for cancer therapies [50]. Among other TICs that have been discovered in different cancer types, the best characterized are those of breast cancer; therefore, we briefly describe their impact on cancer gene therapy. Breast TICs, identified in an enriched $CD44^{+}/CD24^{low}$ subfraction of cells, are often resistant to chemo- and radiotherapy [51,52]. Breast cancer-restricted replicative adenoviruses were developed using the promoter of α-lactalbumin, a key protein in lactogenesis that is overexpressed in about 60% of breast cancer tissues, to target hormone-independent breast cancers [53]. Later, modified oncolytic adenoviruses

expressing E1A under control of the α-lactalbumin promoter completely eradicated $CD44^+CD24^{-/low}$ cells *in vitro* and demonstrated significant anti-tumor activity in $CD44^+CD24^{-/low}$-derived tumors [54]. In addition, the claudin-4 promoter is also another breast cancer-specific promoter identified based on a large number of studies indicating that claudin-4 expression is associated with human cancers, particularly breast cancer [55,56]. Immunohistochemistry staining showed that 93.3% of a cohort of 299 tumors, represented on a tissue microarray, expressed claudin-4 expression, and high claudin-4 expression was also associated with worse breast cancer-specific survival, recurrence-free survival, and overall survival [57]. Interestingly, although the claudin family has been reported to have low expression in certain types of breast cancer referred to as claudin-low subtype of breast cancer [58], claudin-4 is overexpressed in breast cancer cells among the 23 members of the claudin family [55,56,57]. Thus, claudin-4 should not be considered to be in the category of claudin-low, which is characteristic of a subpopulation of breast cancer. In a VISA expression platform (see a later section in this chapter), claudin-4 promoter-driven expression of a therapeutic gene *BikDD* [59,60] reduced the $CD44^+CD24^{-/low}$ population by blocking the activity of multiple proteins in the Bcl-2 family and further increased the benefits of a chemotherapeutic tyrosine kinase inhibitor lapatinib and paclitaxel [61]. This study is currently undergoing Good Laboratory Practice (GLP) toxicity and pharmacokinetics studies in preparation for Investigational New Drug (IND) submission. Our recent study demonstrated that high expression of methytransferase enhancer of zeste homolog 2 (EZH2), which plays an important role in stem cell self-renewal and maintenance, correlated with high grade of human breast tumors [62]. The findings from this study, including identification of the mechanism by which upregulation of EZH2 expression promotes the expansion of breast TICs, suggests that the EZH2 promoter might be worthy of investigation as an alternative approach to drive therapeutic gene expression in breast TICs.

The discovery of trastuzumab (Herceptin) has helped treat breast cancer patients who overexpress human epidermal growth factor receptor 2 (HER-2). For women who are estrogen-receptor- or progesterone-receptor-positive, tamoxifen and aromatase inhibitors prevent their recurrence efficiently. However, for those who have triple-negative breast cancer (TNBC; estrogen-receptor-, progesterone-receptor-, and HER-2-negative), there is no proven targeted therapy currently available. As gene expression profiles of various cancer lines have been well characterized [63], these databases may provide a subtype of gene expression signature to develop TNBC-specific therapeutic and preventative strategies. Combing with current advanced strategy, gene therapy may provide combinatory effect eradicating the difficult-to-treat breast cancers.

Pancreatic Cancer

The mortality rate of pancreatic ductal adenocarcinoma or pancreatic cancer is fourth highest among cancer-related deaths in the USA, and it has been estimated that 45,220 new cases and 38,460 deaths will occur in 2013 [7]. Current treatments for pancreatic cancer consist of surgery, radiation therapy, and chemotherapy but are not curative. Even if patients are diagnosed at earlier stages (Stages I or II), the 5-year survival rate is only about 20–25% [8]. Therefore, alternative treatment strategies such as tumor-targeted gene therapy are being pursued to treat pancreatic cancer.

Several promoters have been reported to drive expression of transgenes in pancreatic cancer cells including c-erbB2, murine pancreatic amylase, carcinoembryonic antigen (see below also), midkine, and MUC1/DF3 [38,64]. In addition, the rat insulin promoter (RIP) has also been utilized to direct suicide gene expression in pancreatic cancer cells that overexpress PDX-1 transcription factor. Systemic delivery of liposome-encapsulated adenovirus (A5-RIP-TK) plus ganciclovirablated human pancreatic cells in SCID mice [65]. The promoter of the cholecystokinin A receptor (*CCKAR*) gene, which encodes a G-protein-coupled receptor, is selectively active in pancreatic cancer cells but not in normal cell lines [66]. CCKAR binds to the peptide hormone cholecystokinin (CCK) and plays a major role in mediating pancreatic growth and enzyme secretion, smooth muscle contraction of the gallbladder and stomach, and secretion from gastric mucosal cells in the gastrointestinal system

[67]. While CCKAR possesses pancreatic cancer specificity, its promoter activity is relatively weak compared to the more powerful nonspecific CMV promoter. A VISA-based non-viral expression system was shown to enhance the activity of this inherently weak promoter (see below; [64]). A phase I clinical trial has been approved by the US Food and Drug Administration and the Recombinant Advisory Committee to assess the safety and tolerability of this CCKAR promoter-based system in patients with advanced pancreatic cancer (ClinicalTrials.gov identifier NCT00968604).

Cancer-Targeting Promoters

In addition to the above-mentioned cancer-specific promoters, there are several that have been characterized to be active in multiple cancer types. Below, we give a few examples of these types of promoters.

Human Telomerase Reverse Transcriptase

Telomerase reverse transcriptase (TERT) is the catalytic subunit of telomerase complex that is crucial for maintaining the length of telomeres at the end of chromosomes to prevent chromosomal termini from end fusion and degradation. hTERT is highly expressed and activated in more than 85% human cancers but usually undetectable or inactive in normal tissues [68]. The activation of hTERT is correlated to its promoter activity that is preferentially activated in cancer cells but not in normal somatic cells [69]. Due to its high tumor specificity, the hTERT promoter has been widely used in cancer gene therapy to selectively target cancer cells without affecting normal cells.

hTERT promoter-driven expression of therapeutic genes has been extensively studied and shown promising tumor-specific therapeutic effects in both viral vectors and non-viral vectors. The expression of apoptosis-related genes, such as *Bax* [70,71], *FADD* [72], *TRAIL* [73,74], *p53* [75], *CASP6* [76] and *CASP8* [77], and *BikDD* [78] under the control of hTERT promoter induced cancer cell-specific apoptosis, leading to tumor growth inhibition in multiple human tumor xenograft models. In addition, hTERT promoter-driven expression of suicide genes such as herpes simplex virus *TK* [79,80,81], bacterial nitroreductase [82], carboxypeptidase G2 [83], cytosine deaminase [84], and horseradish peroxidase [85], when combined with prodrug treatment, also specifically killed tumor cells while keeping normal cells intact, and significantly suppressed tumor growth in animal models. Expression of monocyte chemoattractant protein-1 [86] and sodium iodide symporter [87] under the hTERT promoter have also been reported to efficiently kill tumor cells by enhancing macrophage-mediated tumor cell eradiation and increasing cellular radiotoxicity, respectively. Chimeric hTERT promoter containing the radiation-inducible sequence $CC(A/T)_6GG$ has been shown to greatly enhance hTERT promoter activity and suppress tumor growth upon irradiation treatment in human liver cancer xenograft [85].

Numerous studies have also focused on developing cancer-specific viral vectors to preferentially kill tumor cells but not normal cells. For example, telomerase-specific oncolytic adenoviruses, e.g., Telomelysin and Telomelysin-RGD, are telomerase-dependent, replication-selective adenoviruses in which the hTERT promoter drives the expression of *E1* genes linked with an internal ribosome entry site [88,89]. Telomelysin has been shown to efficiently and specifically kill cancer cells *in vitro* and inhibit tumor growth *in vivo* with multiple human cancer types, including liver cancer [90,91,92], lung cancer [93,94,95], ovarian cancer [96], prostate cancer [97], head and neck cancer [98], esophageal cancer [99], glioblastoma [100], and bone and soft tissue sarcoma [101,102]. Local injection or systemic administration of Telomelysin in animal models revealed that it not only preferentially inhibits primary tumor growth but also specifically targets metastatic tumors by killing metastatic tumor cells and reducing tumor dissemination without observed toxicity to normal tissues [91,96, 103,104,105]. It seems that multiple mechanisms including oncolysis [106], anti-angiogenesis [107], inhibition of DNA-repair machinery [99], and autophagic cell death [100,108] are responsible for the effective tumor-specific cytotoxicity of the telomerase-dependent oncolytic virus. Combination of telomerase-dependent oncolytic virus with other therapeutics such as irradiation [99],

gemcitabine [95,109], docetaxel [110], histone deacetylase (HDAC) inhibitor [94,111], and IL-2 [104] has been shown to induce additive or synergistic cytotoxic effects for tumor cells. In addition, tumor cell-killing efficiency could be greatly increased by cotreatment of telomerase-specific oncolytic virus with the replication-deficient adenovirus expressing either *p53* gene or *NK4* gene driven by the CMV promoter [101,112].

The hTERT promoter-dependent cancer gene therapy holds a promising future with many preclinical studies reporting no observed toxicity to normal tissues. Additionally, a phase I study in multiple human advanced cancers revealed that a single-dose intratumor injection of hTERT promoter-dependent oncolytic virus (up to 1×10^{12} viral particles) was well tolerated and some response was observed to the single-dose administration [113], further supporting the safety of hTERT-dependent cancer gene therapy and its potential use in future clinical trials.

Survivin

Survivin, which belongs to the family of apoptosis inhibitors, is normally expressed during fetal development but is absent in differentiated normal tissues [114]. However, survivin is highly expressed in malignant tissues including lung, breast, colon, and pancreatic carcinomas, melanoma, and non-Hodgkin lymphoma, among others [115]. Replication-selective adenoviruses expressing the *E1A* gene under the survivin promoter demonstrated efficient cancer-specific viral replication and potent therapeutic effects against several cancer types but not normal cells both *in vitro* and *in vivo* [116]. Survivin promoter-based non-viral expression system in a VISA platform (SV-BikDD; see below) coupled with DOTAP/cholesterol liposomes was shown to inhibit tumor growth and prolong survival of mice in lung [117] and ovarian cancer [118] xenograft models. In a rat hematoma model, the bidirectional expression of a therapeutic gene tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and a reporter gene luciferase under the survivin promoter by adenovirus demonstrated potent tumor specificity with the potential to limit hepatotoxicity in HCC patients [119]. Thus, it would be of interest to determine if this cancer-targeting promoter can be applied to human trials.

Carcinoembryonic Antigen

Carcinoembryonic antigen (CEA), a cell-surface glycoprotein, is commonly overexpressed in various types of cancer, including lung, colon, gastric, pancreatic, and gallbladder, among others [120], and has been widely used as a prognostic/diagnostic biomarker. The transcriptional regulatory elements of the CEA promoter have been characterized and applied to several gene therapy studies to restrict gene expression only in CEA-positive tumor cells such as in colon cancer cells [121]. In one of the studies, recombinant adenoviruses expressing *E1A* under the control of the CEA regulatory elements preferentially replicated and killed CEA-producing colorectal cancer cell lines and inhibited tumor growth in xenograft model [121]. The CEA promoter has also been shown to drive double suicide-gene expression (pCEA-TK/CD) to enhance cytotoxicity in lung cancer cells [122]. More recently, Xu et al. developed a tumor-specific adenovirus expressing a heat-shock protein (HSP70) in which viral replication is regulated by the CEA promoter and demonstrated that CEA promoter-regulated adenovirus AdCEAp-HSP70 inhibited tumor growth of human pancreatic xenograft in mice [123]. While these preclinical studies have shown positive results, further studies in the clinical setting would be required to determine the efficacy of this promoter.

Progression-Elevated Gene-3

Progression-elevated gene-3 (PEG-3) was first identified by subtraction hybridization of adenovirus type 5-transformed rat embryos showing higher expression of PEG-3 as a function of transformation by various oncogenes [124]. Subsequent study determined that PEG-3 is a C-terminally truncated mutant form of the rat growth-arrest and DNA-damage-inducible gene-34 (GADD-34) [125]. The promoter of PEG-3 was cloned, characterized, and found to be highly active not only in rodent but also in a wide spectrum of human cancer cells, and its cancer specificity is mediated primarily by transcription factors AP-1 and PEA-3, which are

highly expressed in almost all types of cancers [124]. Using conditionally replication-competent adenovirus which expresses *E1A* under the control of the PEG-3 promoter and also simultaneously expresses a therapeutic gene, Sarkar et al. reported that the virus demonstrated cancer cell-selective growth inhibition and induction of apoptosis *in vitro* and eradication of primary and distant tumors in xenograft mouse models of breast (Ad.PEG-E1A-*mda-7*) [126], pancreas (Ad.PEG-E1A-*IFN-γ*) [127], and prostate (Ad.PEG-E1A-*mda-7*) [128] cancer as well as melanoma (Ad.PEG-E1A-*mda-7*) [129], supporting the potential therapeutic applications of the virus. This cancer-targeting promoter has not yet been tested in a clinical setting, but more recently it has been shown to enable detection of micrometastatic diseases in human melanoma and breast cancer mouse models through radionuclide-based molecular imaging [130]. These encouraging data indicate that this promoter has the potential to facilitate cancer imaging and therapy in a clinical setting in the future.

Engineered Systems that Enhance Promoter Activity

Two-Step Transcription Amplification

While these aforementioned promoters offer tumor/tissue specificity, many of them are much weaker than nonspecific ubiquitous promoters, such as the CMV enhancer/promoter or simian virus 40 (SV40) promoter. One of the most extensively tested and validated methods to boost promoter activity is the two-step transcriptional amplification (TSTA) system (Figure 5.1A [131]). The system expresses a potent activator comprising the yeast GAL4 DNA-binding domain and two viral VP16 activation domains under the tissue-specific promoter. This chimeric GAL4–VP16 activator then binds to the GAL4-binding site, which is present in five copies, to drive expression of transgene. The TSTA system has demonstrated significantly higher activity than the CMV promoter and has been tested in many preclinical cancer models [24]. More recently, Watanabe et al. described a modified TSTA system in which polyglutamine and rat glucocorticoid sequences were inserted between the GAL4 and VP16 sequences and showed that this advanced TSTA system increased hTERT promoter activity by about 15-fold higher than the conventional two-step system [132].

VP16-Gal4-WPRE Integrated Systemic Amplifier

Another recently developed TSTA-based system is VP16-Gal4-WPRE integrated systemic amplifier (VISA) (Figure 5.1B), which not only increased the promoter activity but also prolonged RNA stability of the transgene [66]. In addition to the TSTA module, the VISA expression system contains an RNA-enhancing element from the woodchuck hepatitis virus responsive element that is inserted in the 3′ untranslated region of the gene of interest [66]. The VISA system enhanced the transgene expression to a level comparable or even higher than the CMV promoter in cancer cells but the expression remained low in normal cells. It has been tested in several preclinical cancer models, showing significant tumor-growth inhibition and prolonged animal survival with virtually no toxicities [33,66,78,117,118,133].

Interestingly, the above-mentioned survivin promoter-based non-viral VISA expression system (SV-BikDD), which inhibited tumor growth and prolonged survival of immunocompromised mice with human lung cancer xenograft [117], also elicited a cancer-specific cytotoxic T-lymphocyte response in immunocompetent mice [134]. Mice treated with SV-BikDD further developed systemic antitumor immunity against subsequent exposure to parental tumor cells and activated innate immunity through the proinflammatory cytokines induced by the vector. Importantly, SV-BikDD gene therapy did not elicit an immune response against normal tissues. This unexpected cancer-specific, vector-induced immune response suggests that VISA is favorable for cancer gene therapy. For breast cancer, claudin-4-VISA-BikDD was demonstrated to kill TICs for which there are currently no effective clinical drugs.

With the first VISA-BikDD system for pancreatic cancer (CCKAR-VISA-BikDD) currently being investigated in a phase I clinical trial (ClinicalTrials.gov identifier NCT00968604), the results will be highly anticipated as VISA-BikDD is cancer-specific and has high therapeutic efficacy and very low toxicity (excellent safety profile). A schematic showing how the VISA or TSTA

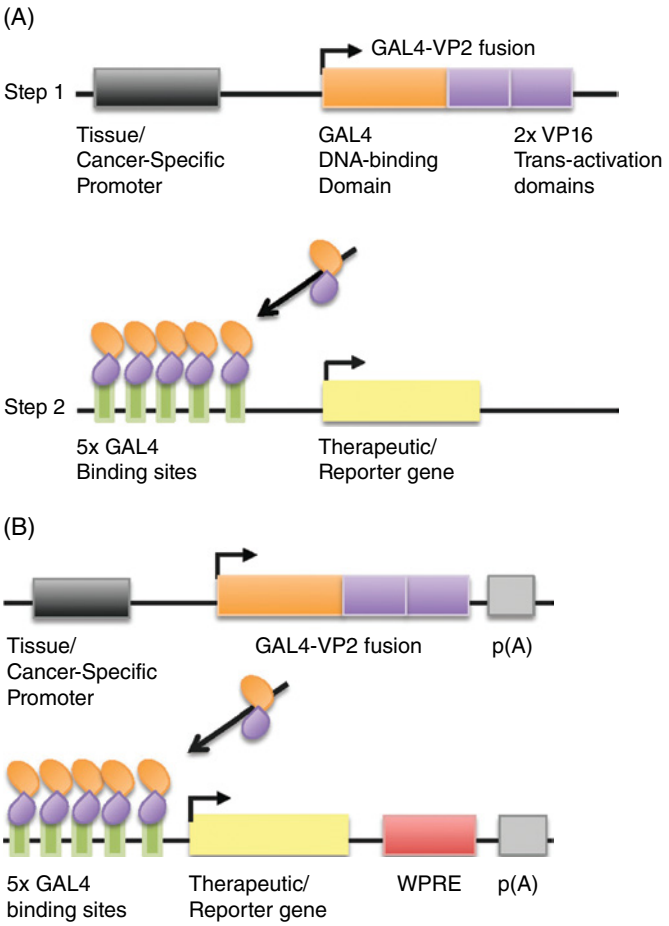


Figure 5.1 Schematic of transcriptional amplification modules. (A) The TSTA system comprises of a two-step transcription process. In the first step of the system, a synthetic transcription factor, GAL4–VP2 is expressed under a tissue-/cancer-specific promoter. This transcription activator then binds to five GAL4 DNA-binding sites in the second step to direct transcription of the downstream therapeutic or reporter gene. Adapted from Figueiredo et al. [135]. (B) The VISA system is comprised of the TSTA module plus WPRE. The combination of transcription amplification plus RNA stabilization enhances and prolongs expression of the target gene.

module can be used to construct promoter-based system to selectively express the gene of interest at high levels in cancer cells only but not normal cells is illustrated in Figure 5.2.

Conclusion

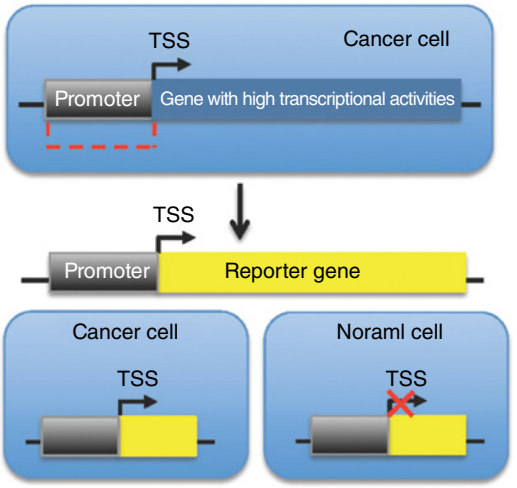
Since therapeutic effects would be detrimental to normal tissues, one of the main focuses of researchers has been to work on improving cancer-targeting specificity to limit therapeutic gene expression only in cancer cells and minimize or avoid expression in normal cells. In doing so, a number of promoters and elements have been successfully used to specifically drive therapeutic genes and efficiently express them in cancer cells by both viral and non-viral vector-mediated approaches. There is also mounting evidence in preclinical studies showing that expression of proapoptotic proteins or prodrug-metabolizing enzymes specifically inhibited tumor growth without toxicity in surrounding normal tissues. Together, these studies support a promising future for targeted cancer gene therapy.

Step 1

Database search
(e.g. Serial analyses of gene expression)
• Identify genes that are overexpressed in a specific cancer type

Step 2

- Clone promoter region (regulatory elements)
- Characterize transcriptional activities based on reporter assays such as luciferase expression
- Compare promoter activities with non-specific strong promoter such as CMV



	Reporter gene expression	
	Cancer cells	Normal cells
CSP/CTP	++	+/–
CMV	++++	++++
CSP/CTP+ VISA/TSTA	++++	+/–

Step 3

- Test therapeutic effects by therapeutic gene *in vitro* and *in vivo*

Figure 5.2 A simplified schematic representation of transcriptionally targeted gene expression by cancer-specific or cancer-targeting promoter. In the first step, genes that are highly expressed in specific cancer types are identified and considered candidates for cancer-specific or cancer-targeting promoters. Then, regulatory elements in the promoter region upstream of the transcriptional start site (TSS) are cloned and characterized. A region containing elements or a combination of regulatory elements carrying the highest transcriptional activities is cloned into a reporter expression vector to test for transcriptional activities in cancer cells. Expression of the reporter gene is limited to cancer cells by presence of specific transcription factors. Reporter gene expression is compared between cancer-specific/-targeting promoter alone, cancer-specific/-targeting promoter plus the VISA/TSTA module, and the CMV promoter. Ideally, to achieve the highest therapeutic window (++++), the promoter activity should be higher than the nonspecific CMV promoter (++++) and show little or no expression (+/–) in normal cells. CSP, cancer-specific promoter; CTP, cancer-targeting promoter.

References

1 Gene Therapy Clinical Trials Worldwide. (2013) The Journal of Gene Medicine. www.abedia.com/wiley/index.html.

2 Vorburger SA, Hunt KK. (2002) Adenoviral gene therapy. *Oncologist* 7: 46–59.

3 Fukazawa T, Matsuoka J, Yamatsuji T, Maeda Y, Durbin ML, Naomoto Y. (2010) Adenovirus-mediated cancer gene therapy and virotherapy (Review). *International Journal of Molecular Medicine* 25: 3–10.

4 Choi JW, Lee JS, Kim SW, Yun CO. (2012) Evolution of oncolytic adenovirus for cancer treatment. *Advanced Drug Delivery Reviews* 64: 720–729.

5 Hsu JL, Chao CH, Xie X, Hung MC. (2012) Advances in liposome-based targeted gene therapy of cancer. In *Recent*

Advances in Cancer Research and Therapy, 1st edn, XY Liu, S Pestka, YF Shi (eds), pp. 113–133. Elsevier: Amsterdam.

6 Lo HW, Hung MC. (2005) Cancer-specific gene therapy. In *Non-Viral Vectors for Gene Therapy*, 2nd edn, L Huang, MC Hung, E Wagner (eds), pp. 235–247. Elsevier: Amsterdam.

7 Siegel R, Naishadham D, Jemal A. (2013) Cancer statistics, 2013. *CA: A Cancer Journal for Clinicians* 63: 11–30.

8 Cancer Facts and Figures (2013) Atlanta, GA: American Cancer Society. www.cancer.org/research/cancerfacts figures/cancerfactsfigures/cancer-facts-figures-2013.

9 Pienta KJ, Bradley D. (2006) Mechanisms underlying the development of androgen-independent prostate cancer. *Clinical Cancer Research* 12: 1665–1671.

10 Balk SP, Ko YJ, Bubley GJ. (2003) Biology of prostate-specific antigen. *Journal of Clinical Oncology* 21: 383–391.

11 Prensner JR, Rubin MA, Wei JT, Chinnaiyan AM. (2012) Beyond PSA: the next generation of prostate cancer biomarkers. *Science Translational Medicine* 4: 127rv3.

12 Cleutjens KB, van der Korput HA, van Eekelen CC, van Rooij HC, Faber PW, Trapman J. (1997) An androgen response element in a far upstream enhancer region is essential for high, androgen-regulated activity of the prostate-specific antigen promoter. *Molecular Endocrinology* 11: 148–161.

13 Wu L, Matherly J, Smallwood A, Adams JY, Billick E, Belldegrun A, et al. (2001) Chimeric PSA enhancers exhibit augmented activity in prostate cancer gene therapy vectors. *Gene Therapy* 8: 1416–1426.

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

15 Xie X, Zhao X, Liu Y, Young CY, Tindall DJ, Slawin KM, et al. (2001) Robust prostate-specific expression for targeted gene therapy based on the human kallikrein 2 promoter. *Human Gene Therapy* 12: 549–561.

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

17 Furuhata S, Ide H, Miura Y, Yoshida T, Aoki K. (2003) Development of a prostate-specific promoter for gene therapy against androgen-independent prostate cancer. *Molecular Therapy* 7: 366–374.

18 Yeung F, Law WK, Yeh CH, Westendorf JJ, Zhang Y, Wang R, et al. (2002) Regulation of human osteocalcin promoter in hormone-independent human prostate cancer cells. *Journal of Biological Chemistry* 277: 2468–2476.

19 Fair WR, Israeli RS, Heston WD. (1997) Prostate-specific membrane antigen. *Prostate* 32: 140–148.

20 Matsubara S, Wada Y, Gardner TA, Egawa M, Park MS, Hsieh CL, et al. (2001) A conditional replication-competent adenoviral vector, Ad-OC-E1a, to cotarget prostate cancer and bone stroma in an experimental model of androgen-independent prostate cancer bone metastasis. *Cancer Research* 61: 6012–6019.

21 Silver DA, Pellicer I, Fair WR, Heston WD, Cordon-Cardo C. (1997) Prostate-specific membrane antigen expression in normal and malignant human tissues. *Clinical Cancer Research* 3: 81–85.

22 O'Keefe DS, Uchida A, Bacich DJ, Watt FB, Martorana A, Molloy PL, et al. (2000) Prostate-specific suicide gene therapy using the prostate-specific membrane antigen promoter and enhancer. *Prostate* 45: 149–157.

23 Lee SJ, Kim HS, Yu R, Lee K, Gardner TA, Jung C, et al. (2002) Novel prostate-specific promoter derived from PSA and PSMA enhancers. *Molecular Therapy* 6: 415–421.

24 Figueiredo ML, Kao C, Wu L. (2007) Advances in pre-clinical investigation of prostate cancer gene therapy. *Molecular Therapy* 15: 1053–1064.

25 Coulter JA, Page NL, Worthington J, Robson T, Hirst DG, McCarthy HO. (2010) Transcriptional regulation of inducible nitric oxide synthase gene therapy: targeting early stage and advanced prostate cancer. *The Journal of Gene Medicine* 12: 755–765.

26 Xie X, Guo J, Kong Y, Xie GX, Li L, Lv N, et al. (2011) Targeted expression of Escherichia coli purine nucleoside phosphorylase and Fludara(R) for prostate cancer therapy. *The Journal of Gene Medicine* 13: 680–691.

27 Trujillo MA, Oneal MJ, McDonough S, Qin R, Morris JC. (2010) A probasin promoter, conditionally replicating adenovirus that expresses the sodium iodide symporter (NIS) for radiovirotherapy of prostate cancer. *Gene Therapy* 17: 1325–1332.

28 Hoofnagle JH. (2004) Hepatocellular carcinoma: summary and recommendations. *Gastroenterology* 127: S319–S323.

29 Abelev GI, Eraiser TL. (1999) Cellular aspects of alpha-fetoprotein reexpression in tumors. *Seminars in Cancer Biology* 9: 95–107.

30 Izumi R, Shimizu K, Kiriyama M, Hashimoto T, Urade M, Yagi M, et al. (1992) Alpha-fetoprotein production by hepatocellular carcinoma is prognostic of poor patient survival. *Journal of Surgical Oncology* 49: 151–155.

31 Watanabe K, Saito A, Tamaoki T. (1987) Cell-specific enhancer activity in a far upstream region of the human alpha-fetoprotein gene. *Journal of Biological Chemistry* 262: 4812–4818.

32 Nakabayashi H, Hashimoto T, Miyao Y, Tjong KK, Chan J, Tamaoki T. (1991) A position-dependent silencer plays a major role in repressing alpha-fetoprotein expression in human hepatoma. *Molecular and Cellular Biology* 11: 5885–5893.

33 Li LY, Dai HY, Yeh FL, Kan SF, Lang J, Hsu JL, et al. (2011) Targeted hepatocellular carcinoma proapoptotic BikDD gene therapy. *Oncogene* 30: 1773–1783.

34 Kwon OJ, Kim PH, Huyn S, Wu L, Kim M, Yun CO. (2010) A hypoxia- and {alpha}-fetoprotein-dependent oncolytic adenovirus exhibits specific killing of hepatocellular carcinomas. *Clinical Cancer Research* 16: 6071–6082.

35 Grunwald GK, Klutz K, Willhauck MJ, Schwenk N, Senekowitsch-Schmidtke R, Schwaiger M, et al. (2013) Sodium iodide symporter (NIS)-mediated radiovirotherapy of hepatocellular cancer using a conditionally replicating adenovirus. *Gene Therapy* 20: 625–633.

36 Zhang KJ, Zhang J, Wu YM, Qian J, Liu XJ, Yan LC, et al. (2012) Complete eradication of hepatomas using an oncolytic adenovirus containing AFP promoter controlling E1A and an E1B deletion to drive IL-24 expression. *Cancer Gene Therapy* 19: 619–629.

37 Dai M, Liu J, Chen DE, Rao Y, Tang ZJ, Ho WZ, et al. (2012) Tumor-targeted gene therapy using Adv-AFP-HRPC/IAA prodrug system suppresses growth of hepatoma xenografted in mice. *Cancer Gene Therapy* 19: 77–83.

38 Haviv YS, Curiel DT. (2001) Conditional gene targeting for cancer gene therapy. *Advanced Drug Delivery Reviews* 53: 135–154.

39 Pedersen N, Pedersen MW, Lan MS, Breslin MB, Poulsen HS. (2006) The insulinoma-associated 1: a novel promoter for targeted cancer gene therapy for small-cell lung cancer. *Cancer Gene Therapy* 13: 375–384.

40 Christensen CL, Gjetting T, Poulsen TT, Cramer F, Roth JA, Poulsen HS. (2010) Targeted cytosine deaminase-uracil phosphoribosyl transferase suicide gene therapy induces small cell lung cancer-specific cytotoxicity and tumor growth delay. *Clinical Cancer Research* 16: 2308–2319.

