Nicola Brunetti-Pierri *Editor*

Safety and Efficacy of Gene-Based Therapeutics for Inherited Disorders

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Editor
Nicola Brunetti-Pierri
Department of Translational Medicine
University of Naples Federico II
Naples
Italy

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Contents

Gene Transfer Strategies and Applications in Genetic Diseases 1
Nicola Brunetti-Pierri

Safety and Efficacy of Retroviral and Lentiviral Vectors for Gene Therapy 9
Daniela Cesana, Monica Volpin, Yasmin Natalia Serina Secanechia and Eugenio Montini

Adenovirus Vector Toxicity 37
Andrew W. Harmon and Andrew P. Byrnes

Helper-Dependent Adenoviral Vectors for Gene Therapy of Inherited Diseases 61
Pasquale Piccolo and Nicola Brunetti-Pierri

AAV Vector-Based Gene Therapy, Progress and Current Challenges 77
Klaudia Kuranda and Federico Mingozzi

Physical Methods of Gene Delivery 113
María José Herrero, Luis Sendra, Antonio Miguel and Salvador F. Aliño

Designer Effectors for Editing and Regulating Complex Genomes 137
Maximilian Müller, Tafadzwa Mlambo and Claudio Mussolino

siRNA Therapeutics to Treat Liver Disorders 159
Chengjung Lai, Cristina Martin-Higueras and Eduardo Salido

Oligonucleotide Therapy 191
Michela Alessandra Denti and Giuseppina Covello

Index 219

Editor and Contributors

About the Editor

Nicola Brunetti-Pierri graduated in medicine from the University of Naples Federico II, Italy. After his residency in pediatrics at Federico II University of Naples, he moved to Baylor College of Medicine, Houston, USA, for a postdoctoral research fellowship and clinical trainings in medical genetics and biochemical genetics. He is currently an associate professor of pediatrics at Federico II University, Naples, Italy, and an associate investigator at the Telethon Institute of Genetics and Medicine (TIGEM), Pozzuoli, Italy. As a pediatrician and geneticist, he studies genetic diseases and inborn errors of metabolism, and his research focuses on developing novel therapies for these disorders.

Contributors

Salvador F. Aliño Departamento de Farmacología, Facultad de Medicina, Universidad de Valencia, Valencia, Spain; Unidad de Farmacología Clínica, Hospital Universitario y Politécnico La Fe, Valencia, Spain

Nicola Brunetti-Pierri Telethon Institute of Genetics and Medicine, Pozzuoli (Napoli), Italy; Department of Translational Medicine, Federico II University of Naples, Naples, Italy

Andrew P. Byrnes Division of Cellular and Gene Therapies, FDA Center for Biologics Evaluation and Research, Silver Spring, MD, USA

Giuseppina Covello RNA Biology and Biotechnology Laboratory, Centre for Integrative Biology, University of Trento, Trento, Italy

Daniela Cesana San Raffaele Telethon Institute for Gene Therapy, San Raffaele Scientific Institute, Milan, Italy

Michela Alessandra Denti RNA Biology and Biotechnology Laboratory, Centre for Integrative Biology, University of Trento, Trento, Italy

Andrew W. Harmon Division of Cellular and Gene Therapies, FDA Center for Biologics Evaluation and Research, Silver Spring, MD, USA

María José Herrero Departamento de Farmacología, Facultad de Medicina, Universidad de Valencia, Valencia, Spain; Instituto de Investigación en Salud La Fe, Valencia, Spain

Klaudia Kuranda University Pierre and Marie Curie-Paris 6, Paris, France

Chengjung Lai Dicerna Pharmaceuticals, Cambridge, MA, USA

Cristina Martin-Higueras Instituto de Tecnologías Biomédicas (ITB), Centro de Investigaciones Biomédicas de Canarias (CIBICAN), Centre for Biomedical Research on Rare Diseases (CIBERER), Universidad La Laguna, Tenerife, Spain

Antonio Miguel Departamento de Farmacología, Facultad de Medicina, Universidad de Valencia, Valencia, Spain

Federico Mingozzi Genethon, Evry, France; University Pierre and Marie Curie-Paris 6, Paris, France; INSERM UMR951, Evry, France

Tafadzwa Mlambo Institute for Cell and Gene Therapy and Center for Chronic Immunodeficiency, University Medical Center Freiburg, Freiburg, Germany; Spemann Graduate School of Biology and Medicine (SGBM), University of Freiburg, Freiburg, Germany; Faculty of Biology, University of Freiburg, Freiburg, Germany

Eugenio Montini San Raffaele Telethon Institute for Gene Therapy, San Raffaele Scientific Institute, Milan, Italy

Claudio Mussolino Institute for Cell and Gene Therapy and Center for Chronic Immunodeficiency, University Medical Center Freiburg, Freiburg, Germany

Maximilian Müller Institute for Cell and Gene Therapy and Center for Chronic Immunodeficiency, University Medical Center Freiburg, Freiburg, Germany; Faculty of Biology, University of Freiburg, Freiburg, Germany

Pasquale Piccolo Telethon Institute of Genetics and Medicine, Pozzuoli (Napoli), Italy

Eduardo Salido Instituto de Tecnologías Biomédicas (ITB), Centro de Investigaciones Biomédicas de Canarias (CIBICAN), Centre for Biomedical Research on Rare Diseases (CIBERER), Universidad La Laguna, Tenerife, Spain; Department of Pathology, Universidad de La Laguna School Medicine, Tenerife, Spain

Yasmin Natalia Serina Secanechia San Raffaele Telethon Institute for Gene Therapy, San Raffaele Scientific Institute, Milan, Italy; Vita Salute San Raffaele University, Milan, Italy

Luis Sendra Departamento de Farmacología, Facultad de Medicina, Universidad de Valencia, Valencia, Spain

Monica Volpin San Raffaele Telethon Institute for Gene Therapy, San Raffaele Scientific Institute, Milan, Italy; Vita Salute San Raffaele University, Milan, Italy

Gene Transfer Strategies and Applications in Genetic Diseases

Nicola Brunetti-Pierri

Abstract Gene-based therapies are emerging as safe and effective treatments for genetic diseases. In this introductory chapter, we provide a general overview of the gene therapy principles and vectors for applications in inherited diseases. Next, we discuss some of the most relevant gene therapy clinical trials.

Keywords Monogenic · Multifactorial · European Medicines Agency (EMA) · Gene addition · Gene reprogramming · Gene supply · Gene repair · Vectors · RNA · Immunodeficiency · Toxicity · Insertional oncogenesis

Introduction

Gene therapy is the treatment or cure of human diseases by transfer of nucleic acids (DNA or RNA) to cells and tissues. Gene therapy covers a broad spectrum of applications ranging from monogenic disorders to acquired diseases such as cancer, infectious, autoimmune, and cardiovascular diseases. Gene therapy has been initially conceived for inherited diseases, but the concept of treating disorders by gene transfer broadened to several other types of acquired or multifactorial disorders. Nevertheless, inherited diseases have set the ground for applications of gene therapy in the clinic and in non-genetic diseases.

N. Brunetti-Pierri (✉)
Telethon Institute of Genetics and Medicine, Via Campi Flegrei 34,
80078 Pozzuoli (Napoli), Italy
e-mail: brunetti@tigem.it

N. Brunetti-Pierri
Department of Translational Medicine, Federico II University
of Naples, Naples, Italy

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Although the concept of gene therapy is simple, its translation into practice has been difficult mostly because of the toxicity deriving from the complex and often unforeseen vector-host interactions (see the chapter 'Adenovirus Vector Toxicity' for adenoviral vector host interactions). The field of gene therapy has been expanding tremendously in the last few years, several obstacles have been overcome, and a growing body of information has been developed for various types of disorders. The first clinical gene therapy trials rose concerns and skepticism over the further employment of this strategy. However, these attitudes are radically changing. A number of phase I/II gene therapy clinical trials have shown remarkable evidence of efficacy and safety for the treatment of various severe inherited diseases of the immune system, blood, brain, and eye such as primary immunodeficiencies, thalassemia, hemophilia, leukodystrophies, and retinal dystrophy. Approvals by the European Medicines Agency of the gene therapy products Glybera and Strimvelis for treatment of lipoprotein lipase deficiency and ADA severe combined immunodeficiency (SCID), respectively, are important first steps in gene-based drug development and have sparkled new enthusiasm in the field [1, 2].

Gene Therapy Principles

Gene therapy approaches can be divided into: (a) *gene addition* (also called *gene replacement* or *gene augmentation*), (b) *gene reprogramming*, (c) *gene supply*, and (d) *gene repair*. *Gene addition* relies on delivery of a corrected copy of the defective gene without removal of the endogenous mutated gene and is well suited for disorders due to loss-of-function mutations. However, this approach is not effective when the gene product requires controlled cell-specific expression or in disorders due to gain-of-function mutations. In contrast, *gene reprogramming* is based on modifications of messenger RNA (mRNA) levels by inhibiting expression of the mutated gene. The *gene supply* is the addition of a gene which does not correspond to the mutated gene, but its expression in the diseased cells or tissues can prevent or arrest disease progression, e.g., neurotrophic factors in neurodegenerative disorders or angiogenic factors for vascular ischemic diseases. In contrast to adding copies of a gene to cells or modifying mRNA levels, *gene repair* seeks to correct mutant sequences in the genomic DNA. This strategy had a tremendous development in the last few years and is based on chimeric proteins composed of a DNA-sequence-specific binding domain and endonucleases inducing site-specific DNA double-strand breaks. These technologies including zinc-finger nucleases (ZFN), transcription activator-like effector nucleases (TALEN), and clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 (reviewed in the chapter 'Designer Effectors for Editing and Regulating Complex Genomes') have been developed more recently and are still relatively immature for clinical applications. Nevertheless, they have potential for becoming the next generation of gene therapy and pioneering applications in the clinic are under development. Targeted genome editing mediated by ZFN performed ex vivo to induce knockout of the *CCR5* gene encoding for human immunodeficiency virus co-receptor has shown safety in a human clinical trial [3].

Several vectors have been investigated for gene therapy, and so far no single vector system has yet emerged as clearly superior to the others for all applications. The choice of the vector delivery system is generally dictated by the nature of the disease and by the target tissue. In its first applications, gene therapy has been performed ex vivo and cells isolated from the organism were genetically modified outside the body and then re-introduced back into the same organism. This approach continues to be used successfully in autologous transplantation of genetically modified cells for therapy of blood and immune disorders. Gene transfer is achieved in vivo by direct administration of the vector to a specific tissue or organ, e.g., systemic intravenous injections or localized delivery, such as airway administration, intramuscular, intracerebral or sub-retinal injections. The choice between ex vivo or in vivo gene transfer depends on the disease and on the target cell or tissue to be treated.

Vectors for Gene Therapy of Human Inherited Diseases

For gene addition, reprogramming or repair, the efficacy of the treatment heavily relies on the ability of vectors to transfer their nucleic acid content to the nucleus of the target cells. Vectors can be derived from viruses or can be non-viral. Viral vectors have been developed by exploiting the natural property of viruses to transfer their genetic material into the nucleus of the infected cells. The host cell transcription and translation machineries are exploited by the vector for production of its transgene (i.e., the therapeutic gene). To turn an infectious agent into a safe and effective gene therapy vector, the viral genes supporting viral replication and expression of cytotoxic viral proteins have to be eliminated, whereas the ability of the virus to infect the target cells has to be retained. Practically, this is achieved by deleting most if not all the coding sequences of the viral genome, leaving intact the sequences that are required *in cis* for packaging of the vector genomes into the viral capsids. Viral vectors are produced in cell factory systems in which the essential components of the virus such as the structural proteins are provided *in trans* to enable vectors to be packaged and to maintain their ability to deliver genes to the target cells. The expression cassette of choice containing at minimum the gene of interest and a promoter is inserted into the viral backbone in place of the deleted sequences. Therefore, viral vectors can only produce a dead-end infection which ultimately results in the transfer of their genetic content to the nucleus of the infected cells [4]. Although deletion of all viral coding sequences reduces the risks of host cell-mediated immune responses against transduced cells expressing de novo viral antigens, immune response can still be mounted against the viral proteins of the transducing vector or the vector encoded transgene product.

Viral vectors have been derived from several viruses, both RNA viruses (e.g., γ-retroviruses and lentiviruses) and DNA viruses (e.g., adenoviruses and adeno-associated viruses). The main drawback of RNA viruses is their uncontrolled integration which holds risks of insertional carcinogenesis, while the major disadvantage of DNA viruses is their immunogenicity. For example, although devoid of all viral

coding sequences, helper-dependent adenoviral vectors still result in capsid-mediated, dose-dependent toxicity following intravascular administration (reviewed in the chapter 'Helper-Dependent Adenoviral Vectors for Gene Therapy of Inherited Diseases').

Non-viral vectors offer a number of advantages over viral-based strategies, including minimal toxicity from the vector, long-term transgene expression, lack of a humoral response to the vector and the consequent ability to repeat dose, and simple, cost-effective production. They could replace many viral vectors if they can be delivered with higher efficacy. However, improvements in formulations or carriers of the gene transfer material resulting in more efficient gene transfer are still needed to make this approach attractive (reviewed in the chapter 'Physical Methods of Gene Delivery').

Targeting the genetic defects at the RNA level has potential for therapy of several inherited disorders. This approach is based on molecules that bind nucleic acids with high specificity and modulate mRNA metabolism [5]. RNA interference (RNAi) can be mediated by various types of RNA molecules, including long double-stranded RNAs (dsRNAs), short interfering RNA (siRNA) (reviewed in the chapter 'siRNA Therapeutics to Treat Liver Disorders'), short hairpin RNA (shRNA), and microRNA (miRNA). These RNAs can be provided as synthetic oligonucleotides or as genetic DNA templates from which the RNAi are transcribed in the target cells (vector-based transcriptional RNAi). As a therapeutic, all four types of RNAi inputs are currently in clinical trials. The toxicity of RNAi therapeutics is related to improper target recognition or immunogenic effects from exogenously introduced RNA. Naked RNAs are indeed relatively unstable, whereas the pharmacokinetic of RNA nanoparticles is much better and their biodistribution is favorable, particularly to the liver. Improved lipid nanoparticle (LNP) and *N*-acetylgalactosamine (GalNAc) chemistry have indeed made liver-targeting RNAi among the most advanced in clinical applications of RNAi therapeutics. In addition, RNA processing can be manipulated selectively oligonucleotides (reviewed in the chapter 'Oligonucleotide Therapy').