41 Fukazawa T, Maeda Y, Sladek FM, Owen-Schaub LB. (2004) Development of a cancer-targeted tissue-specific promoter system. *Cancer Research* 64: 363–369.

42 Fukazawa T, Maeda Y, Matsuoka J, Tanaka N, Tanaka H, Durbin ML, et al. (2009) Drug-regulatable cancer cell death induced by BID under control of the tissue-specific, lung cancer-targeted TTS promoter system. *International Journal of Cancer* 125: 1975–1984.

43 Leinonen HM, Ruotsalainen AK, Maatta AM, Laitinen HM, Kuosmanen SM, Kansanen E, et al. (2012) Oxidative stress-regulated lentiviral TK/GCV gene therapy for lung cancer treatment. *Cancer Research* 72: 6227–6235.

44 Lu C, Stewart DJ, Lee JJ, Ji L, Ramesh R, Jayachandran G, et al. (2012) Phase I clinical trial of systemically administered TUSC2(FUS1)-nanoparticles mediating functional gene transfer in humans. *PLoS One* 7: e34833.

45 Hortobagyi GN, Ueno NT, Xia W, Zhang S, Wolf JK, Putnam JB, et al. (2001) Cationic liposome-mediated E1A gene transfer to human breast and ovarian cancer cells and its biologic effects: a phase I clinical trial. *Journal of Clinical Oncology* 19: 3422–3433.

46 Velculescu VE, Zhang L, Vogelstein B, Kinzler KW. (1995) Serial analysis of gene expression. *Science* 270: 484–487.

47 Lu H, Zhang Y, Roberts DD, Osborne CK, Templeton NS. (2002) Enhanced gene expression in breast cancer cells in vitro and tumors in vivo. *Molecular Therapy* 6: 783–792.

48 Yan C, Wen-Chao L, Hong-Yan Q, Rui Z, Wei-Lin J, Hua H. (2007) A new targeting approach for breast cancer gene therapy using the human fatty acid synthase promoter. *Acta Oncologica* 46: 773–781.

49 Day CP, Rau KM, Qiu L, Liu CW, Kuo HP, Xie X, et al. (2006) Mutant Bik expression mediated by the enhanced minimal topoisomerase IIalpha promoter selectively suppressed breast tumors in an animal model. *Cancer Gene Therapy* 13: 706–719.

50 Al-Hajj M, Clarke MF. (2004) Self-renewal and solid tumor stem cells. *Oncogene* 23: 7274–7282.

51 Li X, Lewis MT, Huang J, Gutierrez C, Osborne CK, Wu MF, et al. (2008) Intrinsic resistance of tumorigenic breast cancer cells to chemotherapy. *Journal of the National Cancer Institute* 100: 672–679.

52 Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. (2003) Prospective identification of tumorigenic breast cancer cells. *Proceedings of the National Academy of Sciences USA* 100: 3983–3988.

53 Li X, Zhang J, Gao H, Vieth E, Bae KH, Zhang YP, et al. (2005) Transcriptional targeting modalities in breast cancer gene therapy using adenovirus vectors controlled by alpha-lactalbumin promoter. *Molecular Cancer Therapeutics* 4: 1850–1859.

54 Bauerschmitz GJ, Ranki T, Kangasniemi L, Ribacka C, Eriksson M, Porten M, et al. (2008) Tissue-specific promoters active in CD44+CD24-/low breast cancer cells. *Cancer Research* 68: 5533–5539.

55 Kominsky SL, Tyler B, Sosnowski J, Brady K, Doucet M, Nell D, et al. (2007) Clostridium perfringens enterotoxin as a novel-targeted therapeutic for brain metastasis. *Cancer Research* 67: 7977–7982.

56 Soini Y. (2004) Claudins 2, 3, 4, and 5 in Paget's disease and breast carcinoma. *Human Pathology* 35: 1531–1536.

57 Lanigan F, McKiernan E, Brennan DJ, Hegarty S, Millikan RC, McBryan J, et al. (2009) Increased claudin-4 expression is associated with poor prognosis and high tumour grade in breast cancer. *International Journal of Cancer* 124: 2088–2097.

58 Hewitt KJ, Agarwal R, Morin PJ. (2006) The claudin gene family: expression in normal and neoplastic tissues. *BMC Cancer* 6: 186.

59 Li YM, Wen Y, Zhou BP, Kuo HP, Ding Q, Hung MC. (2003) Enhancement of Bik antitumor effect by Bik mutants. *Cancer Research* 63: 7630–7633.

60 Li Z, Ding Q, Li Y, Miller SA, Abbruzzese JL, Hung MC. (2006) Suppression of pancreatic tumor progression by systemic delivery of a pancreatic-cancer-specific promoter driven Bik mutant. *Cancer Letters* 236: 58–63.

61 Lang JY, Hsu JL, Meric-Bernstam F, Chang CJ, Wang Q, Bao Y, et al. (2011) BikDD eliminates breast cancer initiating cells and synergizes with lapatinib for breast cancer treatment. *Cancer Cell* 20: 341–356.

62 Chang CJ, Yang JY, Xia W, Chen CT, Xie X, Chao CH, et al. (2011) EZH2 promotes expansion of breast tumor initiating cells through activation of RAF1-beta-catenin signaling. *Cancer Cell* 19: 86–100.

63 Barretina J, Caponigro G, Stransky N, Venkatesan K, Margolin AA, Kim S, et al. (2012) The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity. *Nature* 483: 603–607.

64 Dorer DE, Nettelbeck DM. (2009) Targeting cancer by transcriptional control in cancer gene therapy and viral oncolysis. *Advanced Drug Delivery Reviews* 61: 554–571.

65 Liu SH, Smyth-Templeton N, Davis AR, Davis EA, Ballian N, Li M, et al. (2011) Multiple treatment cycles of liposome-encapsulated adenoviral RIP-TK gene therapy effectively ablate human pancreatic cancer cells in SCID mice. *Surgery* 149: 484–495.

66 Xie X, Xia W, Li Z, Kuo HP, Liu Y, Ding Q, et al. (2007) Targeted expression of BikDD eradicates pancreatic tumors in noninvasive imaging models. *Cancer Cell* 12: 52–65.

67 de Weerth A, Pisegna JR, Huppi K, Wank SA. (1993) Molecular cloning, functional expression and chromosomal localization of the human cholecystokinin type A receptor. *Biochemical and Biophysical Research Communications* 194: 811–818.

68 Hiyama E, Hiyama K. (2003) Telomerase as tumor marker. *Cancer Letters* 194: 221–233.

69 Takakura M, Kyo S, Kanaya T, Hirano H, Takeda J, Yutsudo M, et al. (1999) Cloning of human telomerase catalytic subunit (hTERT) gene promoter and identification of proximal core promoter sequences essential for transcriptional activation in immortalized and cancer cells. *Cancer Research* 59: 551–557.

70 Gu J, Kagawa S, Takakura M, Kyo S, Inoue M, Roth JA, et al. (2000) Tumor-specific transgene expression from the human telomerase reverse transcriptase promoter enables targeting of the therapeutic effects of the Bax gene to cancers. *Cancer Research* 60: 5359–5364.

71 Gu J, Zhang L, Huang X, Lin T, Yin M, Xu K, et al. (2002) A novel single tetracycline-regulative adenoviral vector for tumor-specific Bax gene expression and cell killing in vitro and in vivo. *Oncogene* 21: 4757–4764.

72 Koga S, Hirohata S, Kondo Y, Komata T, Takakura M, Inoue M, et al. (2001) FADD gene therapy using the human telomerase catalytic subunit (hTERT) gene promoter to restrict induction of apoptosis to tumors in vitro and in vivo. *Anticancer Research* 21: 1937–1943.

73 Lin T, Gu J, Zhang L, Huang X, Stephens LC, Curley SA, et al. (2002) Targeted expression of green fluorescent protein/tumor necrosis factor-related apoptosis-inducing ligand fusion protein from human telomerase reverse transcriptase promoter elicits antitumor activity without toxic effects on primary human hepatocytes. *Cancer Research* 62: 3620–3625.

74 Jacob D, Davis J, Zhu H, Zhang L, Teraishi F, Wu S, et al. (2004) Suppressing orthotopic pancreatic tumor growth with a fiber-modified adenovector expressing the TRAIL gene from the human telomerase reverse transcriptase promoter. *Clinical Cancer Research* 10: 3535–3541.

75 Yamasaki Y, Tazawa H, Hashimoto Y, Kojima T, Kuroda S, Yano S, et al. (2012) A novel apoptotic mechanism of genetically engineered adenovirus-mediated tumour-specific p53 overexpression through E1A-dependent p21 and MDM2 suppression. *European Journal of Cancer* 48: 2282–2291.

76 Komata T, Kondo Y, Kanzawa T, Hirohata S, Koga S, Sumiyoshi H, et al. (2001) Treatment of malignant glioma cells with the transfer of constitutively active caspase-6 using the human telomerase catalytic subunit (human telomerase reverse transcriptase) gene promoter. *Cancer Research* 61: 5796–5802.

77 Komata T, Kondo Y, Kanzawa T, Ito H, Hirohata S, Koga S, et al. (2002) Caspase-8 gene therapy using the human telomerase reverse transcriptase promoter for malignant glioma cells. *Human Gene Therapy* 13: 1015–1025.

78 Xie X, Li L, Xiao X, Guo J, Kong Y, Wu M, et al. (2012) Targeted expression of BikDD eliminates breast cancer with virtually no toxicity in noninvasive imaging models. *Molecular Cancer Therapeutics* 11: 1915–1924.

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

80 Yao X, Yoshioka Y, Morishige T, Eto Y, Narimatsu S, Mizuguchi H, et al. (2010) Adenovirus vector covalently conjugated to polyethylene glycol with a cancer-specific promoter suppresses the tumor growth through systemic administration. *Biological & Pharmaceutical Bulletin* 33: 1073–1076.

81 Shen CX, Wen Z, Qian YH, Mu SF, Guan XF. (2010) Targeted gene therapy of nasopharyngeal cancer in vitro and in vivo by enhanced thymidine kinase expression driven by human TERT promoter and CMV enhancer. *Journal of Experimental & Clinical Cancer Research* 29: 94.

82 Plumb JA, Bilsland A, Kakani R, Zhao J, Glasspool RM, Knox RJ, et al. (2001) Telomerase-specific suicide gene therapy vectors expressing bacterial nitroreductase sensitize human cancer cells to the pro-drug CB1954. *Oncogene* 20: 7797–7803.

83 Schepelmann S, Ogilvie LM, Hedley D, Friedlos F, Martin J, Scanlon I, et al. (2007) Suicide gene therapy of human colon carcinoma xenografts using an armed oncolytic adenovirus expressing carboxypeptidase G2. *Cancer Research* 67: 4949–4955.

84 Yu ST, Li C, Lu MH, Liang GP, Li N, Tang XD, et al. (2012) Noninvasive and real-time monitoring of the therapeutic response of tumors in vivo with an optimized hTERT promoter. *Cancer* 118: 1884–1893.

85 Xiong J, Sun WJ, Wang WF, Liao ZK, Zhou FX, Kong HY, et al. (2012) Novel, chimeric, cancer-specific, and radiation-inducible gene promoters for suicide gene therapy of cancer. *Cancer* 118: 536–548.

86 Nakamura M, Kyo S, Kanaya T, Yatabe N, Maida Y, Tanaka M, et al. (2004) hTERT-promoter-based tumor-specific expression of MCP-1 effectively sensitizes cervical cancer cells to a low dose of cisplatin. *Cancer Gene Therapy* 11: 1–7.

87 Riesco-Eizaguirre G, De la Vieja A, Rodriguez I, Miranda S, Martin-Duque P, Vassaux G, et al. (2011) Telomerase-driven expression of the sodium iodide symporter (NIS) for in vivo radioiodide treatment of cancer: a new broad-spectrum NIS-mediated antitumor approach. *Journal of Clinical Endocrinology and Metabolism* 96: E1435–E1443.

88 Fujiwara T, Urata Y, Tanaka N. (2007) Telomerase-specific oncolytic virotherapy for human cancer with the hTERT promoter. *Current Cancer Drug Targets* 7: 191–201.

89 Taki M, Kagawa S, Nishizaki M, Mizuguchi H, Hayakawa T, Kyo S, et al. (2005) Enhanced oncolysis by a tropism-modified telomerase-specific replication-selective adenoviral agent OBP-405 ('Telomelysin-RGD'). *Oncogene* 24: 3130–3140.

90 Irving J, Wang Z, Powell S, O'Sullivan C, Mok M, Murphy B, et al. (2004) Conditionally replicative adenovirus driven by the human telomerase promoter provides broad-spectrum antitumor activity without liver toxicity. *Cancer Gene Therapy* 11: 174–185.

91 Kishimoto H, Urata Y, Tanaka N, Fujiwara T, Hoffman RM. (2009) Selective metastatic tumor labeling with green fluorescent protein and killing by systemic administration of telomerase-dependent adenoviruses. *Molecular Cancer Therapeutics* 8: 3001–3008.

92 Lin WH, Yeh SH, Yang WJ, Yeh KH, Fujiwara T, Nii A, et al. (2013) Telomerase-specific oncolytic adenoviral therapy for orthotopic hepatocellular carcinoma in HBx transgenic mice. *International Journal of Cancer* 132: 1451–1462.

93 Uchino J, Takayama K, Harada A, Kawakami Y, Inoue H, Curiel DT, et al. (2005) Infectivity enhanced, hTERT promoter-based conditionally replicative adenoviruses are useful for SCLC treatment. *Cancer Gene Therapy* 12: 737–748.

94 Watanabe T, Hioki M, Fujiwara T, Nishizaki M, Kagawa S, Taki M, et al. (2006) Histone deacetylase inhibitor FR901228 enhances the antitumor effect of telomerase-specific replication-selective adenoviral agent OBP-301 in human lung cancer cells. *Experimental Cell Research* 312: 256–265.

95 Liu D, Kojima T, Ouchi M, Kuroda S, Watanabe Y, Hashimoto Y, et al. (2009) Preclinical evaluation of synergistic effect of telomerase-specific oncolytic virotherapy and gemcitabine for human lung cancer. *Molecular Cancer Therapeutics* 8: 980–987.

96 Takakura M, Nakamura M, Kyo S, Hashimoto M, Mori N, Ikoma T, et al. (2010) Intraperitoneal administration of telomerase-specific oncolytic adenovirus sensitizes ovarian cancer cells to cisplatin and affects survival in a xenograft model with peritoneal dissemination. *Cancer Gene Therapy* 17: 11–19.

97 Huang P, Watanabe M, Kaku H, Kashiwakura Y, Chen J, Saika T, et al. (2008) Direct and distant antitumor effects of a telomerase-selective oncolytic adenoviral agent, OBP-301, in a mouse prostate cancer model. *Cancer Gene Therapy* 15: 315–322.

98 Nakajima O, Matsunaga A, Ichimaru D, Urata Y, Fujiwara T, Kawakami K. (2009) Telomerase-specific virotherapy in an animal model of human head and neck cancer. *Molecular Cancer Therapeutics* 8: 171–177.

99 Kuroda S, Fujiwara T, Shirakawa Y, Yamasaki Y, Yano S, Uno F, et al. (2010) Telomerase-dependent oncolytic adenovirus sensitizes human cancer cells to ionizing radiation via inhibition of DNA repair machinery. *Cancer Research* 70: 9339–9348.

100 Yokoyama T, Iwado E, Kondo Y, Aoki H, Hayashi Y, Georgescu MM, et al. (2008) Autophagy-inducing agents augment the antitumor effect of telerase-selve oncolytic adenovirus OBP-405 on glioblastoma cells. *Gene Therapy* 15: 1233–1239.

101 Sasaki T, Tazawa H, Hasei J, Kunisada T, Yoshida A, Hashimoto Y, et al. (2011) Preclinical evaluation of telomerase-specific oncolytic virotherapy for human bone and soft tissue sarcomas. *Clinical Cancer Research* 17: 1828–1838.

102 Li G, Kawashima H, Ogose A, Ariizumi T, Xu Y, Hotta T, et al. (2011) Efficient virotherapy for osteosarcoma by telomerase-specific oncolytic adenovirus. *Journal of Cancer Research and Clinical Oncology* 137: 1037–1051.

103 Hashimoto Y, Tazawa H, Teraishi F, Kojima T, Watanabe Y, Uno F, et al. (2012) The hTERT promoter enhances the antitumor activity of an oncolytic adenovirus under a hypoxic microenvironment. *PLoS One* 7: e39292.

104 Huang P, Kaku H, Chen J, Kashiwakura Y, Saika T, Nasu Y, et al. (2010) Potent antitumor effects of combined therapy with a telomerase-specific, replication-competent adenovirus (OBP-301) and IL-2 in a mouse model of renal cell carcinoma. *Cancer Gene Therapy* 17: 484–491.

105 Kojima T, Watanabe Y, Hashimoto Y, Kuroda S, Yamasaki Y, Yano S, et al. (2010) In vivo biological – purging for lymph node metastasis of human colorectal

cancer by telomerase-specific oncolytic virotherapy. *Annals of Surgery* 251: 1079–1086.

106 Mullen JT, Tanabe KK. (2002) Viral oncolysis. *Oncologist* 7: 106–119.

107 Ikeda Y, Kojima T, Kuroda S, Endo Y, Sakai R, Hioki M, et al. (2009) A novel antiangiogenic effect for telomerase-specific virotherapy through host immune system. *Journal of Immunology* 182: 1763–1769.

108 Tazawa H, Yano S, Yoshida R, Yamasaki Y, Sasaki T, Hashimoto Y, et al. (2012) Genetically engineered oncolytic adenovirus induces autophagic cell death through an E2F1-microRNA-7-epidermal growth factor receptor axis. *International Journal of Cancer* 131: 2939–2950.

109 Onimaru M, Ohuchida K, Nagai E, Mizumoto K, Egami T, Cui L, et al. (2010) Combination with low-dose gemcitabine and hTERT-promoter-dependent conditionally replicative adenovirus enhances cytotoxicity through their crosstalk mechanisms in pancreatic cancer. *Cancer Letters* 294: 178–186.

110 Fujiwara T, Kagawa S, Kishimoto H, Endo Y, Hioki M, Ikeda Y, et al. (2006) Enhanced antitumor efficacy of telomerase-selective oncolytic adenoviral agent OBP-401 with docetaxel: preclinical evaluation of chemovirotherapy. *International Journal of Cancer* 119: 432–440.

111 Watanabe Y, Hashimoto Y, Kagawa S, Kawamura H, Nagai K, Tanaka N, et al. (2012) Enhanced antitumor efficacy of telomerase-specific oncolytic adenovirus with valproic acid against human cancer cells. *Cancer Gene Therapy* 9: 767–772.

112 Onimaru M, Ohuchida K, Mizumoto K, Nagai E, Cui L, Toma H, et al. (2010) hTERT-promoter-dependent oncolytic adenovirus enhances the transduction and therapeutic efficacy of replication-defective adenovirus vectors in pancreatic cancer cells. *Cancer Science* 101: 735–742.

113 Nemunaitis J, Tong AW, Nemunaitis M, Senzer N, Phadke AP, Bedell C, et al. (2010) A phase I study of telomerase-specific replication competent oncolytic adenovirus (telomelysin) for various solid tumors. *Molecular Therapy* 18: 429–434.

114 Ambrosini G, Adida C, Altieri DC. (1997) A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. *Nature Medicine* 3: 917–921.

115 Mita AC, Mita MM, Nawrocki ST, Giles FJ. (2008) Survivin: key regulator of mitosis and apoptosis and novel target for cancer therapeutics. *Clinical Cancer Research* 14: 5000–5005.

116 Kamizono J, Nagano S, Murofushi Y, Komiya S, Fujiwara H, Matsuishi T, et al. (2005) Survivin-responsive conditionally replicating adenovirus exhibits cancer-specific and efficient viral replication. *Cancer Research* 65: 5284–5291.

117 Sher YP, Tzeng TF, Kan SF, Hsu J, Xie X, Han Z, et al. (2009) Cancer targeted gene therapy of BikDD inhibits orthotopic lung cancer growth and improves long-term survival. *Oncogene* 28: 3286–3295.

118 Sher YP, Chang CM, Juo CG, Chen CT, Hsu JL, Lin CY, et al. (2013) Targeted endostatin-cytosine deaminase fusion gene therapy plus 5-fluorocytosine suppresses ovarian tumor growth. *Oncogene* 32: 1082–1090.

119 Ahn BC, Ronald JA, Kim YI, Katzenberg R, Singh A, Paulmurugan R, et al. (2011) Potent, tumor-specific gene expression in an orthotopic hepatoma rat model using a Survivin-targeted, amplifiable adenoviral vector. *Gene Therapy* 18: 606–612.

120 Hammarstrom S. (1999) The carcinoembryonic antigen (CEA) family: structures, suggested functions and expression in normal and malignant tissues. *Seminars in Cancer Biology* 9: 67–81.

121 Li Y, Chen Y, Dilley J, Arroyo T, Ko D, Working P, et al. (2003) Carcinoembryonic antigen-producing cell-specific oncolytic adenovirus, OV798, for colorectal cancer therapy. *Molecular Cancer Therapeutics* 2: 1003–1009.

122 Qiu Y, Peng GL, Liu QC, Li FL, Zou XS, He JX. (2012) Selective killing of lung cancer cells using carcinoembryonic antigen promoter and double suicide genes, thymidine kinase and cytosine deaminase (pCEA-TK/CD). *Cancer Letters* 316: 31–38.

123 Xu C, Sun Y, Wang Y, Yan Y, Shi Z, Chen L, et al. (2012) CEA promoter-regulated oncolytic adenovirus-mediated Hsp70 expression in immune gene therapy for pancreatic cancer. *Cancer Letters* 319: 154–163.

124 Su ZZ, Sarkar D, Emdad L, Duigou GJ, Young CS, Ware J, et al. (2005) Targeting gene expression selectively in cancer cells by using the progression-elevated gene-3 promoter. *Proceedings of the National Academy of Sciences USA* 102: 1059–1064.

125 Su ZZ, Emdad L, Sarkar D, Randolph A, Valerie K, Yacoub A, et al. (2005) Potential molecular mechanism for rodent tumorigenesis: mutational generation of Progression Elevated Gene-3 (PEG-3). *Oncogene* 24: 2247–2255.

126 Sarkar D, Su ZZ, Vozhilla N, Park ES, Gupta P, Fisher PB. (2005) Dual cancer-specific targeting strategy cures primary and distant breast carcinomas in nude mice. *Proceedings of the National Academy of Sciences USA* 102: 14034–14039.

127 Sarkar D, Su ZZ, Vozhilla N, Park ES, Randolph A, Valerie K, et al. (2005) Targeted virus replication plus immunotherapy eradicates primary and distant pancreatic tumors in nude mice. *Cancer Research* 65: 9056–9063.

128 Sarkar D, Lebedeva IV, Su ZZ, Park ES, Chatman L, Vozhilla N, et al. (2007) Eradication of therapy-resistant human prostate tumors using a cancer terminator virus. *Cancer Research* 67: 5434–5442.

129 Sarkar D, Su ZZ, Park ES, Vozhilla N, Dent P, Curiel DT, et al. (2008) A cancer terminator virus eradicates both

primary and distant human melanomas. *Cancer Gene Therapy* 15: 293–302.

130 Bhang HE, Gabrielson KL, Laterra J, Fisher PB, Pomper MG. (2011) Tumor-specific imaging through progression elevated gene-3 promoter-driven gene expression. *Nature Medicine* 17: 123–129.

131 Iyer M, Wu L, Carey M, Wang Y, Smallwood A, Gambhir SS. (2001) Two-step transcriptional amplification as a method for imaging reporter gene expression using weak promoters. *Proceedings of the National Academy of Sciences USA* 98: 14595–14600.

132 Watanabe M, Ueki H, Ochiai K, Huang P, Kobayashi Y, Nasu Y, et al. (2011) Advanced two-step transcriptional amplification as a novel method for cancer-specific gene expression and imaging. *Oncology Reports* 26: 769–775.

133 Xie X, Hsu JL, Choi MG, Xia W, Yamaguchi H, Chen CT, et al. (2009) A novel hTERT promoter-driven E1A therapeutic for ovarian cancer. *Molecular Cancer Therapeutics* 8: 2375–2382.

134 Sher YP, Liu SJ, Chang CM, Lien SP, Chen CH, Han Z, et al. (2011) Cancer-targeted BikDD gene therapy elicits protective antitumor immunity against lung cancer. *Molecular Cancer Therapeutics* 10: 637–647.

135 Figueiredo ML, Kao C, Wu L. (2007) Advances in preclinical investigation of prostate cancer gene therapy. *Molecular Therapy* 15: 1053–1064.

6

CHAPTER 6

MicroRNAs as Drugs and Drug Targets in Cancer

Hui Ling and George A. Calin
The University of Texas MD Anderson Cancer Center, Houston, TX, USA

Introduction

MicroRNAs (miRNAs) were first discovered in 1993 by Victor Ambros and Gary Ruvkun's groups when they found a small noncoding RNA (ncRNA) transcript that controls developmental timing in *Caenorhabditis elegans* by regulating the lin-14 protein [1]. Since identification of the first miRNA, the last two decades have witnessed an exponentially increasing number of miRNA discoveries from a large variety of species. According to a recent miRBase database release (version 19), more than 2000 human mature miRNAs have been identified and catalogued. These small ncRNAs do not code for peptides but work as master regulators of protein-coding genes through posttranscriptional regulation. It has been estimated that more than 30% of mammalian genes are regulated by miRNAs. Therefore, aberrant miRNA expression could have broad effects on the whole genome and play dynamic roles in various disease types. Abnormal miRNA expression, commonly seen in many human diseases such as cancer, provides a unique opportunity for manipulation of miRNA expression or function in favor of patients suffering from the diseases.

MiRNA and its Functioning Mechanism

Mature miRNAs are evolutionarily conserved single-stranded ncRNAs of 19–24 nucleotides in length. MiRNAs are initially transcribed from the genome by RNA polymerase II as primary transcripts (pri-miRNAs) of various lengths (usually 1000–3000 nucleotides). The pri-miRNAs are processed by the ribonuclease (or RNase) III Drosha–DGCR8 nuclear complex into hairpin RNAs of 60–100 nucleotides, named precursor miRNAs (pre-miRNAs). The pre-miRNAs are exported to the cytoplasm by exportin-5 and further processed by the ribonuclease enzyme Dicer into double-stranded mature miRNAs of about 22 nucleotides. This miRNA duplex unwinds and the mature single-strand incorporates into the RNA-induced silencing complex (RISC). By Watson–Crick complementarities between the seed sequence of miRNA (positions 2–8) and the 3′ untranslated region (UTR) of its target messenger RNAs (mRNAs), miRNA recognizes and recruits sequence-specific mRNAs onto RISC. The miRNA–mRNA interaction prevents mRNA translation or enhances mRNA degradation, in either case leading to diminished expression levels of target protein (see Figure 6.1 for an illustration of miRNA biogenesis).

It should be noted that miRNAs could also be produced by alternative biogenesis pathways which are Drosha- or Dicer-independent [2]. The short RNA duplexes mapped to short hairpin introns, named as mirtrons, are initially produced by splicing and debranching, not by Drosha cleavage. After trimming by exosomes, mirtrons are further cleaved by Dicer to produce mature products [2]. In the case

Cancer Gene Therapy by Viral and Non-viral Vectors, First Edition. Edited by Malcolm K. Brenner and Mien-Chie Hung.

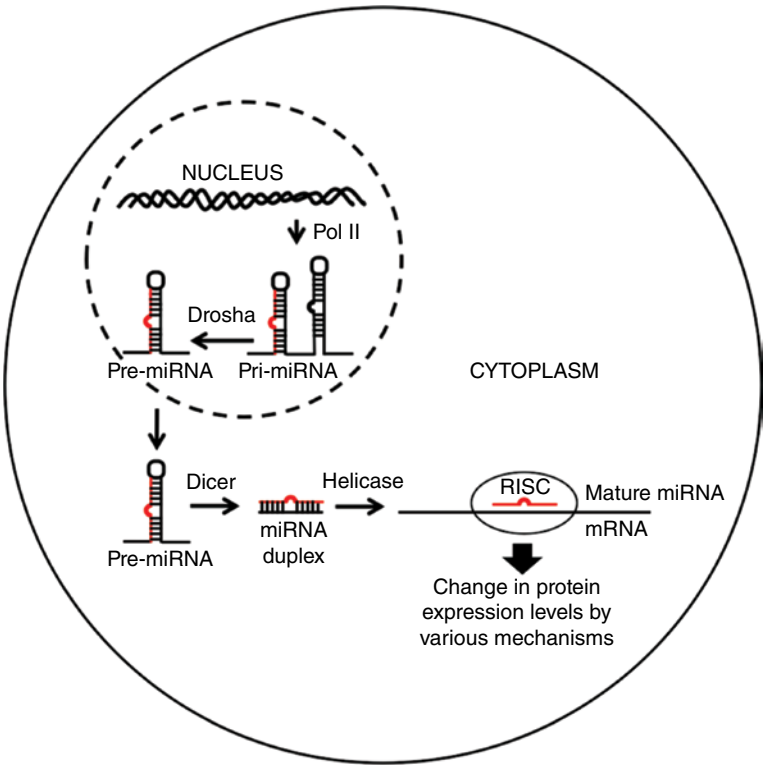


Figure 6.1 MiRNA biogenesis. MiRNAs are transcribed from the genome by RNA polymerase II (Pol II) into pri-miRNAs, which are then processed by Drosha into hairpin structures called pre-miRNAs. The pre-miRNAs are transported by exportin-5 from nucleus to the cytoplasm, where Dicer further processes them into miRNA duplex. The miRNA duplex unwinds and the mature miRNAs are incorporated onto the RISC. By base pairing with the target mRNAs, miRNA affects protein production either by interfering with protein translation or by changing the mRNA's stability.

of miR-451 biogenesis, pri-miR-451 is cleaved by Drosha–DGCR8 into a ≈ 18 bp duplex structure, and further processed by Ago2 to produce mature miR-451 without involvement of Dicer [3]. Furthermore, while the above-mentioned canonical mechanism of miRNA regulation is generally accepted, emerging evidence suggests a more diverse regulatory mechanism of miRNA on protein-coding genes. Tay et al. demonstrated that the miRNA gene regulation can occur by binding to the mRNA-coding sequence, and this regulatory mechanism plays essential roles in embryonic stem cell differentiation [4]. Lytle et al. showed that the interaction of miRNA with the 5′-UTR of a protein-coding gene mediates gene silencing as efficiently as with the canonical interaction with 3′-UTR [5]. Orom et al. found that miR-10a binds to the 5′-UTR of the targeted protein-coding genes and enhances the translation of these genes [6]. Shin et al. demonstrated that centered pairing, which is independent of seed sequence pairing, also leads to mRNA destabilization or repression of translation [7].

Besides the miRNA–mRNA interaction in the cytoplasm, an additional gene-regulation mechanism exists for the rare cases where miRNAs are predominantly located in the nucleus. Recent studies suggest that the miRNAs in the nucleus may affect gene transcription by acting on the promoter levels. As an example, miR-373 binds to the CDH1 promoter and stimulates its transcription [8]. Additionally, miRNAs can directly interact with proteins, as in the case of miR-328, which acts as a decoy to release heterogeneous nuclear ribonucleoprotein (hnRNP) E2-mediated translational inhibition [9]. In a recent study, nuclear miRNAs induced by DNA double-strand breaks were found to facilitate repair of DNA damage through chromatin modifications [10].

Because of their short length and diverse regulatory mechanisms, each miRNA can have hundreds or thousands of targets and the coding genome is probably under tight control of miRNAs. These miRNA genes may be involved in many physiological processes and pathways, such as the fate of B-cell lineages (miR-181), B-cell survival (miR-15a and miR-16-1), cell-proliferation control (miR-125b and let-7), brain patterning (miR-430), pancreatic cell insulin secretion (miR-375), and adipocyte development (miR-145) [11].

In a specific disease setting, the interaction and regulatory mechanism between miRNA and protein-coding genes can be more complex. Recent studies suggest the existence of sophisticated networks composed of multiple miRNAs and protein-coding genes in cancer pathogenesis. We reported miRNA/TP53 circuitry in B-cell chronic lymphocytic leukemia (CLL) comprising five miRNAs (miR-15a/miR-16-1, and miR-34a, -34b, and -34c) and four coding genes including the transcription factor TP53, the anti-apoptotic oncogenes *BCL2* and *MCL1*, and the 70-kDa zeta-associated protein (Zap70) [12]. This circuitry signature was proven to be a powerful predictor of CLL patient survival. Because of the complex interaction between multiple miRNAs and protein-coding genes, targeting only a specific miRNA or protein-coding gene may not effectively revert this aberrant network in this situation.

MiRNA and Cancers

In search of possible tumor suppressors in the 13q14 region that is frequently deleted in CLL, George Calin and Carlo Croce screened for new regulatory elements in this region. In 2002, they found that the miRNA cluster miR-15a/-16-1 is exactly located in the 13q14 region, and the expression of this cluster was downregulated in 69% of CLL cases analyzed [13]. This was the first report of the involvement of miRNAs in cancer. Subsequent studies demonstrated that the miR-15a/-16-1 cluster acts as a tumor suppressor by targeting the anti-apoptotic gene *BCL2* [14] and this miRNA cluster controls the expression of about 14% of all the genes in the human genome [15]. The role of miR-15a/-16-1 in CLL was supported by other group's study using the New Zealand black mouse model [16]. In this study, a point mutation in the 3′ flanking region of miR-16-1 and decreased miR-16-1 expression were identified in the spontaneous mouse model of human CLL.

Following the initial finding of miR-15a/-16-1 involvement in cancer, the Croce group further identified that many of the known miRNAs are located in the frequently altered cancer risk regions: chromosomal loci prone to deletions or amplification [17]. Profiling studies showed a widespread alteration in miRNA expression levels in many types of cancer, and these miRNA signatures can be used as diagnostic and prognostic tools [18]. For instance, a signature of aberrant expression of 11 miRNAs was found to correlate well with the survival rate of patients with acute myelogenous leukemia [19]. In another study, an miRNA expression pattern was identified to be associated with prognosis and therapeutic outcome in colon adenocarcinoma [20]. Most impressively, miRNA signatures are more accurate in identifying the origin of metastatic cancer than mRNA patterns [21].

Although miRNAs were initially thought to be tumor suppressors, many miRNAs were soon discovered to play oncogenic roles in initiating and promoting carcinogenesis (see Table 6.1, which summarizes the involvement of miRNAs in cancer). miR-21 was the first oncogenic miRNA that have been identified to be elevated in many types of cancers. Ectopic overexpression of miR-21 leads to pre-B-cell lymphoma in transgenic mouse model [22]. miR-21 was also demonstrated to enhance K-Ras-dependent lung tumorigenesis by targeting negative regulators of the Ras/mitogen-activated extracellular-signal-regulated kinase (ERK) activating kinase (MEK)/ERK pathway [23]. It has also been reported that miR-21 induced invasion, intravasation, and metastasis in colorectal cancer by negatively regulating the tumor-suppressor PDCD4 [24]. Another example of miRNA with oncogenic function is the miR-17-92 cluster located at 13q22, which is frequently upregulated in a wide range of cancer types, including lymphoma, lung, breast, stomach, colon, and pancreatic cancer, through amplification or transcriptional activation [25]. These miRNAs are direct Myc targets and they cooperate with the *Myc* oncogene in facilitating carcinogenesis [26]. The oncogenic effect of miR-17-92 cluster has been validated in various

Table 6.1 Overview of the major miRNAs involved in cancer.

miRNA	*Genomic location*	*Oncogene/tumor suppressor*	*Associated cancers*	*Regulation in cancer*	*Targeted protein-coding genes*
miR-155	21q21	Oncogene	CLL, lymphoma, lung cancer	Up	*SHIP1*, *CEBPB*
miR-17-92	13q22	Oncogene	Lymphoma, lung cancer, breast cancer	Up	*E2F1*, *BIM*, *PTEN*
miR-21	17q23	Oncogene	Lymphoma, lung cancer, colorectal cancer	Up	*PTEN*, *PDCD4*, *TPM1*
let-7 family	11q24	Tumor suppressor	Lung cancer, breast cancer	Down	*CCND1*, *CDC25a*, *CDK6*, *HOXA9*, *IMP-1*, *Myc*, *RAS*, *TLR4*
miR-15a/-16-1	13q14	Tumor suppressor	CLL	Down	*BCL2*, *MCL1*
miR-29	7q32 and 1q30	Tumor suppressor	Breast cancer, CLL	Down	*MCL1*, *DNMTs*
miR-34a	1p36 and 11q23	Tumor suppressor	Colorectal cancer, lung cancer, breast cancer	Down	*CDK4*, *CDK6*, *Myc*

animal models. Transfection of miR-17-92 cluster promotes the development of B-cell lymphomas in Myc transgenic mice [27]. In another study using a conditional knockout model, Mu et al. demonstrated that miR-17-92 cluster is required to suppress Myc-induced apoptosis and deletion of this cluster decelerates Myc-induced lymphoma [26]. miR-155 is another typical miRNA with oncogenic function. Overexpression of miR-155 alone is sufficient to cause lymphoblastic leukemia as demonstrated using a transgenic mouse model [28]. This was the first report that a single miRNA can induce tumor development and this finding indicates that miRNAs can be the driving forces of cancer initiation. miR-155 was also found to modulate mismatch repair and genomic stability [29]. A more recent study identified that miR-155 reduces transcriptional activity of B-cell lymphoma 6 (BCL6) by targeting histone deacetylase 4 (HDAC4) [30].

The tumor-suppressor miRNAs include miR-15a/-16-1, miR-34 family, miR-200c, and let-7 family. These miRNAs are downregulated by various mechanisms such as deletion, mutation, epigenetic silencing, and transcriptional repression. For example, miR-34a is positively regulated by TP53, is negatively regulated by Myc, is silenced by aberrant CpG methylation, and is located at 1p36, a chromosomal region frequently lost in neuroblastomas [31]. The miR-34 family is part of the p53 tumor-suppressor network [32]. Ectopic miR-34 expression induces cell cycle arrest and apoptosis, while reduced miR-34 expression confers resistance to p53-induced apoptosis [31]. The miR-34 expression can also be independent of p53. For instance, B-Raf-induced senescence caused strong induction of miR-34 expression by a mechanism independent of p53, and the induced miR-34 represses the *Myc* oncogene [33]. In prostate cancer, miR-34 was found to be potential inhibitor of cancer stem cells and tumor progression by directly targeting CD44 [34]. The p53 pathway also activates the expression of miR-200c, which plays an essential role mediating the regulatory effect of p53 on epithelial–mesenchymal transition and stem cell properties [35]. let-7 family members are typical tumor-suppressor miRNAs. Low let-7 expression has been associated with shorter postoperative survival of lung cancer patients [36]. The tumor-suppressor role of let-7 was also demonstrated in animal models where overexpression of let-7 reduced lung cancer formation [37].

MiRNAs are also intensively involved in tumor metastasis. Studies from Weinberg group demonstrated that miR-9 [38] and miR-10b [39] promote breast cancer metastasis to the lung in mouse models. Induced by the transcription factor Twist, the miR-10b upregulates expression of a prometastatic gene *RHOC* through inhibition of HOXD10 translation. miR-9 is transcriptionally activated by Myc/MYCN and it negatively regulates E-cadherin

to promote cancer metastasis. Similarly, the Weinberg group also identified a miRNA with function of reducing tumor metastasis [40]. They found an inverse correlation between the expression level of miR-31 and metastasis in breast cancer patients. Experimental expression of miR-31 reduced tumor metastasis, while inhibition of miR-31 function caused nonaggressive breast cancer cells to metastasize. Study of the mechanism showed that miR-31 negatively regulates a series of metastasis-promoting genes including *RHOA*. Other research groups identified the miR-200 family as suppressors of cancer metastasis. By targeting ZEB1 and ZEB2, miR-200 inhibits epithelial–mesenchymal transition and hinders tumor migration and invasion [41]. However, a recent finding suggests that miR-200 promotes colonization of metastatic breast cancer cells that entered the circulation [42]. A more comprehensive understanding of miR-200 in metastasis is needed, considering the multifaceted mechanisms at different metastatic stages.

It should be pointed out that, depending on cellular context, the same miRNA could behave like an oncogene in one cell type while working as a tumor-suppressor in another. For example, in acute myeloid leukemia and aggressive CLL, the miR-29 family exerts tumor-suppressor function by regulating epigenetic changes through targeting DNA methyltransferases [43]. In contrast, in indolent CLL [44] or breast cancer [45] miR-29 promotes cancer development. Similarly, miR-221 regulates the *KIT* oncogene to inhibit erythroleukemic cell growth [46], but exerts oncogenic function by targeting the DNA-damage-inducible transcript 4 in hepatocellular carcinoma [47]. This dual-faceted activity of miRNA has therapeutic implications, since the same miRNA can have different targets in different cell types of the same organism, and consequently opposite effects. Further complicating the issue of miRNA targeting, the mature products generated from each strand of the same hairpin RNA structure, termed 5p and 3p, can target different mRNAs and display distinct functions. Our recent study showed that miR-28-5p and miR-28-3p have different effects in colorectal cancer: overexpression of miR-28-5p reduced cell proliferation, migration, and invasion *in vitro* by targeting CCND1 and HOXB3, whereas miR-28-3p increased cell migration and invasion *in vitro* by altering NM23-H1 expression [48]. Such information indicates that the identification of the specific roles of each strand is mandatory if a pre-miRNA is to be used and both strands are produced. If opposite effects are observed, the specific strand of mature miRNA should be preferably delivered and the pre-miRNA could be avoided in the design of miRNA therapeutics.

Besides genomic amplification/deletion and transcriptional regulation, the miRNA expression and function are also deregulated by other mechanisms. For instance, expression levels of Drosha and Dicer were lower in ovarian cancer than the normal tissue counterpart, and this could lead to widespread miRNA downregulation [49]. Other defects in miRNA biogenesis machinery such as genetic effect in exportin-5 could also reduce mature miRNA production by trapping the pre-miRNA in the nucleus [50]. Moreover, alteration of the target mRNA 3′-UTR was observed in cancer cells, and this shortening of UTRs in cancer cells activates oncogenes because of the loss of an miRNA-binding site, and thus loss of miRNA regulation of these oncogene targets [51]. The mechanism of miRNA regulation was further expanded by the discovery of competitive endogenous RNAs (or ceRNAs). These are transcribed pseudogenes that can modulate miRNA activity through competing for miRNA binding with mRNA targets [52,53,54]. These defects, although with different mechanisms, invariably lead to deregulated miRNA expression and function, favoring cancer development.

MiRNA in Other Diseases

As master regulators of the human genome, miRNAs also play dynamic roles in other disease types such as heart disease, Alzheimer's disease, diabetes, virus replication, and inflammatory disease. For instance, miR-1, miR-21, miR-23, miR-133, and miR-208a are important regulators in cardiomyocyte hypertrophy and heart failure [55]. miR-146a is involved in innate immune response and plays roles in virus infection [56]. miR-210 is ubiquitously expressed in cell hypoxia and acts as a master regulator of the gene-expression networks that are activated under conditions of hypoxia [57]. miR-375 regulates glucose-induced insulin

secretion by targeting myotrophin [58]. Reduced miR-107 expression in Alzheimer's disease may contribute to the progression of this disease by a consequent derepression of targeting β-site amyloid precursor protein-cleaving enzyme 1 (BACE1) [59]. miR-122 is specifically expressed in human liver and it can facilitate replication of the hepatitis C virus (HCV) [60].

MiRNA Therapeutics

Rationale for miRNA-Targeting Treatments

The aberrant expression and intense involvement of miRNA in cancer and other diseases offer an excellent opportunity of targeting miRNA for therapeutic treatment of human diseases. Generally, two main strategies can be employed to manipulate miRNA function: the restoration of miRNAs that are downregulated, and silencing of miRNAs that are upregulated. Unlike chemotherapeutic agents, miRNAs are "natural" products of human cells, and theoretically produce fewer side effects. On the other hand, a single miRNA targets multiple genes and a mild change in the miRNA expression or function can exert widespread effects on many protein-coding genes and thus change the phenotype. It is possible to use miRNA-targeting tools to regulate multiple genes from the same pathways at different levels and prevent compensatory mechanisms such as mutation in the targeted oncogenes, which could cause resistance to many of the current treatments. One typical example is miR-181, which regulates T-cell receptor sensitivity by targeting multiple phosphatases [61]. It is hard to achieve a similar effect by using small interfering RNAs. From another angle, multiple miRNAs can target the same genes by binding to distinct complementary sequences, and producing a combined effect on the target genes. For instance, Myc can be negatively regulated by either let-7 or miR-34. Similarly, PTEN is a common target for both miR-21 and the miR-17-92 cluster. Current strategies for miRNA targeting comprise the use of synthetic mimics to restore miRNA expression and the use of antisense oligonucleotides to block the oncogenic miRNAs (illustrated in Figure 6.2).

Restoring miRNA Function

Disruption of normal physiological homeostasis induces human disease. Tumors occur as a result of an imbalance between oncogenic and

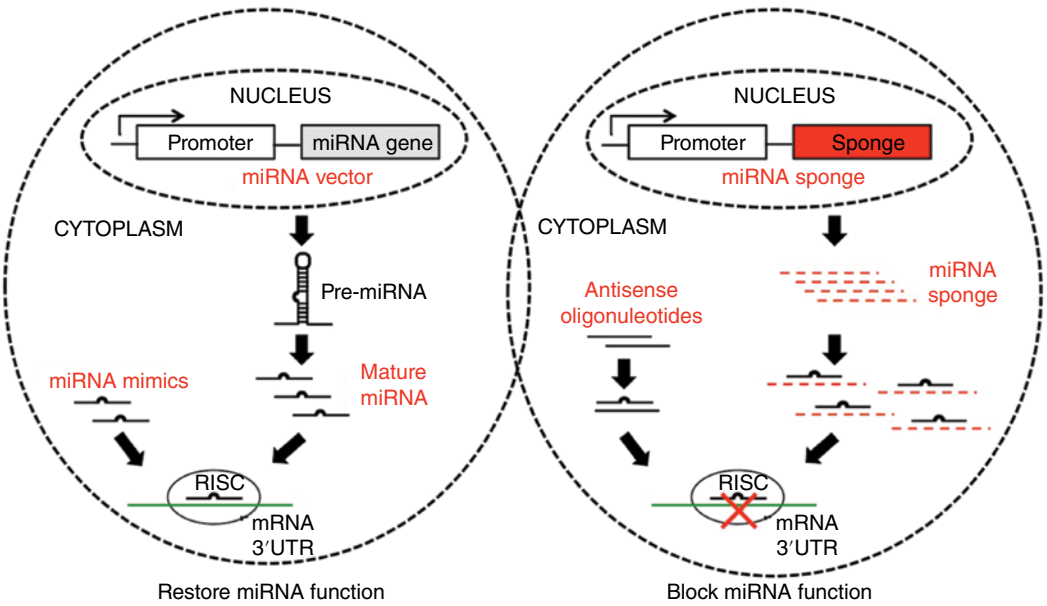


Figure 6.2 Strategies of miRNA therapeutics. Generally, two strategies can be employed to modulate miRNA expression or function: using miRNA mimics or viral constructs to restore miRNA function, and using antisense oligonucleotides (including antagomirs and locked nucleic acids) or miRNA sponge constructs to block miRNA function.

tumor-suppressive potentials. As we have learned from the protein-coding tumor-suppressor genes, resupply of the biomolecules that the body lacks may prevent or reverse the progression of pathogenesis. Therefore, approaches that can restore miRNA expression or miRNA function are promising alternatives to currently available treatments.

The reintroduction of miRNA function can be achieved by indirect upregulation of miRNA production. For instance, hypomethylating agents such as decitabine or 5-azacytidine have been approved for the treatment of myelodysplastic syndromes. These drugs reinduce the expression of multiple mRNAs and ncRNAs including miRNAs [62,63]. However, this effect is not specific. In addition, the set of genes regulated by these agents is dependent on the context. Enoxacin (Penetrex), a fluoroquinolone used as an antibacterial compound, is another example of drugs enhancing miRNA production [64]. By binding to the miRNA biosynthesis protein TRBP, this drug upregulates tumor-suppressor miRNAs such as let-7, miR-18, and miR-125, and reduces tumor growth in various mouse models. These examples highlight the key role of disrupted miRNA expression patterns in human disease, and suggest a unique therapeutic strategy for restoring the full spectrum of dysregulated miRNAs.

A more specific replacement of miRNA function could be achieved by miRNA mimics (miR-mimics). The miR-mimics are small, double-stranded RNAs mimicking mature endogenous miRNAs after infection into cells. The mimics can be delivered by nanoparticles or liposomes. By coating the nanoparticles with antibodies that recognize tumor-specific antigens, the nanoparticle delivery allows precise delivery of the miRNA of interest into targeted cancer cells. This method has been successfully used to inhibit neuroblastoma tumor growth by targeted delivery of miR-34a using nanoparticles coated with a neuroblastoma-specific antibody in a murine orthotopic xenograft model [65]. In another study, systematically delivered neutral lipid emulsions of miR-34a and let-7 mimics significantly reduced the tumor burden in a K-Ras-activated mouse model of lung cancer [66]. It has also been reported that formulation with atelocollagen facilitated miR-34a delivery into tumors and inhibited xenografted colon cancer growth in mice [67]. Most impressively, a formulated miR-34 mimic called MRX34, developed by Mirna Therapeutics, produced complete tumor regression in two separate orthotopic mouse models of liver cancer. MRX34 displayed therapeutic activity in models of lung and metastatic colorectal cancer as well. No immunostimulatory activity and toxicity to normal tissues were observed in the treatments using MRX34 (www.mirnatherapeutics.com).

The specific restoration of miRNA function can also be realized by miRNA-expressing vectors. For instance, the adenovirus-associated virus (AAV) can be used to overexpress a tumor-suppressor miRNA. These vectors have high efficiency of transduction, and they do not integrate into genomes and have minimal toxicity [68]. miR-26 is highly expressed in normal liver tissues, but the expression of miR-26 is lost in hepatocellular carcinoma. Kota et al. cloned miR-26 into an AAV vector and systematically delivered this miRNA by intravenous injection into a mouse model of hepatocellular carcinoma [69]. They found that the ectopic expression of miR-26 inhibits cancer cell proliferation, induces tumor-specific apoptosis, and prevents disease progression without observable toxicity. This was the first study using AAV to deliver miRNA to the liver, and it also showed for the first time that restoring the expression of a miRNA effectively blocks tumor progression *in vivo*. The AAV vector has also been applied to experimental treatment of other diseases. Adenoviral overexpression of miR-1 in the heart attenuated cardiomyocyte hypertrophy *in vitro* and *in vivo* [70]. With engineered promoters, the AAV system also allows for targeted delivery of miRNA in a tissue- or tumor-specific fashion. Chen et al. constructed a miR-26a expression vector that driven by a dual promoter for α-fetoprotein (hAFP) and human telomerase reverse transcriptase (hTERT). They observed reduced liver tumor growth, with tissue- and tumor-specific expression of miR-26a [71]. However, it should be noted that there are possible hazards associated with a viral system. The delivery material can be integrated into the host genome (in the case of retroviral and lentiviral vectors) or replicate as an autonomous unit (in the case of adenoviral vectors). In addition, the AAV may produce a strong immunological response that could be harmful to the subject. Finally, the viral system may have the risk of insertional mutagenesis and activation of proto-oncogenes.

Blocking miRNA Function

Silencing of oncogenic miRNAs presents the other aspect of miRNA-based therapeutics. Various methods can be used to knock down miRNA expression levels or block miRNA function. These include the use of antisense oligonucleotides, antagomirs, locked nucleic acids (LNAs), and miRNA sponges. Whereas the efficacy of restoring of miRNA function can be easily determined by measuring miRNA expression levels, the effect of functional blocking of miRNA usually cannot be assessed in this way. This is because the blocking of miRNA function by various anti-miRNA approaches may not necessarily reduce its expression. Instead, these effects are better reflected by changes in the expression of the miRNA-targeted mRNAs and translated proteins. Typically a luciferase or green fluorescence protein (GFP) reporter fused to miRNA-binding sites is used to indicate if this miRNA function was successfully blocked. Ideally, these effects should be analyzed by genome-wide technologies; the other choice for monitoring the therapeutic efficacy of antagonists is a phenotypic readout (such as cell proliferation or cell survival) in the *in vitro* and *in vivo* models [72].

Anti-miRNA oligonucleotides

Anti-miRNA oligonucleotides (or AMOs) are single-stranded antisense oligonucleotides that can be used to block miRNA function [73]. As single-stranded oligonucleotides, AMOs are easily degraded by nucleases. To increase the stability and increase its binding affinity to the specific miRNA, several chemical modifications have been applied to this type of anti-miRNA (anti-miR) molecule. The 2′ position of the ribose moiety represents the most frequently modified site. Chemical modifications include 2′-O-methylation (2′-O-Me), 2′-O-methoxyethylation (2′-O-MOE), and 2′-fluoro substitution (2′-F) [73].

LNA anti-miRNAs

LNAs are RNA analogues where the ribose is locked in a C3′-endo conformation by the introduction of a 2′-O,4′-C methylene bridge (see Figure 6.3 for LNA structure). This structural modification greatly improves the binding affinity of the oligonucleotide and its complementary miRNA. The duplex formation is thermodynamically strong, which renders a prolonged anti-miRNA effect. Because of the higher binding affinity, it is possible to achieve a similar silencing effect using a lower dose of LNA anti-miRNA. Systematic delivery of an unconjugated saline formulation of LNA-anti-miR-122 by either intraperitoneal or intravenous injection leads to dose-dependent silencing of liver-specific miR-122 in mice [74]. Compared with other blocking strategies such as antagomiRs and 2′-modified anti-miRNAs, LNA has the advantage of being administrated as a single intraperitoneal injection with dose-dependent silencing of target miRNAs. In another study, an LNA-anti-miR-33 was shown to increase the expression levels of high-density lipoprotein cholesterol *in vivo*, suggesting potential therapeutic value in cardiovascular disease [75]. The 8-mer LNA anti-miRs, specifically designed to target the 5′-seed region of miRNAs, are new additions to this category of therapeutic agents. A recent study by Zhang et al. showed that an 8-mer LNA-anti-miR-155 oligonucleotide targeting miR-155 inhibits Waldenstrom macroglobulinemia and CLL cell proliferation *in vitro*, while systemic delivery of anti-miR-155 significantly decreased tumor growth *in vivo* [76].

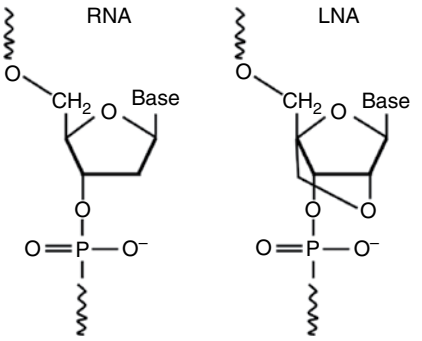


Figure 6.3 The chemical structures of RNA and LNA. By forming a C3′-endo conformation by the introduction of a 2′-O,4′-C methylene bridge, the LNA modification greatly increases the stability and specificity of the antisense oligonucleotide.

SPC3649, an LNA-based antisense molecule against miR-122, was the first miRNA-targeting agent to enter clinical trials. Developed by Santaris Pharma A/S for the treatment of HCV, this agent has been evaluated in Phase 1 and Phase 2a clinical trials [77]. The rationale for these studies is based on the observation that liver-specific miR-122 recognizes two target sites in the 5′-noncoding region of the HCV genome, and upregulates viral RNA

replication. In the clinical trials, SPC3649 significantly reduced HCV expression and the treatment was well tolerated in human subjects [77]. The study using HCV-infected chimpanzees showed that during a 12-week treatment period with SPC3649 no viral rebound was detected [78]. This suggests a lack of adaptive mutations in the interaction sites between miR-122 and the HCV mRNA.

Antagomirs

Antagomirs are synthetic antisense oligonucleotides characterized by a 2′-O-Me modification on the ribose sugar, the terminal phosphorothioate linkage, and the cholesterol conjugation at the 3′ end. Antagomirs were first developed by Krutzfeldt et al., who injected antagomiR-16, −192, and −194 into the tail vein of mice and observed a diffuse silencing of the respective miRNAs in the liver, lung, kidney, heart, intestine, fat, skin, bone marrow, muscle, ovaries, and adrenal glands [79]. Antagomirs targeting miR-122 showed effectiveness by reducing liver-specific miR-122, increasing the expression of mRNA targets regulated by miR-122, and significantly reducing cholesterol levels in the serum of treated mice [79]. The Weinberg group used antagomirs to investigate the function of miRNA in tumor metastasis [80]. In a breast cancer metastasis model, 4T1 cells implanted into the mammary fat pad of immunocompetent, syngeneic BALB/c mouse rapidly metastasized to the lungs. Antagomirs targeting miR-10b administered 2 days after 4T1 cell implantation effectively caused an 86% decrease in the number of lung metastases. This anti-metastasis effect was fully replicated by using anti-miR-10b sponges. However, antagomiR-10b had no effect on late metastatic stages, when the tumor cells had already disseminated. These findings indicate that miR-10b is specifically involved in the early stages of tumor metastasis. Importantly, in this study only a very minor toxic effect, limited to a slight decrease in the number of white blood cells and increased bilirubin levels in the serum, was observed in animals. Findings from these studies suggest that antagomirs could be an effective and safe approach for therapeutic application.

MiRNA sponges

MiRNA sponges are artificial transgenes composed of multiple complementary sequences, which are able to bind and sequester miRNAs by base pairing (see Figure 6.4 for an illustration of the sponge mechanism). The first miRNA sponge was used by Care et al. to study miR-133 function in cardiac hypertrophy [81]. The miR-133 sponges containing multiple miR-133 target sequences cloned into an AAV vector induced marked and sustained cardiac hypertrophy in mouse. In another study, a lentiviral sponge for miR-326 was able to reduce the generation of Th-17T cells secreting interleukin (IL)-17, and consequently alleviating experimental autoimmune encephalomyelitis in mice [82]. Importantly, miRNA sponges can be designed to simultaneously silence multiple different miRNAs. For example, Kluiver and colleagues developed combination sponges of the miR-17-92 cluster, which were able to silence each member of this cluster; that is., miR-17, miR-18a, miR-19, and miR-92a. Compared with individual miRNA sponges, these combination sponges showed a stronger inhibitory effect on proliferation of WEHI-231 B-cell lymphoma cells [83].

There are several advantages of miRNA sponges over chemically modified antisense oligonucleotides. First, the property of the interaction sites on sponges, which are specific to the miRNA seeding sequence, allows blockade of a whole family of related miRNAs. Second, the inhibitory potency of miRNA sponges on miRNA function is higher than miRNA antisense oligonucleotides. In some cases where miRNA-target interaction causes miRNA degradation, the expression level of miRNA is reduced even to an extent not detectable by northern blotting [84]. Third, the fused reporter gene such as GFP or luciferase to the sponge vector can monitor the sponge transgenes. In addition, it allows for tissue-specific targeted delivery by including regulatory elements in the sponge promoter. Finally, the sponges can be stably transfected and thus the inhibitory effect can be sustained for a long time without multiple administration. The disadvantage of sponges is increased risk of insertional mutagenesis in target cells due to the strong promoter activity required for overexpression of sponges.