Overview of Clinical Applications

Development of clinical gene therapy has been hampered by several drawbacks including the death of one patient in the clinical trial for ornithine transcarbamylase (OTC) deficiency [6, 7] and the development of leukemia in patients with X-linked severe combined immunodeficiencies (SCID) and Wiskott–Aldrich syndrome which were treated with retroviral ex vivo gene therapy [8, 9]. The trial for OTC deficiency, the first with direct administration of a gene transfer vector (an earlier adenoviral vector) in patients with a genetic disease, resulted in lethal toxicity and death of one of the two subjects injected with the highest dose [10]. The patient developed coagulopathy and hyperammonemia, followed by respiratory distress syndrome and multiple organ failure within 24 h from vector infusion [6]. It remains unclear whether the underlying more severe OTC deficiency, genetic susceptibility to enhanced innate immune response, or previous exposure to the adenovirus contributed to the fatal outcome. Nevertheless, it became clear that systemic injection of

adenoviral vectors could be associated with dose-dependent toxicity, mediated by the vector capsid proteins that activate a potent inflammatory response [6, 11, 12]. In the case of severe forms of SCID, while they have obviously raised concerns about the safety of the gene transfer, the development of leukemia is arguably an acceptable risk given the clear demonstration of sustained clinical benefit by gene therapy. Moreover, the use of vectors based on lentivirus shows a better preclinical safety profile, more efficient gene delivery and can overcome several of the limitations of γ-retroviral vectors [13, 14] (reviewed in the chapter 'Safety and efficacy of retroviral and lentiviral vectors for gene therapy').

After these major adverse events, encouraging results from a gene therapy trial using serotype 8 adeno-associated virus (AAV8) in hemophilia B patients [15] sparked new enthusiasm for development of clinical trials for in vivo liver-directed gene therapy. A single intravenous infusion of vector in 10 patients with severe hemophilia B resulted in a dose-dependent increase in circulating factor IX (FIX) to therapeutic levels that were sustained over a period of about 3 years. Importantly, clinical efficacy was achieved in the high-dose group, as shown by reduction in the use of prophylactic FIX concentrates and decreased bleeding episodes. However, a mild increase in alanine aminotransferase (ALT) due to a cytotoxic T lymphocyte (CTL) immune response occurred between 7 and 10 weeks post-vector administration in 4 of the 6 patients in the high-dose group but was resolved after prednisolone treatment [15, 16]. Although the increase in ALT was mild and transient, this trial once again highlighted the obstacles posed by the immune response against viral vectors directly injected in humans. Moreover, AAV vectors have shown to be highly effective in ocular gene transfer for Leber congenital amaurosis type 2 (LCA2), an inherited form of blindness due to mutations in *RPE65* gene. Three clinical studies carried out independently by different groups showed that a single sub-retinal injection of AAV2 vector carrying *RPE65* improved and sustained vision in treated regions of the retina [17–19]. Recent long-term evaluations of patients from two of the three aforementioned trials found a decline in retinal sensitivity, visual acuity, and functional gain over time that was not been observed in the third study [20, 21]. Differences in vector design, final formulation, immunomodulatory regimens used, and surgical delivery may have contributed to the different outcomes. Nevertheless, based on these promising studies, AAV-mediated liver and retinal gene therapy has been investigated and it is currently under clinical evaluations in several inherited disorders (reviewed in the chapter 'AAV Vector-Based Gene Therapy, Progress and Current Challenges').

Conclusions

The concept of treating genetic diseases by gene therapy has long appealed researchers because it promises to treat their primary gene defects. In the last few years, gene therapy has become widely accepted and has emerged as a novel form of medicine with potential impact on several human disorders. However, these

studies have also revealed several problems, such as unexpected toxicity and insertional oncogenesis. These issues require a careful and thorough evaluation to define more precisely the risks related to gene therapy. These risks have to be compared with those related to current treatments, whenever these are available.

Despite the toxicity emerged so far and the lack of long-term safety data for several of these approaches, the progress made in both the preclinical and clinical arenas clearly supports further development of gene therapies. The experience in clinical gene therapy clearly indicates that a careful risk/benefit assessment must be made for each condition incorporating the underlying pathophysiology, long-term prognosis, inherent and potential unforeseen risks of the different gene transfer approaches. This book aims at bringing together expert in the field to dissect the toxicity issues related to each of the main vectors used in gene therapy. These are exciting times for gene therapy, and we can expect to see more clinical studies in a variety of inherited disorders are evaluated during the next decade. These studies are likely to provide better therapies for severe and devastating genetic disorders and will provide a better knowledge about the risks related to each approach.

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Safety and Efficacy of Retroviral and Lentiviral Vectors for Gene Therapy

Daniela Cesana, Monica Volpin, Yasmin Natalia Serina Secanechia and Eugenio Montini

Abstract In hematopoietic stem progenitor cell (HSPC) gene therapy (GT) applications for the treatment of genetic diseases, retroviral vectors (RVs) are used to efficiently transduce and integrate therapeutic genes in the genome of patient-derived HSPCs, which, upon reinfusion, reconstruct the entire hematopoietic system and restore the correct hematopoietic functions, or deliver the therapeutic factor to different tissues. However, in initial HSPC-GT clinical trials using early-generation γ-RVs, vector insertions near proto-oncogenes triggered their overexpression and induced leukemia in some of the transplanted patients. These unexpected adverse events have prompted the development of highly sensitive preclinical assays to test the genotoxic potential of different GT vector types and designs, and the development of powerful PCR-based techniques, combined with next generation sequencing (NGS) and bioinformatics analyses, have allowed to study integration sites (ISs) present in leukemic and dominant expanding cells, identify the genes targeted by insertions and to investigate the clonal composition of complex vector-marked cell populations. The positive safety data obtained from the testing in highly sensitive preclinical models and, successively, in clinical trials, of the more advanced lentiviral vectors (LVs) with self-inactivating (SIN) long terminal repeats (LTRs), have reduced the concerns related to insertional mutagenesis, encouraging the adoption of this vector platform in GT protocols for the treatment of many other diseases. Nevertheless, the evidences collected from genotoxicity assays and a β-thalassemia clinical trial, during which a vector-driven clonal dominance event has occurred, point to the fact that even SIN LVs insertions are not entirely neutral, and thus to the importance of a continuous effort to improve both the design of GT vectors, and the sensitivity of preclinical assays aimed at assessing their residual genotoxicity.

D. Cesana · M. Volpin · Y.N. Serina Secanechia · E. Montini (✉)
San Raffaele Telethon Institute for Gene Therapy, San Raffaele Scientific Institute, Milan, Italy
e-mail: montini.eugenio@hsr.it

M. Volpin · Y.N. Serina Secanechia
Vita Salute San Raffaele University, Milan, Italy

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Keywords Genotoxicity · Insertional mutagenesis · Hematopoietic stem cell gene therapy · Viral integration · Retroviral vectors · Common integration sites · Clonal dominance

Hematopoietic Stem Progenitor Cell Gene Therapy with Gamma Retroviral Vectors

Retroviruses belong to a family of enveloped viruses with single-stranded positive-sense RNA genome that, once entered the host cell cytoplasm, is reverse-transcribed into a DNA intermediate by the activity of the viral enzyme reverse transcriptase to produce DNA from its RNA genome. This new DNA is then integrated in semi-random positions of the host cell genome by the viral enzyme integrase. The stably integrated provirus will thus produce the viral proteins and RNA genomes to produce new viral particles and reinitiate the infectious cycle.

RVs have been the classical gene delivery vehicles for hematopoietic cells, including stem cells [1]. The ability of retroviruses to stably integrate in the host cell genome allows to take advantage of the cellular transcriptional machinery for the permanent genetic modification of the host cells and their progeny. Moloney murine leukemia virus (MoMLV) was in fact the first retroviral genome to be engineered to carry a foreign gene into a host cell [2]. In this initial work, MoMLV was used to transfer a copy of the herpes simplex virus thymidine kinase into murine hematopoietic stem progenitor cells (HSPCs) that were subsequently able to rescue irradiated recipient mice from lethality upon transplantation [2]. Few years later, MLV vectors were demonstrated able to mediate gene transfer also into human bone marrow stem progenitor cells [3], successfully mediating the transfer of neomycin and methotrexate resistance genes, and conferring functional drug resistance to the recipient cells. Early gene therapy clinical trials exploiting gamma (γ) Retroviral Vectors (RVs) were developed for the treatment of primary immunodeficiencies (Table 1). For these patients, HLA-matched bone marrow transplantation or T-lymphocyte infusion were the only available treatment options, and the well-known risks associated with these procedures, coupled to the lack of HLA-matched donors, pressed to find alternative curative solutions for these patients [4].

The keystone for using γ-RVs for gene therapy clinical applications was set as the first trials with these vectors revealed a tangible clinical application: in 1990 Blaese et al. [5] started a clinical trial for the treatment of adenosine deaminase (ADA) gene deficiency, a metabolic disorder characterized by the accumulation of toxic metabolites causing near to total absence of lymphocytes in the affected subjects. In this opening study, patient-derived T-lymphocytes were modified ex vivo with a γ-RV carrying the functional copy of ADA and infused back into the patients. Although this first attempt did not revert the immunodeficiency, it demonstrated the feasibility and safety of the procedure [5, 6].

Table 1 Summary of γ-retroviral and lentiviral vector gene therapy trials

Disease	Viral vector	Total *n* patients	Vector-induced adverse events	CIS genes
ADA-SCID	γ-RV	42	None	
X-SCID	γ-RV	23	Leukemia	*LM02*
X-SCID	SIN.RV	9	None	
X-CGD	γ-RV	3	Myelodysplasia	*EVI1*, *PRMD16*, *SETBP1*
WAS	γ-RV	10	Leukemia	*LM02*
WAS	SIN.lV	14	None	
ALD	SIN.LV	4	None	
MLD	SIN.lV	20	None	
β-Thalassemia	SIN.lV	10	None	

The total number of patients per disease with the same vector treatment is shown
γ-RV γ-retroviral vector with active LTR design; *SIN.RV* γ-retroviral vector with SIN LTR design; *SIN.LV* lentiviral vector with SIN LTR design

Since the treatment of the first patients, improvements in gene therapy protocols made significant advances, smoothing ground for fruitful trials that followed.

Indeed, noteworthy success was achieved in a later clinical trial [7], which used improved gene transfer protocols taking advantage of hematopoietic stem/progenitors cells (HSPC) as cell target to achieve the correction of ADA-deficiency (Table 1). In this setting, gene therapy with genetically modified HSPC would warrant a constant supply of gene-corrected cell progeny, able to restore the healthy phenotype in treated patients. Combined with a non-myeloablative conditioning regimen and the withdrawal of enzyme replacement treatment, this strategy advantaged the gene-corrected cells over the enzyme-deficient ones, contributing to a first perceptible efficacy of the treatment. With a slight variation in conditioning regimen and vector constructs, similar protocols have been used by different centers for ADA treatment [8–12]. Overall, a total of forty-two patients with ADA-deficiency have been treated by gamma retroviral HSC gene therapy worldwide so far. In highlighting that no adverse events have been reported in any of the treated patients of these trials, thirty-one became independent from life-long pharmacological enzyme replacement therapy. The strategy of using γ-RV-based gene therapy clinical trials with HSPCs was exploited also for additional trials to cure different primary immunodeficiencies such as X-linked severe combined immunodeficiency (SCID-X1) [13, 14], X-linked chronic granulomatous disease (X-CGD) [15–19], and Wiskott–Aldrich syndrome (WAS) (Table 1) [20–22].

SCID-X1 is caused by mutations of the interleukin 2 receptor subunit gamma (IL2RG) gene, which encodes for the common gamma chain (γc) subunit shared in the interleukin (IL)-2, 4, 7, 9, 15, and 21 receptor complexes. SCID-X1 accounts for 40–50% of all SCID cases [23]. SCID-X1 patients present profound immunological defects caused by low numbers or complete absence of T and NK cells, and

presence of nonfunctional B-cells [24]. The observation that spontaneous somatic reversion of the mutation in the γc-encoding IL2RG gene in lymphocyte progenitors resulted in the restoration of immunological competence in some patients [25] suggested that γ-RV-meditated gene transfer of a normal γc transgene into lymphocyte progenitors could provide a selective advantage over their non-transduced counterparts, after autologous transplantation into patients. Aimed at restoring the healthy immunological phenotype, different clinical trials have been performed, treating, from 1999 to 2006, a total of twenty-three SCID-X1 patients, of which twenty recovered immunological functions after the gene therapy treatment [13, 14, 26]. Despite the successes, as explained in detail below, leukemogenesis occurred in a number of cases as the result of vector insertions that deregulated the expression of nearby cellular oncogenes in transduced cells.

Chronic granulomatous diseases encompass a group of pathologies caused by defects in the nicotinamide dinucleotide phosphate (NADPH) oxidase complex, vital for antimicrobial activity of phagocytes. In X-CGD, this defect relies on mutations in the CYBB gene, the enzymatic center of NADPH oxidase [27, 28]. Initial clinical trials with gamma retroviral vectors for X-CGD had limited success compared to the abovementioned trials, since they reached only transitory functional correction of less than 0.5% of peripheral blood granulocytes [29–31], and although following trials extended restoration of functional neutrophils up to 30%, the long-term engraftment of gene-corrected cells fell short, and myelodysplastic cell clones were selected as the result of vector insertions near a specific oncogene [15–19].

For Wiskott–Aldrich syndrome (WAS), a severe X-linked disorder caused by mutations in the leukocyte migration involved gene WAS, a phase I/II clinical trial was initiated in 2007 [21]. Nine out of the ten patients treated in this trial had sustained engraftment and correction of WAS protein expression in platelets, lymphoid and myeloid cells, resulting in partial or complete resolution of immunodeficiency [22].

Taken together, these clinical trials demonstrate that vector-mediated stable gene transfer into hematopoietic stem and progenitor cells can provide clinical benefits to many patients.

Amidst of its advantages, vector integration, in altering the host genetic code, is an intrinsic mutagenic event that can lead to cell damage with deleterious consequences. As a result of harmful vector integration events, lymphoproliferative or myelodysplastic disorders have been reported in X-SCID, X-CGD and WAS clinical trials (Table 1) [18, 19, 21, 22, 32–34].

In the X-SCID trial, five successfully treated patients developed leukemia. In four out of five cases, analysis of malignant clones found integrations close to the *Lim Domain Only-2* (*LMO-2*) proto-oncogene (Table 1) [33, 34]. These vector integrations caused increased gene expression and consequent enhanced protein production. Although the *IL2Rγc*-deficient background and the transgene itself have been hypothesized as potential cofactors of clonal expansion [35], the vector-mediated aberrant expression of this proto-oncogene is still considered the main cause of oncogenesis.

A similar scenario hit WAS clinical trial when the same locus was found to be targeted in patients' expanded clones, triggering, in seven treated subjects, hematologic malignancies (Table 1). Four patients developed T-cell acute lymphoblastic leukemia (T-ALL), two primary T-ALL with secondary acute myeloid leukemia (AML) and one patient displayed Primary AML [21, 22].