Combination Strategies

Patients benefit from combined treatment with multiple chemotherapeutic drugs. The combination regime is not only able to improve the efficacy, but

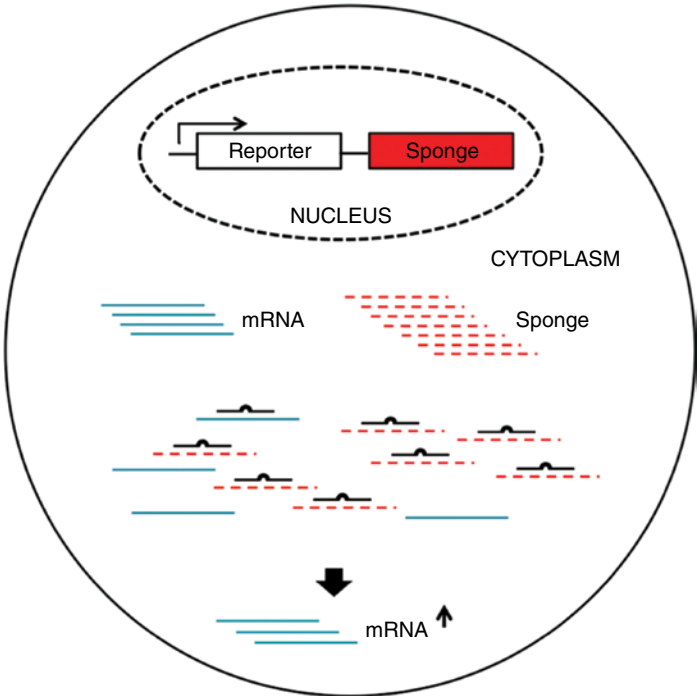


Figure 6.4 The mechanism of miRNA sponges. MiRNA sponges are transcribed at high levels by using expression vectors in the nucleus. After release into the cytosol, these sponges compete with the miRNA-targeting mRNAs for the binding of miRNAs. As a result, the mRNAs are released from the inhibition by miRNAs, and consequently the expression levels of proteins translated by these mRNAs are upregulated.

also reduces the side effect caused by a single agent. Similarly, the various miRNA agents can be combined together, or combined with small interfering RNAs or chemotherapeutic agents to achieve better therapeutic effects, while maintaining the lower toxicity and milder side effects. For instance, two combination strategies could be envisioned for CLL treatment. The first strategy focuses on a major molecular alteration clearly involved in CLL pathogenesis, and uses multiple agents to achieve an additive effect of inhibition. For instance, oblimersen sodium, an antisense oligonucleotide designed to specifically bind to human BCL2 mRNA [85], can combine with miRNAs targeting BCL2, such as miR-15a and miR-16, for treatment of indolent CLL. The second strategy is to use multiple miRNAs targeting defective protein-coding genes in the same pathway. For example, combined use of miR-15a and miR-16 (targeting BCL2) and miR-29 family (targeting MCL1) may be more efficient and robust in restoring the proper apoptotic pathways than a single agent. In the case of liver cancer, where high expression of miR-21 and loss of miR-26 expression are frequently observed, a combination of chemotherapy with a miR-21 inhibitor and miR-26 mimics may bring beneficial effects to the patients [86]. Kim et al. showed that the combination of 5-fluorouracil (5-FU) with an adenoviral vector expressing miR-145 exerts a stronger inhibitory effect on breast cancer growth *in vitro* and *in vivo* compared with the 5-FU treatment alone [87]. Some miRNAs are involved in the resistance mechanism to chemotherapy. For instance, downregulation of miR-128b and miR-221 is implicated in glucocorticoid resistance in MLL-AF4 acute lymphocytic leukemia cells [88]. Therefore the restoration of these miRNA expression levels may sensitize the chemotherapeutic treatment, and represent a potentially promising therapeutic advance in combination with chemotherapy.

Challenges

Although miRNA-based therapeutics are definitely promising and represent a novel category of future drugs, there are many issues to be solved before the successful development of these therapeutics into clinical application. One challenge is the successful delivery of the therapeutic agents to the target sites. Many factors in the human body, such as degradation of miR-mimics or anti-miRs by nucleases, renal clearance, and failure to cross the capillary endothelium could limit the bioavailability of these miRNA-targeting agents. For instance, unconjugated RNAs can be easily filtered by the kidneys and excreted. Since macrophages and monocytes can remove complex RNAs from extracellular spaces, systematic delivery of miRNA therapeutic agents can be compromised by the host immune system. Chemical modifications, which are needed to increase the stability of the nucleotides, may produce off-target effects or compromise efficiency. Another challenge is how to evaluate the safety of miRNA-based treatments. The virus delivery system can cause mutagenesis and/or promote harmful immune response. As such, the toxicity and side effects of the delivery system need to be carefully evaluated. One advantage of miRNA is its multifaceted functions: this also likely to lead to unexpected off-target effects that are more difficult to predict. This is a practical issue that needs to be carefully considered, especially for a disease such as cancer that needs long-term administration of therapeutic agents.

Outlook

Encouragingly, several miRNA-based therapeutics have entered or are ready to enter clinical trials. The miR-122 antagonist SPC3649 has passed the phase I and phase IIa clinical trials for treatment of HCV infection, with remarkable efficacy and safety profile [77]. The recently completed phase IIa trial showed that SPC3649 robustly reduced the virus level in a dose-dependent manner. Impressively, four out of nine patients treated at the highest dose (7 mg/kg) with SPC3649 became HCV RNA undetectable [77]. The effectiveness of LNA-anti-miR-122 treatment proved the plausibility of miRNA-based therapeutical strategies in clinical usage. More recently, MRX34, a liposome-formulated miR-34 mimic developed by Mirna Therapeutics, is scheduled for a phase I clinical trial in patients with advanced or metastatic liver cancers (see [89]). Several other pharmaceutical companies have also enthusiastically pursued the commercial development of miRNA therapeutics. MiRagen Therapeutics is currently focusing on cardiovascular and muscle diseases by means of miRNA inhibition and replacement. Regulus Therapeutics is actively researching anti-miRs for treatment of diseases such as fibrosis, HCV infection, atherosclerosis, and cancer. We anticipate that future development will improve the efficacy and specificity of anti-miRs and miR-mimic agents, and reduce the toxicity and off-target effects. We believe that the powerful and innovative approaches based on miRNA research will provide renewed hope in the war against cancer.

References

1 Lee RC, Feinbaum RL, Ambros V. (1993) The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell* 75(5): 843–854.

2 Yang JS, Lai EC. (2011) Alternative miRNA biogenesis pathways and the interpretation of core miRNA pathway mutants. *Molecular Cell* 43(6): 892–903.

3 Yang JS, Maurin T, Robine N, Rasmussen KD, Jeffrey KL, Chandwani R, et al. (2010) Conserved vertebrate mir-451 provides a platform for Dicer-independent, Ago2-mediated microRNA biogenesis. *Proceedings of the National Academy of Sciences USA* 107(34): 15163–15168.

4 Tay Y, Zhang J, Thomson AM, Lim B, Rigoutsos I. (2008) MicroRNAs to Nanog, Oct4 and Sox2 coding regions modulate embryonic stem cell differentiation. *Nature* 455(7216): 1124–1128.

5 Lytle JR, Yario TA, Steitz JA. (2007) Target mRNAs are repressed as efficiently by microRNA-binding sites in the 5′ UTR as in the 3′ UTR. *Proceedings of the National Academy of Sciences USA* 104(23): 9667–9672.

6 Orom UA, Nielsen FC, Lund AH. (2008) MicroRNA-10a binds the 5'UTR of ribosomal protein mRNAs and enhances their translation. *Molecular Cell* 30(4): 460–471.

7 Shin C, Nam JW, Farh KK, Chiang HR, Shkumatava A, Bartel DP. (2010) Expanding the microRNA targeting code: functional sites with centered pairing. *Molecular Cell* 38(6): 789–802.

8 Place RF, Li LC, Pookot D, Noonan EJ, Dahiya R. (2008) MicroRNA-373 induces expression of genes with

complementary promoter sequences. *Proceedings of the National Academy of Sciences USA* 105(5): 1608–1613.

9 Eiring AM, Harb JG, Neviani P, Garton C, Oaks JJ, Spizzo R, et al. (2010) miR-328 functions as an RNA decoy to modulate hnRNP E2 regulation of mRNA translation in leukemic blasts. *Cell* 140(5): 652–665.

10 Wei W, Ba Z, Gao M, Wu Y, Ma Y, Amiard S, et al. (2012) A role for small RNAs in DNA double-strand break repair. *Cell* 149(1): 101–112.

11 Ling H, Zhang W, Calin GA. (2011) Principles of microRNA involvement in human cancers. *Chinese Journal of Cancer* 30(11): 739–748.

12 Fabbri M, Bottoni A, Shimizu M, Spizzo R, Nicoloso MS, Rossi S, et al. (2011) Association of a microRNA/TP53 feedback circuitry with pathogenesis and outcome of B-cell chronic lymphocytic leukemia. *Journal of the American Medical Association* 305(1): 59–67.

13 Calin GA, Dumitru CD, Shimizu M, Bichi R, Zupo S, Noch E, et al. (2002) Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proceedings of the National Academy of Sciences USA* 99(24): 15524–15529.

14 Cimmino A, Calin GA, Fabbri M, Iorio MV, Ferracin M, Shimizu M, et al. (2005) miR-15 and miR-16 induce apoptosis by targeting BCL2. *Proceedings of the National Academy of Sciences USA* 102(39): 13944–13949.

15 Calin GA, Cimmino A, Fabbri M, Ferracin M, Wojcik SE, Shimizu M, et al. (2008) MiR-15a and miR-16–1 cluster functions in human leukemia. *Proceedings of the National Academy of Sciences USA* 105(13): 5166–5171.

16 Raveche ES, Salerno E, Scaglione BJ, Manohar V, Abbasi F, Lin YC, et al. (2007) Abnormal microRNA-16 locus with synteny to human 13q14 linked to CLL in NZB mice. *Blood* 109(12): 5079–5086.

17 Calin GA, Sevignani C, Dumitru CD, Hyslop T, Noch E, Yendamuri S, et al. (2004) Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. *Proceedings of the National Academy of Sciences USA* 101(9): 2999–3004.

18 Calin GA, Ferracin M, Cimmino A, Di Leva G, Shimizu M, Wojcik SE, et al. (2005) A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. *New England Journal of Medicine* 353(17): 1793–1801.

19 Garzon R, Garofalo M, Martelli MP, Briesewitz R, Wang L, Fernandez-Cymering C, et al. (2008) Distinctive microRNA signature of acute myeloid leukemia bearing cytoplasmic mutated nucleophosmin. *Proceedings of the National Academy of Sciences USA* 105(10): 3945–3950.

20 Schetter AJ, Leung SY, Sohn JJ, Zanetti KA, Bowman ED, Yanaihara N, et al. (2008) MicroRNA expression profiles associated with prognosis and therapeutic outcome in colon adenocarcinoma. *Journal of the American Medical Association* 299(4): 425–436.

21 Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, et al. (2005) MicroRNA expression profiles classify human cancers. *Nature* 435(7043): 834–838.

22 Medina PP, Nolde M, Slack FJ. (2010) OncomiR addiction in an in vivo model of microRNA-21-induced pre-B-cell lymphoma. *Nature* 467(7311): 86–90.

23 Hatley ME, Patrick DM, Garcia MR, Richardson JA, Bassel-Duby R, van Rooij E, et al. (2010) Modulation of K-Ras-dependent lung tumorigenesis by MicroRNA-21. *Cancer Cell* 18(3): 282–293.

24 Asangani IA, Rasheed SA, Nikolova DA, Leupold JH, Colburn NH, Post S, et al. (2008) MicroRNA-21 (miR-21) post-transcriptionally downregulates tumor suppressor Pdcd4 and stimulates invasion, intravasation and metastasis in colorectal cancer. *Oncogene* 27(15): 2128–2136.

25 Olive V, Jiang I, He L. (2010) mir-17-92, a cluster of miRNAs in the midst of the cancer network. *International Journal of Biochemistry and Cell Biology* 42(8): 1348–1354.

26 Mu P, Han YC, Betel D, Yao E, Squatrito M, Ogrodowski P, et al. (2009) Genetic dissection of the miR-17 ~ 92 cluster of microRNAs in Myc-induced B-cell lymphomas. *Genes and Development* 23(24): 2806–2811.

27 He L, Thomson JM, Hemann MT, Hernando-Monge E, Mu D, Goodson S, et al. (2005) A microRNA polycistron as a potential human oncogene. *Nature* 435(7043): 828–833.

28 Costinean S, Zanesi N, Pekarsky Y, Tili E, Volinia S, Heerema N, et al. (2006) Pre-B cell proliferation and lymphoblastic leukemia/high-grade lymphoma in E(mu)-miR155 transgenic mice. *Proceedings of the National Academy of Sciences USA* 103(18): 7024–7029.

29 Valeri N, Gasparini P, Fabbri M, Braconi C, Veronese A, Lovat F, et al. (2010) Modulation of mismatch repair and genomic stability by miR-155. *Proceedings of the National Academy of Sciences USA* 107(15): 6982–6987.

30 Sandhu SK, Volinia S, Costinean S, Galasso M, Neinast R, Santhanam R, et al. (2012) miR-155 targets histone deacetylase 4 (HDAC4) and impairs transcriptional activity of B-cell lymphoma 6 (BCL6) in the Emu-miR-155 transgenic mouse model. *Proceedings of the National Academy of Sciences USA* 109(49): 20047–52.

31 Hermeking H. (2010) The miR-34 family in cancer and apoptosis. *Cell Death and Differentiation* 17(2): 193–199.

32 Chang TC, Wentzel EA, Kent OA, Ramachandran K, Mullendore M, Lee KH, et al. (2007) Transactivation of miR-34a by p53 broadly influences gene expression and promotes apoptosis. *Molecular Cell* 26(5): 745–752.

33 Christoffersen NR, Shalgi R, Frankel LB, Leucci E, Lees M, Klausen M, et al. (2010) p53-independent upregulation of miR-34a during oncogene-induced senescence represses MYC. *Cell Death and Differentiation* 17(2): 236–245.

34 Liu C, Kelnar K, Liu B, Chen X, Calhoun-Davis T, Li H, et al. (2011) The microRNA miR-34a inhibits prostate cancer stem cells and metastasis by directly repressing CD44. *Nature Medicine* 17(2): 211–215.

35 Chang CJ, Chao CH, Xia W, Yang JY, Xiong Y, Li CW, et al. (2011) p53 regulates epithelial-mesenchymal transition and stem cell properties through modulating miRNAs. *Nature Cell Biology* 13(3): 317–323.

36 Takamizawa J, Konishi H, Yanagisawa K, Tomida S, Osada H, Endoh H, et al. (2004) Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. *Cancer Research* 64(11): 3753–3756.

37 Esquela-Kerscher A, Trang P, Wiggins JF, Patrawala L, Cheng A, Ford L, et al. (2008) The let-7 microRNA reduces tumor growth in mouse models of lung cancer. *Cell Cycle* 7(6): 759–764.

38 Ma L, Young J, Prabhala H, Pan E, Mestdagh P, Muth D, et al. (2010) miR-9, a MYC/MYCN-activated microRNA, regulates E-cadherin and cancer metastasis. *Nature Cell Biology* 12(3): 247–256.

39 Ma L, Teruya-Feldstein J, Weinberg RA. (2007) Tumour invasion and metastasis initiated by microRNA-10b in breast cancer. *Nature* 449(7163): 682–688.

40 Valastyan S, Reinhardt F, Benaich N, Calogrias D, Szasz AM, Wang ZC, et al. (2009) A pleiotropically acting microRNA, miR-31, inhibits breast cancer metastasis. *Cell* 137(6): 1032–1046.

41 Korpal M, Lee ES, Hu G, Kang Y. (2008) The miR-200 family inhibits epithelial-mesenchymal transition and cancer cell migration by direct targeting of E-cadherin transcriptional repressors ZEB1 and ZEB2. *Journal of Biological Chemistry* 283(22): 14910–14914.

42 Korpal M, Ell BJ, Buffa FM, Ibrahim T, Blanco MA, Celia-Terrassa T, et al. (2011) Direct targeting of Sec23a by miR-200s influences cancer cell secretome and promotes metastatic colonization. *Nature Medicine* 17(9): 1101–1108.

43 Fabbri M, Garzon R, Cimmino A, Liu Z, Zanesi N, Callegari E, et al. (2007) MicroRNA-29 family reverts aberrant methylation in lung cancer by targeting DNA methyltransferases 3A and 3B. *Proceedings of the National Academy of Sciences USA* 104(40): 15805–10.

44 Santanam U, Zanesi N, Efanov A, Costinean S, Palamarchuk A, Hagan JP, et al. (2010) Chronic lymphocytic leukemia modeled in mouse by targeted miR-29 expression. *Proceedings of the National Academy of Sciences USA* 107(27): 12210–12215.

45 Gebeshuber CA, Zatloukal K, Martinez J. (2009) miR-29a suppresses tristetraprolin, which is a regulator of epithelial polarity and metastasis. *EMBO Reports* 10(4): 400–405.

46 Felli N, Fontana L, Pelosi E, Botta R, Bonci D, Facchiano F, et al. (2005) MicroRNAs 221 and 222 inhibit normal erythropoiesis and erythroleukemic cell growth via kit receptor down-modulation. *Proceedings of the National Academy of Sciences USA* 102(50): 18081–18086.

47 Pineau P, Volinia S, McJunkin K, Marchio A, Battiston C, Terris B, et al. (2010) miR-221 overexpression contributes to liver tumorigenesis. *Proceedings of the National Academy of Sciences USA* 107(1): 264–269.

48 Almeida MI, Nicoloso MS, Zeng L, Ivan C, Spizzo R, Gafa R, et al. (2012) Strand-specific miR-28–5p and miR-28-3p have distinct effects in colorectal cancer cells. *Gastroenterology* 142(4): 886–96 e9.

49 Merritt WM, Lin YG, Han LY, Kamat AA, Spannuth WA, Schmandt R, et al. (2008) Dicer, Drosha, and outcomes in patients with ovarian cancer. *New England Journal of Medicine* 359(25): 2641–2650.

50 Melo SA, Moutinho C, Ropero S, Calin GA, Rossi S, Spizzo R, et al. (2010) A genetic defect in exportin-5 traps precursor microRNAs in the nucleus of cancer cells. *Cancer Cell* 18(4): 303–315.

51 Mayr C, Bartel DP. (2009) Widespread shortening of 3'UTRs by alternative cleavage and polyadenylation activates oncogenes in cancer cells. *Cell* 138(4): 673–684.

52 Sumazin P, Yang X, Chiu HS, Chung WJ, Iyer A, Llobet-Navas D, et al. (2011) An extensive microRNA-mediated network of RNA-RNA interactions regulates established oncogenic pathways in glioblastoma. *Cell* 147(2): 370–381.

53 Karreth FA, Tay Y, Perna D, Ala U, Tan SM, Rust AG, et al. (2011) In vivo identification of tumor- suppressive PTEN ceRNAs in an oncogenic BRAF-induced mouse model of melanoma. *Cell* 147(2): 382–395.

54 Tay Y, Kats L, Salmena L, Weiss D, Tan SM, Ala U, et al. (2011) Coding-independent regulation of the tumor suppressor PTEN by competing endogenous mRNAs. *Cell* 147(2): 344–357.

55 Small EM, Frost RJ, Olson EN. MicroRNAs add a new dimension to cardiovascular disease. *Circulation* (2010) 121(8): 1022–1032.

56 Taganov KD, Boldin MP, Chang KJ, Baltimore D. (2006) NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. *Proceedings of the National Academy of Sciences USA* 103(33): 12481–12486.

57 Chan SY, Loscalzo J. (2010) MicroRNA-210: a unique and pleiotropic hypoxamir. *Cell Cycle* 9(6): 1072–1083.

58 Poy MN, Eliasson L, Krutzfeldt J, Kuwajima S, Ma X, Macdonald PE, et al. (2004) A pancreatic islet-specific microRNA regulates insulin secretion. *Nature* 432(7014): 226–230.

59 Wang WX, Rajeev BW, Stromberg AJ, Ren N, Tang G, Huang Q, et al. (2008) The expression of microRNA miR-107 decreases early in Alzheimer's disease and may accelerate disease progression through regulation of beta-site amyloid precursor protein-cleaving enzyme 1. *Journal of Neuroscience* 28(5): 1213–1223.

60 Jopling CL, Yi M, Lancaster AM, Lemon SM, Sarnow P. (2005) Modulation of hepatitis C virus RNA abundance by a liver-specific MicroRNA. *Science* 309(5740): 1577–1581.

61 Li QJ, Chau J, Ebert PJ, Sylvester G, Min H, Liu G, et al. (2007) miR-181a is an intrinsic modulator of T cell sensitivity and selection. *Cell* 129(1): 147–161.

62 Saito Y, Liang G, Egger G, Friedman JM, Chuang JC, Coetzee GA, et al. (2006) Specific activation of microRNA-127 with downregulation of the proto-oncogene BCL6 by chromatin-modifying drugs in human cancer cells. *Cancer Cell* 9(6): 435–443.

63 Lujambio A, Ropero S, Ballestar E, Fraga MF, Cerrato C, Setien F, et al. (2007) Genetic unmasking of an epigenetically silenced microRNA in human cancer cells. *Cancer Research* 67(4): 1424–1429.

64 Shan G, Li Y, Zhang J, Li W, Szulwach KE, Duan R, et al. (2008) A small molecule enhances RNA interference and promotes microRNA processing. *Nature Biotechnology* 26(8): 933–940.

65 Tivnan A, Orr WS, Gubala V, Nooney R, Williams DE, McDonagh C, et al. (2012) Inhibition of neuroblastoma tumor growth by targeted delivery of microRNA-34a using anti-disialoganglioside GD2 coated nanoparticles. *PLoS One* 7(5): e38129.

66 Trang P, Wiggins JF, Daige CL, Cho C, Omotola M, Brown D, et al. (2011) Systemic delivery of tumor suppressor microRNA mimics using a neutral lipid emulsion inhibits lung tumors in mice. *Molecular Therapy* 19(6): 1116–1122.

67 Tazawa H, Tsuchiya N, Izumiya M, Nakagama H. (2007) Tumor-suppressive miR-34a induces senescence-like growth arrest through modulation of the E2F pathway in human colon cancer cells. *Proceedings of the National Academy of Sciences USA* 104(39): 15472–15477.

68 Michelfelder S, Trepel M. (2009) Adeno-associated viral vectors and their redirection to cell-type specific receptors. *Advances in Genetics* 67: 29–60.

69 Kota J, Chivukula RR, O'Donnell KA, Wentzel EA, Montgomery CL, Hwang HW, et al. (2009) Therapeutic microRNA delivery suppresses tumorigenesis in a murine liver cancer model. *Cell* 137(6): 1005–1017.

70 Ikeda S, He A, Kong SW, Lu J, Bejar R, Bodyak N, et al. (2009) MicroRNA-1 negatively regulates expression of the hypertrophy-associated calmodulin and Mef2a genes. *Molecular and Cellular Biology* 29(8): 2193–2204.

71 Chen L, Zheng J, Zhang Y, Yang L, Wang J, Ni J, et al. (2011) Tumor-specific expression of microRNA-26a suppresses human hepatocellular carcinoma growth via cyclin-dependent and -independent pathways. *Molecular Therapy* 19(8): 1521–1528.

72 Garzon R, Marcucci G, Croce CM. (2010) Targeting microRNAs in cancer: rationale, strategies and challenges. *Nature Reviews Drug Discovery* 9(10): 775–789.

73 Weiler J, Hunziker J, Hall J. (2006) Anti-miRNA oligonucleotides (AMOs): ammunition to target miRNAs implicated in human disease? *Gene Therapy* 13(6): 496–502.

74 Elmen J, Lindow M, Silahtaroglu A, Bak M, Christensen M, Lind-Thomsen A, et al. (2008) Antagonism of microRNA-122 in mice by systemically administered LNA-antimiR leads to up-regulation of a large set of predicted target mRNAs in the liver. *Nucleic Acids Research* 36(4): 1153–1162.

75 Najafi-Shoushtari SH, Kristo F, Li Y, Shioda T, Cohen DE, Gerszten RE, et al. (2010) MicroRNA-33 and the SREBP host genes cooperate to control cholesterol homeostasis. *Science* 328(5985): 1566–1569.

76 Zhang Y, Roccaro AM, Rombaoa C, Flores L, Obad S, Fernandes SM, et al. (2012) LNA-mediated anti-miR-155 silencing in low-grade B-cell lymphomas. *Blood* 120(8): 1678–1686.

77 Lindow M, Kauppinen S. (2012) Discovering the first microRNA-targeted drug. *Journal of Cell Biology* 199(3): 407–412.

78 Lanford RE, Hildebrandt-Eriksen ES, Petri A, Persson R, Lindow M, Munk ME, et al. (2010) Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection. *Science* 327(5962): 198–201.

79 Krutzfeldt J, Rajewsky N, Braich R, Rajeev KG, Tuschl T, Manoharan M, et al. (2005) Silencing of microRNAs in vivo with 'antagomirs'. *Nature* 438(7068): 685–689.

80 Ma L, Reinhardt F, Pan E, Soutschek J, Bhat B, Marcusson EG, et al. (2010) Therapeutic silencing of miR-10b inhibits metastasis in a mouse mammary tumor model. *Nature Biotechnology* 28(4): 341–347.

81 Care A, Catalucci D, Felicetti F, Bonci D, Addario A, Gallo P, et al. (2007) MicroRNA-133 controls cardiac hypertrophy. *Nature Medicine* 13(5): 613–618.

82 Du C, Liu C, Kang J, Zhao G, Ye Z, Huang S, et al. (2009) MicroRNA miR-326 regulates TH-17 differentiation and is associated with the pathogenesis of multiple sclerosis. *Nature Immunology* 10(12): 1252–1259.

83 Kluiver J, Gibcus JH, Hettinga C, Adema A, Richter MK, Halsema N, et al. (2012) Rapid generation of microRNA sponges for microRNA inhibition. *PLoS One* 7(1): e29275.

84 Sayed D, Rane S, Lypowy J, He M, Chen IY, Vashistha H, et al. (2008) MicroRNA-21 targets Sprouty2 and promotes cellular outgrowths. *Molecular Biology of the Cell* 19(8): 3272–3282.

85 Frankel SR. (2003) Oblimersen sodium (G3139 Bcl-2 antisense oligonucleotide) therapy in Waldenstrom's

macroglobulinemia: a targeted approach to enhance apoptosis. *Seminars in Oncology* 30(2): 300–304.

86 Lujambio A, Lowe SW. (2012) The microcosmos of cancer. *Nature* 482(7385): 347–355.

87 Kim SJ, Oh JS, Shin JY, Lee KD, Sung KW, Nam SJ, et al. (2011) Development of microRNA-145 for therapeutic application in breast cancer. *Journal of Controlled Release* 155(3): 427–434.

88 Kotani A, Ha D, Hsieh J, Rao PK, Schotte D, den Boer ML, et al. (2009) miR-128b is a potent glucocorticoid sensitizer in MLL-AF4 acute lymphocytic leukemia cells and exerts cooperative effects with miR-221. *Blood* 114(19): 4169–4178.

89 Bader AG. (2012) miR-34 - a microRNA replacement therapy is headed to the clinic. *Frontiers in Genetics* 2(3): 120.

PART III

Principles of Clinical Trials in Gene Therapy

7

CHAPTER 7

Regulatory Issues for Manufacturers of Viral Vectors and Vector-transduced Cells for Phase I/II Trials

Adrian P. Gee and Zhuyong Mei
Baylor College of Medicine, Houston, TX, USA

Introduction

In the USA responsibility for the regulation of emerging clinical therapies rests with the Food and Drug Administration (FDA). As a part of its surveillance activities the FDA will review new therapies to determine whether they fall under existing regulations or may require the development of new laws. This has been true for gene therapies that either involve the direct administration of viral vectors or use gene-modified cells as effectors of the treatment. This chapter focuses on the regulations that apply to the manufacture of viral vectors and vector-transduced therapeutic cells and the impact these have on operations in an academic facility.

Development of Good Manufacturing Practices

The primary regulations that have an impact on manufacturers of therapeutic products are the current Good Manufacturing Practice (cGMP) laws. The history of the development of cGMP illustrates how the FDA institutes and modifies regulations [1]. In 1902 US Congress passed the Biologics Control Act in response primarily to the death of 12 children who died from live tetanus bacilli present in a preparation of diphtheria antitoxin. This legislation required inspection of manufacturers and testing of their products for strength and purity. Most of us are also familiar with the potentially toxic or addictive miracle cures and the adulteration of foodstuffs with foreign materials that were pedaled in the nineteenth century. In 1906 Congress passed the Pure Food and Drug Act which forbade the selling of adulterated food and required truthful labeling. Overseen initially by the Chemistry Bureau at the Department of Agriculture, the FDA was eventually established to assume this responsibility.

In 1933 the FDA mounted an exhibition dubbed "America's Chamber of Horrors" which illustrated deficiencies in the 1906 Act. These included an eyebrow and eyelash dye, "Lash Lure," that caused blindness, "Raditor," a radium-containing tonic, and "Banbar," a cure for diabetes. Two years later a toxic solvent (similar to antifreeze) was an ingredient in "elixir of sulfanilamide" that directly lead to the death of more than 100 people. Congress, in response, passed the Federal Food, Drug and Cosmetic Act in 1938. This required demonstration of safety by the manufacturer prior to selling the product. In 1941,

Cancer Gene Therapy by Viral and Non-viral Vectors, First Edition. Edited by Malcolm K. Brenner and Mien-Chie Hung.

however, a similar tragedy occurred when nearly 300 people were poisoned by a sulfa drug that was tainted with phenobarbital. This led to the introduction by the FDA of improved manufacturing and quality control requirements, which ultimately evolved into Good Manufacturing Practice (GMP) regulations [1]. These were made final in 1978 with publication of GMP for drugs [Title 21 Code of Federal Regulations (CFR) Parts 210 and 211; 21CFR210, 21CFR211] [2] and for medical devices (21CFR820). Additional cGMPs are available for blood and blood-derived products (21CFR606), biological products (21CFR600), food for human consumption (21CFR110), and dietary supplements (21CFR111). The FDA has determined that vectors and transduced cells are classified as pharmaceuticals and that 21CFR210 and 21CFR211 apply.

Similar GMP regulations have been developed in Europe, Canada, Australia, and many other countries. In an attempt to standardize these, the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, or ICH (www.ich.org/), was established in 1990 in Brussels. This has lead to the development of joint international guidances and recommendations that will facilitate transfer of finished products between countries.

cGMP Requirements

An understanding of cGMP regulations is essential for any academic institution contemplating manufacturing viral vectors and/or vector-transduced cells for clinical applications. Most such institutions will be involved in only phase I and early phase II clinical trials, rather than progressing to a phase III trial designed for licensure of the product. Under such circumstances full GMP compliance during manufacturing is not expected by the FDA. The regulations are applied on a sliding scale or continuum model in which increasingly stringent compliance is required as products progress from phase I to the initiation of phase III trials. This does not appear to be the case in the countries of the European Union. While useful, this concept has led to some confusion, in that it was not clear what level of compliance is expected at different points on the continuum. For clarification, in July 2008 the FDA published a guidance document, *cGMP for Phase I Investigational Drugs* [3], that describes which components of cGMP are to be in place for a phase I study. A common misconception is that cGMP regulations solely address design of the manufacturing facility and mandate that a clean room must be used. Little could be further from the truth. cGMP regulations are designed to produce a controlled and auditable system to ensure the preparation of a safe (and effective) therapeutic product. Although facility requirements are addressed, they form only a small part of the regulations.

The prime principles of cGMP are as follows.

- Manufacturing procedures must be defined and controlled.
- Critical processes are validated to ensure consistency and compliance with specifications [4].
- Changes to procedures are evaluated and validated to ensure no adverse effects on product quality.
- Procedures (Standard Operating Procedures, SOPs) are controlled, clear, and in unambiguous language.
- Staff must be trained to carry out and document procedures.
- Records are made concurrently during manufacturing to demonstrate that all of the steps in the SOP were taken.
- Any deviations from SOPs must be documented, investigated, and the efficacy of corrective actions confirmed.
- Manufacturing records must permit tracing and tracking of the complete history of a product batch, and must be retained and accessible.
- There must be procedures for recall of the product and to deal with complaints.

How to comply with these principles is covered in the following sections.

cGMP Compliance

Organization and Personnel

A primary requirement here is that there shall be a quality control unit (QCU) with the responsibility to approve or reject materials, containers, closures, packaging, labeling, etc., and who have the authority to review manufacturing records. The QCU must have adequate laboratory space for testing and other activities and access to in-process samples. They have the responsibility to approve or reject procedure and specifications that relate to product identity,

strength, quality, and purity. The QCU shall also have written policies and procedures.

For small academic centers it may prove difficult to employ staff solely responsible for quality control. In that case options include using the institutional QCU, educating another staff member on the job responsibilities so that they can perform these activities when required, or as a last resort, using manufacturing staff, in which case there should be a reasonable gap in time between manufacturing and quality control review, and the staff member should not, if at all possible, review his or her own manufacturing activities.

Staff Qualifications and Responsibilities

There must be an adequate number of staff to perform the required manufacturing procedures. Staff members must have education, training, and experience to allow them to perform their assigned activities. Their training must also include education on GMP (performed by qualified staff) on a regular and continuing basis. The training must be at a level that ensures that the product meets the required safety, identity, strength, quality, and purity requirements.

Staff must have good health and sanitation habits and must wear clean clothing and protective apparel suitable for the work that they perform. They must not enter areas where they are not authorized and any staff member with evidence of illness that may adversely affect the product must be excluded from direct contact with the components.

In practice, these regulations require a staff training program that is fully documented and conducted by qualified individuals. This usually involves the trainee reading the appropriate SOP, observing the procedure several times, and performing the same procedure under supervision several times. GMP training can consist of initial documented review of all SOPs that pertain to facility operations (gowning, equipment management, accessioning of components, labeling, etc.), followed in subsequent years by a review presentation and/or GMP quiz. Gowning policies are facility-dependent and may range from simple clean laboratory coats to full sterile gowning. The policy must be written and staff trained to gown correctly. Restricted access can be achieved by securing areas against unauthorized entry using badge or barcode readers.

Buildings and Facilities

Facilities must be designed to be of suitable size and location to facilitate cleaning, maintenance, and proper operation. They must have adequate space to accommodate all operations and be designed to minimize contamination and cross-contamination. The areas for specific operations must be defined and should include areas for receipt of materials, storage of rejected components, released components, in-process material, packaging and labeling operations, quarantine storage prior to and after release, control and laboratory operations, and aseptic technique. Aseptic areas should include hard working surfaces that are easily cleanable, for example stainless steel or epoxy, controls for temperature and humidity, an air supply filtered through high-efficiency particulate air (HEPA) filters under positive pressure, an environmental monitoring system, a cleaning and disinfection system for both the room and equipment, and a system maintaining equipment used to produce aseptic conditions. There are additional requirements for lighting, ventilation, plumbing, hand washing and toilet facilities and sewage, sanitation, and pest control that are standard for most modern buildings.

The reader will see that the requirements lack written specifications and in many cases the FDA will look to the individual facility to justify the choices that they have made, preferably supported by data. Size and construction details are based upon the operations to be performed and should be driven by anticipated capacity and the requirement to divide the space into defined areas. Finishes range from fixed epoxy countertops to full mobile cabinetry with stainless steel working surfaces. The choice may be dictated by product type and budget. Wall coatings should be epoxy painted and seamless vinyl floors are acceptable. Temperature and humidity controls are now standard for all buildings. The requirement for HEPA-filtered air should be discussed with the FDA. In contrast to traditional pharmaceutical manufacturing, which is performed by "open" operations in a clean room, vector and transduced-cell manufacturing is traditionally done inside a Class 100 (where 100 refers to ≤100 particles of ≥0.5 μm in size/cubic foot of air. Equivalent to International Standards Organization (ISO) Class 5) biological safety cabinet [BSC], and should use closed or functionally closed systems wherever

possible. This considerably reduces the risk of contamination from the environment. Nevertheless, larger academic GMP facilities have opted to manufacture within a BSC placed in a clean room. In the USA, in most cases the clean room will be rated at Class 10,000 (ISO 7), whereas in Europe a Class 1000 (ISO 6) room would be required.

Plans for new facilities should be shown to the facilities staff at the FDA's Center for Biologics Evaluation and Research (CBER) for review before construction begins. This will obviate any potentially expensive downstream changes. The FDA is primarily concerned with contamination, cross-contamination, containment, and flow paths for staff, supplies, and waste in the facility plan. There are many ways to address these concerns and none is officially sanctioned. The applicants must be prepared to discuss the rationale for the approach that they have selected, and the possible alternatives if it is not acceptable.

Cleaning and maintenance procedures are important and the FDA usually expects to see validation of the selected disinfectants against likely contaminating organisms. Cleaning and disinfectant agents should be rotated monthly to avoid development of resistance. Cleaning staff must have documented training and there must be written records of cleaning of rooms and equipment. There should also be written SOPs for pest control.

Equipment

The regulations require that equipment must be of appropriate design, size, and location for the intended use and for cleaning and maintenance. It must be constructed of material that is nonreactive with components in the case of direct contact and must not be a potential source of cross-contamination. There must be detailed cleaning procedures that will not affect the product or components, methods to prevent cleaned equipment from contamination, and a documented examination of the equipment for cleanliness immediately before use. The procedures should also indicate the cleaning schedule, the materials used in cleaning, and who is assigned to perform the cleaning. Written records of cleaning, maintenance, and inspection must be maintained.

Suitability of equipment can be demonstrated by performing a qualification procedure consisting of five components: design qualification, installation qualification, operational qualification, performance qualification, and requalification. The design qualification sets the customer's specification requirements, which should be in alignment with regulatory requirements. The installation qualification can be designed so that the equipment is located according to the regulations to facilitate servicing and cleaning. The operational qualification demonstrates that the equipment operates as per the manufacturer's specifications and the performance qualification confirms that it meets the specifications established by the customer. Requalification is performed whenever the equipment is affected in a substantial way, such as by moving, maintenance, etc. Equipment records should document cleaning before and after use and the identity of the product that was processed by that equipment. The normal procedure is to establish an equipment log in which all of this information is stored and which is also used to document service, maintenance, repairs, and calibration. A system for equipment identification, for example a serial number or barcode, must be implemented so that the specific pieces used during manufacture of a particular lot of product can be recorded.

Component Receipt, Testing, and Storage

A written procedure should be available for determining the identity, storage, handling, testing, and approval process of incoming components. Components should be examined visually for damage and contamination and then stored in quarantine until tested and released.

For materials used in the manufacturing of products for phase I studies this is usually addressed in the Investigational New Drug (IND) application. The FDA is provided with a list of materials that will be used, usually together with the certificates of analysis. The applicant should select materials of clinical/pharmaceutical grade wherever possible. If not available, the purest grade should be selected and, where appropriate, additional testing performed. The FDA will respond as to the suitability of the reagents, and, if approved, these should be used throughout manufacturing. Alternatively they may ask for substitution of certain items, or that additional testing be performed and the results submitted. It is important to list all materials and

reagents that will come into contact with the product during manufacturing and storing. Once approved, the usual procedure will be to use these items exclusively and to have on file the Certificate of Analysis (CofA) for each lot used. Upon receipt the items should be examined for contamination and damage, and checked for expiration date. The CofA should be checked against that submitted in the IND application to ensure that the specifications have not changed. Items that have a pending CofA or require additional testing before use should be clearly marked as being "in quarantine" and physically segregated from released materials. The procedures used for ordering, receipt, quarantine, and release of materials should be clearly documented in an SOP. Storage should always be off the floor and more than 45 cm from the ceiling and can be maximized by high-density shelving that uses a track system.

Facilities should determine whether it is sufficient to rely solely on a CofA for release or whether additional testing should be performed for critical reagents. If this is the case, representative samples of the component should be tested according to a validated testing procedure (preferably in compliance with FDA recommendations) and the results submitted to Quality Assurance for review and approval or disapproval. Items failing testing must be segregated from incoming, quarantined, and released components and destroyed or returned to the manufacturer.

SOPs and Deviations

Each facility must have written SOPs that cover all aspects of their operations. These must be reviewed and released by the QCU. Any deviations from these procedures must be documented, reviewed, and remedial actions taken to prevent future occurrence. The efficacy of these actions should be reviewed and documented by the QCU.

Development of SOPs is an art rather than a science. For a new facility the task appears daunting as each new SOP inevitably results in the need to write two more. An excellent guide to SOP writing is available from the Environmental Protection Agency [5]. Styles differ, but the traditional format includes sections devoted to Purpose, Scope, Definitions, Materials and Equipment, Procedure, Expected Results, and References. The aim is to provide sufficient detail so that a person with the appropriate level of education should be able to use the SOP as a step-by-step instructional manual. It is particularly important to balance specificity and generality. Highly specific procedures with a lot of detail may help in training but are likely to generate many deviations due to failure to follow instructions that are included, but do not allow for the natural variability inherent in the procedure. A good practice, even when manufacturing for phase I studies, is to establish a list of approved components and materials that have been qualified for use in manufacturing the product(s). This facilitates the rapid development of the Materials and Equipment section. It is important to indicate the expected results and provide some troubleshooting advice if these are not achieved. SOPs are usually initially drafted by the Principal Investigator in collaboration with the QCU. They should undergo a formal review and release procedure that is documented on the written copy. Once released, they must be protected from unauthorized changes using a formal document-management procedure. Electronic versions can be provided in a read-only or PDF format. SOPs should be reviewed at least biennially or more frequently if changes are needed. This review must be documented. Worksheets should follow the steps of the SOP closely and should be subject to the same management and review process.

The number of deviations from a procedure is closely related to how well the SOP is written. Deviations are either unplanned (errors, accidents, etc.), or planned (e.g. changes to culture procedures due to variations in cell growth rates). Once an unplanned deviation is detected it must be documented. Worksheets (paper or electronic) are issued by the QCU and completed by the staff member primarily involved in the deviation. These are reviewed by the immediate supervisor for any additional comments and returned to the QCU for review and implementation of a corrective action plan. The efficacy of that plan should be determined and documented.

The release of SOPs should be linked to the staff training system. There should be a formal procedure for training (see above) and this should be implemented each time the procedure is reviewed or changed. Training and retraining of staff must be documented at least annually. The FDA also requires

some form of staff proficiency and competency evaluation. Although formal external proficiency testing programs are available for some disciplines, for example flow cytometry and hematology, this is not true for vector and transduced-cell product manufacturing. An alternative is to document reviewing of the number of variances generated by each staff member annually and to provide a quiz covering the procedures on which he or she is trained.

Packaging and Labeling

There must be formal procedures in place to document the review and approval of packaging and labeling materials before use. Where on-demand label printing is used a template of the specific label should be developed and approved by the customer and the QCU. A predetermined number of labels is then printed and reviewed by the QCU for accuracy, legibility, and alignment. The approved number of labels is issued to manufacturing for use, and any remaining or damaged labels are returned for destruction. All steps in this procedure must be documented. If preprinted labels are used these should be proofed upon receipt and stored securely and in a manner to prevent mix-ups. These are then issued as described above. Copies of final product labels should be submitted to the FDA as part of the Chemistry, Manufacturing, and Control (CMC) section of the IND application. This will ensure that the required language and statements, for example "investigational use only," appear on the labels. If the complete information cannot be accommodated on the label, a larger label can be generated and this becomes part of the information that travels with the product at the time of administration.

The choice of containers is strictly regulated for licensed pharmaceuticals. In the case of products used in phase I studies some flexibility is allowed by the FDA depending on the nature and volume of the product. The container and closure should be clearly described in the CMC so that it can be appropriately reviewed. Containers and closures should be visually examined before and after use. The filling procedure should be validated for a predetermined number of vials. This is normally performed by substituting tissue culture medium or the product vehicle. Representative samples should be tested for sterility and endotoxin levels. This is also true for the actual finish-and-fill operation.

Expiration Dating

Expiration dating is standard and required for drug products; however, the GMP regulations recognize the difficulty in assigning an expiration date to a new investigational product. The expiration date is determined on an ongoing basis by performing stability studies, in which samples of the product are tested (for potency, sterility, container integrity, etc.) at regular predetermined intervals. Each facility should develop formal stability testing procedures for the products that it manufactures. It is advisable to review these with the FDA prior to implementation. If the product requires manipulation before administration (e.g. thawing), the stability of the final formulation should also be evaluated. Stability testing should be initiated as early as possible after finish and fill have been finalized. This will support the expiration date that must be assigned later in the development of the product.

Holding and Distribution

Final products awaiting release by the QCU must be held in quarantine in a manner that would prevent their accidental distribution. Release from quarantine must be documented. Investigational products must be stored under appropriate environmental conditions and their release documented, so that a complete inventory is available at all times.

Laboratory Controls

Scientifically sound and appropriate procedures must be used to assure that in-process samples and final products meet the required standards for identity, quality, potency, and purity. Written procedures for testing should be generated and a testing plan developed and approved. The samples used should be representative of the material and testing devices should have been calibrated. The FDA and other organizations provide multiple guidance and other documents on testing methods applicable to master and working cell banks [6] (Figures 7.1 and 7.2), adenoviral and retroviral vectors, and cell therapy products [7,8] (Figures 7.3 and 7.4). The proposed release tests should be described in the CMC section of the IND application (see also Chapter 8 in this volume). The FDA has written two guidances for writing the CMC section of the IND, one related to transduced cells and the other to vectors [9]. These should be used as a template by CMC authors,

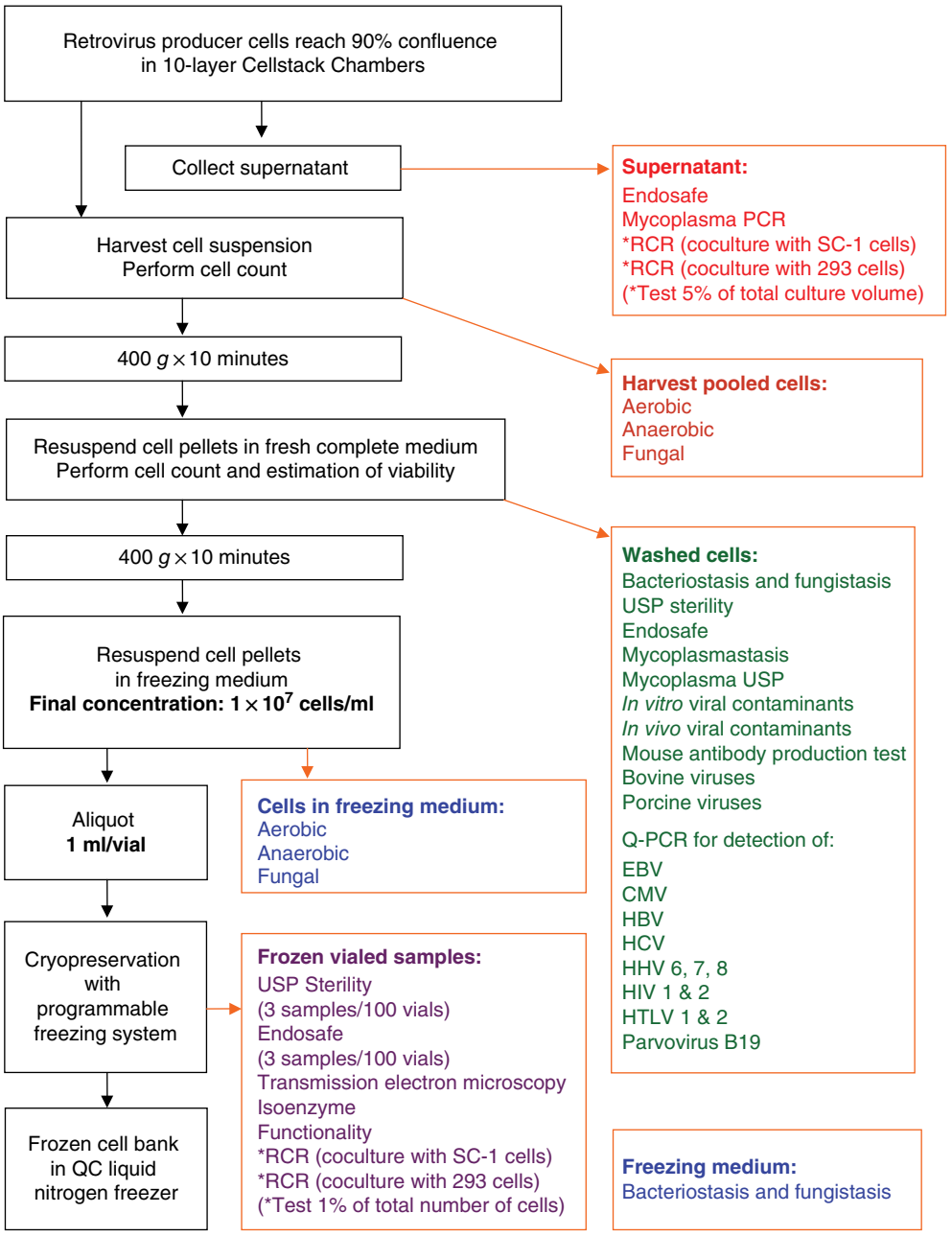


Figure 7.1 Producer line Master Cell Bank production and testing. Manufacturers are cautioned to contact the FDA to determine current testing requirements. QC, Quality Control; RCR, replication-competent retrovirus; USP, *United States' Pharmacopoeia*.

as they describe what information the FDA expects to receive and how it should be formatted. The section on testing indicates the types of tests that should be performed (purity, identity, etc.) and requires information on time of testing and the sensitivity and specificity of the test method. CFR-compliant test methods should be used whenever possible. If another method is preferred its use must be justified, and the FDA may require its formal validation. In the case of testing on the final product there

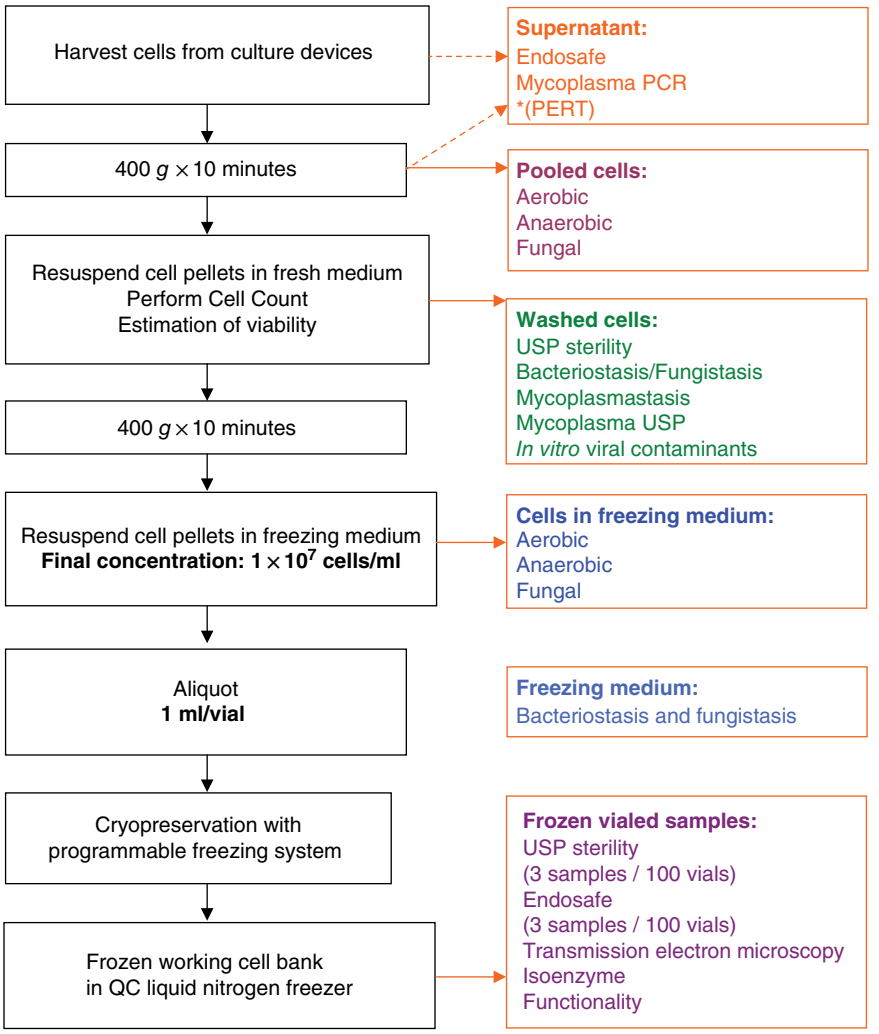


Figure 7.2 Retrovirus producer working cell bank freezing and testing. Manufacturers are cautioned to contact the FDA to determine current testing requirements.* Indicates required for vaccine product. PERT, product-enhanced reverse transcriptase; QC, Quality Control; USP, *United States' Pharmacopoeia*.

should be written release specifications for each test and the product cannot be released without meeting all of the predetermined specifications. This information is normally presented in the form of a written CofA, which details the tests to be performed, the test method to be used (and its sensitivity), the specifications or results that must be achieved for release, and the actual test results. This document is normally generated by the QCU after review of the manufacturing and testing data, and at that point the material can be released for use. Failure to meet a test specification must be addressed. It is not acceptable to retest the product until the desired result is achieved. Instead, a written procedure for out-of-specification results should be written and followed. The FDA has provided a guidance on how to address out-of-specification results and this should be used as the template for the SOP.

Reserve Samples

The FDA requires retention of reserve samples of the product and specifies the amount to be retained and the retention times. It is advisable to archive samples for both stability testing and retesting in the case of any adverse responses, and to retain some material indefinitely.

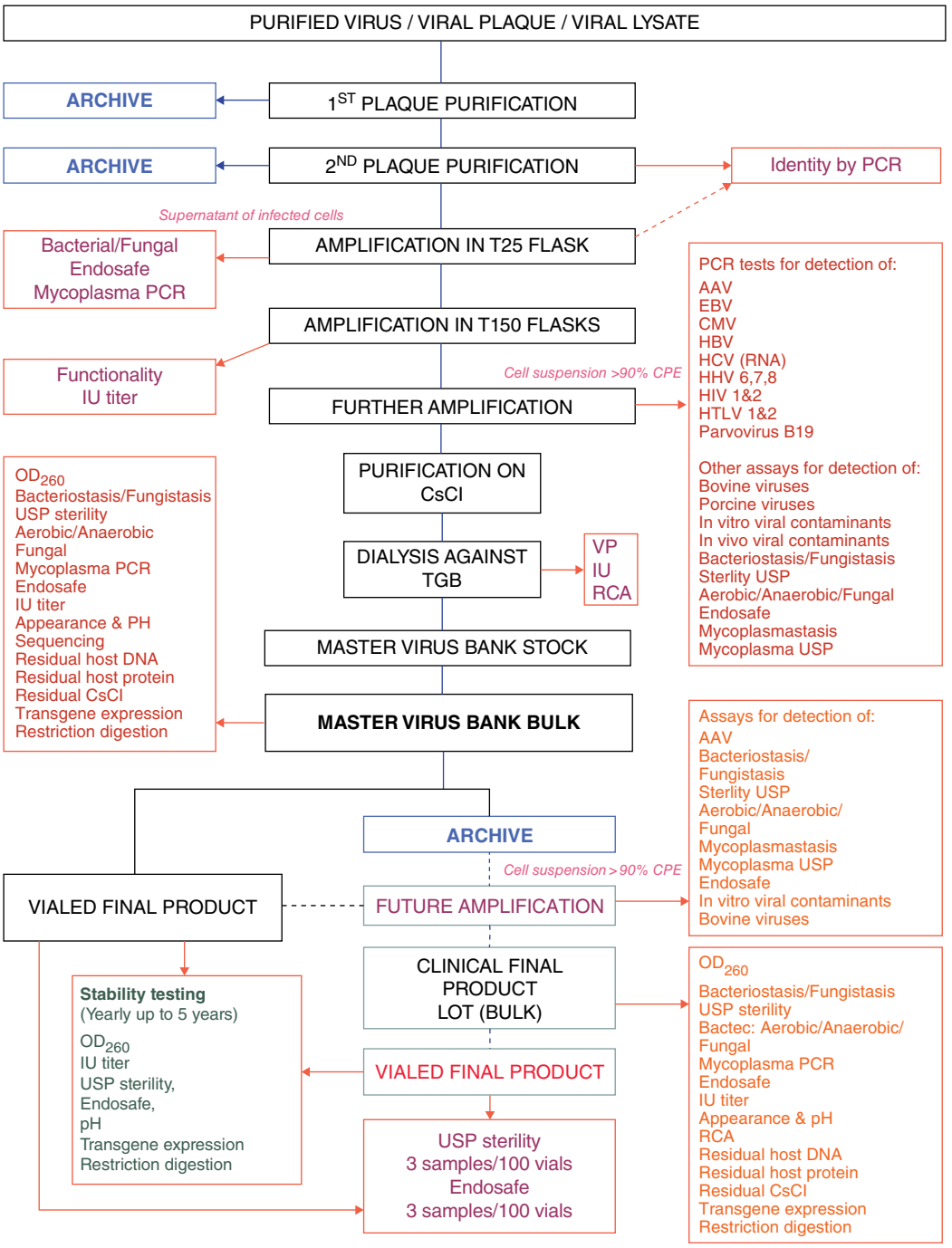
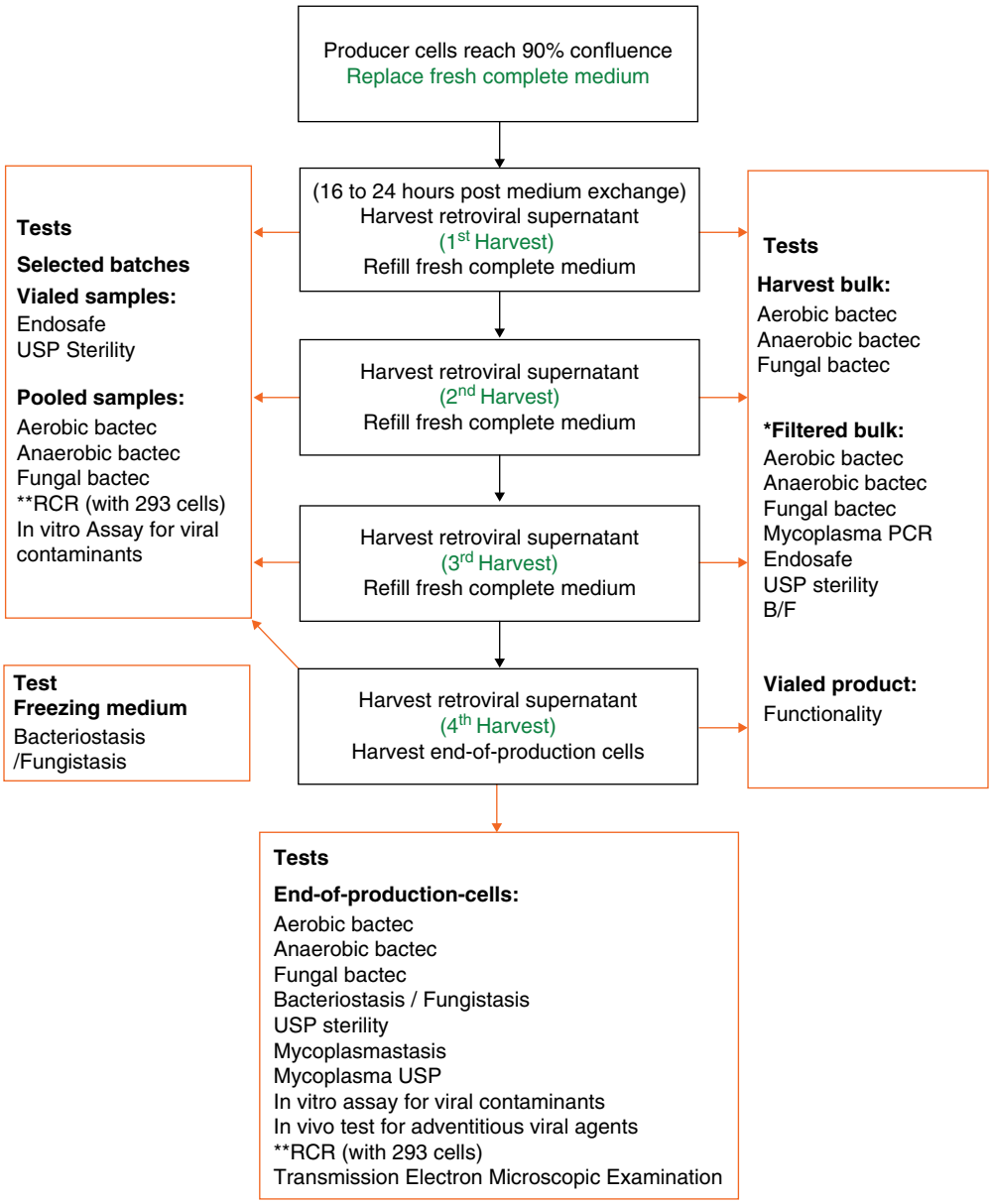


Figure 7.3 Major steps in the manufacture and testing of an adenoviral vector. Manufacturers are cautioned to contact the FDA to determine current testing requirements. In this schema part of the Master Virus Bank has been used as the final clinical product. TGB, Tris/glycerol buffer; USP, *United States' Pharmacopoeia*; VP, viral particles.