Myelodysplastic disorders originated in three patients that enrolled in the X-CGD clinical trial. Aberrantly expanded clones where characterized by vector-driven upregulation of *MDS-EVI1*, *PRDM16* and *SETBP1* proto-oncogenes (Table 1) [18, 19].

Mechanisms of Insertional Mutagenesis

γ-RVs conventionally used in gene therapy applications were derived from slow transforming retroviruses, a class of oncogenic viruses capable of inducing, after a moderately long period of latency, the development of tumors in infected animals. Differently from other oncogenic viruses, carrying coding sequences for proto-oncogenes [36] or for viral proteins able to interfere with cellular tumor suppressor genes [37], the capacity of γ-retroviruses to promote malignant transformation is strictly dependent on their integration into the host's cellular genome. This event allows the provirus to interact in various ways with the genomic elements surrounding the site of integration, potentially leading to alterations in physiological cellular gene expression, a phenomenon referred to as "*insertional mutagenesis*" [38, 39].

Retroviral integration can cause upregulation of cellular genes as well as their disruption by three different mechanisms, involving enhancer and promoter elements contained within the proviral long terminal repeats (LTRs), splicing signals and/or polyadenylation sites present in the vector [38–40].

Integrations, either upstream or downstream a cellular gene, can trigger insertional mutagenesis through a mechanism of *enhancer activation* [38, 39]. In this scenario, gene transcription, driven by the gene's endogenous promoter, is augmented by the activity of the enhancers present in the nearby integrated proviral genome (Fig. 1a). This effect can be exerted not only on genes that are most proximal to the insertion site, but also on genes lying far apart on the linear genome and that are brought in spatial vicinity *via* the generation of chromatin loops in the nucleus [41].

Retroviral integrations can lead to the overexpression of genes also by the mechanism of *promoter insertion* (Fig. 1b), which takes place when integrations are in the same transcriptional orientation of the gene, and the proviral promoter elements within the LTR replace the cellular promoter in driving gene transcription [38, 39]. Although the enhancer/promoter elements of both LTRs have the potential to initiate gene transcription, this phenomenon is frequently driven by the 5′ LTR, since the 3′ LTR is inactivated through a phenomenon called *promoter occlusion* [36].

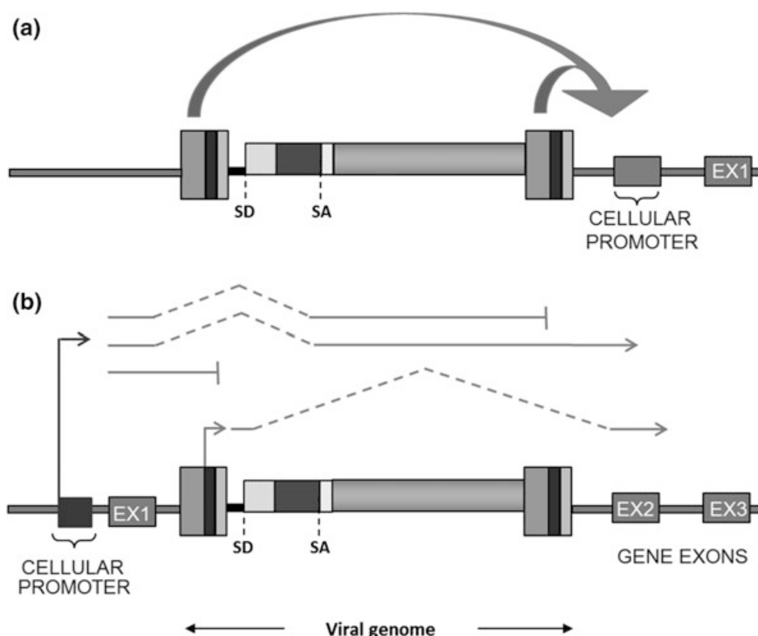

Fig. 1 Insertional mutagenesis mechanisms by retroviral insertions. **a** Enhancer-mediated gene activation events; **b** promoter insertion, transcript truncation and altered splicing mechanisms. *E/P* viral enhancer/promoter elements; *pA* viral polyadenylation signal; *SD* and *SA* splice donor and splice acceptor signals, respectively

The interference of *cis*-acting elements between virus and host genome can lead to formation of dangerous transcription-signals that deceive the cellular transcription machinery. Aberrant splicing and read-through mechanisms, overcoming the LTR-polyadenylation site (polyA), allow generating aberrant fusion transcripts containing vector and cellular sequences (Fig. 1b). Specifically, when starting from the 5′ LTR, aberrant transcripts are fused to cellular sequences by using canonical or cryptic viral splice donor (SD) sites and cellular splice acceptors (SA) signals [38, 39].

Intragenic insertions, which are integrations landing inside the host genomic transcriptional units, can trigger insertional mutagenesis by disrupting coding domains, thus leading to gene inactivation. Given the presence in the R region of the LTR of a canonical polyA signal in the same orientation as the viral transcription, and of a cryptic polyA signal in antisense orientation, proviral intragenic integrations in both orientations can elicit the premature termination of gene transcription (Fig. 1b).

Such events may occur in concert with aberrant splicing, enhancer or promoter activation. Viral insertions may also target the 3′ UTR region of genes, resulting in the deletion of mRNA-destabilizing motifs such as miRNA target sequences or

AUUUA hairpins, leading to the increase of the mRNA expression levels and consequently increased protein levels [39].

Common Integration Sites: The Hallmarks of Insertional Mutagenesis

Owing to their oncogenic potential, slow transforming retroviruses have long been exploited as mutagenic agents in forward genetic screens aimed at identifying novel cancer genes [38, 39, 42–44]. Such screens were originally performed either by infecting newborn mice with replication competent retroviruses, or by using constitutively infected recombinant inbred mouse strains, in which the virus is vertically transmitted [43]. In both cases, retroviral infection in early life allows the establishment of a life-long viremia during which multiple rounds of proviral integration in millions of cells and within the same cellular genome can occur. This may result in the deregulation of multiple growth-regulatory genes that can in turn confer a selective advantage to cells which, upon the acquisition of additional mutations, can become fully transformed and malignant [43].

The type of tumor developed depends on the specific tissue tropism of the virus used; for instance, the mouse mammary tumor virus (MMTV) and the Moloney murine leukemia virus (MoMLV), extensively employed in such studies, induce mammary and hematopoietic tumors, respectively [44]. Compared to other mutagens, such as chemicals and radiations, retroviruses display the feature of integration that can be advantageously used as a molecular tag, allowing the mutated genes to be easily identified by retrieving the viral integration site (IS) and mapping its sequence to the reference genome.

Genomic loci targeted by proviral insertions in multiple independent tumors, at a frequency higher than expected by chance, and being consequence of clonal selection triggered by integrations conferring a growth and proliferative advantage to the cells, are termed common insertion sites (CIS).

Targeting of genes responsible for malignant events occurred during the initial γ-RV-based GT clinical trials. Genes, such as *LMO2* in the X-SCID and WAS trials, *MDS1-EVI1* in the X-CGD and WAS trials and *PRDM16* and *SETPB1* in X-CGD trial, were found to be highly targeted by vector integrations in malignant and pre-malignant clones retrieved from different patients (Table 1). Hence, the identification and monitoring of CIS in vivo in GT patients is of great importance in clinical applications, to monitor the safety outcome of GT procedures.

Since some genomic regions can be frequently targeted as a result of retroviral integration biases, the classical methods to identify CIS based on comparison with simulated random integration distributions, founding on the assumption that retroviral integrations occur randomly into the genome, had to be revisited [39]. The total number of insertions of the analyzed dataset must be considered since the higher the number of overall integrations, the greater is the probability that these

could target by chance the same genomic region. Differently, oncogenic CIS tend to be highly clustered within narrow genomic regions targeting a single gene [45]. Furthermore, the orientation of the vector integration compared to the targeted gene can be an important feature defining genotoxic CIS, since it provides important insights on the probable mechanisms of genotoxicity leading to deregulation. As HIV-1/LV integrations have the tendency to distribute over megabase-wide genomic areas, an additional analysis step to avoid the overestimation of potential cancer-associated CIS has been proposed [45]. This approach takes advantage of the Grubbs test for outliers to define if integrations within a CIS gene are significantly enriched compared to genes contained in the flanking genomic regions, in which case the identified CIS will be considered the result of a selection process; conversely, it will be considered the product of an integration bias [45].

Overall, the different analytical strategies that have been used over the years to identify CIS can be classified into two main groups: (i) whole genome scanning, in which CIS are computed by parsing all IS in the whole covered genome independently from the functional or genomic annotation, and (ii) gene-centric approach, in which all IS are associated with the closest gene and CIS are computed with respect to the corresponding gene size. The first group includes the majority of the methods, from the first approaches based on genomic sliding window [46] to Kernel convolution-based methods [47], Poisson distribution statistics [48], Monte Carlo-based methods [49], and, more recently, on scan statistics [50], whereas the Grubbs test for outliers [45, 51, 52] is classified in the gene-centric group.

State of the Art Methods for Integration Sites Retrieval

Methods

Gene therapy safety studies and retroviral integration analyses require identifying the genomic site of the integrated provirus. To this purpose, during the last decade, different techniques have been developed and optimized. All these methods rely on polymerase chain reaction (PCR) for the isolation and amplification of proviral-host genome junctions, starting amplifications from known sequences in the proviral LTR, followed by sequencing and mapping to the reference genome. Among these methods, ligation-mediated PCR (LM-PCR) [53, 54] and linear amplification-mediated PCR (LAM-PCR) [55–57] have been the most frequently exploited ones. In both approaches, restriction enzymes are used to fragment DNA and a common oligonucleotide sequence (a DNA-linker cassette) is ligated to the resulting fragments, followed by exponential amplification with primers complementary to LTR and linker cassette sequences.

In LM-PCR, genomic DNA is directly subjected to the above-described flow, while LAM-PCR involves a prior step of linear amplification and second strand synthesis, preceding the enzymatic restriction (Fig. 2).

Identification of vector integration sites is crucial for evaluating the efficacy and the safety of gene therapy clinical trials. Moreover, in clonal tracking studies, like the ones performed for the monitoring of HSPC-GT patients, the total number of reads derived from the same sequence, called sequence count, can be used to calculate the relative abundance of clones "marked" by a specific integration within the pool of vector-marked clones [51, 52, 56–60]. This strategy is based on the assumption that the more one integration site is sequenced, the more the clone harboring the specific integration is abundant in the sample.

However, the use of restriction enzymes for DNA fragmentation in LM and LAM-PCR can introduce biases, which impact the sensitivity of these techniques and the abundance estimates obtained from sequence counts [61]. Specifically, the non-uniform distribution of restriction sites throughout the genome leads to the generation, upon digestion, of fragments of different lengths. During the PCR, shorter fragments can be preferentially amplified over longer ones, thus misrepresenting the relationship between the real frequency of an integration and its calculated estimate. Additionally, integration loci lacking close restriction sites may be missed, since those fragments will result being too long to be amplified by standard polymerases [61], or, even in case of successful amplification, the long products could be too long to be analyzed by specific sequencing platforms [62]. Furthermore, some of the generated fragments might be too short to be

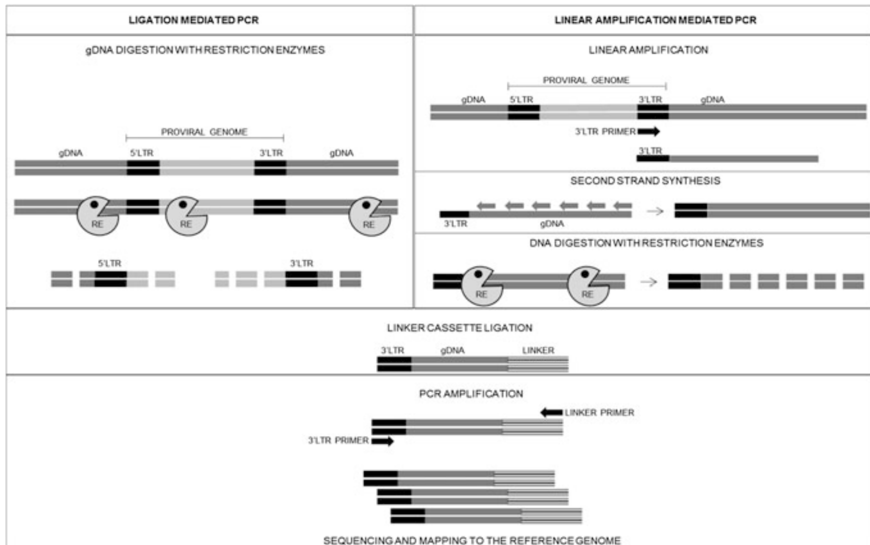

Fig. 2 Schematic representation of: ligation-mediated (*LM*) PCR; *left panel* and linear amplification-mediated (*LAM*) PCR; *right panel*. *RE* Restriction enzymes; *gDNA* genomic DNA

unequivocally mapped to the reference genome after sequencing [61]. To overcome these technical limitations, restriction-free LM and LAM-PCR methods have been introduced [22, 61–65]. The use of sonication to fragment DNA allows controlling DNA-shearing, achieving an evenly sized distribution of the fragments. This permits to take advantage of novel strategies for clonal abundance quantification, which do not rely on the sequence count, and should therefore improve the accuracy of such measurements [58, 62, 63, 65].

Although of similar size, being the sites of genomic DNA fragmentation random, the sonicated fragments will display different shearing ends. The number of different break points (called: shear sites) associated with each integration site reflects the abundance of cells containing that specific insertion. Ligating the linker cassette directly to the sonicated fragments prior to the amplification steps generates unique ligation points (LPs), which serve as molecular barcodes to identify individual cells [58, 62, 63, 65].

One limitation of this technique is that the maximum number of clones with the same integration that can be distinguished corresponds to the maximum fragment length generated by sonication, leading in some cases to underestimate the abundance of large clones [58, 65–67]. To overcome this, the use of adaptors that contain random barcode sequences has been proposed, so that individual cells can be identified by coupling unique shear sites to unique barcodes [65, 67].

Applications

Following HSPC gene therapy, gene-corrected stem cells are expected to give rise to a gene-corrected hematopoietic cell progeny, with a plethora of vector-marked cells harboring different integration sites, which is referred to as to as *polyclonal* integration pattern [4]. Vector integration studies can be exploited to follow the clonal composition of hematopoietic reconstitution over time after gene therapy and are known as *clonal tracking studies* [18, 22, 51, 52, 60, 66]. In allowing the detection of clonal dominance events, which might represent early steps of tumorigenesis, these studies are important safety readouts of gene therapy trials. Pioneering work has been performed by large-scale mapping analysis of retroviral insertion sites achieved in the trial for X-CGD disease [18], where the clones that became malignant were found to be the most represented among gene-corrected cells already five months after gene therapy, and before symptoms-based diagnosis of myelodysplastic syndromes and leukemia [18, 19].