*Before vialing, retroviral supernatant is filtered through a filtration assembly.
**RCR samples: 5% of production-run supernatant; 1% or 10^8 cells from the end of production cell suspension.

Figure 7.4 Major steps in the manufacture and testing of a retroviral vector. Manufacturers are cautioned to contact the FDA to determine current testing requirements. RCR, replication-competent retrovirus; USP, *United States' Pharmacopoeia*.

Records and Reports

For licensed drugs with no assigned expiration date, the records must be retained for at least 3 years after distribution of the batch. For pharmaceuticals there are detailed requirements on record formats, retention, and notification of "responsible officials" of the manufacturing facility if investigations are carried out. There must be an annual evaluation of

the quality standards of the product to determine whether any changes need to be made. For academic manufacturing facilities it is advisable to retain all records indefinitely whenever possible. The record format is quite flexible in that electronic, microfiche, or true copies of original documents are all acceptable. Whatever format is used, the records must be easily retrievable and the appropriate equipment available to read them.

The GMP regulations describe in detail the use of master production and control records. These are the approved final procedures and worksheets for manufacturing the product. They constitute the master copy from which the batch product record (BPR) is generated, signed, dated, and checked out to manufacturing staff. Such a formal procedure is not usually required for manufacturing phase I products; however, the required components in the BPR should be incorporated into whatever worksheets are used to document manufacturing and testing. These include documentation of each significant step in production, including manufacturing, processing, packaging, and holding. The information should include dates, equipment used, weights and measures of the components used, sampling performed, results of laboratory tests, details of any deviations, and the identity of persons performing each of the steps. Laboratory testing procedures should be documented similarly.

Distribution Records

Records must be maintained of the distribution of the released product. These should include the amount, lot number, date of distribution, and the name and address of the consignee. For investigational drugs it is important to maintain an accurate inventory of the product to ensure that complete accountability is maintained.

Complaints

There must be in place a procedure to deal with complaints about the product. The procedure must include QCU review of the complaint and determination as to whether it constitutes a serious or unexpected adverse experience that must be reported to the FDA.

Practical Compliance: One Center's Experience

At first reading the GMP regulations are somewhat intimidating; however, as stated previously, full compliance is not expected when manufacturing a product for a phase I clinical trial [3]. The expected level of compliance at phase I is described in FDA guidance [2]. As an illustration, in this section we describe the operation of the GMP facilities at Baylor College of Medicine in Houston, TX, USA.

The Center for Cell and Gene Therapy at Baylor operates two cGMP facilities, one for viral vector manufacturing (the Vector Production Facility, VPF) and one for generation of cellular therapy products (the Cell Processing Facility, CPF). The organization of the space is shown in the floor plan in Figure 7.5. The two facilities differ in design. The CPF consists of manufacturing Class 10,000 (ISO 7) clean rooms leading from a central corridor. The rooms are at positive pressure with respect to the corridor which is at negative pressure with respect to the cell storage area. There are 20–30 air changes per hour (acph) and 50% recirculation of the air. The VPF clean rooms (Figure 7.6) are located between clean and dirty corridors such that Class 10,000 (ISO 7) air moves from the clean corridor, through the manufacturing suite, to the dirty corridor. There are 60 acph and the air is 100% exhausted to the outside. All manufacturing is performed in externally ducted Class 100 (ISO 5) BSC. The choice to use clean rooms was made as an additional safety barrier between the BSCs and the outside environment. Cell therapy products are currently manufactured in small batches that are usually designated for a specific individual and each is individually tested. In contrast, vectors more closely resemble traditional pharmaceuticals that are made in larger amounts and which may be administered to multiple recipients. Under these circumstances, in the VPF we felt it more appropriate to choose the conventional unidirectional flow of air, staff, material, and waste (characteristic of drug manufacturing facilities). All staff are gowned (Tyvek suits, shoe covers, head covers, etc.), but masks are only required in the VPF. The status of the environment in each area is checked (particle and viable counts) at least weekly and during each vector

Figure 7.5 Floor plan of the cGMP facilities at the Baylor Center for Cell and Gene Therapy. The Vector Production Facility has unidirectional flow of staff, materials, and waste, whereas the Cell Processing Facility is a single-corridor design with multidirectional flow.

manufacturing procedure (particle and viable counts, touch plates, and fallout plates in the BSC). There are specified Alert and Alarm levels with associated actions to be taken. The Alert level is set below the Alarm level and indicates impending problems that require immediate attention and corrective action. If an Alarm level is reached, the facility should be closed until remedial action is completed and the facility is back within normal specifications.

Manufacturing in the CPF is continuous, in that multiple patient products for a specific protocol, are in culture all the time in any one room. This requires a detailed changeover procedure that is strictly implemented to prevent contamination and cross-contamination.

In the VPF campaign manufacturing is used. The rooms are stripped of reagents and materials, cleaned, restocked, monitored, and then assigned for the manufacture of a specified vector. Once that production is completed the changeover procedure is repeated. In each suite the ceilings are solid and contain at least two terminal HEPA filters. Air returns are at floor level. All work surfaces are stainless steel and all cabinetry is on wheels. The floors are seamless vinyl and are coved at the walls. Cleaning is performed by trained hospital staff using rotation of validated disinfectants.

Master and working cell and vector banks are generated for the production of adenoviral and retroviral vectors. The Master Cell Bank (MCB) is the stock of material that has undergone the most

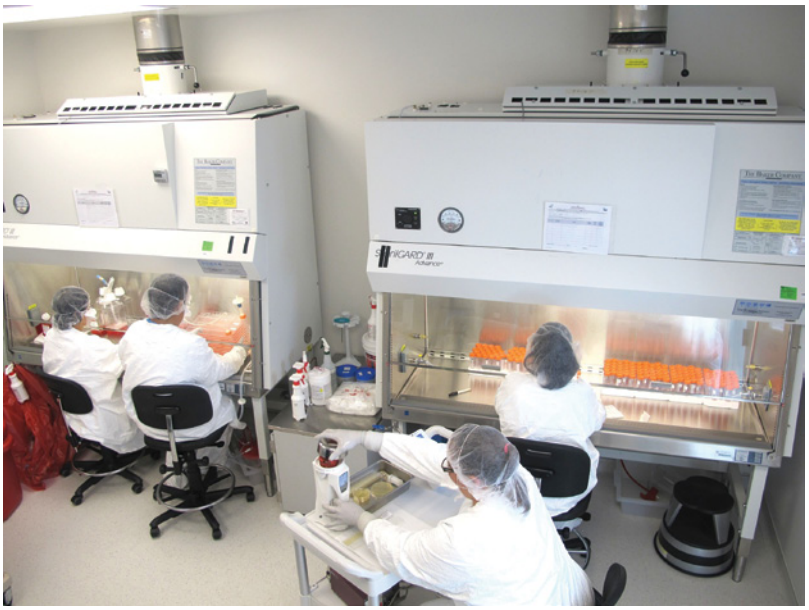


Figure 7.6 Vector manufacturing. Showing the interior of a Class 10,000 (ISO 7) manufacturing suite during retroviral vector production. Staff at the left-hand BSC are harvesting the supernatant. The staff member at the right-hand BSC is labeling the tubes for storage of the supernatant. The staff member in the foreground is performing in-production environmental monitoring.

comprehensive testing and from which Working Banks (WB) are derived. The MCB should be regarded as the reserve and should not used as the stock from which the product is routinely derived. The product is manufactured using the WB which is, in turn, manufactured from the MCB. Multiple WBs can be prepared from the MCB, and each requires only abbreviated testing, rather than the comprehensive testing performed on the MCB. The generation and testing of cell banks have been discussed in guidance documents from the FDA [6]. These should be used as the initial template and discussed with the FDA at the pre-IND meeting to determine their adequacy and whether there have been changes to the recommendations. Procedures have been improved and standardized over the last 12 years to a level where manufacturing master files could be generated for filing with the FDA. The flow charts in use currently at the Center for Cell and Gene Therapy are shown in Figures 7.1, 7.2, 7.3 and 7.4.

Standardized worksheets in the form of a BPR are used to document manufacturing and testing. These closely follow the manufacturing SOP, and contain copies of labels, all associated testing documentation and results, storage of in-process material, and records of correspondence with the customer. To ensure their integrity, numbered BPRs are issued by the QCU to manufacturing staff when preparing licensed products. A similar procedure is recommended, although not mandated, when producing phase I products. The completed BPR and attachments are reviewed by Quality Assurance who also generate the CofA. This is reviewed by the VPF Technical Director; however, formal release is through the QCU. At this time, transfer of the released product to Quality Control is documented and the product is distributed from controlled storage.

Generation of vector-transduced cells takes place in the CPF. The starting cells are obtained from donors whose eligibility has been formally determined [10]. These are cultured according to SOPs specific for each protocol. At the time of transduction a request is placed with Quality Control to provide the required vector and its release is documented and cross-checked. The CofA for the vector is included in the production records. Transduction and remaining culture and testing procedures are

performed in accordance with the SOP. Most products are cryopreserved, which allows time for complete testing prior to release and distribution. Sterility testing requires 7–28 days depending upon the system used; however, for noncryopreserved cellular products the normal release criteria include a STAT Gram stain for sterility, endotoxin testing using a rapid test system such as Endosafe, viability, and sometimes phenotype. Tests with a longer turnaround time must still be performed and reported and an action plan must be in place should any of them return a positive result. Specific tests may be required for virus-transduced cells, for example replication-competent retrovirus, and again the reader is cautioned to contact the FDA to determine the current requirements. Testing results are pulled by the QCU, who review the production records, generate the CofA, and release the product for use.

If vectors are to be directly administered a dosing and preparation worksheet is developed in collaboration with the Principal Investigator for the clinical study. This details the dose, lot number, volume, and method of administration. In most cases the dose is prepared by VPF staff from a vial of vector released by Quality Control. Preparation is documented on the specific worksheet and cross-checked by a second staff member. The vector is transported to the intended recipient on cold packs and must be administered within a specified time. Any unused material is returned to Quality Control and its destruction documented.

Conclusions

Many challenges face the manufacturer of viral vectors. These are not only technical but also regulatory. The GMP regulations are not that complex once you become familiar with "FDA speak." The issue is understanding when and where they must be applied during manufacturing products for phase I studies. The FDA is aware that this difficulty exists and is more than willing to provide assistance in the form of informal interactions, or the more formal pre-IND meetings. Given these opportunities, and the numerous guidances that have been provided by CBER, the onus is now upon the manufacturer to make the best use of these resources.

Acknowledgments

The authors are grateful to the following for support of Vector Production Facility operations: Texas Children's Hospital, Department of Pediatrics; The Methodist Hospital, Houston; The Dan L. Duncan Cancer Center at Baylor College of Medicine NIH-NCI CA125123 Baylor College of Medicine Cancer Center; The Cancer Prevention and Research Institute of Texas RP110553 Cellular Therapy of Cancer; NIH-NCI PO1 CA094237 Enhancing T Cell Therapy of Cancer; NIH-NCI P50 CA126752 SPORE in Lymphoma; NIH-NHLBI N01-HB-10-03 Production Assistance for Cellular Therapies (PACT Program – Cell Processing Facilities); and Leukemia and Lymphoma Society R7016-09 Specialized Center of Research; Cellular Therapy of Lymphoma. We are also grateful for Sara Richman for assistance with the manuscript and to Crystal Silva-Lentz for the photograph in Figure 7.6.

References

1 US Food and Drug Administration (2013) *FDA History*: www.fda.gov/AboutFDA/WhatWeDo/History/default.htm.
2 Code of Federal Regulations (2012) *cGMP in manufacturing, Processing, Packing, or Holding of Drugs and Finished Pharmaceuticals*. Title 21, Parts 210 and 211.
3 US Department for Health and Human Services (2008) *Guidance: cGMP for Phase I Investigational Drugs*. US Department for Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research, Center for Biologics Evaluation and Research, Office of Regulatory Affairs.
4 US Department for Health and Human Services (2011) *Guidance for Industry: Process Validation: General Principles and Practices*. US Department for Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research, Center for Biologics Evaluation and Research, Center for Veterinary Medicine
5 Environmental Protection Agency (2007) *Guidance for Preparing Standard Operating Procedures (SOPs)*. EPA QA/G-6. www.epa.gov/quality/qs-docs/g6-final.pdf.
6 US Food and Drug Administration (1993) *Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals*. US Food and Drug Administration, Center for Biologics Evaluation and Research.
7 US Department for Health and Human Services (2009) *Guidance for Industry: Supplemental Guidance on Testing for Replication Competent Retrovirus in Retroviral vector Based Gene Therapy Products and During Follow-up of*

Patients in Clinical Trials. US Department for Health and Human Services, Food and Drug Administration, Center for Biologics Evaluation and Research. July 2009.

8 NIH Report (2001) National Institutes of Health Report: assessment of adenoviral vector safety and toxicity: report of the National Institutes of Health Recombinant DNA Advisory Committee. *Human Gene Therapy* 13: 3–13.

9 US Department for Health and Human Services (2008) *Guidance for FDA Reviewers and Sponsors; Content and Review of Chemistry, Manufacturing and Control (CMC) Information for Human Somatic Cell Therapy Investigational New Drug Applications (INDs)*. US Department for Health and Human Services, Food and Drug Administration, Center for Biologics Evaluation and Research.

10 US Food and Drug Administration (2009) *Donor Eligibility Final Rule and Guidance Questions and Answers*. www.fda.gov/biologicsbloodvaccines/tissuetissueproducts/questionsabouttissues/ucm102842.htm.

8

CHAPTER 8

US Regulations Governing Clinical Trials in Gene Therapy

Bambi Grilley
Baylor College of Medicine, Houston, TX, USA

Introduction

Clinical trials conducted in the USA that utilize gene therapy require review from many different regulatory agencies and review groups. Specifically, most gene therapy studies will require review from at least one Institutional Review Board (IRB), the Food and Drug Administration (FDA), at least one Institutional Biosafety Committee (IBC), National Institutes of Health (NIH) Office of Biotechnology Activities (OBA), and the Recombinant DNA Advisory Committee (RAC). While studies that include agents that are not FDA-approved will undergo review by the FDA and at least one IRB prior to implementation, the involvement of two additional review processes is exclusive to gene therapy research. These additional reviews, both nationally (NIH OBA and RAC) and at a local level (IBC), were deemed necessary by the Federal government due to the unique scientific, medical, ethical, and social considerations inherent in this type of research. Notably, recombinant DNA research uses techniques that are novel and include potential irreversible risks to the subject and their progeny (in cases of germline alteration). In this chapter we will discuss the regulatory processes needed prior to initiation of a gene therapy clinical trial in the USA as well as continuing submission requirements for ongoing trials. Finally, the chapter will discuss development of a gene therapy clinical trial with a focus on components of the protocol document and consent form that are unique to gene therapy. It is important to note that these types of trials are regulated differently throughout the world and that if a study is to be conducted outside of the USA a thorough review of regulations governing that country will be required.

Submission to regulatory agencies and review boards can occur simultaneously or sequentially. However, several factors make sequential submission preferable. First of all, the NIH and FDA have agreed that submission of any gene-transfer project to the NIH OBA must occur prior to submission of an Investigational New Drug (IND) application to the FDA [1]. Second, the IBC cannot release final approval of a project until the RAC review process has been completed [2]. And finally, simultaneous submissions can be problematic if different reviewers have different or conflicting thoughts regarding the project. For these reasons a sequential submission process is preferable. Figure 8.1 is a sequential submission flowchart. This chapter will follow this flow chart when discussing regulatory requirements for each regulatory/review group.

NIH OBA and RAC

The NIH established the RAC on October 7, 1974 to address public concerns regarding the safety of manipulating genetic material through the use of recombinant DNA techniques. The purpose of

Cancer Gene Therapy by Viral and Non-viral Vectors, First Edition. Edited by Malcolm K. Brenner and Mien-Chie Hung.

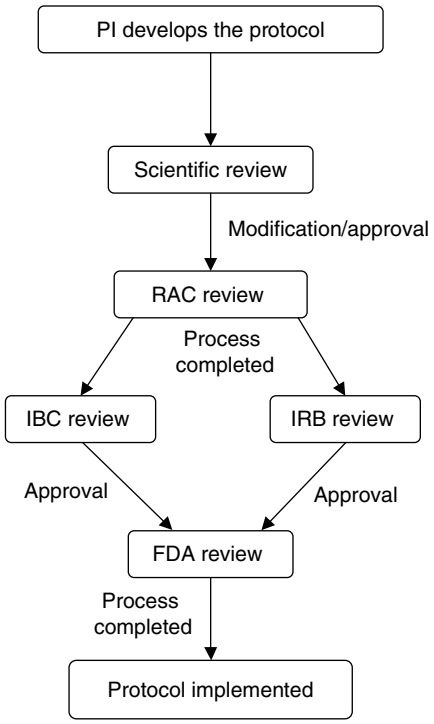


Figure 8.1 Flow chart for development and review of a gene therapy clinical trial. PI, Principal Investigator.

the RAC is to allow public discussion of scientific, ethical, and legal issues related to recombinant DNA technology. The RAC developed a document called the *NIH Guidelines for Research Involving Recombinant DNA Molecules (NIH Guidelines)* [3] that specifies practices for constructing and handling recombinant DNA. Included in the *NIH Guidelines* is Appendix M, which is titled *Points to Consider in the Design and Submission of Protocols for the Transfer of Recombinant DNA Molecules into One or More Human Research Participants (Points to Consider)* [4]. This *Points to Consider* document guides development of recombinant DNA projects and includes key information regarding submission requirements for the RAC.

Compliance with the *NIH Guidelines* is mandatory for investigators at institutions receiving NIH funds for research involving recombinant DNA. It is not mandatory for independent companies and institutions not receiving NIH funding; however, the RAC will review voluntary submissions from these entities.

The Principal Investigator (PI) is responsible for submitting documents to the NIH OBA. Documents required for submission are:

- a cover letter on institutional letterhead, signed by the PI(s), that:
  - acknowledges that the documentation submitted to NIH OBA complies with the requirements set forth in Appendix M-I-A, *Requirements for Protocol Submission*; http://oba.od.nih.gov/oba/rac/Guidelines/NIH_Guidelines.htm#_Toc351276403,
  - identifies the IBC and IRB responsible for local review and approval of the protocol, and
  - acknowledges that no research participant will be enrolled (see definition of enrollment in Section I-E-7; http://oba.od.nih.gov/oba/rac/Guidelines/NIH_Guidelines.htm#_Toc351276221) until the RAC review process has been completed (see Appendix M-I-B, *RAC Review Requirements*; http://oba.od.nih.gov/oba/rac/Guidelines/NIH_Guidelines.htm#_Toc351276404), IBC approval (from the clinical trial site) has been obtained, IRB approval has been obtained, and all applicable regulatory authorizations have been obtained;
- a scientific abstract;
- a nontechnical abstract;
- the proposed clinical protocol, including tables, figures, and relevant manuscripts;
- the proposed informed consent document;
- curriculum vitae of the PI(s).

After submission, the NIH OBA will confirm receipt of the submission within 3 days. A summary of the submission will be sent to RAC members for review. RAC members may send queries to the PI for further clarification about the project or send suggestions to the PI regarding the project or the clinical trial documents. The PI will be notified of the outcome of this preliminary review within 15 working days of receipt of the submission. The outcome will include a recommendation regarding the need for public review of the project.

Materials submitted to NIH OBA are considered to be in the public domain and access to these documents is made available to the public with many of the documents being posted on the NIH OBA website. The RAC has quarterly meetings that are open to the public in an effort to encourage an open dialogue about the various aspects of gene

therapy research and specific projects under review. Selected clinical trials are discussed at these meetings. Clinical trials are selected for discussion at these public meetings based on whether or not the reviewers feel the specific trial presents a unique scientific, social, or ethical issue. In situations where public review is required several RAC members will be asked to provide an in-depth review of the project. Queries arising from that review will be sent to the PI for a written response prior to the meeting. At the meeting the PI and appropriate colleagues will be allowed 15–20 min to present the project and some face-to-face discussion of outstanding issues will occur.

The outcome of RAC review, whether occurring following a public discussion or through the more expedited process, is advisory in nature and does not include approval or disapproval. Additionally, the outcome of RAC review, including all recommendations, will be sent to the FDA, Office of Human Subject Protections (OHRP), the identified IRB for the project, and the identified IBC for the project. In this way RAC review serves to help advise other entities that will be involved in the review of the project.

Following completion of all steps in the regulatory process the first subjects can be enrolled. Within 20 days of consenting the first subject, the PI is required to submit the following documents to the NIH OBA:

- a copy of the informed consent document approved by the IRB;
- a copy of the protocol approved by the IBC and IRB;
- a copy of the final IBC approval from the clinical trial site;
- a copy of the final IRB approval;
- a brief written report that includes the following information:
  - how the investigators responded to each of the RAC's recommendations on the protocol (if applicable),
  - any modifications to the protocol as required by FDA;
- applicable NIH grant number(s);
- the FDA IND number; and
- the date of initiation of the trial.

After the study is initiated there is a requirement for ongoing submission of amendments, adverse events, and annual reports to NIH OBA. Amendments must be sent to NIH OBA as part of the annual report; however, it might be simpler for the investigator to send such amendments in real time and simultaneous with submissions to the FDA. Serious adverse events that are unexpected and possibly associated with the gene-transfer product must be submitted to OBA within 15 calendar days of notification, unless they are fatal or life-threatening, in which case they must be reported within 7 calendar days. NIH OBA prefers that such submissions be sent using the GeMCRIS system located at http://oba.od.nih.gov/rdna/adverse_event_oba.html. Further information regarding the definitions of adverse events (referred to by the FDA as adverse reactions) will be discussed in the section of this chapter dealing with FDA submissions. Annual reports must be submitted within 60 days of the annual anniversary of the date the IND went into effect. The submission to NIH OBA should include a summary of the trial including:

- the title and purpose of the trial;
- clinical site;
- the PI;
- clinical protocol identifiers, including the NIH OBA protocol number, NIH grant number(s) (if applicable), and the FDA IND application number;
- participant population (such as disease indication and general age group; e.g. adult or pediatric);
- the total number of participants planned for inclusion in the trial, the number entered into the trial to date, the number whose participation in the trial was completed, and the number who dropped out of the trial with a brief description of the reasons;
- the status of the trial; e.g. open to accrual of subjects, closed but data collection ongoing, or fully completed, and
- if the trial has been completed, a brief description of any study results.

The submission to NIH OBA should also include a progress report on the status of the study, including:

- a narrative or tabular summary showing the most frequent and most serious adverse experiences by body system;
- a summary of all serious adverse events submitted during the past year;

- a summary of serious adverse events that were expected or considered to have causes not associated with the use of the gene-transfer product such as disease progression or reactions felt to be related to concurrent medications;
- if any deaths have occurred, the number of participants who died during participation in the investigation and causes of death; and
- a brief description of any information obtained that is pertinent to an understanding of the gene-transfer product's actions, including, for example, information about dose response, information from controlled trials, and information about bioavailability.

If amended copies of the clinical trial have not been submitted in real time the annual report should also include a copy of the updated clinical trial including a technical and nontechnical abstract.

The majority of information required for annual reporting is included in the IND renewal prepared for the FDA. For simplicity, the investigator should consider sending a copy of the IND renewal with additional information (specifically the NIH OBA protocol number) to the NIH OBA simultaneously with submission to the FDA [3,4,5].

Moving back to a discussion of initial approvals of a gene therapy project, following completion of the RAC review process the clinical trial should be submitted to the IRB and IBC. Many institutions prefer that these submissions be simultaneous since the review processes are complementary.

IBC

IBC review is intended to provide local review of all experiments involving the deliberate transfer of recombinant DNA or RNA derived from recombinant DNA into any human research participant. Regulations for IBC review are also included in the *NIH Guidelines* as specified above. IBC approval must be obtained from each institution at which recombinant DNA material will be administered to human subjects. IBC applications are generally submitted by the PI. Requirements for submission of a project to the IBC will vary among institutions; however, in all cases the IBC is required to review:

- the source of the DNA;
- the nature of the inserted DNA sequences;
- the vectors to be used;
- information on whether an attempt will be made to obtain expression of a foreign gene (and if so the protein that will be produced); and
- the containment conditions that will be implemented.

The IBC must ensure that all aspects of RAC Appendix M are addressed, that the containment levels are appropriate, that appropriate trained personnel are involved in the project, and that proper plans exist for handling accidental spills and personnel contamination resulting from the recombinant DNA research. Finally, prior to approval, the IBC must consider the issues raised during RAC review as well as response to that review as prepared by the PI [2]. RAC review must occur prior to final IBC approval and IBC approval must be obtained before the study is initiated [5].

The *NIH Guidelines* specify that the investigator is to remain in communication with the IBC throughout the conduct of the project [Section IV-B-7-c-(4); http://oba.od.nih.gov/oba/rac/Guidelines/NIH_Guidelines.htm#_Toc351276263]. In addition, the IBC is responsible for ensuring compliance with all surveillance, data reporting, and adverse event reporting requirements set forth in the *NIH Guidelines* [Section IV-B-2-b-(1); oba.od.nih.gov/oba/rac/Guidelines/NIH_Guidelines.htm#_Toc351276263]. To facilitate capture of this information many IBCs require that changes in the clinical trial, serious adverse events, and annual reports be sent to the committee. As previously stated, submission requirements for different IBCs can vary significantly and the PI should ensure that they are familiar with the IBC requirements at each institution where the recombinant DNA will be administered.

IRB

The IRB is intended to provide local review of human subject research. The review is intended to protect the rights, welfare, and wellbeing of subjects involved in research. IRB review and related requirements for obtaining informed consent from research subjects are codified in regulations Title 45 Code of Federal Regulations (CFR) part 46 (45CFR46; *Protection of Human Subjects [HHS]*), 21CFR50 (*Protection of Human Subjects [FDA]*), and 21CFR56 (*Institutional Review Boards [FDA]*). For the most part the regulations governing Human

Subject Protection are harmonized between the US Department of Health and Human Services (HHS) and FDA and any differences should not impact the vast majority of gene-transfer studies. We therefore will not deal with any discrepancies between those regulations in this chapter. The federal agency involved in oversight of Human Subject Protection is the Office for Human Research Protection (OHRP), which is part of the HHS. The OHRP website (www.hhs.gov/ohrp/index.html) contains a significant amount of documents and information regarding the regulations and guidances covering human subject research and IRB review and oversight.

As with IBC approval, IRB approval must be obtained from each institution at which recombinant DNA material will be administered to human subjects. Also similarly, requirements for submission of a project to the IRB will vary among institutions. In all cases the IRB will review the clinical trial and the consent form for the clinical trial. IRB applications are submitted by the PI. The IRB will review the project with a focus on the following aspects:

- risks to subjects are minimized;
- risks to subjects are reasonable in relation to anticipated benefits;
- selection of subjects is equitable;
- informed consent will be sought from each prospective subject or the subject's legally authorized representative, and appropriately documented;
- when appropriate, the research plan makes adequate provision for monitoring the data collected to ensure the safety of subjects;
- when appropriate, there are adequate provisions to protect the privacy of subjects and to maintain the confidentiality of data;
- appropriate safeguards are included to protect subjects likely to be vulnerable to coercion or undue influence;
- when the research involves pregnant women, fetuses, or neonates; prisoners; or children, the research satisfies the additional requirements for IRB approval under HHS regulations at subparts B, C, or D, respectively, of 45CFR46 [6].

Unlike IBC review there is no regulatory requirement for IRB review to follow RAC review; however, many institutions prefer that IRB review occur after RAC review and either simultaneously or following IBC review so that IRB members may benefit from the review of experts in the area of gene therapy studies.

Following initial approval the IRB is required to review amendments, unanticipated problems (including serious adverse events/reactions) and annual reports regarding conduct of the study.

The IRB must approve any modifications of previously approved research including any changes to the consent form prior to implementation of those modifications. The only acceptable exception to this regulation is a modification that the investigator deems necessary to eliminate apparent immediate risk of harm to subjects [7].

IRBs are required to review "unanticipated problems involving risks to subjects or others." Such unanticipated problems are generally required to meet all of the following criteria:

- *unexpected* (in terms of nature, severity, or frequency) given existing information about the product, study, and subject population;
- *related or possibly related*, meaning that there is a reasonable possibility that the incident, experience, or outcome may have been caused by the procedures involved in the research; and
- suggests that the research places subjects or others at a *greater risk of harm* (including physical, psychological, economic, or social harm) than was previously known or recognized [7].

Unanticipated problems include but are not limited to adverse events/reactions (further discussion of adverse reactions is included in the FDA section of this chapter). However, unanticipated problems can also include problems with study conduct such as dosing errors and consent issues and noncompliance with regulations. As with all IRB policies specific definitions and methods of reporting are determined by the specific IRB. A good reference regarding unanticipated problems is available from OHRP and is entitled *Guidance on Reviewing and Reporting Unanticipated Problems Involving Risks to Subjects or Others and Adverse Events* [8].

The IRB must review and approve each ongoing project not less than once per year [9]. Continuing review and approval of the research project is required for the duration of time that the study is ongoing; that is, until research-related interactions and interventions with human subjects or the obtaining and analysis of identifiable private information described in the IRB-approved research plan have been completed.

Submission requirements for this annual review will vary. However, in general, such submissions will include information on the progress of the research (including the number of subjects treated) and issues related to the research (with a focus on any unanticipated problems or new developments in regards to the project or the field of study). In particular the IRB will focus on any new information that might impact the determinations made during the initial review of the project [6,9].

FDA

Last but not least is submission of the IND to the FDA. It is preferable to make this the last stage of the process for several reasons including the requirement that RAC review occurs prior to FDA review, that the FDA will ask for a copy of the IRB approval letter, and that submitting to the FDA last ensures that the most final version of the clinical trial is being reviewed and discussed during the IND process. The sponsor is responsible for submitting to the FDA.

The FDA is divided into different centers for review of the various products it regulates. For medical products the primary centers used to regulate products include:

- Center for Drug Evaluation and Research (CDER),
- Center for Biologics Evaluation and Research (CBER), and
- Center for Devices and Radiological Health (CDRH).

The majority of gene therapy clinical trials will be submitted to and reviewed by CBER.