Similarly, in the γ-RV-based clinical trial for WAS [22], the progressive expansion of the clones that triggered leukemia could be followed during time by IS retrieval. Therefore, longitudinal tracking studies aimed at quantifying over time the clonal abundance of cell clones harboring different integration sites are extremely important to monitor the safety of HSPC gene transfer applications.

To assess clonal diversity in each lineage over time, an index, measuring the entropy of the integration data sets, the Shannon Diversity Index, is used, and takes

in account the total number of integrations and their relative contribution to the clonal output over time [22, 51, 52, 60]. Analyses of the clonal contribution of gene-marked cells to hematopoiesis in gene therapy patients in the LV-based clinical trials for WAS and MLD showed that no clonal dominance events had occurred during the time of follow-up [51, 52] with almost all cell clones marked by a specific integration accounting for only a fraction of less than 5% of the total clones, while the few clones displaying a higher percentage at a specific time point then disappeared or were strongly reduced at later time points [51, 52]. In line with this, the Shannon Diversity Index calculated for each patient at respective time points in both trials revealed that hematopoietic reconstitution after transplantation became increasingly polyclonal in all hematopoietic compartments, providing a further indication of the safety of these treatments [51, 52].

Beyond the safety assessment purposes, given the powerful information that can be earned from clonal tracking studies, such investigations can be also exploited to study the dynamics and lineages in hematopoietic reconstitution [51].

Based on results from the WAS clinical trial, Aiuti and coworkers [51] were able to propose a model in which the hematopoietic output after transplantation by long-term HSPCs occurs in a defined time window after gene therapy, generating a diverse clonal repertoire in the blood progeny. As an example, the diversity of the HSPC (CD34+ cells) compartment of patient 1 from the WAS trial progressively decreased, initially reaching its minimum at six months post gene therapy, and rebounded over time after that time point, while diversity in the other hematopoietic lineages increased stably over time. This trend is believed to result from an initial contribution of multiple progenitor cells to hematopoiesis that are subsequently supplanted by engrafted long-term HSCs upon exhaustion of short-term progenitors.

Long-term reconstituting HSCs are expected to share identical integrations among bone marrow CD34+ progenitors and multiple myeloid and lymphoid lineages persisting overtime, whereas integrations shared only among some hematopoietic lineages, but not others, might indicate lineage-restricted progenitors [51, 52, 59]. The output of short-lived mature myeloid cells in the peripheral blood long-term after HSC-GT has been exploited as a readout to estimate the number of transduced HSCs contributing to human hematopoiesis in vivo [51, 52]. Lineage tracking studies could also provide insights in the lifespan and fluctuations in lineage output of hematopoietic progenitors [59].

Assessing Retroviral Vector Genotoxicity

Despite the well-known oncogenic potential of the parental viruses, the risk of tumor development associated with gene therapy-grade retroviral vectors was considered to be remote, owing to the replication-defective nature of the vectors and to the unlikelihood that few vector integrations could activate multiple proto-oncogenes within the same cell, promoting neoplastic transformation.

Furthermore, the results of preclinical studies performed on animal models did not highlight the occurrence of such adverse events [68–74].

However, unexpected malignant events occurred during the early γ-RV-based GT clinical trials (Table 1), and the identification of vector-induced insertional mutagenesis as their major drivers highlighted the need for a deeper understanding of the mechanisms and the intrinsic vector features underlying vector genotoxicity, which is instrumental for designing safer vectors for clinical applications.

Several models, both in vitro and in vivo, have thus been proposed over the years to address the mutagenic risk and transforming potential of integrative vectors (Fig. 3).

In Vitro Assays

Cell-based assays are a convenient means to perform rapid functional screens, by scoring for macroscopic gain of functions induced by vector treatment. Molecular analyses can then be performed, to identify the genes targeted by integrations, and the mechanisms by which they were deregulated (Figs. 3a, 4b).

One of the strategies takes advantage of growth factor-dependent cell lines, such as interleukin-3 (IL-3) (Fig. 3b) [75, 76]. Cellular transduction with the vector to be tested, followed by growth factor withdrawal from the culture medium, gives rise to growth factor-independent clones generated by vector-induced insertional mutagenesis. Using this assay, the mutagenic potential of matched design γ-RVs and LVs has been compared, revealing that, while both vectors are able to generate insertional mutants at similar frequencies, they do so by different mechanisms: Moloney leukemia-virus (MLV)-derived γ-RV tested induced the overexpression of the *IL-3* gene or other cancer-associated genes by enhancer insertion, whereas LV intragenic insertions lead to aberrant fusion transcripts starting at the proviral 5'-LTR encoding for the growth hormone receptor [75].

A similar strategy is employed in the in vitro immortalization (IVIM) assay based on transduction of primary hematopoietic stem and progenitor cells isolated from untreated adult mice (Fig. 3a) [77]. Cell transformation is detected by culturing transduced cells under myeloid differentiation conditions, followed by a replating step in limiting dilution that suppresses the residual self-renewal ability of the cells, unless insertional upregulation of cellular proto-oncogenes occurred. To allow direct comparison of the mutagenic potential of different vectors, an index is calculated by correcting the replating efficiency for the post-transduction vector copy number (i.e. the average number of integrated vectors per cellular genome). Comparative studies using the IVIM assay have shown that, compared to conventional γ-RVs, third-generation SIN LVs have a lower transforming potential and that improvement in vector design such as the use of moderate cellular enhancer/promoters or chromatin insulators can reduce the transforming capacity and thus the vector genotoxic potential [78–83].

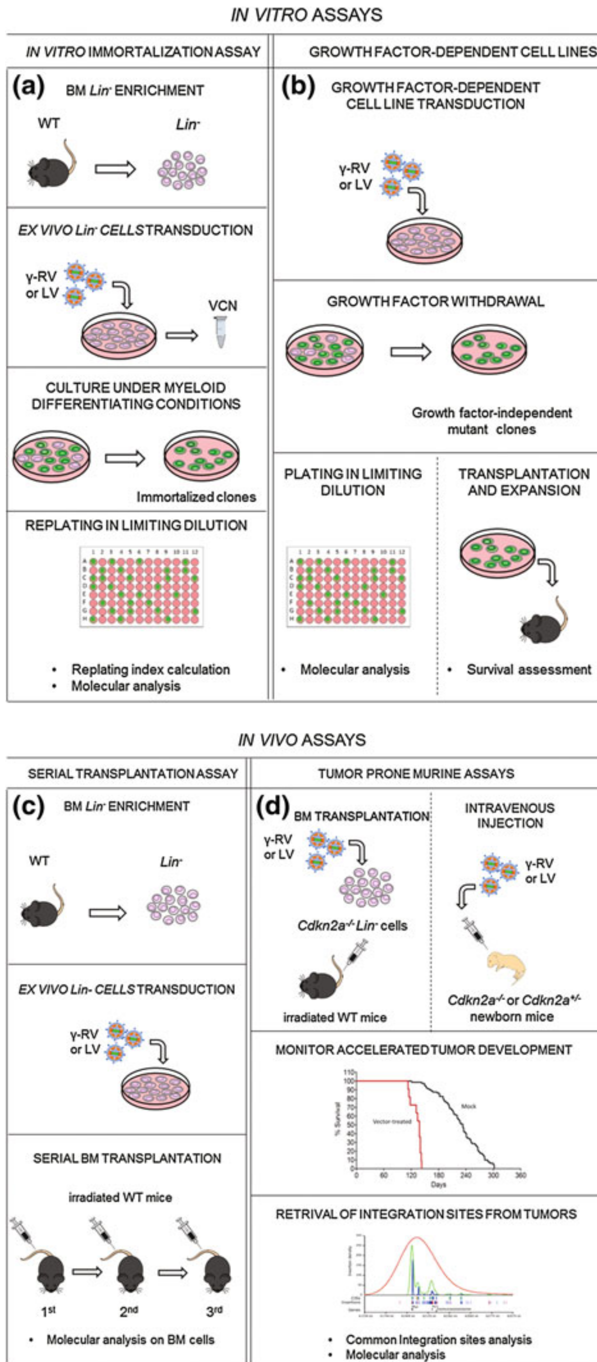

Fig. 3 **a** In vitro immortalization assay (*IVIM*); **b** IL-3 independence growth assay with plating in limiting dilution or expansion in mice; **c** in vivo serial transplantation assay with lineage negative (Lin^{-}) wt murine cells; **d** tumor-prone mice genotoxicity assay with transduction and transplantation of *Cdkn2a*$^{-/-}$ Lin^{-} cells in wt recipients or direct vector injection in newborn *Cdkn2a*$^{-/-}$ mice

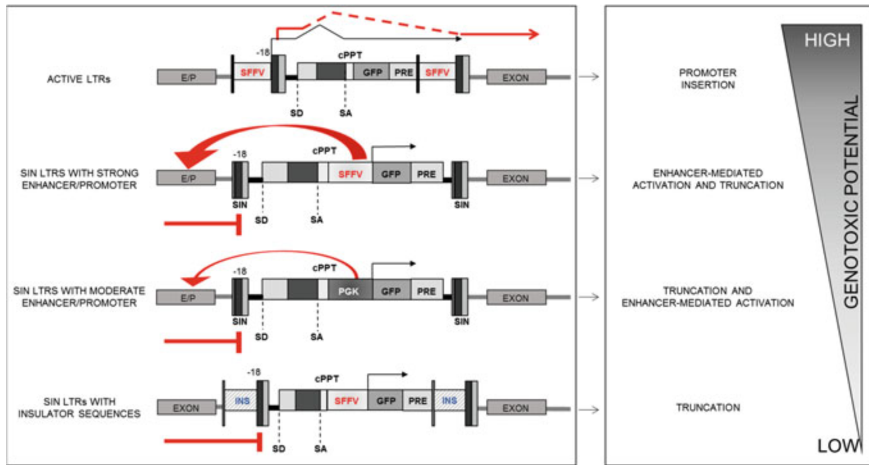

Fig. 4 Genotoxicity mechanisms induced by different vector designs: From *top* to *bottom*: active LTR lentiviral vector with strong enhancer/promoters performing promoter insertion; SIN.LV with strong enhancer/promoter activating oncogenes by enhancer-mediated mechanisms and truncation transactivation; SIN LV with moderate enhancer/promoter causing gene truncation and enhancer-mediated genotoxicity; SIN.LV with strong enhancer/promoter in internal position bracketed by chromatin insulators in LTRs induces mostly gene truncation events. *SFFV* spleen focus forming virus enhancer/promoter elements; *GFP* green fluorescent protein; *PRE* post-transcriptional regulatory element; *PGK* phospho-glycerate kinase promoter; *INS* chromatin insulator sequences

In Vivo Assays

In vivo genotoxicity assays are important to investigate genotoxicity since different components like tumor development, immune response, cellular microenvironment and interactions between different cell-types cannot be readily assessed in in vitro models. Both murine and non-human primates in vivo models have been largely exploited in genotoxicity studies, in order to assess not only the mutagenicity of gene therapy vector integrations, but also their actual oncogenic potential, and their impact on the hematopoiesis of animals subjected to HSPC gene therapy-like procedures [76, 84–90].

Initial mouse-based studies relied on the transplantation of transduced wild-type Lineage negative (Lin$^-$) murine HSPCs into primary recipients, followed by serial transplantation of bone marrow-derived cells from these primary recipients into secondary and tertiary animals, in order to promote potential leukemic progression (Fig. 3c). This strategy has allowed detecting and investigating the molecular bases of vector-mediated oncogenesis in a number of different studies providing experimental evidence that the overexpression of oncogenes, such as the murine *Evi1*, is the major driver of γ-RV-induced cellular transformation [76, 91] and that the onset of γ-RV-associated leukemia, at least in this model, necessitates the cooperation of

multiple vector insertions in growth-regulatory genes within the same clone [87]. However, the predictive power of safety tests based on the use of wild-type murine models is limited by the difficulty of inducing cell transformation, which requires time to occur since the acquisition of multiple mutations is needed, so that even serial transplantation can result inefficient at promoting the development of leukemia [87]. Non-human primates have also been used as models to study the genotoxicity of retroviral vectors in the context of HSPC-GT [86, 90, 92, 93]. These models too, however, seem to lack enough sensitivity to detect the oncogenic potential of retroviral vectors, including the ones that have caused overt malignancies in humans, and to compare the genotoxic potential of vectors with different designs, since a malignant event has been reported only in one rhesus macaque that had been transplanted with γ-RV-transduced autologous HSPCs six years earlier, while the long-term follow-up of a large cohort of animals in two other studies has revealed that all of them had retained a completely normal hematopoiesis during time [86, 90, 92, 93].

To increase the sensitivity to vector genotoxicity, reducing the time required to obtain safety readouts in vivo, genotoxicity assays based on the use of mouse strains with a predisposition to tumor development have been developed (Fig. 3d) [84, 88, 89]. Specifically, these studies have taken advantage of *Cdkn2a*$^{-/-}$ mice [94], in which the knock out of the Cdkn2a locus results in the combined deficiency of the p53 and Rb pathways, rendering the mice more susceptible, compared to wild-type mice, to mutagenic insults such as those that can be delivered by a genotoxic vector. *Cdkn2a*$^{-/-}$ genotoxicity assays have been performed either by transducing and transplanting tumor-prone Lin^{-} cells into wild-type recipients [88, 89], or by directly injecting the vector intravenously into newborn *Cdkn2a*$^{-/-}$ mice [84]. Since all animals develop hematologic malignancies, the readout for vector genotoxicity is the accelerated tumor onset in vector-treated mice compared to mock-treated animals, which increases according to the mutagenic potential of the tested vectors. The genes involved in tumorigenesis can then be identified by harvesting malignant tissues from the mice and performing integration site studies for CIS identification and molecular analyses. Studies using the *Cdkn2a*$^{-/-}$ transplantation model have established a correlation between γ-RV dosage and the risk of malignant transformation, since γ-RV-treatment triggered a significant dose-dependent acceleration of tumor onset in mice compared to Mock and provided a strong evidence of the safety of third-generation SIN LVs, which, instead, did not cause accelerated tumor onset [89]. Experiments in the same model provided a formal proof of the predominant role of active proviral LTRs in mediating vector-induced tumorigenesis, showing that the re-introduction of strong enhancer/promoter elements into LV's LTRs renders these vectors extremely genotoxic, and validating the safety of the SIN configuration in both retroviral and lentiviral vector platforms [88]. The direct-injection-based *Cdkn2a*$^{-/-}$ genotoxicity assay has allowed detecting the residual oncogenic potential of SIN LVs, which resulted undetectable by the *Cdkn2a*$^{-/-}$ transplantation assay [84]. The sensitivity of the injection-based assay has allowed to compare and rank the genotoxic potential of LVs with different designs, revealing that both the mechanism of

insertional mutagenesis and the genetic drivers of oncogenesis strongly depend on the specific features of each vector [84].

The combination of in vitro and in vivo approaches has also been exploited, to test the ability of different insulator sequences to reduce the mutagenic and oncogenic potential of γ-RVs (Fig. 3b) [95]. In this assay, IL-3-dependent cell lines were transduced either with insulated vectors, or non-insulated, match-design, vectors and, after selection, the expanded clones were transplanted into B lymphocyte-deficient C3H/HeJ mice, which were monitored for tumor development. Mice transplanted with mock-treated cells did not develop tumors, whereas the readout for the insulating activity was both the reduction in the frequency of animals developing tumors and the delay on tumor onset in mice receiving cells transduced with insulated vectors, compared to the ones receiving cells transduced with the non-insulated counterparts.

Factors Influencing Genotoxicity

Different factors may influence the success of gene therapy applications, such as the patients' age and health-status, as well as the specific type of disease. Besides patient-related aspects, the outcome of the vector treatment can be influenced by three main vector-related factors: (a) the genomic integration profile, (b) the vector design and (c) the vector dose.

Vector Genomic Integration Profile

The integration pattern of retroviruses is not uniform throughout the host genome, and different retrovirus families display diverse integration preferences, as distinctive fingerprints of their identity [86, 96–103].

First studies on HIV integration revealed preferences of lentiviruses—and their derived vectors—to target genes with a greater percentage opposed to the theoretical of randomly targeted transcriptional units present in the human genome. For these vectors, gene-dense regions and gene-rich chromosomes are the main targets, and integrations are consistently present along the entire length of the transcriptional unit [101, 102]. Differently, MLV integrations displayed a lower preference to integrate within genes [103] and a marked bias to integrate near the transcriptional start site (TSS) of expressed genes, where a bimodal integration distribution around the transcription start was observed for γ-RVs, probably due to physical inaccessibility of specific positions within the promoter when bound by transcription factors [100]. MLV integrations cluster around CpG islands, likely due to MLV bias for TSS, and correlate with epigenetic markers for active promoters and enhancers, like H3k4me1or H3K9ac. Differently, LVs insertion pattern target actively transcribed regions marked by H3K36me3 [86, 92, 100, 101].

Overall, the integration pattern of γ-RV and LV vectors is defined as semi-random and indicates that some intrinsic cellular characteristics are particular appealing to integration of specific viral vectors [97]. The interaction of the pre-integration complex with cellular intrinsic nuclear factors, tethers the complex to the preferred integration loci. LEDGF/p75 and bromo/extraterminal-domain (BET) proteins have recently been shown to be involved in lentiviral and γ-RV tethering, respectively. Specifically, the chromatin-binding domain of LEDGF/p75 involved in interactions with chromatin marks of actively transcribed gene bodies, such as H3K36me3, is responsible for LV bias of integrations. To similar extent but with a different result, BET proteins interacting with chromatin marks enriched around the genes' TSS, are responsible of γ-RV integrations around these genomic features [104]. The knowledge of the molecular players involved in integration sites preferences can allow engineering vectors to fuse to alternative binding domains allowing for safer integration site choice.

Vector Design

Different features present in the vector are able to deregulate host cellular transcripts (Fig. 4). Vector features, like the LTRs, the promoters, the transgene plus additional regulatory elements, can be modified or adjusted in order to improve the vector safety profile.

The vector type itself can influence genotoxicity: with the IVIM assay Baum and colleagues compared γ-RV and LV vectors with the same design and showed that the latter induced mutants with a threefold lower incidence compared to γ-RVs [80]. Moreover, in vivo assays with tumor-prone mice showed a cumulative higher genotoxic potential of RVs compared to LVs [89]. In mice carrying vector copy number (VCN)-matched integrations of γ-RV and LV with the same design, it was found that γ-RV was significantly more genotoxic than LV. It was estimated that a ten-fold higher integration load of the design-matched LV would be required to have the same oncogenic risk, meaning that the relative risk differs between these two vector types [88]. As previously described, vector LTRs can deregulate neighboring genes through enhancer-mediated effect and through aberrant transcript mechanisms (Fig. 1). γ-RVs' transforming potential can be significantly reduced by removing the strong retroviral enhancer/promoter sequences from the LTR and placing them in single copy in internal position [80, 88] and the use of SIN LTR is able to reduce the genotoxic potential of γ-RV and LV vectors carrying strong enhancer/promoter sequences (Fig. 4).

The internal promoter in SIN vectors can be either a moderate cellular promoter or a promoter of viral-origin and with different strengths. A safe vector design should avoid strong viral promoters and support the use of moderate cellular promoters, like elongation factor 1α (EF1α) and phospho-glycerate kinase (PGK) promoters [83]. Indeed, a LV with active proviral LTRs was found to be highly genotoxic in *Cdkn2a*$^{-/-}$ mice and induced tumors by predominantly

activating *Braf* proto-oncogene, through the promoter insertion mechanism (Fig. 4) [84]. Differently, the SIN LV carrying the same enhancer/promoter elements in internal position caused tumorigenesis mainly by activating a different proto-oncogene, *Map3k8*, through a combination of enhancer-mediated overexpression and transcript truncation using cryptic vector splice acceptor sites and/or the LV polyadenylation site present in the LTR (Fig. 4) [84]. The same SIN LV backbone carrying the moderate PGK promoter in internal position, while still being able to cause the enhancer-mediated overexpression of *Map3k8*, triggered tumorigenesis also by inactivating tumor suppressor genes like *Pten*, indicating that the propensity of SIN LVs to induce enhancer-mediated activation of oncogenes or to inactivate tumor suppressor genes depends on the strength of the internal enhancer/promoter used (Fig. 4) [84]. Indeed, when blunting the interaction between the internal vector enhancers and the surrounding cellular genes with chromatin insulators, inactivation of tumor suppressor genes became even more predominant (Fig. 4) [84]. Furthermore, these results highlighted that tumor suppressor disruption endured as escape genotoxicity mechanism that cannot be prevented when using integration competent retroviral vectors (Fig. 4) [84].

Vector Dose

A single mutation event is rarely able to induce neoplastic transformation, suggesting that also in vector-induced genotoxicity, the collaboration with other mutations is needed. The increase in vector load with the consequent increase of vector integrations in the cells results in an enhancement of the risk of targeting cancer-related genes in vitro and in vivo [87–89]. Wild-type mice transplanted with bone marrow-derived Lin^{-} cells transduced with high or low doses of γ-RV sporadically developed leukemia only in the high dose vector treatment group [105]. Other genotoxicity studies performed by transplanting wild-type mice with vector-transduced tumor-prone *Cdkn2a*$^{-/-}$ bone marrow-derived Lin^{-} cells showed a better correlation between vector dose and genotoxicity [88, 89]. γ-RVs or LVs with active LTRs were able to trigger a dose-dependent acceleration of tumor onset although to a different extent. The vector dose needed for detecting genotoxicity in this highly sensitive genotoxicity assay was in part dependent on the vector integration profile (dictated by the vector type). Indeed, the innate tendency of γ-RV to integrate near the TSS and growth-promoting genes resulted in 10-fold higher risk of leukemia compared to an LV with a matched design. Most importantly, the design of the vector was the most relevant factor modulating the genotoxic potential of vector integration, since SIN LTR RVs or LVs even at high dose did not accelerate tumor onset [88] highlighted that different doses of a genotoxic vector lead to different genotoxic readout. Mice that received tumor-prone cells transduced at high vector dose died significantly earlier not only compared to mock-treated mice, but also compared to mice that had received low-dose transduced cells. Moreover, by stratifying the mice according to the

retrieved VCN in the tumors, it was demonstrated that the ones with high copy number (>6) died significantly earlier compared to the one with lower VCN (1–6). The same studies have revealed that LVs with active LTRs require a 10-fold higher integration load compared to γ-RVs with active LTRs to achieve the same oncogenic risk, likely reflecting the differences in the integration preferences of the two vectors, which may increase the probability of oncogene activation and, consequently, cancer development by γ-RVs as compared to LVs.

Lentiviral Vector-Based Clinical Trials

The unexpected adverse events in γ-RV gene therapy trials highlighted that clinical benefits of HSPC gene therapy were offset by limitations and risks associated with γ-RV-based gene therapy applications. On one side, the occurrence of leukemia posed major issues concerning the safety of these applications, which, together with the limiting unfeasibility to transduce non-dividing cells, promoted the use of different vectors to deliver the corrected gene copy to the diseased cells.

Lentiviral vectors allured scientists for such purposes, since they subsume important features of retroviral vectors—as the ability to stably integrate within the host genome—as well as grant advantages of reaching higher vector titers and ability to transduce non-dividing cells. Genotoxicity studies also showed that these vectors harbor a reduced genotoxic potential compared to analogous γ-RV constructs [75, 80, 89, 105]. Thus, gene therapy clinical trials using LVs as vehicles to deliver therapeutic genes expanded beyond primary immunodeficiencies, such as the LV-based clinical trial for WAS [52], toward the treatment of numerous monogenic disorders (Table 1).

A LV-based clinical trial for X-linked adrenoleukodystrophy (ALD), a severe demyelinating disease caused by ABCD1 gene mutations, showed disease correction by engineered HSPC cell progeny able to replace diseased microglia (Table 1) [106]. Along the same line, a clinical trial for the correction of a lysosomal storage disorder caused by Arylsulfatase A (*ARSA*) deficiency, namely metachromatic leukodystrophy (MLD) was performed (Table 1) [51]. Interestingly, MLD gene therapy patients greatly profited from gene therapy edited HSPC by means of corrected microglia replacement and cross-correction phenomenon, a mechanism by which gene-corrected monocyte-derived cells release the therapeutic enzyme, whose' uptake from enzyme-deficient cells of the central nervous system allows restoration of enzymatic function although these cells do not directly express the therapeutic transgene [4, 51].

Beta-thalassemia, caused by mutations in the beta chains of hemoglobin leading to decreased or absent globin protein and, consequently, anemia, was also a target disease of LV-based gene therapy trials (Table 1) [66, 107]. One of the beta-thalassemia treated patients experienced a transient and benign clonal dominance event, attributed to lentiviral vector-induced overexpression of *HMGA2* gene. Molecular investigations revealed LV integrations in *HMGA2* engendering a

chimeric transcript between the third exon of *HMGA2* and a cryptic splice-site located inside the 3′ end of the vector construct causing, by the vector polyadenylation signal, premature truncation and loss of host microRNA *Let-7* regulatory sequences in charge of physiological degradation of *HMGA2* transcript [66]. Present in over 60% of vector-marked nucleated blood cell population, this overt clonal expansion was undermined by untransduced cells that continued to dominate on hematopoiesis so that positive clones for this insertion site represented only near to 3% of the total nucleated blood cells' population and over time this extent reduced. Nevertheless, this patient turned independent from transfusion treatments and never displayed oncologic malignancies [66]. More recently, clinical trials have initiated for the treatment of ADA-SCID and X-CGD using LVs (Table 1) [6, 9]. In the current short time treatment follow-up interval, lack of transplantation-related side effects as well as absence of vector-related oncogenic events was reported.

Overall, no severe adverse events have been reported for any of the LV-based gene therapy trials so far, and most patients displayed hematopoietic gene modified cells reconstitution underlying clinical benefit. Comparison of γ-RV and LV-based trials for WAS bests recapitulates the safety of the different vector platforms for gene therapy applications. While γ-RV integrations next to *LMO2* proto-oncogene in patients conferred growth advantage to these clones, driving leukemia occurrence, vector integration sites studies for WAS LV-based clinical trial confirmed absence of recurrent integrations targeting potential oncogenes [22, 52]. Moreover, no evidences of clonal expansion was detected, since CIS harboring cell clones were not the most abundant at any given time point during the first three years of follow-up [51, 52]. Indeed, CIS found in the LV-based trial, e.g. *KDM2A* or *PACS1*, are LV insertion hot spots likely being the result of vector integration biases at the time of transduction and not consequence of in vivo genetic selection [45, 52, 99].

Concluding Remarks

The successful results of gene therapy are embodied by the clinical benefits and positive long-term follow-up of treated patients. With gene therapy becoming a curative treatment option for many patients with severe diseases, improvements both in vector engineering and in genotoxicity assessment will help sustaining further improved therapies to safely cure patients. Ultrasensitive genotoxicity assays and powerful technologies for safety testing and clonal monitoring have shown that retroviral vector insertions are not neutral to the host genome, since they can alter the mRNAs structure and stability or expression levels of targeted genes in human and mouse HSPCs cells and even promote cancer formation [18, 19, 21, 22, 33, 34, 66, 84, 108]. Several novel vector designs and novel genetic elements are being developed to improve the safety of vector integration and tested in different genotoxicity assays. However, when more advanced vector designs with lower

genotoxic potential or with low vector doses requirements will be available, even the currently most sensitive tumor-prone mouse models may not be sensitive enough to score for possible residual insertional mutagenesis events. Therefore, the development of increasingly sensitive genotoxicity assays, and the assessment of mutagenicity and oncogenicity of vector treatments is still a crucial, outstanding issue for the whole gene therapy field.

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Adenovirus Vector Toxicity

Andrew W. Harmon and Andrew P. Byrnes

Abstract Adenovirus (Ad) vectors are one of the most commonly used classes of vectors being used in gene therapy clinical trials. However, vector-induced toxicity remains a significant barrier to safe, high-dose systemic therapy with Ad vectors. This review will describe what is known about the mechanisms of Ad-induced toxicity after administration of vector by the intravenous route, as well as how these toxicities can be mitigated. Given the hepatotropic nature of many commonly used Ad serotypes, the liver is a key site of virus-induced toxicity. Both innate and adaptive immunity contribute to hepatotoxicity. Intravenous delivery of Ad can also induce other rapid innate toxicities, including thrombocytopenia, systemic inflammation, fever and shock. Recent progress in understanding Ad biology has enabled improvements in vector safety and gene delivery efficiency in animal models, including genetic and chemical modification of the Ad vector itself, new ways to administer vector and pre-treatment with drugs that suppress innate and adaptive immune responses.