Before we proceed to discuss submission of an IND, it is important to note that in some situations there will be contact with the FDA prior to submission of documents to the RAC, IRB, or IBC. Specifically, in some situations the sponsor may want to meet with the FDA early in the product- and protocol-development process to discuss ways to optimize product development while minimizing protocol delays. The FDA has a specific mechanism for facilitating such discussions, referred to as the pre-IND meeting, also known as a Type B meeting.

A sponsor who wishes to have a pre-IND meeting with the CBER component of the FDA submits a meeting request in triplicate to the appropriate applications division director. The meeting request should include adequate information for the FDA to determine the utility of the meeting and to identify Agency staff necessary to discuss proposed agenda items. The meeting request should include the following information:

- product name;
- chemical name and structure;
- proposed indication(s);
- that it is a Type B meeting;
- a brief statement of the purpose of the meeting;
- a list of the specific objectives/outcomes expected from the meeting;
- a preliminary proposed agenda, including estimated amounts of time needed for each agenda item and designated speaker(s);
- a draft list of specific questions, grouped by discipline;
- a list of all individuals (including titles) who will attend the proposed meeting from the sponsor's or applicant's organization and consultants;
- a list of Agency staff requested by the sponsor or applicant to participate in the proposed meeting, if known;
- the approximate date on which supporting documentation (i.e. the information package as described below) will be sent to the review division;
- suggested dates and times (i.e. morning or afternoon) for the meeting.

The FDA will respond to the sponsor or applicant within 14 days of receipt of the meeting request. If the FDA agrees to the meeting, the written response (i.e. letter or fax) should include the date, time, length, and place of the meeting as well as the expected FDA participants.

The sponsor should submit the information package to the FDA so that it is received at least 4 weeks prior to the formal meeting.

To facilitate the FDA's review the sponsor or applicant should organize the contents of the information package according to the proposed agenda. A fully paginated document with a table of contents, appropriate indices, appendices, cross-references, and tabs differentiating sections is recommended. Hard copies of the information package should be provided for each FDA participant, with an extra five copies for consultation. The project manager or division contact can advise on the numbers of copies needed. The cover letter accompanying the information package should clearly identify the date, time, and

subject of the meeting. Although the contents of the information package will vary depending on the product, indication, phase of drug development, and issues to be discussed, information packages generally should include the following:

- product name and application number (if applicable);
- chemical name and structure;
- proposed indication(s);
- dosage form, route of administration, and dosing regimen (frequency and duration);
- a brief statement of the purpose of the meeting. This statement could include a discussion of the types of completed or planned studies or data that the sponsor or applicant intends to discuss at the meeting, the general nature of the critical questions to be asked, and where the meeting fits in overall development plans;
- a list of the specific objectives/outcomes expected from the meeting;
- a proposed agenda, including estimated amounts of time needed for each agenda item and designated speaker(s);
- a list of specific questions grouped by discipline;
- clinical data summary (as appropriate);
- preclinical data summary (as appropriate);
- chemistry, manufacturing, and controls information (as appropriate).

At the end of the meeting, the FDA chair or facilitator should summarize all important discussion points, decisions, recommendations, agreements, disagreements, and action items for the benefit of all meeting attendees. Attendees should be provided the opportunity to comment. If there are any differences of opinion regarding the outcome of the discussion, the chair or facilitator should ensure that the issues are resolved to the extent practicable. The FDA recorder should document this summary as the official minutes. The official minutes should be issued to all FDA attendees (with copies to appropriate files) and to the sponsor or applicant within 30 days of the formal meeting. Sponsors or applicants may provide the assigned project manager with a draft of the firm's minutes in writing, or may identify at the end of the meeting the critical outcomes they believe should be included in the meeting documentation. Draft minutes provided by the sponsor or applicant are useful only if submitted promptly; if so submitted, they will be considered by the review division during the preparation of the official minutes. If, after receiving the official minutes from FDA, a sponsor or applicant of a Prescription Drug User Fee Act (PDUFA) product wishes additional clarification of the minutes or issues related to the meeting, it may contact the project manager of the FDA component for guidance or to arrange a teleconference with the appropriate Agency staff.

Sponsors and applicants may notify FDA of significant differences in understanding regarding the content of the official minutes of a meeting. If sponsors or applicants wish to effect a change in the official minutes, they may send a letter to the Division Director, with a copy to the project manager, citing their recommendations and rationale. The concerns of the sponsor or applicant should be taken under consideration by the review division, and the project manager should issue an appropriate response in writing. If FDA agrees to change the official minutes, such changes should be documented in an addendum to the official minutes. If sponsors or applicants are not satisfied with the response provided by the FDA component, they may elect to pursue the Agency's procedures for internal review and dispute resolution (21CFR10.75, 21CFR312.48, 314.103) [10].

The IND submission should include:

- form 1571: the initial submission will be serial number 000;
- a cover letter;
- table of contents that is inclusive of the entire submission and includes page numbers;
- introductory statement and general investigational plan: a brief summary of the product and the overall plan for investigating the product;
- Investigator's Brochure (IB):
  - the regulations allow for an exemption from an IB for single-site sponsor investigator submissions if preparation of an IB is impractical. In such situations an expanded background section of the clinical trial containing relevant information can be substituted [11],
  - if required, the components of the IB include: description of drug substance and formulation, summary of pharmacological and toxicological effects in animals and humans (as available), summary of pharmacokinetics and biological disposition of the drug in animals and humans

(as available), and description of known and anticipated risks,
  - the IB must be updated as new data become available but at a minimum it must be updated annually;
- clinical protocol:
  - objectives,
  - background,
  - product information,
  - eligibility criteria,
  - treatment plan,
  - monitoring criteria,
  - criteria for response assessment,
  - statistical analysis,
  - adverse reaction reporting,
  - informed consent document;
- Chemistry, Manufacturing, and Control (CMC) data:
  - a description of the drug substance,
  - a list of all components of the drug product,
  - a brief general description of the composition, manufacture, and control of any placebo used in the trial,
  - a copy of all labels,
  - environmental analysis: in most cases this is handled as a claim for categorical exclusion under 21CFR25;
- pharmacology and toxicology data: *in vitro* and animal data;
- previous human experience;
- form 1572 and related documents: curriculum vitae for the PI and a biosketch for all coinvestigators;
- other additional information as needed (21CFR312.23);
- form 3674: Certification of Compliance, under 42 USC §282(j)(5)(B), with requirements of the ClinicalTrials.gov data bank. It is important to note that in order to register the study with ClinicalTrials.gov prior to submission of the IND, the IND number in the ClinicalTrials.gov system will be listed as pending. Following IND activation the number will need to be updated [12].

In addition, for gene-transfer studies the following issues must be addressed.

- A summary of product manufacturing quality assurance (QA) and quality control (QC) programs. This should consist of a brief (approximately three pages) description of the system for preventing, detecting, and correcting deficiencies that may compromise product integrity or function, or may lead to the possible transmission of adventitious infectious agents. Each individual who has authority over the QA and QC programs should be included with a list of their duties. The date of the last QA and QC audits of the manufacturing operations and those of contract manufacturers, vendors or other partners should also be included.
- For each clinical trial contained in the IND, the sponsor is required to submit a two- to three-page summary of the procedures in place to ensure:
  - there is adequate monitoring of the clinical investigations to demonstrate the trial(s) are conducted in accordance with regulatory requirements and Good Clinical Practices (GCPs), and the clinical trial; that the rights and wellbeing of human subjects are protected; and that data reporting, including safety reporting to the sponsor, the IRB, and NIH is accurate and complete; and
  - that the sponsor has adequate oversight of the clinical investigation, as outlined in 21CFR312, subpart D. Included in the summary should be an organizational chart identifying each individual responsible for oversight of clinical studies and his or her duties. If any of these obligations have been transferred to a Contract Research Organization (CRO) this should be indicated and verification that these obligations are being met must be included. A summary of the CRO's oversight procedures should be included [13].

The document developed to address issues of compliance with GCP and oversight of clinical trials is commonly referred to as a monitoring plan or a Gene Therapy Monitoring Plan (GTMP). A basic format for the plan could include:

- a basic overview of the research program (or institution) covered by the plan;
- a point-by-point response to the specific issues raised by the FDA including:
  - adherence to study eligibility,
  - adherence to the treatment plan,
  - adherence to clinical trial-specified data collection for safety and efficacy,
  - adherence to reporting of adverse events to IRB with authority, sponsor, or FDA (if sponsor is

also investigator), and NIH OBA including adherence to required time frames,
  - adherence to informed consent requirements (including verification that written and other informed consent materials have been approved by IRB; are signed prior to entry into study; and appropriate witnesses documented),
  - verification that any modification to the study plan has been submitted to FDA and the IRB with authority prior to implementation and that IRB has approved the changes,
  - audit of study reports by verification of the accuracy of the information submitted to FDA, IRB, and NIH by comparison against primary source documents; maintains a complete set of source documents,
  - procedures for correction of errors in the study reports and records, which includes date of correction, individual making correction, and reason; the mechanism must preserve the original record,
  - procedures identify individuals responsible for performing monitoring and completion of study report forms and identify individuals with authority for final verification (sign-off) of study records (usually the PI),
  - procedures for receipt and tracking of investigational drug products and the individual responsible for tracking drug product,
  - procedures for closing study sites/removing investigators for failure to adhere to the protocol,
  - organizational chart to identify the individuals and/or organization with responsibility for monitoring the clinical study or program,
  - organizational chart to summarize the duties of each individual who has monitoring/oversight responsibilities,
  - organizational chart to provide the reporting relationship between the study monitor/monitoring organization and the IND sponsor,
  - organizational chart and summary of responsibilities to indicate that the sponsor has adequate oversight;
- a list of key positions and related personnel, including: IND holder, PI, statistician, laboratory director, laboratory QA and QC personnel, laboratory research personnel, research nurse/research coordinators, clinical research regulatory staff, and clinical research QA and QC personnel;
- the qualifications of key personnel should also be included in the GTMP;
- the long-term follow-up plan for subjects enrolled on gene transfer protocols;
- the mechanism to update the Cell/Gene Therapy Monitoring Plan.

Development of Standard Operating Procedures (SOPs) for clinical research not only standardizes research practices within a research group, it can also significantly simplify submission and implementation of clinical trials and INDs. Common SOPs used to support a GTMP are included in Box 8.1. Most sites will have other SOPs that are utilized at the site but that are not necessary to submit to the FDA in support of an IND.

A very important part of the GTMP relates to the actual monitoring of the study. FDA expects a Clinical Research Quality Assurance Program (QA program), a Clinical Research Quality Control Program (QC program), and a Data Safety Monitoring Plan (DSMP).

In general, the QA program serves a retrospective monitoring function for specific studies. QA activities serve to evaluate and ensure that the conduct of clinical trials complies with all federal regulations and International Conference on Harmonization (ICH) GCPs. QA should also ensure that institutional and programmatic SOPs establishing the processes used for conduct of clinical trials are being followed and improve the processes established in the SOPs when necessary.

In general, the QC program serves a prospective function in training and evaluating clinical research personnel and their compliance with federal regulations, the ICH GCP, and institutional SOPs. The secondary purpose of QC activity is to provide training to new clinical research operations personnel and continuing education to all clinical research personnel. The third purpose of QC activity is to define and implement or improve the operational processes established in the SOPs.

A final component of study monitoring is DSMP. The concept of data and safety monitoring dates back to as early as 1978, when the NIH Clinical Trials Committee proposed a policy that would have required the establishment of a data and safety monitoring committee for every clinical trial sponsored by NIH. In June 1998, the NIH released their *Policy for Data and Safety Monitoring* [14].

Box 8.1 SOPs included in a GTMP

Document Archiving for Clinical Research Clinical trials This SOP should include paper and electronic storage guidelines for regulatory binders and research charts. Included in this SOP should be instructions on how regulatory and research charts are organized. In addition, this SOP should include instructions on storage of documents while the study is active and when it is closed. Similarly, there should be clear instructions on storage of research charts for subjects who are on and off study during the time the study is active and after the study is closed. This SOP should be compliant with 21CFR312.57.

Protocol Review of Cancer-Related Clinical Research Protocols This SOP should codify investigator (or institutional) policies regarding internal and external review of clinical trials prior to implementation. If the institution requires that a certain "order" be observed regarding submission that should be included in this SOP.

Regulatory Review This SOP should provide more detailed information regarding the submission requirements to each review agency including at a minimum the IRB, IBC, FDA, and NIH OBA. This SOP should be consistent with all external and internal submission requirements including 21CFR312 and the *NIH Guidelines*.

Obtaining and Documenting Informed Consent This SOP should include detailed information about the components of the consent form in compliance with 21CFR50, 45CFR46, and applicable local IRB requirements. As appropriate the SOP should specify who can obtain consent (by role) and how such consent is documented. If it is likely due to the subject population that there will be special issues related to obtaining consent, those should be discussed in the document. Examples of such special issues include the need to obtain assent/parental permission and the use of translators in the consent process.

Product Infusion Specific guidelines for product delivery, infusion, and premedication as specified by the institution/research group. As possible, standards for treatment of common product-related adverse events should be included.

Collection and Confirmation of Study Data This SOP should codify issues such as what is considered to be a source document, how to complete a case report form (CRF), and how to confirm subject eligibility. The SOP should specify who collects study data and who reviews/confirms study data (by role).

Adverse Experience Reporting This SOP should codify who is responsible for capturing adverse experiences (AEs), how such data are captured, and who is responsible for review/confirmation of AE data. As possible the SOP should codify grading scales used for toxicity. If due to the disease(s) treated there are specific types of AEs that should be excluded from assessment, that should be codified in this SOP as well. The SOP should discuss how serious adverse events/unanticipated problems are reported to regulatory agencies and review groups. Such reporting should be in compliance with 21CFR312, and *NIH Guidelines* Appendix M-1-C, and instructional IRB and IBC guidelines. The SOP should include instructions for reporting via the NIH GeMCRIS system.

Long-Term Follow-Up for Gene Transfer Studies This SOP should codify institutional or research group policies for long-term follow-up of study subjects in compliance with guidance documents from the FDA and *NIH Guidelines* Appendix M-II-B-3.

Laboratory Quality Assurance Program A description of the quality management program for the manufacturing facility. Included are details of the program used to assure the quality of products prepared in the facility and how these products are to be released for clinical use or returned to inventory for potential reissue.

Clinical Research Quality Control and Quality Assurance Program A description of the quality management program(s) for the conduct of clinical research at the institution or within the research group. The program should be developed to confirm compliances with appropriate CFRs, ICH GCP, institutional review groups, and site-specific SOPs.

Data Safety Monitoring Plan A description of the mechanism implemented by the institution or study group to evaluate study progress including risk/benefit outcomes and consideration of new scientific or therapeutic developments that may impact the risks/benefits of the study. The plan should specify the types of studies requiring review, provide guidelines for the frequency of review, and suggest specific mechanisms of review based on study type. The spectrum of data review can range from review of data by the PI to review of data by a fully convened, study-specific Data Safety Monitoring Board (DSMB) with incremental modifications based on study risk and type in between these two ends of the spectrum. Types of data usually reviewed include the expected number of subjects to be treated, the number of subjects currently enrolled, interim analysis information (as appropriate; has one been done and what was the outcome of that analysis?), toxicity data, and any new scientific discoveries that would impact the conduct of the study. For multisite studies data safety monitoring must be inclusive of all data from all sites.

Note: some SOPs may be split into more than one SOP dependent on the amount of information to be presented and the complexity of the topic.

In 2000 the NIH released further guidance on data and safety monitoring for phase I and II studies [15]. Since then, many of the institutes and centers within NIH have established their own guidelines for data and safety monitoring. Investigators receiving funding for clinical research projects through any institute or center of the NIH are obligated to follow the data and safety monitoring guidelines established by the specific institute or center. The various guidelines vary in size and scope with the National Heart, Lung, and Blood Institute (NHLBI) having a robust set of policies/guidelines covering not only the requirements for data and safety monitoring but also how to establish an independent Data and Safety Monitoring Board (DSMB) and what the responsibilities of a DSMB include.

The FDA released a guidance on data monitoring committees in March 2006 [16]. Most of that guidance is similar to the policies/guidance of the NIH. However, the scope of the guidance is much broader than that of NIH in that it applies to all clinical research, not just that funded by the NIH.

There are some areas in which the various policies and guidelines may vary. Such variations can include the number of required reviewers, the expertise of the reviewers, and the minimal required frequency of the meetings.

At their most basic, all of the policy and guidance related to data and safety monitoring agree on the following principles:

- all clinical trials (phases I, II, and III) require monitoring, and
- monitoring should be commensurate with risks, and
- monitoring may be conducted in various ways depending on the size and scope of the research effort with the continuum ranging from review of data by the PI to the requirement for establishment of a DSMB.

Factors that have an impact on which method of monitoring is appropriate include the phase of study, the treatment modality (high-risk treatments may require a DSMB), and the disease/study population (inclusion of vulnerable populations may necessitate the use of a DSMB). Phase I and II studies are usually small, and monitoring can be difficult. Review of the data by the study investigator or by an independent individual is usually considered to be adequate. Phase III studies on the other hand are usually large, multicenter studies, which may include different arms of treatment and in some cases include randomization. These studies require review by a DSMB. In some cases (such as a phase I study in a vulnerable patient population) it

may be more appropriate to establish a monitoring committee. This committee would allow for a more broad-based review of the data without meeting the requirements of a DSMB. Some investigators (or groups of investigators) that conduct considerable research in the same area(s) may establish standing committees to serve as a data monitoring committee.

Data and safety monitoring is the responsibility of the sponsor. In the case of investigator-initiated studies, the NIH (as applicable) and FDA expect that the PI will develop and implement a data and safety monitoring plan for the research. At a minimum, the plan must include a description of the reporting mechanisms of adverse events to the IRB, the FDA, and the NIH. The plan should include a detailed description of who will be responsible for monitoring the study data. The plan should be submitted to the IRB for review and approval before the study is initiated.

Possible outcomes of a DSMB review include determining that:the study is safe to proceed as written;

- the study requires modification in order to be safe to proceed; or
- the study is not safe to proceed or that study objectives have been met; in either case the DSMB would determine that the study should be closed.

Written documentation of the recommendations of the DSMB should be supplied to the PI and to the study sponsor. The sponsor is responsible for notifying the FDA (as appropriate) and all responsible IRBs of any recommendations from the DSMB.

The IND submission should be sent to the FDA:

- with a 1571 numbered serially using a single three-digit serial number starting with 000;
- each submission should include an original and two copies [17].

Following initial submission, each IND will be assigned a project manager. The assignment is communicated to the IND sponsor via the IND acknowledgment letter sent by the FDA when a new IND is submitted. The project manager serves as a liaison between the FDA and the IND sponsor. As such, it is important that after the project manager is assigned queries and correspondence should be addressed to that project manager.

Following submission, the IND and clinical trial will be assigned to a review team that includes:

- the project manager;
- a CMC reviewer;
- a nonclinical pharmacology/toxicology reviewer;
- a clinical reviewer; and
- other reviewers as needed (e.g. statistics, epidemiology, site inspectors, patient representative).

Each of these reviewers may contact the IND sponsor directly with questions and queries regarding the submission.

As with RAC submissions the FDA does not approve or disapprove INDs or submissions. An IND goes into effect 30 days after the FDA receives the IND unless the FDA notifies the sponsor that the IND is on clinical hold. The most common reasons for a clinical hold include that subjects would be exposed to unreasonable or significant risk or that the IND does not contain sufficient information to assess the risks to the subject [18,19].

The IND should be updated for:

- protocol amendments including submission of new protocols on an existing IND;
- information amendments including CMC and pharmacology changes;
- IND safety reports;
- annual reports [17,20,21,22].

Protocol amendments to existing studies and new protocols added to an existing IND may be initiated provided the protocol has been submitted to the FDA for review and the protocol has been approved by the responsible IRB. Protocol amendments intended to add new investigators may be batched and submitted at 30 day intervals [17]. Similarly, the FDA requests that information amendments be batched and as feasible submitted not more than every 30 days [20].

The regulations defining IND safety reports help to guide adverse event/reaction reporting for many regulatory groups. The sponsor must notify the FDA by phone or facsimile transmission within 7 days of receiving notification of any unexpected fatal or life-threatening experience associated with the use of the drug. In this regulation the following definitions exist:

- adverse event means any untoward medical occurrence associated with the use of a drug in humans, whether or not considered drug-related;

- suspected adverse reaction means any adverse event for which there is a reasonable possibility that the drug caused the adverse event,
- unexpected means the specificity or severity of the event is not consistent with the current IB or application on file with the FDA; and
- life-threatening means that the event placed the subject at immediate risk of death from the reaction (this is not inclusive of events that had they occurred in a more severe form might have caused death) [21].

Events that are serious and unexpected but not fatal or life-threatening should be submitted to the FDA by the sponsor within 15 days of notification. In addition to fatal and life-threatening events, the FDA includes the following three additional types of events in the category of serious:

- inpatient hospitalization or prolongation of an existing hospitalization;
- a persistent significant disability/incapacity (a disruption of a person's ability to conduct normal life functions); or
- a congenital anomaly/birth defect.

The FDA does allow that other important medical events may be considered serious if, based on medical judgment, they may jeopardize the subject and may require medical or surgical intervention to prevent one of the outcomes above. An example of such an event is an allergic bronchospasm. It is important to note that any findings from tests in laboratory animals that suggest a significant risk in humans should be reported to the FDA within 15 days of sponsor notification [21]. One issue that is specific to gene therapy studies relates to events identified during long-term follow-up. Such events may require expedited reporting to the FDA if they meet criteria specified above [23]. Adverse reactions requiring expedited reporting to the FDA can be submitted on FDA form 3500A, in a narrative form, or using the GeMCRIS report used to submit such events to NIH OBA [21].

More globally, adverse events/reactions of interest (and those that are to be excluded) should be collected and reported as defined in the clinical trial. Toxicities should be graded using the grading system specified in the clinical trial. A summary of adverse events that do not meet the criteria for expedited reporting as specified above should be reviewed by the sponsor and relevant adverse events/reactions should be submitted to the FDA, at least annually [21,22,24,25].

The IND sponsor is required to submit within 60 days of the anniversary date that the IND went into effect, a brief report of the progress of the investigation. If there is more than one study being conducted under the IND all study reports should be submitted on the IND anniversary regardless of when the studies were submitted to the FDA. The annual report should be organized by study and each study report should include the following:

- title of the study, purpose of the study, and a brief statement about the subject population;
- total number of subjects planned for inclusion, the number entered tabulated by age, gender, and race, the number whose participation was completed as planned, and the number who dropped out of the study for any reason;
- if the study is completed, or if interim results are available, any study results;
- a narrative or tabular summary showing the most frequent and most serious adverse experiences by body system;
- a summary of all IND safety reports during the past year;
- a summary of all subjects who died during participation in the study and the cause of death;
- a summary of subjects who dropped out of the investigation in association with an adverse experience (AE) and whether the AE was felt to be related to the study drug;
- a brief description of information obtained that is pertinent to understanding the drug's actions;
- a list of preclinical studies and findings from the past year;
- a summary of significant manufacturing or microbiological changes;
- a description of the general investigational plan for the next year;
- a revised IB (as appropriate);
- any significant updates in product development including updates from foreign marketing of the product;
- outstanding business with the FDA [22].

In addition, for gene-transfer studies the FDA requires annual submission of the following information:an updated GTMP;

- a list of all lots of all gene therapy products, cell banks (CBs), and viral banks (VBs), ever produced or generated in your facility for potential use in nonclinical or clinical studies of human gene therapy. Included should be the date of manufacture for each, their use (e.g. nonclinical or clinical), and their interrelationships, i.e. which CBs and/or VBs were used to prepare each CB, VB, or product lot;
- a list of all IND files that cross-reference the IND(s) or master file(s). In addition, the report should include confirmation of all IND(s) or master files that have been cross-referenced in support of the IND;
- all lot release data and characterization testing for each lot of product used in clinical trials, and testing information for all master CB, working CB, master VB, and/or working VB used during manufacture of your lots. When possible, this information should be submitted in tabular form including the lot number or identifier, date of manufacture, test, test method, the sensitivity and specificity of test methods when appropriate, specification, and test result;
- any lots of product produced for, but not used in, clinical studies and a description of the reason they were not used [13].

Protocol

The format of a gene therapy protocol and the related consent form will start with the basic framework of all such clinical trial documents. However, gene therapy protocols have some specific issues that must be addressed in the document.

Within the protocol itself the issue of long-term follow-up will need to be addressed. Basic guidelines for such follow-up are included in FDA guidance (see [23,26]). The protocol and the consent form will need to address issues such as the length of time subjects will be followed and the procedures involved in long-term follow-up. Appendix M [4] also requires that at the time of death, regardless of cause, an autopsy will be requested from the family. This issue of autopsy should also be included in the protocol and consent form [4].

The FDA does not require long-term follow-up observations following exposure to gene-transfer products when the risk of delayed adverse events is low. The FDA recommends that the sponsor consider existing preclinical and clinical data when assessing the risk of delayed adverse events. Table 8.1 includes guidance from the FDA on assessing the risk of delayed adverse events. In addition the FDA provides Table 8.2, which indicates the integration risk of commonly used vectors and the potential need for long-term follow-up. In those studies where the vector being utilized would indicate that long-term follow-up is required, the FDA recommends that 15 years is a suitable time for such follow-up. However, the FDA does allow the sponsor to propose a different plan for follow-up. Any such plan should be included in the IND submission with appropriate justification [26].

For studies utilizing retroviral vectors, the FDA recommends that monitoring include analysis of patient samples pretreatment and at 3 months, 6 months, and 1 year after treatment, and yearly thereafter. If all posttreatment assays are negative during the first year, the subsequent yearly samples should be archived. If any posttreatment samples are positive, further analysis of the RCR and more extensive patient follow-up should occur. In addition, it is important to note that positive results should be reported to the FDA as an IND safety report as per 21CFR312.32 and as discussed previously in this chapter. Further analysis of the RCR and more extensive patient follow-up should occur; however, the appropriate course of action should be discussed with CBER. Archived samples should be stored with appropriate safeguards as well as with a system that allows for linkage of the sample to study subject information and production lot records [23].

In addition to the annual sample collection, the FDA recommends that a brief clinical history be obtained annually. This history should assess outcomes suggestive of retroviral diseases focusing on emergence of:

- new malignancy(ies);
- new incidence or exacerbation of a pre-existing neurologic disorder;
- new incidence or exacerbation of a prior rheumatologic or other autoimmune disorder; and
- new incidence of a hematologic disorder [26].

The *NIH Guidelines* require that at the time of death, no matter what the cause, permission for

Table 8.1 Assessment of risk of delayed adverse events in a gene therapy study.

Question 1: Is your gene therapy product used only for *ex vivo* modification of cells?
If the answer is "no," go to Question 2. If the answer is "yes," go to Questions 3 and 4.

Question 2: Do preclinical study results show persistence of vector sequences?
If the answer is "no," the risk of gene therapy-related delayed adverse events is low, and long-term follow-up observations may not be needed. If the answer is "yes," go to Questions 3 and 4.

If it is unknown whether your vector persists, for the purpose of assessing risk, we recommend that you either assume that it does persist, or perform a preclinical study to assay for vector persistence in a relevant animal species. Please refer to Section IV.B, "Considerations for Preclinical Study Design to Assess Vector Biodistribution and Persistence," for help with preclinical trial design and details on the use and expected sensitivity of PCR assay for biodistribution studies. In assays performed after the final administration of vector, persistence is indicated by detectable levels of vector sequences above the threshold level in the PCR assay and absence of an apparent downward trend over several time points. In contrast, persistence is unlikely if you cannot detect vector sequences with a sensitive PCR assay or if the assay for vector sequences demonstrates a downward trend over time. We encourage you to consult with the Office of Cellular, Tissue and Gene Therapies (OCTGT; an office of the CBER) for specific advice about determination of persistence and biodistribution in your test system.

Question 3: Are vector sequences integrated?
If the answer is "no," go to Question 4. If the answer is "yes," we would require that clinical protocols with the product include clinical long-term follow-up observations.

Question 4: Does the vector have potential for latency and reactivation?
If the answer is "no," the risk is low that exposure to your gene-transfer technology will be followed by gene therapy-related delayed adverse events. Long-term follow-up observations may not be needed. If the answer is "yes," we would require that all your clinical protocols with the product include clinical long-term follow-up observations.

Source: US Food and Drug Administration [26].

Table 8.2 Integration properties of current commonly used gene therapy vectors in clinical trials.

Vector type	*Propensity to integrate*	*Long-term follow-up observations*
Plasmid	No	No
Poxvirus	No	No
Adenovirus	No	No
Adeno-associated virus	No	No
Herpes virus	No, but may undergo latency/ reactivation	Yes
Gammaretrovirus	Yes	Yes
Lentivirus	Yes	Yes

Source: US Food and Drug Administration [26].

an autopsy be requested from the families of subjects who have received a gene therapy product (Appendix M-III-B-2-c; oba.od.nih.gov/oba/rac/Guidelines/NIH_Guidelines.htm#_Toc351276263).

With this long-term follow-up, it is important that the protocol be clear regarding data collection and reporting criteria. The distinction between off-treatment and off-study criteria is therefore crucial. Standard criteria that can be utilized are outlined in the following subsections.

Off-Treatment Criteria

- Any patient who develops irreversible, life-threatening, or Grade 3–5 toxicity considered to be primarily related to the *investigational agent* will be taken off treatment. In such patients the toxicities will be followed until resolution and the patient will not be eligible to receive subsequent treatment with the investigational agent. However, response data will continue to be collected as applicable. Or,
- Any patient who receives therapy for relapse of their primary disease will be taken off treatment, adverse event data collection will cease, and the patient will not be eligible to receive subsequent treatment with the *investigational agent*. However, response data will continue to be collected as applicable.

Off-Study Criteria

- Patients who complete study-specified procedures;
- patients who refuse further study follow-up;
- patients who are lost to follow-up; or
- patients who die.

Consent Form

In the same way that a gene therapy protocol must include specific components that are particular to the science, the related consent form must include certain specific components as well. The consent form should include information about the gene (and the risks of the gene), the vector (and the risks of the vector), and the fact that this type of intervention is potentially irreversible. The NIH OBA has developed a document entitled *NIH Guidance on Informed Consent For Gene Transfer Research* [27] that contains considerable information on this issue. The document does include sample wording that can be used to describe the gene therapy or the type of vector to be used.

Some of the key points are summarized here.

- As possible a separate consent form should be used for the gene-transfer component of the research. If that is not feasible, the issues specific to the gene transfer (including the vector) should be included in separate sections throughout the consent form.
- NIH OBA recommends that investigators emphasize the speculative nature of benefit from gene therapy protocols.
- Risks of gene therapy studies should include (as appropriate) malignancies also seen in some other CD34 studies using retroviruses:
  - the added vector and/or gene could create changes in cells that could lead to cancer. In studies using retroviral vectors this section should include some discussion of the leukemia-like malignancy that occurred in French subjects enrolled on the X-linked severe combined immunodeficiency study and/or similar findings from other more recent or more related studies;
  - the added vector and/or gene could create permanent changes in cells that could be passed on to children conceived and born during or after study participation;
  - the added vector and gene could go to unexpected cells or tissues in the body;
  - the added vector could become able to reproduce itself, and the added vector and gene could be passed on to close contacts like an infection.
- Related to the risk of reproductive harm, the need for contraception should be discussed. Dependent on the risks it might be best to consider developing separate discussions of these issues in the consent form for male subjects as compared to female subjects.
- The anticipated length and related procedures for long-term follow-up should be included in the consent form.
- Subjects should be told that at the time of death their families will be asked to allow for autopsy.
- Subjects should be told that it is possible that the media might be interested in the study and in them, but that the investigators will protect their confidentiality [27].

Multisite Protocols

For all sites involved in clinical research projects, IRB and IBC review and approval is required prior to initiating any study subject interventions at that site. Although it is the responsibility of the local PI to obtain local IRB and IBC approval, it is the responsibility of the sponsor (the IND holder) to ensure that the approvals are in place.

Only one submission (IND) is made to the FDA for each protocol, but separate documentation is required for each additional site.

For each site, form 1572 and curriculum vitae or biosketches for all investigators listed on form 1572 must be obtained and submitted to the FDA. In addition, the following documents must be obtained and filed for each site:

- medical licenses (for physicians);
- proof of training in Human Subject Protection for all personnel involved in the clinical research;
- conflict-of-interest forms (3454 and 3455) for all investigators listed on form 1572;
- laboratory normals and certifications for each laboratory utilized by that site for research subjects;
- IRB approvals of initial submissions, amendments, AEs, and annual renewals;

- proof of training in protocol-specific activities for all personnel involved in the clinical research.

As with IND submissions, only one submission is made to the NIH OBA (RAC); however, if additional sites are to be included in the clinical trial it is necessary to notify NIH OBA of those sites and include in the submission:

- a copy of their IBC approval;
- IRB approval;
- their IRB-approved consent form;
- curriculum vitae of the investigator(s); and
- any applicable grant information.

This information can be submitted to NIH OBA by the PI of the new site or by the initial PI. In most cases ongoing communication with NIH OBA is handled by the initial PI.

Additionally, the monitoring activities and plans must be inclusive of all sites. QA/QC activities as discussed elsewhere in this chapter must include plans for monitoring research activities at all sites. The FDA has released a guidance document [28] that can be helpful in developing an approach to the required monitoring. Finally, data safety monitoring as discussed earlier in the chapter must include review of data from all sites.

Conclusion

Regulatory requirements for gene therapy studies are complex. All aspects of the project, from writing the protocol and consent, through study conduct, and long-term follow-up, must be developed and implemented with careful consideration of the gene-transfer aspects of the project. Larger programs can benefit through standardization of processes, development of SOPs, and utilization of a dedicated regulatory group familiar with the specific issues particular to gene therapy clinical trials.

As a rule of thumb, for a protocol that includes a novel gene or novel vector, or presents unique ethical issues, the regulatory processes as described above may take on average between 1.5 and 2 years to complete. For protocols that do not present these challenges, especially protocols being added to existing IND, the processes may be completed in 3–6 months.

The amount of infrastructure required to support each project will vary based on factors including the number of sites involved in the study, the length of time required to complete the study, the number of adverse reactions that occur in a study, and the number of times a study is amended. In each case, an increase in any one of those factors increases the amount of infrastructure that is required for successful completion of the project. The design and reporting structure for the required infrastructure can vary considerably from institution to institution. One example of the organizational chart developed to provide support for gene-transfer studies is shown as Figure 8.2.

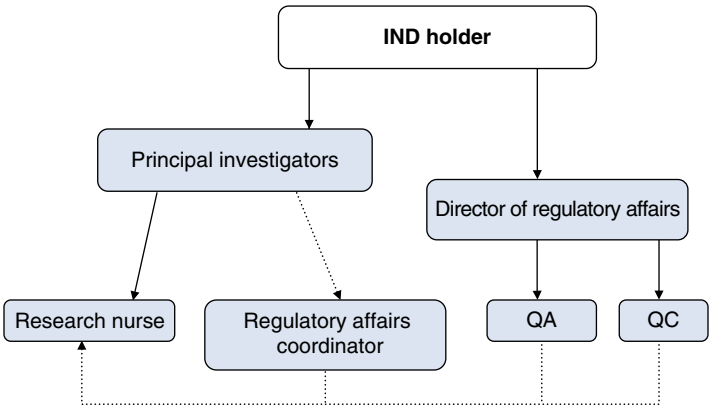


Figure 8.2 An example of the organizational chart developed to provide support for gene-transfer studies.

References

1 National Institutes of Health, Office of Biotechnology Activities (1999) *Gene Therapy Letter*. http://oba.od.nih.gov/oba/rac/genetherapyltr.pdf.

2 National Institutes of Health, Office of Biotechnology Activities (2009) *IBC Frequently Asked Questions of Interest to IBCs*. http://oba.od.nih.gov/oba/ibc/FAQs/IBC_Frequently_Asked_Questions7.24.09.pdf.

3 National Institutes of Health, Office of Biotechnology Activities (2013) *NIH Guidelines for Research Involving Recombinant DNA Molecules (NIH Guidelines)*. http://oba.od.nih.gov/rdna/nih_guidelines_oba.html.

4 National Institutes of Health, Office of Biotechnology Activities (2013) *Points to Consider in the Design and Submission of Protocols for the Transfer of Recombinant DNA Molecules into One or More Human Research Participants (Points to Consider)*. Appendix M of the NIH Guidelines. http://oba.od.nih.gov/oba/rac/Guidelines/NIH_Guidelines.htm#_Toc351276401.

5 National Institutes of Health, Office of Biotechnology Activities (2013) *RAC Frequently Asked Questions*. http://oba.od.nih.gov/rdna/rdna_faq.html.

6 US Department of Health and Human Services (2010) *Protection of Human Subjects: Criteria for IRB Approval of Research*. Title 45, Code of Federal Regulations, Part 46.111. www.hhs.gov/ohrp/humansubjects/guidance/45cfr46.html.

7 US Department of Health and Human Services (2010) *Protection of Human Subjects: Assuring Compliance with this Policy*. Title 45, Code of Federal Regulations, Part 46.103. www.hhs.gov/ohrp/humansubjects/guidance/45cfr46.html.

8 US Department of Health and Human Services (2007) *Guidance on Reviewing and Reporting Unanticipated Problems Involving Risks to Subjects and Others and Adverse Events*. www.hhs.gov/ohrp/policy/advevntguid.html#AB.

9 US Department of Health and Human Services (2010) *Protection of Human Subjects: IRB Review of Research*. Title 45, Code of Federal Regulations, Part 46.109. www.hhs.gov/ohrp/humansubjects/guidance/45cfr46.html.

10 US Food and Drug Administration (2000) *Guidance for Industry: Formal Meetings with Sponsors and Applicants for PDUFA Products*. www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM079744.pdf.

11 US Food and Drug Administration (2013) *Investigational New Drug Application: Informing Investigators*. Title 21, Code of Federal Regulations, Part 312.55. www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=312&showFR=1.

12 US Food and Drug Administration (2009) *Certifications to Accompany Drug, Biological Product, and Device Applications/Submissions*. www.fda.gov/RegulatoryInformation/Guidances/ucm125335.htm.

13 US Food and Drug Administration (2000) *Dear Gene Therapy IND or Master File Sponsor Letter*. www.fda.gov/BiologicsBloodVaccines/SafetyAvailability/ucm105882.htm.

14 National Institutes of Health (1998) *NIH Policy for Data and Safety Monitoring*. http://grants.nih.gov/grants/guide/notice-files/not98-084.html.

15 National Institutes of Health (2000) *Further Guidance on a Data and Safety Monitoring for Phase I and Phase II Trials*. http://grants.nih.gov/grants/guide/notice-files/NOT-OD-00-038.html.

16 US Food and Drug Administration (2013) *Guidance for Clinical Trial Sponsors - Establishment and Operation of Clinical Trial Data Monitoring Committees*. www.fda.gov/RegulatoryInformation/Guidances/ucm127069.htm.

17 US Food and Drug Administration (2013) *Investigational New Drug Application: Protocol Amendments*. Title 21, Code of Federal Regulations, Part 312.30. www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=312&showFR=1.

18 US Food and Drug Administration (2013) *Investigational New Drug Application: General Requirements for Use of an Investigational New Drug in a Clinical Investigation*. Title 21, Code of Federal Regulations, Part 312.40. www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=312&showFR=1.

19 US Food and Drug Administration (2013) *Investigational New Drug Application: Clinical Holds and Requests for Modification*. Title 21, Code of Federal Regulations, Part 312.42. www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=312&showFR=1.

20 US Food and Drug Administration (2013) *Investigational New Drug Application: Information Amendments*. Title 21, Code of Federal Regulations, Part 312.31. www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=312&showFR=1.

21 US Food and Drug Administration (2013) *Investigational New Drug Application: IND Safety Reporting*. Title 21, Code of Federal Regulations, Part 312.32. www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=312&showFR=1.

22 US Food and Drug Administration (2013) *Investigational New Drug Application: Annual Reports*. Title 21, Code of Federal Regulations, Part 312.33. www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=312&showFR=1.

23 US Food and Drug Administration (2009) *Guidance for Industry: Supplemental Guidance on Testing for Replication Competent Retrovirus in Retroviral Vector Based Gene Therapy Products and During Follow-up of Patients in Clinical Trials Using Retroviral Vectors*.

www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/CellularandGeneTherapy/ucm072961.htm.

24 US Food and Drug Administration (2011) *E2F Development Safety Update Report*. www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm073109.

25 US Food and Drug Administration (2012) *Safety Reporting Requirements for INDs and BA/BE Studies*. www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM227351.pdf.

26 US Food and Drug Administration (2012) *Guidance for Industry: Gene Therapy Clinical Trials – Observing Subjects for Delayed Adverse Events*. www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/CellularandGeneTherapy/ucm072957.htm.

27 National Institutes of Health, Office of Biotechnology Activities (n.d.) *NIH Guidance on Informed Consent for Gene Transfer Research*. http://oba.od.nih.gov/oba/rac/ic/index.html.

28 US Food and Drug Administration (2013) *Guidance for Industry: Oversight of Clinical Investigations – A Risk-Based Approach to Monitoring*. www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM269919.pdf.

9

CHAPTER 9

Remaining Obstacles to the Success of Cancer Gene Therapy

Malcolm K. Brenner
Baylor College of Medicine, Houston, TX, USA

Introduction

Cancer gene therapy has been long in development but short on success. For gene-modified cell therapies of cancer, the approach seems finally to be moving from the category of "promising" to "effective;" the first ever genetically modified dendritic cell vaccine, Provenge, was shown to be both safe and effective for the treatment of advanced prostate cancer [1], while autologous T cells that were genetically modified to express chimeric antigen receptors (CARs) directed to the tumor-associated antigen CD19 [1,2,3] have produced dramatic responses in patients with acute and chronic B-cell malignancies. It has, however, taken longer for approaches using direct delivery of transgenes – the focus of the current volume – to become licensed agents or to show equivalent dramatic benefits. Nonetheless, the first randomized study of an oncolytic virus/vaccine has now shown clear evidence for a dose-dependent clinical benefit [4] and as will be apparent from many of the chapters in this book, a multitude of other nucleotide-delivery approaches are in early- to late-stage clinical development. As we progressively define the strengths and weaknesses of each direct approach we can reasonably expect progress to continue and more licensed agents to emerge.

Gene therapies are appealing because they are targeted, and so have the potential for higher specificity and lower toxicity than the blunt instruments of chemotherapy, radiotherapy, and surgery. Why then has it proved so stubbornly difficult to ensure these agents become licensed and a standard of care for cancer? There are undoubtedly scientific complexities and barriers to success, and these have been outlined in the relevant chapters. But there are also more conceptual and structural/economic barriers to successful exploitation, and these must be clearly identified and understood if we are ever to take full advantage of gene therapy for cancer.

Drug-Development Pathways

Most gene therapeutics are examples of complex biologics or, in European Union parlance, advanced therapy medicinal products (ATMPs) [5]. As a consequence their development is distinct from the standard pharmaceutical model. While these distinctions are most apparent when gene transfer is used to modify cells *ex vivo* and the altered cell rather than the transgene/vector is the final product, differences in the drug-development pathway are evident for almost all the complex biologics described in this volume, except perhaps for the oligonucleotides described in Chapters 4 and 6.

The standard pharmaceutical business model for small-molecule drugs is to spend increasing amounts of money on linear phases of drug development; phase I (toxicity), phase II (early efficacy), phase III

Cancer Gene Therapy by Viral and Non-viral Vectors, First Edition. Edited by Malcolm K. Brenner and Mien-Chie Hung.

(larger-scale, usually randomized efficacy studies), and sometimes phase IV (postregistration/postmarketing studies). If the toxicity of a drug is too great, or it fails to reach efficacy endpoints at any stage, it is usually abandoned. If the drug is successful, however, companies then recoup the – often vast – sums of money spent on development by selling cheap-to-manufacture goods with exceedingly high margins. For most complex biological agents, however, the cost of goods remains high, even after approval and scale-up. Worse, the same complexity that renders these agents difficult and expensive to make also means that it is often necessary to perform iterative early-phase clinical studies with each product, making minor modifications to every subcomponent of the agent to enhance overall safety and potency, rather than the more conventional linear drug development. This process, with its unpredictable timelines and unknowable duration, makes financial structuring of the project difficult for biotechnology and major pharmaceutical companies alike. The problem is compounded because the associated intellectual property embedded in a complex biologic is usually equally complex and diversely held, leading to lengthy negotiations over cross-licensing and the payment of stacked royalties, complexities that may prove insuperable when multiple investigators, companies, and countries are involved. Finally, the very specificity of these therapies means that only a small subset of patients with any given cancer may be suited to treatment, making every gene therapy an orphan drug. In combination, these market issues can lead to an unaffordable pricing structure with little appeal to major pharmaceutical companies.

Offsetting the above commercial constraints is the increasing acceptance of the idea among physicians and drug companies alike that the future of cancer drug development will consist of creating medicines, such as gene therapies, that are highly effective cures for identified subsets of patients rather than unpredictable palliatives that can be used with only modest benefit for a larger number of patients. Once these potent targeted therapies show superior therapeutic activities it will be possible to complete pivotal (licensing) studies faster and with far smaller numbers of patients than required for conventional drugs. Since these new and targeted agents will have vastly improved pharmaco-economics in terms of quality and length of life, they can be priced at a premium and the more limited development costs will be readily recovered.

Broadening the Appeal of Complex Biologics

To change the status of complex biologics for cancer and make them broadly applicable we need to accomplish four tasks. The first is to show that these more complex biological approaches have significantly greater qualitative benefits compared to any other available therapies. In other words, these therapies must clearly be shown to ameliorate, and ideally cure, diseases that are otherwise not amenable to conventional treatment. Simply briefly extending life or slowing disease progression by a few weeks will almost always (*pace* Provenge) be insufficient to convince individuals and companies to commit the necessary resources. Moreover, there needs to be at least some evidence of these qualitatively superior activities even in phase I (safety) clinical studies, otherwise phase II (efficacy) studies will rarely be implemented. An excellent recent example of this requirement for superior results during phase I study is the recently reported studies of CD19 CAR T cells, which helped dramatize the potency of the approach for a wider public [2]. The combination of these data with other dramatic results for complex biologics in cancer [3,4,5,6,7,8,9,10,11] has formed a cluster of success that has finally helped convince the broader community of the potential value of the approach.

The second task is to make complex biologics as broad as possible, by designing them in such a way as to allow a single vector/oncolytic virus or transgene to be minimally modified (for example, by changing the vector targeting or route of administration) to permit application to as many disorders as possible. Such a building-block approach means that it should not be necessary to completely restart the product-development clock every time a slightly different patient population is targeted.

It is also necessary to ensure that complex biologics are manufactured by processes that are both scalable and robust. By including process development from the earliest phases of clinical testing of complex biologics we can avoid a requirement for building ever larger teams of highly skilled and experienced research technicians and investigators to implement any study for each individual at every center, and will

have the option of rapid transition into larger pivotal or licensing studies.

Finally, gene transfer must be shown to be safe. As the disproportionate response to the adenoviral-vector-associated fatality at U.Penn revealed over a decade ago [6], there is a low societal tolerance for severe adverse effects from genetic modifications. Indeed, such intolerance may be well justified since gene transfer, unlike most small-molecule drugs, may produce unwanted consequences that persist and indeed progressively worsen over time. Although not addressed specifically in earlier chapters in this book, investigators have therefore developed suicide, or safety, gene systems which allow an "exit strategy" from a gene therapy should severe adverse events occur. The first and most widely used suicide gene is the herpes simplex virus-derived thymidine kinase gene (HSV *TK*). Incorporation of this gene in a vector can destroy gene-modified cells and prevent toxic effects from vector replication or abnormalities in function of the transduced cell. For example, the adoptive transfer of T cells after allogeneic stem cell transplantation has accelerated immune reconstitution, reducing viral disease and perhaps leukemic relapse [7]. If these cells are transduced to express HSV *TK*, then any graft-versus-host disease (GvHD) resulting from their alloreactivity can be abrogated by administration of nucleoside analogues, such as ganciclovir, which are phosphorylated by the HSV *TK* and block DNA synthesis. This suicide gene approach has successfully removed alloreactive cells and ablated GvHD, while sparing the desired antiviral and anti-leukemic activity of the adoptively transferred T cells [7]. The overall safety and efficacy of this approach is now being established in a pivotal multicenter phase II clinical trial.

Despite the undoubted value of the HSV *TK*/ganciclovir suicide system, the approach has limitations that may limit the ultimate range of applications. Because cell toxicity is produced by inhibiting DNA synthesis, actively dividing cells are the most susceptible. This selectivity may be beneficial, for example by sparing non-alloreactive (that is, nondividing) T cells when treatment is given to terminate GvHD and thereby sparing T cells that may be directed to viruses or other infectious organisms, but under other circumstances, and for other cell types, the discrimination may be less helpful. For example, the system cannot readily eliminate postmitotic cell populations, and the mode of action means cell killing may be delayed for days or even weeks, a major concern if toxicities are acute and severe. As an alternative, therefore, investigators have developed the inducible caspase-9 system that relies on dimerization of a semi-synthetic inducible caspase-9 molecule using an otherwise bioinert small molecule, leading in turn to cleavage and activation of endogenous caspase 3 and the rapid onset of apoptosis [8]. This approach works within minutes of administration of the dimerizing agent and may be effective irrespective of whether the target cell population is dividing or postmitotic [9].

Clinical Trial Design and Execution

None of the requirements for successful biologic development and licensing described above will be met unless there are concomitant improvements to clinical trial design and evaluation in patients with cancer, since there are currently significant challenges using available approaches. Endpoints that are standard for most small-molecule therapeutic studies in cancer, such as tumor shrinkage at 4–6 weeks, may be unsuited to some complex biologic agents that may induce initial tumor inflammation with apparent progression by imaging, or may produce a prolonged stabilization of tumor without shrinkage or eradication, so that patients live with, rather than die from, their disease. While such benefits to long-term survival may be significant, they greatly increase the cost and time of clinical studies, as well illustrated by the development of Provenge/Sipuleucel-T [10], so far the only gene/cell therapeutic to have received a product license in the USA. In addition, many gene therapies function by recruiting the immune system, and may be particularly unsuited to evaluation in the classical phase I setting of advanced disease, since immunity will likely be disrupted by both the disease and its treatment.

Because of their lack of similarity to conventional pharmaceuticals (see Drug-Development Pathways above), many of these initial clinical studies will continue to be executed by academic investigators and then (ideally) seamlessly selected for commercial development. Such a process requires a cadre of clinical research investigators with access to an infrastructure that is adequate to follow the Good

Manufacturing Practices required to prepare and distribute the cellular and vector products. This infrastructure must also ensure that even early-phase studies are performed to meet Good Clinical Practice standards, a requirement made more difficult by the unusually complex regulatory requirements surrounding gene-transfer studies (see Chapters 7 and 8). The magnitude of the manufacturing and clinical support required means that individual investigators in the field cannot be expected to develop the necessary infrastructure. Fortunately, in the USA the National Institutes of Health uses a variety of mechanisms to support the manufacturing of vectors and of cells for small-scale clinical trials and the impact of these infrastructure-support programs on investigator-initiated trials of T-cell therapies has been substantial. The European Union and Japan have developed alternative approaches that are also government-sponsored but there is no doubt that their further enhancement would accelerate progress in this field.

Conceptual Limitations

Although gene therapy investigators working with inherited monogenic disorders share most of the development-related problems described above for cancer gene therapy investigators, their achievements have clearly been more substantive [11,12]. Other, conceptual, factors must therefore be invoked to explain the more modest accomplishments of cancer gene therapy. The gene therapy approaches described in this book are directed at the tumor cells and their microenvironment and/or host cells to increase resistance to the disease (Table 9.1). Although the successes of gene therapy for direct correction of monogenic disorders would seem to favor direct attack on the tumor cells for cancer as well, this has in reality proved exceedingly difficult. In the successfully treated inherited monogenic disorders, a single transgenic construct can be used to replace or supplement a well-defined single genetic abnormality. While cancer is indeed an (acquired) genetic disorder, the abnormalities are polygenic. Moreover, there is substantial genetic heterogeneity [13,14], not just between tumors in different individuals but also in tumors at different sites within the same patient. Unless it were possible to identify a universal corrective gene, efforts to use gene transfer to correct abnormal genetic function in cancer cells would require access to multiple transgenes, individually tailored for optimum use between and even within a single individual. Moreover, clinical correction of most monogenic disorders often requires only a small proportion of the targeted cells to be successfully transduced with the relevant transgene. In disorders in which the missing gene produces a secreted protein (e.g. hemophilia B), less than 10% of normal levels of the product may restore health, and if the transgene is secreted from each cell at supranormal levels then the number of cells actually transduced may be far lower than 10% of the targeted population [15]. For treatment of malignancy, of course, such low levels of corrective or destructive gene transfer to tumor tissue would have negligible impact on the course of the disease. At present, therefore, direct gene transfer to tumor cells will likely only be a curative option when a high proportion of tumor cells is transduced, for example by a conditionally replication-competent viral vector that is then able to induce an antitumor immune response able to kill the remaining tumor cells that have failed to be infected.

Table 9.1 Therapeutic applications of cancer gene therapy.

Therapeutic application	*Comments*
Gene repair	Correction of genetic defects associated with the malignant process; hampered by low efficiency of gene transfer
Prodrug-metabolizing enzyme gene therapy	Renders the tumor cells sensitive to corresponding cytotoxic agent
Viral oncolysis	Delivery of viruses which selectively replicate in tumor cells
Modulation of the tumor microenvironment	Inhibiting angiogenesis or tumor cell proteinases
Drug-resistance gene therapy	Prevention of toxic side effects of chemotherapeutic agents
Immunotherapy with genetically modified cells	Generating or boosting immune responses to tumor antigens by genetically modifying effector cells, antigen-presenting cells, or tumor cells

Table 9.2 Advantages and disadvantages of cancer gene therapy vectors.

Vector	*Advantages*	*Disadvantages*	*Cancer gene therapy application*
Adenovirus	Poxviruses and vaccinia virus	High titer, broad tropism Accepts large insert size	Complex viral genome Replicates in target cell Immunogenic*
Adeno-associated virus	High titer, broad tropism Efficient gene transfer Transduces nondividing cells Limited immunogenicity	Limited insert size	PDME gene therapy Modulation of the tumor microenvironment Immunotherapy
Herpesvirus	High titer Accepts large insert size	Complex viral genome May be cytotoxic to cells	Viral oncolysis PDME gene therapy
Lentivirus	Stable genome integration† Integrates into nondividing cells Long-term gene expression Low immunogenicity	Risk of insertional mutagenesis Inefficient gene delivery *in vivo*	Gene repair PDME gene therapy Drug-resistance gene therapy Gene marking Immunotherapy
Poxviruses and vaccinia virus	High titer, broad tropism Accept large insert size	Complex viral genome Replicate in target cell Immunogenic*	Viral oncolysis Immunotherapy
Retrovirus	Stable genome integration† Long-term gene expression Low immunogenicity	Integrations only in dividing cells Limited insert size Risk of insertional mutagenesis Inefficient gene delivery *in vivo*	Gene repair PDME gene therapy Drug-resistance gene therapy, Gene marking Immunotherapy
Non-viral DNA delivery	Non-viral Accepts large insert size Low immunogenicity	Transient gene expression Inefficient gene delivery *in vivo*	Gene repair Modulation of the tumor microenvironment Immunotherapy

*Immunogenicity is considered advantageous for many cancer immunotherapy applications.
†Integration of vector sequences into the genome is a characteristic of retroviruses, lentiviruses, and to a limited extent of adeno-associated viruses; depending on the application this may be an advantage or disadvantage.
PDME, prodrug-metabolizing enzyme.

Since most gene-transfer vectors (Table 9.2) have insufficient infectivity or inadequately broad biodistribution to be able to transfer the desired corrective or destructive genes to every cancer cell, it will likely be necessary either to combine these approaches with targeted small molecules or similarly recruit an effective innate/adaptive immune response that will amplify and sustain any of the approaches to gene therapy described in this volume. Ultimately, it is likely that multiple non-cross-resistant therapies – including gene therapy – will need to be combined to trigger a cascade of events that will eradicate, or at least fully stabilize, the tumor.

Conclusions

Although just two licensed cancer gene therapy products have appeared over the past 20 years, there is now a confluence of forces that should ensure substantially greater success over the next 5 years. Among commercial groups, there is an understanding that advances in our understanding of cancer biology make it imperative to develop more individualized targeted therapies that will have high efficacy in identifiable patient subpopulations, and whose superior pharmaco-economics will justify sufficient reimbursement to permit

development and distribution. These advances, coupled with an improved understanding of vaccine technologies (including virotherapies) and the availability of monoclonal antibodies able to block signals that downregulate the immune response, form a potent triangulation of forces. The major challenge facing the field may therefore soon become the need to ensure there is a sufficiently large cadre of skilled therapists available to deliver these novel products as they emerge from clinical trials.

References

1 Kalos M, Levine BL, Porter DL, Katz S, Grupp SA, Bagg A, June CH. (2013) T cells with chimeric antigen receptors have potent antitumor effects and can establish memory in patients with advanced leukemia. *Science Translational Medicine* 3: 95ra73.

2 Brentjens RJ, Davila ML, Riviere I, Park J, Wang X, Cowell LG, et al. (2013) CD19-targeted T cells rapidly induce molecular remissions in adults with chemotherapy -refractory acute lymphoblastic leukemia. *Science Translational Medicine* 5: 177ra38.

3 Kochenderfer JN, Dudley ME, Feldman SA, Wilson WH, Spaner DE, Maric I, et al. (2012) B-cell depletion and remissions of malignancy along with cytokine-associated toxicity in a clinical trial of anti-CD19 chimeric-antigen-receptor-transduced T cells. *Blood* 119: 2709–2720.

4 Heo J, Reid T, Ruo L, Breitbach CJ, Rose S, Bloomston M, et al. (2013) Randomized dose-finding clinical trial of oncolytic immunotherapeutic vaccinia JX-594 in liver cancer. *Nature Medicine* 19: 329–336.

5 Maciulaitis R, D'Apote L, Buchanan A, Pioppo L, Schneider CK. (2012) Clinical development of advanced therapy medicinal products in Europe: evidence that regulators must be proactive. *Molecular Therapy* 20: 479–482.

6 Raper SE, Chirmule N, Lee FS, Wivel NA, Bagg A, Gao GP, et al. (2003) Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. *Molecular Genetics and Metabolism* 80: 148–158.

7 Ciceri F, Bonini C, Stanghellini MT, Bondanza A, Traversari C, Salomoni M, et al. (2009) Infusion of suicide-gene-engineered donor lymphocytes after family haploidentical haemopoietic stem-cell transplantation for leukaemia (the TK007 trial): a non-randomised phase I-II study. *Lancet Oncology* 10: 489–500.

8 Straathof KC, Pule MA, Yotnda P, Dotti G, Vanin EF, Brenner MK, et al. (2005) An inducible caspase 9 safety switch for T-cell therapy. *Blood* 105: 4247–4254.

9 Di Stasi A, Tey SK, Dotti G, Fujita Y, Kennedy-Nasser A, Martinez C, et al. (2011) Inducible apoptosis as a safety switch for adoptive cell therapy. *New England Journal of Medicine* 365: 1673–1683.

10 Kantoff PW, Higano CS, Shore ND, Berger ER, Small EJ, Penson DF, et al. (2010) Sipuleucel-T immunotherapy for castration-resistant prostate cancer. *New England Journal of Medicine* 363: 411–422.

11 Aiuti A, Cattaneo F, Galimberti S, Benninghoff U, Cassani B, Callegaro L, et al. (2009) Gene therapy for immunodeficiency due to adenosine deaminase deficiency. *New England Journal of Medicine* 360: 447–458.

12 Hacein-Bey-Abina S, Hauer J, Lim A, Picard C, Wang GP, Berry CC, et al. (2010) Efficacy of gene therapy for X-linked severe combined immunodeficiency. *New England Journal of Medicine* 363: 355–364.

13 Yates LR, Campbell PJ. (2012) Evolution of the cancer genome. *Nature Reviews Genetics* 13: 795–806.

14 Swanton C. (2012) Intratumor heterogeneity: evolution through space and time. *Cancer Research* 72: 4875–4882.

15 Nathwani AC, Tuddenham EG, Rangarajan S, Rosales C, McIntosh J, Linch DC, et al. (2011) Adenovirus-associated virus vector-mediated gene transfer in hemophilia B. *New England Journal of Medicine* 365: 2357–2365.

Index

Cancer Gene Therapy by Viral and Non-viral Vectors, First Edition. Edited by Malcolm K. Brenner and Mien-Chie Hung.