Keywords Adenovirus vector · Gene therapy · Hepatotoxicity · Shock · Thrombocytopenia · Biodistribution · Innate immunity · Adaptive immunity · Animal models

Introduction

Adenovirus (Ad) vectors have a versatile ability to deliver therapeutic genes to a wide variety of tissues, and Ad vectors have been used in hundreds of gene therapy clinical trials. However, the potential of Ad vectors is hampered by their toxicity,

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A.W. Harmon · A.P. Byrnes (✉)
Division of Cellular and Gene Therapies, FDA Center for Biologics Evaluation and Research, Silver Spring, MD, USA
e-mail: andrew.byrnes@fda.hhs.gov

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which can be life-threatening at very high doses. First isolated from human adenoid tissue in 1953 [1], there are currently 66 unique serotypes of human Ad, grouped into seven species (A through G). The majority of knowledge about Ad has been derived from research on species C viruses, with Ad2 and Ad5 being the most heavily studied. Ads have many advantages as vaccine and gene therapy vectors—they have a stable genome, generous packaging capacity of up to 38 kb, can be prepared at high titers and are able to efficiently transduce both dividing and nondividing cells. Additionally, Ads are non-integrating viruses, and therefore, the risk of insertional mutagenesis is minimal with Ad vectors [2].

The ability to transduce a variety of cell types and produce strong expression of therapeutic proteins has made Ad one of the most widely used gene transfer vectors. Since 1989, there have been approximately 500 clinical trials using Ad vectors (The Journal of Gene Medicine Clinical Trial Site) [3]. Currently, the most common clinical use of Ad vectors is in trials for cancer gene therapy, and many of these studies administer the vector by intratumoral injection. While local injection of Ad vectors is feasible for delivery to many solid tumors, systemic administration would be a better way to reach multiple metastases or inaccessible tumors. Unfortunately, Ad is rapidly cleared from the circulation, and systemic exposure to high doses of Ad vector triggers dangerous toxicities.

This chapter will focus on intravenous (IV) delivery of Ad vectors. The toxicities associated with IV delivery have been extensively studied in animal models, and this route has considerable promise if toxicities can be understood and controlled. We will focus on discussing clinically observed toxicities, rather than molecular mediators of toxicity such as pro-inflammatory cytokines. Although Ad-induced cytokines and chemokines play important roles during innate immune responses to vectors, these mediators have been extensively reviewed elsewhere [4–7].

While Ad-induced toxicity remains a significant barrier to high-dose IV administration of vector, recent advances in the understanding of Ad biology have led to vectors that have more efficient delivery and better safety, at least in animal models. In the final section of this chapter, some of these new strategies will be reviewed with a focus on how they may ameliorate the toxicity of Ad vectors.

Vector Clearance and Biodistribution Affect Toxicity

Selective clearance of vector by certain organs can profoundly influence the types of toxicities that occur after Ad vector administration. Following intravenous injection, Ad vectors travel to the heart and through the lungs before being pumped to peripheral tissue via the arterial vasculature [8]. Studies in mice have shown that systemically administered Ad is rapidly cleared from the bloodstream with a half-life of less than two minutes [9]. Studies in both rodents and non-human primates have identified the main culprit in Ad clearance as the resident macrophage of the liver: the Kupffer cell (KC) [10–14].

KCs adhere to the endothelial walls of hepatic sinusoids and account for 80–90% of the tissue macrophages in the body. KCs clear bacteria and viruses from the circulation, including microbes that enter the bloodstream from the gut and are then carried to the liver via the portal vein. In addition to being highly phagocytic, activated KCs also amplify innate immune responses by producing pro-inflammatory mediators and recruiting neutrophils to the liver [15].

Macrophages in the spleen also contribute to clearance of systemically administered Ad [11, 14]. Together, KCs and splenic macrophages constitute the reticuloendothelial system (RES), which is primarily responsible for the clearance of particulate materials from the bloodstream. Because Ad is cleared so efficiently by the RES, it is difficult for vector to reach target cells unless the dose is high enough to saturate the RES [12, 16].

Organ transduction by species C Ads, such as Ad5, is strongly hepatotropic. Virions in the liver that manage to escape clearance by KCs may enter the subendothelial space of Disse and then transduce hepatocytes [17–20]. This hepatotropism is a double-edged sword: it is highly desirable if the liver is the intended target of gene therapy, but problematic when attempting to reach other organs or tissues. In both cases, hepatotropism may result in clinically significant liver toxicity.

Differences among Animal Models

Establishing appropriate preclinical gene therapy models can be complicated by differences among animal species as well as differences between animals and humans. Some vector-induced toxicities are strongly species-dependent; for example, mice can tolerate doses of Ad vector that induce severe shock in rats [21], pigs [22] and baboons [23].

Differences among species in RES clearance may result in species-specific toxicity. For rodents and primates, Ad clearance is mediated primarily by KCs in the liver, but Ad clearance in pigs is predominantly in the lungs [24]. The RES in pigs, sheep, goats and cats is comprised not only of phagocytes in the liver and spleen, but also additional macrophages anchored to the endothelium of pulmonary capillaries [25]. These cells, called pulmonary intravascular macrophages (PIMs), can clear circulating material from the blood in much the same manner as KCs, resulting in biodistribution to the pulmonary capillaries [26]. In pigs, this pulmonary vector clearance correlates with severe Ad-induced toxicity, including extravasation of blood cells from the pulmonary circulation, congestion of the tracheobronchial lymph nodes, decreased respiratory rates and pulmonary hypotension [22]. These observations suggest that shifts in vector biodistribution among animal models can profoundly alter vector-induced toxicity.

In addition to clearance of Ad by the RES, erythrocytes in the blood have also been reported to be a significant sink for Ad vectors. Ad5 binds directly to the Coxsackie and adenovirus receptor (CAR) on human erythrocytes [27, 28]. When

opsonized by complement, Ad5 can also bind to complement receptor 1 (CR1) on erythrocytes [27]. Notably, rhesus macaque erythrocytes do not express CAR, while mouse erythrocytes express neither CAR nor CR1 [27]. The dramatic impact of these receptor differences on vector biodistribution and pharmacokinetics has been demonstrated in mice. Transgenic mice that express CAR on erythrocytes have prolonged vector circulation in the blood and significantly reduced liver transduction by Ad, as compared to wild-type mice that lack CAR on erythrocytes [27, 28]. Interestingly, transgenic expression of CR1 on mouse erythrocytes leads to substantially faster vector clearance, but hepatic transduction is reduced compared to wild-type mice [27]. It has not been reported whether these differences in pharmacokinetics and transduction efficiency modulate Ad-induced toxicity, but such information will be necessary to determine whether these transgenic model systems might be better preclinical models than wild-type mice.

Species-specific differences in hepatic sinusoids can affect the amount of vector that transduces hepatocytes. In order to reach hepatocytes, vectors in the circulation must pass through small pores in the sinusoidal endothelium (fenestrations). Species-specific differences in fenestration size have been reported in rats, mice, rabbits and humans [29, 30]. Substantial differences in fenestration size can also occur between different strains of rabbits [31] and even between individual rabbits of the same strain [30]. Snoeys et al. [29] noted a correlation between small fenestration size and decreased hepatocyte transduction, suggesting that fenestration size is a major limiting factor for vector hepatotropism. Rabbits (which have small fenestrations) show greater Ad-induced anemia and thrombocytopenia than mice (which have larger fenestrations), but it is unclear whether differences in fenestration size directly contribute to differences in toxicity [29].

Disease Can Affect Vector Biodistribution and Toxicity

Diseases such as cancer can alter the biodistribution of Ad vectors. Pande et al. [32] demonstrated that tumor-bearing mice exhibit a significantly expanded RES. This change in turn led to increased activation of macrophages by Ad and increased severity of Ad-induced shock. Liver cirrhosis is another disease that alters the RES. In cirrhotic rats, the RES shifts from the liver to the lungs due to development of PIMs in the pulmonary capillaries [33]. These cirrhosis-induced PIMs are much like the PIMs in the lungs of pigs, and thus cirrhotic rats show significantly increased Ad vector biodistribution to the lungs, and correspondingly decreased clearance by the liver [34]. IV injection of cirrhotic rats with Ad vector causes fatal pulmonary edema and hemorrhages that are not observed in normal rats with healthy livers [35].

Direct evidence of disease-induced PIMs in humans is difficult to obtain, but certain diseases are known to cause shifts in the RES to the lungs [36]. The human RES can be evaluated by IV injection with ^{99m}Tc colloidal tracers, and patients suffering from liver dysfunction and cancer show enhanced clearance of these

tracers in the lungs [37]. Higher severity of malignancy correlates with higher lung clearance of IV-injected particles [38]. These clinical observations suggest that disease-induced variations in the RES may have the potential to change vector biodistribution. Animal models that have similar RES alterations might play a useful role in predicting Ad toxicity in humans.

Shock

Severe local inflammatory reactions against viruses and other pathogens may evolve into uncontrolled systemic reactions. If untreated, a systemic reaction can develop into shock. Shock is defined as poor blood flow and insufficient oxygenation of tissues, and shock can lead to life-threatening organ damage. Intravenous delivery of Ad vector can cause rapid shock in animal models, which can be fatal at very high doses of vector. It is worth noting that Ad-induced shock occurs even in animals that have never been exposed to Ad. This toxicity is therefore distinct from anaphylaxis, which requires prior antigen exposure and the development of specific antibodies that sensitize to shock [39].

Behavioral symptoms of Ad-induced shock in laboratory animals include lethargy, difficulty breathing and reduced activity. Several studies have reported such behavioral symptoms within minutes after systemic administration of high doses of Ad5 vectors and other Ad serotypes [40, 41, 22, 42, 43]. In mice, shock-related pathology can sometimes occur even without visible signs of distress [21].

Vector-induced cardiovascular toxicity is a major contributor to Ad-induced shock. Following IV injection of Ad, acute systemic hypotension has been observed in mice, rats and pigs [44, 22, 21], as well as in a single human subject who received a very high dose of vector [45]. In addition to hypotension, Ad-induced shock can also involve additional cardiovascular toxicities, including bradycardia and dysregulation of the cardiac conduction system [44, 42].

Rodent models have been invaluable for determining how Ad vectors cause shock. Hemodynamic defects do not depend on viral replication, vector transduction or the presence of B and T cells [42]. However, induction of shock in mice requires intact vector, since IV administration of either heat-inactivated Ad or purified capsid proteins fails to elicit a hemodynamic response [44]. The RES also plays a key role in Ad-induced shock. In mice and rats, hemodynamic responses to Ad are abolished if KCs and splenic macrophages are depleted using clodronate liposomes prior to vector administration [44, 42, 21].

During shock, leakage of plasma from the vasculature into the surrounding tissue results in hemoconcentration and tissue edema. Platelet Activating Factor (PAF) is a phospholipid that mediates certain types of shock [46], and IV injection of PAF rapidly triggers vascular leakage, hypotension and other symptoms of shock [47]. In rats, IV Ad vector causes elevation of PAF in the circulation within minutes [21]. Pre-treatment of rats with PAF receptor antagonists abolishes Ad-induced shock

symptoms and pathology. The RES is centrally involved in Ad-induced shock, since splenectomy or RES macrophage depletion blocks the ability of Ad to induce PAF and shock. In sum, these results indicate that PAF is an essential mediator of Ad-induced shock and that induction of PAF by Ad depends on the RES.

Although PAF is a key mediator of Ad-induced shock, it is likely that induction of shock by Ad also involves other mediators downstream of PAF. For example, PAF induces nitric oxide, which is essential for PAF-induced vascular leakage [48]. Interestingly, it has been reported that IV administration of Ad increases levels of phosphorylated nitric oxide synthetase [42] and that antagonizing nitric oxide prevents Ad-induced mortality in tumor-bearing mice [32]. The work of Pande et al. also indicates that additional inflammatory mediators such as prostaglandins or leukotrienes contribute to Ad-induced shock. Inhibiting PAF, nitric oxide and other related mediators is a potential future approach to limiting the risk of shock following Ad gene therapy.

Thrombocytopenia

Platelets are important for Ad-induced toxicity in at least two ways. First, studies in mice have shown that platelet depletion prior to Ad can decrease the severity of vector-induced hepatotoxicity, indicating that platelets help to mediate Ad-induced organ toxicity [49, 50]. Second, Ad-induced loss of platelets (thrombocytopenia) can result in coagulation defects. Systemic injection of Ad vector causes thrombocytopenia in humans [51] as well as in animal models such as mice [52], rabbits [53] and non-human primates [54, 11, 55]. While modest decreases in platelet count are often asymptomatic, significant platelet loss can inhibit clotting and has the potential to cause complications such as internal bleeding. In non-human primates, Lozier et al. [54] showed that Ad-induced thrombocytopenia is dose-dependent and rapid (within hours after injection), followed by a slow recovery. In their study, clotting times were prolonged by as much as twofold in animals receiving the highest dose (3.8×10^{12} particles/kg) of Ad. This suggests, not surprisingly, that vector-induced thrombocytopenia can cause clotting deficiencies.

Thrombocytopenia can be caused either by increased clearance of platelets from the circulation or by decreased production of platelets, which are released by megakaryocytes in the bone marrow. In non-human primates, IV injection of Ad causes platelet counts to decline much faster than the normal half-life of these cells [54, 55]. It is therefore evident that Ad triggers thrombocytopenia by increasing the clearance of platelets from the circulation, rather than by disrupting bone marrow function. In mice, IV injection of Ad actually causes a delayed increase in the number of megakaryocytes [56], likely as a compensatory reaction to thrombocytopenia.

The mechanisms through which Ad induces thrombocytopenia are not completely understood. One hypothesis is that Ad may directly interact with platelets,

resulting in activation and removal of platelets. A number of studies have found that Ad binds to and activates human and mouse platelets in vitro [56–58]. However, other studies have shown that interaction of Ad with platelets is insufficient to induce platelet aggregation [59, 54].

How does Ad interact with platelets? Othman et al. [58] have proposed that Ad may bind directly to platelets through receptors such as CAR. Several groups have reported conflicting results regarding expression of CAR on human platelets, finding that 72% [58], 3.5% [60] and 0% [61] of platelets express CAR. Moreover, direct evidence of Ad binding to CAR on platelets has not been reported. Another potential receptor interaction is with platelet integrins. The Ad penton base has an arginine-glycine-aspartic acid (RGD) motif that facilitates viral internalization by binding to cellular integrins [62]. Platelets express $\alpha_{IIb}\beta3$ integrin (CD41) and other integrins that bind RGD motifs found in adhesive proteins such as fibrinogen [63]. Shimony et al. [61] observed that an engineered vector with an additional RGD motif in the fiber had an increased ability to bind to platelets, but there is currently no direct evidence that vectors with wild-type capsids use integrins as receptors on platelets. One major issue with analyzing Ad–platelet interactions in vitro is that such experiments do not adequately capture the complex interactions that occur in vivo, including opsonization of Ad by plasma proteins, rapid clearance of Ad from the circulation and interactions of platelets with leukocytes and endothelial cells. Arguing against the importance of CAR and integrins, a study in cynomolgous monkeys found that neither receptor interaction is essential for Ad-induced thrombocytopenia [64].

It has been suggested that platelets may contribute to Ad clearance, but evidence for this scenario is inconclusive. Othman et al. [58] found that IV injection of mice with Ad induced formation of platelet-leukocyte aggregates. Stone et al. [65] reported direct Ad5-platelet interactions in mice and observed that platelet depletion prior to IV Ad results in a significant reduction of Ad5 accumulation in the liver. However, the transgenic mice in this study expressed the human complement receptor CD46 on the surface of their platelets, which might have affected the results. A subsequent study in wild-type mice failed to observe any effect of platelets on vector clearance in the liver [66].

It is likely that activated endothelial cells contribute to Ad-induced thrombocytopenia. It is well recognized that Ad vectors rapidly activate endothelial cells in the liver and other organs [67, 42]. Endothelial activation can lead to increased exposure of von Willebrand factor (vWF), a multimeric glycoprotein that binds platelets and collagen with high affinity [68]. Shortly after IV administration of Ad, circulating levels of vWF become significantly elevated in rhesus macaques [54] and mice [58]. vWF knockout mice exhibit attenuated thrombocytopenia after IV injection of vector, providing direct evidence that vWF plays a role in Ad-induced platelet loss [58]. Thus, there is evidence that Ad can cause thrombocytopenia indirectly, through activation or damage of endothelial cells.

In sum, platelets are involved in clinically significant Ad-induced toxicity, and a better understanding of the mechanism for thrombocytopenia would be helpful when considering ways to mitigate this toxicity. Although there is evidence that Ad

activates platelets directly in vitro, it remains unknown whether this interaction occurs in vivo or contributes meaningfully to thrombocytopenia. It seems possible that Ad-induced thrombocytopenia might be mediated indirectly, by activation of other cell types such as endothelial cells.

Hepatotoxicity

The hepatotropic nature of commonly used serotypes such as Ad5 means that the liver is a key site of vector-induced toxicity. Ad uptake by the liver results in two phases of hepatotoxicity. The first phase is caused when the vector triggers a rapid innate inflammatory response, and the second phase is due to a T lymphocyte-driven adaptive immune response against foreign proteins in transduced hepatocytes.

Liver toxicity can be assessed clinically by measuring the levels of hepatocyte enzymes in the blood. These cytoplasmic enzymes leak from hepatocytes after disruption of their plasma membranes [69]. Aspartate transaminase (AST) and alanine transaminase (ALT) are both expressed by hepatocytes, but AST is also expressed widely by cells of the heart, skeletal muscle, brain and blood [70]. Given the higher specificity of ALT for hepatocytes, elevation of plasma ALT is a more reliable indicator of hepatotoxicity than elevation of AST.

Following systemic administration of Ad, elevated levels of serum ALT have been observed in mice [71, 72, 73, 18, 67, 11], non-human primates [23, 54, 74] and humans [51, 45]. This hepatotoxicity is mediated by both innate and adaptive immune responses, leading to two distinct phases of tissue damage.

Innate Immunity Leads to Early Hepatotoxicity

Ad vectors rapidly activate the innate immune system, and the resulting inflammation and tissue damage is the single most significant barrier to high-dose systemic therapy. Systemic administration of Ad rapidly induces pro-inflammatory cytokines and chemokines [67, 75, 11, 14]. Pro-inflammatory chemokines play an important role in the amplification of toxicity, as they attract circulating innate immune cells to transmigrate into the liver parenchyma. Muruve et al. [75] found that Ad-induced hepatocyte injury is partially mediated by neutrophil infiltration and that the chemokine MIP-2 plays a major role in the recruitment of neutrophils to the liver. Activated neutrophils are toxic to hepatocytes because they produce reactive oxygen species and release stored toxic proteins via degranulation [76]. In addition to causing toxicity, innate immune responses accelerate vector clearance. Although IV-injected Ad vector rapidly localizes to the liver, 90% of Ad vector DNA is eliminated from the liver within 24 h. This loss of vector DNA is due to innate immune responses that occur independently of T cells and transgene expression from the vector [13].

In vivo studies have demonstrated that innate inflammatory responses against Ad are triggered by the vector itself and do not depend on the expression of vector-encoded gene products. Ad vectors can be rendered transcription-defective through exposure to UV light. In mice and non-human primates, UV inactivation of Ad does not blunt innate inflammatory responses [67, 75, 11, 14]. When mice and non-human primates are injected with helper-dependent Ad (HDAd) vectors, which do not contain any viral genes, innate immune responses and hepatotoxicity are as robust as with E1-deleted vectors [23, 77]. These observations suggest that the Ad capsid, rather than the Ad genome, is the primary trigger for the innate immune response and hepatotoxicity.

In addition to being toxic to hepatocytes, Ad vectors induce striking and unusual toxicity in KCs [78, 42]. In mice, KCs became permeable to propidium iodide within 10 min of Ad injection, indicating loss of plasma membrane integrity [79, 80, 78]. Within an hour of Ad injection, KCs become necrotic, showing complete disruption of cytoplasmic, nuclear and plasma membrane compartments [78]. Studies with the Ad2 ts1 mutant, which binds normally to cells but is unable to lyse endosomes or escape into the cytoplasm, have shown that Ad must enter the cytoplasm in order to cause KC necrosis [81]. A recent study indicates that cellular interferon-regulatory factor 3 (IRF3) is required for Ad-induced KC necrosis [79]. Although IRF3 is a transcription factor, KC necrosis does not depend on activation of transcription by IRF3, demonstrating a novel unknown activity of this protein. Interestingly, Di Paolo et al. [79] found that IV administration of a pathogenic bacterium, *Listeria monocytogenes*, also causes KC necrosis in an IRF3-dependent manner and that killing of KCs by *Listeria* requires the membrane lytic protein listeriolysin O. Together, these findings suggest that KC necrosis may be triggered by membrane lysis, but the exact pathway for this unusual cellular toxicity remains unclear.

Interestingly, IV Ad also triggers the destruction of certain subsets of macrophages in the marginal zone of the spleen, including macrophages that express CD14 and MARCO [82, 11]. However, the mechanism of cellular toxicity differs for splenic macrophages and hepatic KCs; Di Paolo et al. [82] demonstrated that macrophages within the spleen are not immediately disrupted by Ad and are instead eliminated by newly recruited polymorphonuclear leukocytes. Mechanistically, the recruitment of PMNs to the spleen depends on Ad-induced chemokines and complement activation [82].

Adaptive Immunity Leads to Late Hepatotoxicity

After initial Ad-induced hepatotoxicity resolves, a second wave of hepatotoxicity may occur 1–2 weeks after vector administration. This second phase of hepatotoxicity is caused by adaptive immune responses directed against hepatocytes that

express vector-encoded proteins. In mice, the resulting toxicities include hepatitis, lymphocytic infiltration, transaminitis, loss of lobular structure, hepatocyte necrosis and turnover of hepatocytes [83–87]. In addition to causing hepatotoxicity, T cell-mediated destruction of transduced cells also results in loss of vector-encoded transgene expression [83, 84, 86]. In rhesus macaques, infusion of Ad via the portal vein or bile duct also causes T cell proliferation and lymphocyte infiltration in the liver [88]. The most severe hepatitis in these non-human primates was observed between 1 and 3 weeks post-Ad infusion, with liver pathology similar to that seen in mice.

In athymic mice that lack T cells, IV administration of Ad causes much less liver pathology than in wild-type mice [86]. Mice that selectively lack $CD4^+$ T cells also exhibit decreased T cell infiltration and less toxicity in both the lung and liver [89, 90, 87]. First generation E1-deleted Ad vectors express low levels of viral proteins [91, 86], and as a result, T cells target not only vector-encoded transgenes, but also Ad proteins [83–87]. Jooss et al. [92] used in vitro and in vivo T cell activation assays to identify the vector proteins that are targets of cellular immune responses in mice. Vector expression of hexon, fiber and the reporter protein β-galactosidase induced robust T cell responses. Penton was also capable of inducing T cell infiltration, but to a lesser extent than hexon or fiber. Experiments using UV-inactivated Ad demonstrated a requirement for the de novo synthesis of these proteins for T cell targeting [92].

Vectors with extensive early region deletions (and thus lower expression of viral proteins) induce significantly attenuated T cell responses and diminished liver toxicity [72, 93, 94, 95]. Furthermore, HDAd vectors devoid of all viral coding sequences have been shown to evade the adaptive immune system and to produce sustained transgene expression in the livers of mice [96, 77, 97] and non-human primates [98–100]. Thus, even though HDAd vectors still induce robust innate immune responses, T cell responses against these vectors are significantly attenuated.

Other Innate Immune Toxicities

In addition to the toxicities described above, several other types of toxicities have been observed in human trials but have not been as extensively studied in animal models. Many patients develop fever soon after local or systemic administration of Ad vector [101, 51, 45, 102]. Fever is often accompanied by flu-like symptoms including myalgia, rigors and weakness. Ad-induced fever has received little study in animal models, but significant Ad-induced elevations in body temperature occur in rats and rabbits [103–105]. In rats, local administration of Ad within the brain causes a rapid and significant increase in body temperature [103]. Treatment with IL-1 receptor antagonist prior to vector injection abolishes the fever response, indicating that IL-1 plays an essential role in Ad-induced fever. In mice,

interestingly, Ad has been reported to cause transient hypothermia, likely related to shock [44].

Administration of a high dose of Ad caused systemic inflammatory response syndrome (SIRS) in a patient undergoing Ad-mediated therapy for a partial ornithine transcarbamylase (OTC) deficiency [101]. SIRS is a life-threatening condition that is associated with high levels of cytokines and coagulopathy, leading to disseminated intravascular coagulation (DIC) [106]. DIC involves decreases in plasma fibrinogen levels and elevation of fibrin breakdown products such as D-dimers [107]. In mice, Ad causes elevated D-dimers [56], but in rhesus macaques, Ad causes only a minimal rise in D-dimers, along with a considerable increase in plasma fibrinogen [54]. These varied results suggest that Ad may be capable of inducing more than one type of coagulopathy, or that there are differences in coagulopathy between animal models and humans.

Bone marrow suppression was also noted in the OTC clinical trial, including leukopenia and red cell aplasia [101]. Similar Ad-induced bone marrow toxicities have been reproduced in animal models. Systemic administration of Ad in rhesus macaques and mice results in bone marrow hypoplasia, leukopenia and suppression of granulocyte-macrophage progenitor development [11, 52, 108]. Currently, the mechanisms underlying Ad-induced bone marrow toxicity are unknown, and further studies in animal models are warranted.

The Effect of Preexisting Immunity on Toxicity

While the vast majority of gene therapy studies in animals are performed in naïve animals, a large percentage of humans have been previously exposed to Ad during childhood infections [109–111]. Therefore, seronegative laboratory animals may not be representative of human patients, who will exhibit varying levels of immunity to Ad, including neutralizing antibodies against the virus. Antibody-mediated vector neutralization can significantly limit the efficiency of gene transfer [112, 113].

The effect of preexisting antibodies can be modeled either by pre-immunizing animals with Ad or by transferring Ad-specific antibodies to naïve animals. Rabbits that have received anti-Ad antibodies show an enhanced fever response to Ad [105]. Pre-immunization studies in mice and rhesus monkeys have demonstrated that previous exposure to Ad does not block Ad-induced innate immune toxicities [52, 108]. Twenty-four hours after vector administration, both naïve and pre-immunized animals have thrombocytopenia and elevated levels of inflammatory cytokines. Pre-immunization partially protected mice from Ad-induced hepatitis, but was associated with increased rapid mortality after IV injection of Ad, indicating that pre-immunization can exacerbate certain toxicities [52]. These deaths occurred within hours after IV Ad injection. Local intratumoral administration of vector has also been found to cause increased hepatotoxicity and mortality in pre-immunized mice compared to naïve controls [114]. It remains unclear how

pre-immunization increases lethal sensitivity to Ad vectors, and thus these observations are currently difficult to translate into predictions for clinical studies.

Although the early innate immune response to Ad remains robust in pre-immunized animals, the later wave of toxicity caused by the adaptive immune response can be severely attenuated [52]. One likely reason for lower toxicity is that preexisting antibodies neutralize vector and reduce transduction. In pre-immunized mice, Ad-induced plasma ALT elevations resolve in two days, while ALT levels in naïve mice continue to rise until day five and remain elevated for over two weeks [52].

Pre-immunization can also partially protect against Ad-induced bone marrow dysregulation. In monkeys and mice, pre-immunization can ameliorate Ad-induced bone marrow toxicity, leukopenia and suppression of granulocyte-macrophage progenitor development [52, 108]. However, pre-immunized animals are susceptible to Ad-induced suppression of erythropoiesis, an effect that is not seen when Ad is administered to naïve animals. Of note, red cell aplasia was observed in the OTC deficiency clinical trial patient discussed above, who had preexisting neutralizing antibodies against Ad [101]. Further mechanistic studies would be helpful in understanding the impact of preexisting immunity on Ad vector toxicity.

Looking Forward

As the studies discussed above illustrate, vector-induced toxicity limits the clinical utility of current Ad gene therapy vectors. Innate immune mechanisms not only lead to toxicity, but also to destruction of vector and transduced cells. Adaptive immunity limits the duration of transgene expression and has the potential to induce additional toxicities in transduced tissues. Several approaches to creating safer and more efficient vectors have been reported, including modification of the vector itself, altering vector administration protocols and pre-treatment with drugs that attenuate the innate and adaptive immune responses.

Genetic Modification and Use of Uncommon Serotypes

As discussed earlier, the effectiveness of first generation Ad vectors can be hampered by low levels of viral gene expression that trigger adaptive immune responses against the vector. The creation of gutless HDAd vectors eliminates any possibility of viral gene expression, and is one method of decreasing toxicities associated with adaptive immune responses [115]. However, HDAd vectors are still fully capable of inducing innate immune responses [77].

Engineering vectors that are capable of evading adaptive immunity is also complicated by the high seroprevalence of commonly used Ads such as Ad5 and Ad2. Because of this prior exposure, adaptive immune mechanisms may already be

primed to respond during initial administration of the vector. There is therefore considerable incentive to develop vectors that are based on rare Ad serotypes. Compared to Ad5, these "alternative" vectors may differ in their biodistribution profiles and ability to induce innate immune responses [40, 116, 43].

Capsid chimeras have also been investigated as a method for mitigating Ad-induced toxicity and avoiding preexisting immunity to common Ad serotypes. This approach can change vector biodistribution and immunogenicity [117]. Chimeras can be made by swapping hexon, penton or fiber from one serotype to another. For example, shortening the length of the fiber shaft on Ad5 can result in decreased liver tropism, reduced inflammatory responses and less toxicity to KCs [118, 119, 81, 43]. Modifications made to the fiber knob [120] and hexon [121] have also been reported to decrease vector hepatotoxicity in vivo.

De-targeting Vectors from the RES

Blocking the ability of the RES to take up Ad vectors is one of the potential avenues for decreasing innate inflammatory responses and increasing the amount of vector available for productive gene transfer. Scavenger receptors (SRs) on the KC membrane are key mediators of Ad vector clearance [122, 123, 124, 66]. SRs facilitate the binding and internalization of negatively charged materials including DNA, damaged erythrocytes, endotoxin, bacteria and viruses. In the case of Ad, SRs on the surface of RES macrophages may recognize the overall negative charge of the Ad capsid. In mice, pre-treatment with the negatively charged SR ligand polyinosinic acid (poly(I)) increases hepatocyte transduction by Ad and greatly inhibits KC uptake [122, 125, 123, 66]. However, polyanionic SR ligands are unlikely to be clinically useful, as studies in mice have shown that poly(I) can synergistically increase the toxicity of high doses of vector [124, 66]. In addition to SRs, studies in mice have demonstrated that opsonization of Ad, via natural antibodies and complement C3, can contribute to KC uptake of virus [80, 66]. Antibodies against the complement receptor CRIg can partially protect against KC death following Ad injection [80].

Since it will be challenging to inhibit all of the varied mechanisms that contribute to clearance of Ad by the RES, altering the vector seems to be a more feasible strategy. Changes to fiber have little or no effect on clearance of vector from the circulation by KCs [81], but alterations to hexon may have more potential impact. Khare et al. [121] found that a chimeric Ad5 vector with Ad6 hexon has reduced clearance by KCs and reduced hepatotoxicity. Another promising strategy for de-targeting Ad from KCs and reducing Ad-induced toxicity is chemical shielding of the vector capsid. Chemical modification is performed after virus purification; therefore it does not require any modification of virus production protocols. Currently, the most popular synthetic polymers for the modification of Ad are polyethylene glycol (PEG) and poly–*N*–(2-hydroxypropyl)methacrylamide (poly-HPMA) [126]. PEG is an uncharged, hydrophilic, linear polymer that can be

synthesized at different lengths based on the number of subunit (CH_2CH_2O) repeats. Poly-HPMA is also a hydrophilic synthetic polymer. HPMA is multivalent due to its chemical structure allowing for each polymer chain to bind to the viral capsid at multiple positions.

PEGylation of Ad alters vector clearance and improves toxicity profiles in mice. When intravenously injected, PEGylated Ad is able to evade uptake by the RES [127, 128]. PEGylated vector exhibits prolonged circulation times in the blood [9] and is protected from neutralizing antibodies [129]. PEG-shielded Ad induces less thrombocytopenia, less inflammation and lower transaminase levels [129, 127, 56]. Shielding of Ad with 2-HPMA has also been shown in mice to decrease innate toxicity and increase the circulation time of vector [130].

In addition to attenuating innate immune responses and avoiding preexisting antibodies, PEGylation also decreases adaptive immunity against Ad. Compared to unshielded vector, mice injected with PEGylated vector show significantly decreased T cell infiltration in the liver after IV administration of Ad [127].

Initial strategies for shielding Ad vectors involved indiscriminate attachment of PEG to lysine residues on the surface of Ad. While studies have shown that the extent of lysine PEGylation can be controlled so that it does not significantly compromise transduction [129, 128], Prill et al. [131] have demonstrated that inserting a single cysteine within hypervariable region 5 (HVR5) of hexon leads to a more targeted platform for shielding of the Ad capsid. With this system, polymers can be attached covalently to a single hexon residue via thioether bonds. Polymers can also be reversibly attached via disulfide bonds, allowing for shedding of shielding polymers during intracellular trafficking [132]. Another distinct advantage of vector shielding is its potential to be used in combination with other strategies to increase the safety profile of Ad. In mice, for example, the inflammatory response induced by PEGylated Ad can be further decreased if mice are pre-treated with glucocorticoids [127].

Altered Vector Administration Protocols

An additional strategy for decreasing toxicity is to increase the efficiency of Ad delivery to target tissues, so that less vector is required for efficient gene transfer. In the context of hepatic gene therapy, fenestrations in endothelial cells represent a significant bottleneck for systemically administered vector to reach hepatocytes.

Increased intravascular pressure during IV vector delivery leads to a transient increase in the size of liver fenestrae, allowing more efficient escape of Ad from the hepatic vasculature [133]. Blood pressure can be systemically increased by hydrodynamic injection: rapid IV administration of a large volume of fluid. In mice, delivery of Ad using hydrodynamic injection significantly increases hepatocyte transduction and decreases non-hepatic vector dissemination [134]. This study also demonstrated that hydrodynamic injection of vector induces lower levels of pro-inflammatory cytokines compared to conventional IV injection. Hydrodynamic

injection causes an irregular heart rhythm, although this irregularity resolves within a few minutes [133]. Hydrodynamic injection also causes significant increases in plasma transaminase levels, indicating that the procedure itself can induce acute hepatotoxicity [135].

Rapid systemic injection of large volumes of fluid is not feasible in larger animal models or human patients. However, intrahepatic pressure can be transiently increased by placement of balloon occlusion catheters in the inferior vena cava. In baboons, this pseudo-hydrodynamic procedure results in higher hepatocyte transduction and prolonged transgene expression without long-term liver toxicity [99, 135]. Similarly to hydrodynamic injection in mice, pseudo-hydrodynamic injection in non-human primates causes a transient increase in plasma transaminase levels [135]. However, this increase is modest compared to what is observed during hydrodynamic injection, suggesting that this modified procedure results in less acute liver toxicity.

Transient Immune Suppression

Pre-treatment with immunosuppressive agents is a strategy that can either be used alone or combined with other approaches, especially if the immunosuppressive agent is already approved for human use. In mice, administration of the glucocorticoids dexamethasone (Dex) or methylprednisolone (MP) significantly inhibits Ad-induced thrombocytopenia and reduces pro-inflammatory cytokines and chemokines [127, 136]. Seregin et al. [136] also observed significantly decreased neutrophil infiltration and endothelial activation within the liver, although hepatocyte toxicity was not assessed. In rats, Dex significantly reduces hemoconcentration associated with Ad-induced shock, but does not prevent Ad-induced hypotension [21]. In addition to inhibiting innate immune responses to Ad vectors, treatment with Dex also blunts adaptive humoral immunity directed against both the Ad capsid and transgene products [136].

Glucocorticoid pre-treatment also decreases inflammatory responses associated with local vector administration in mice, including vector delivery to the lungs [137] and salivary glands [138]. In a small clinical trial to treat mesothelioma, MP decreased the acute inflammatory response to intrapleural Ad vector, but had no effect on humoral or cellular immunity against Ad [139].

Nonsteroidal immunosuppressive treatments can attenuate Ad-induced adaptive immune responses in mice. Pre-treatment with calcineurin inhibitors decreases lymphocyte infiltration and extends transgene expression following intramuscular Ad vector injection [140, 141]. Cyclophosphamide treatment prior to intratracheal or IV administration of Ad significantly attenuates T cell responses and neutralizing antibodies against Ad [142]. Cyclophosphamide also decreases Ad-induced liver inflammation and reduces apoptotic and mitotic hepatocytes [142]. In rhesus monkeys, administration of a combination of cyclophosphamide and prednisone

significantly reduced antibody and T cell responses and led to sustained transgene expression [88].

Conclusions

Over the last two decades, great strides have been made in understanding how Ad tropism and toxicity are shaped by interactions with specific host factors. Nevertheless, vector-induced toxicity remains a significant limitation for high-dose systemic therapy with Ad vectors. In addition to hepatotoxicity, vectors may cause thrombocytopenia, systemic inflammation and shock. Improvements in vector design may help to evade the innate and adaptive immune responses that are responsible for many of these toxicities. It will be helpful to ensure that animal models adequately represent what will be encountered in the clinic, taking into account the possible impact of factors such as disease and preexisting immunity. Ultimately, a deeper understanding of Ad vector biology will be valuable for designing and testing improved Ad vectors.

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Helper-Dependent Adenoviral Vectors for Gene Therapy of Inherited Diseases

Pasquale Piccolo and Nicola Brunetti-Pierri

Abstract Helper-dependent adenoviral (HDAd) vectors that are devoid of all viral coding sequences are attractive vectors for gene therapy because they efficiently transduce a variety of cell types, have a large cloning capacity, have low risks of insertional carcinogenesis, and drive long-term transgene expression without chronic toxicity. The main limitation of HDAd vectors is the host innate inflammatory response elicited by capsid proteins that occurs shortly after intravascular administration and result in dose-dependent acute toxicity. Major efforts focused on elucidating adenoviral vector–host interactions have unraveled multiple factors involved in the acute toxicity. In this chapter, we provide a review of the most significant and advanced studies on the strategies to overcome the issue of acute toxicity and on the applications of these vectors for gene therapy of inherited diseases.

Keywords Replication · Encapsidation · Sub-acute toxicity · Systemic dissemination · Intrahepatic injection · Kupffer cells

Introduction

Helper-dependent adenoviral (HDAd) vectors, also known as "gutless" or "gutted" vector, are devoid of all viral coding sequences and only include the non-coding viral sequences required for vector genome replication and encapsidation. The deletion of large portions of the viral genome leaves abundant space to accommodate large genes and expression cassettes. Although multiple methods have been

P. Piccolo · N. Brunetti-Pierri
Telethon Institute of Genetics and Medicine,
Via Campi Flegrei 34, 80078 Pozzuoli (Napoli), Italy

N. Brunetti-Pierri (✉)
Department of Translational Medicine, Federico II University
of Naples, Naples, Italy
e-mail: brunetti@tigem.it

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developed [1–4], the most efficient and used method for HDAd vector production is based on the Cre/loxP system [5]. In this system, the HDAd genome is first constructed into a bacterial plasmid and released by restriction enzyme digestion. The HDAd genome is then transfected into 293 cells expressing the Cre recombinase (293Cre) that are infected with a helper virus (HV) (Fig. 1). The HDAd genome includes the expression cassette of interest and ~500 bp of *cis*-acting adenoviral sequences required for vector DNA replication (ITRs) and packaging (ψ). Moreover, a small segment of non-coding adenovirus sequence from the E4 region adjacent to the right ITR can be included to increase vector yield, possibly by enhancing packaging of HDAd genomes [6]. Stuffer DNA is often required to bring the size of the HDAd genome up to the packaging requirements of the viral capsid which are between 27.7 and 37.8 kb [7, 8]. The large cloning capacity of HDAd allows the inclusion of transgenes in their native genomic context that can result in higher levels and longer duration of expression of the transgenes compared to their cDNA counterparts [9–11]. The HV is an E1-deleted adenovirus that bears a packaging signal flanked by loxP sites that is excised from the HV genome by Cre-mediated site-specific recombination between loxP sites in infected 293Cre cells (Fig. 1). This makes the HV genome unpackagable but still able to trans-complement replication and encapsidation of the HDAd genome. Methods for rapid and robust large-scale production of high-quality HDAd with very low HV contamination have been developed [12].

In addition to the adenovirus serotype 5 (Ad5)-based HV, several other HV based on other serotypes (serotypes 1, 2, and 6) have been generated [13–15]. Therefore, genetically identical HDAd vectors with different capsids can be generated by switching the HV serotype used for vector production. Because there are ~50 human serotypes of adenoviruses, it may be possible to generate a panel of multiple HV serotypes to produce HDAd with different capsids but the same genome. Should transgene expression fade over time, re-administration of the same vector will be ineffective because the first administration elicits a neutralizing anti-adenovirus antibody response that prevents target cell transduction. Switching vector serotype may be a strategy to overcome the neutralizing anti-adenovirus antibody response. Available HDAd vector serotypes can be administered sequentially when transgene expression from previous vector administration is lost [10, 13–15].

Host Response and Interactions with HDAd Vectors

HDAd vectors elicit no chronic toxicity and result in long-term transgene expression and phenotypic correction in a wide variety of small and large animal models [16]. However, acute toxicity elicited by systemic administration of adenovirus-based vectors is an obstacle for clinical applications of HDAd vectors [17]. Major efforts have been focused on understanding the factors triggering the acute toxic response with the goal of developing strategies to blunt or to minimize

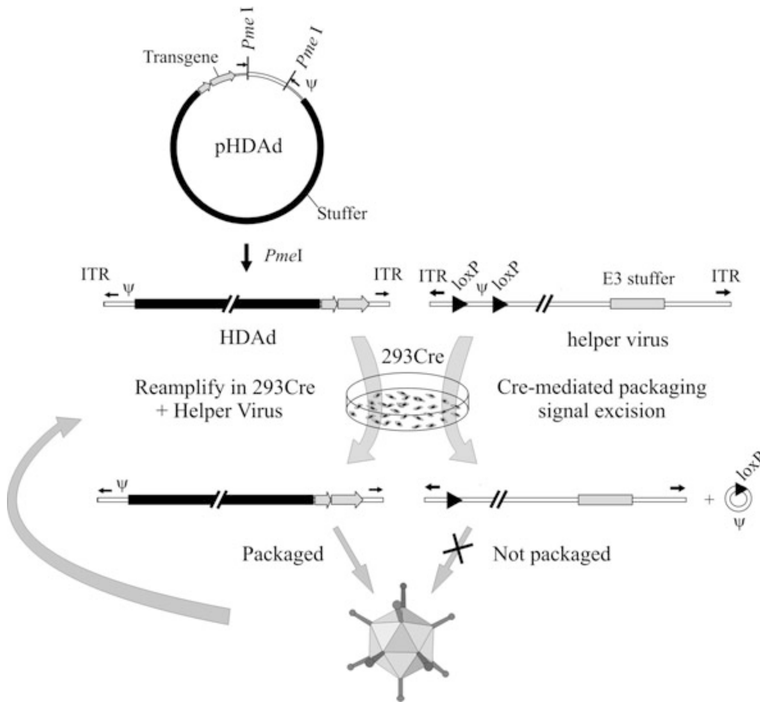

Fig. 1 The HDAd genome contains only ~500 bp of cis-acting adenoviral sequences needed for replication (ITRs) and packaging (ψ). The rest of the genome includes the desired transgene and "stuffer" sequences. The HDAd genome constructed as a bacterial plasmid (pHDAd) is released by restriction enzyme digestion (e.g., *Pme*I). To rescue the HDAd, the released genome is transfected into 293 cells expressing Cre and infected with a helper virus bearing a packaging signal (ψ) flanked by loxP sites. Cre-mediated excision of ψ makes the helper virus genome unpackagable but still able to replicate and provide all the necessary *trans*-acting factors for propagation of the HDAd. The titer of the HDAd vector is increased by serial co-infections of 293Cre cells with the HDAd and the helper virus. Adapted from [5]. Copyright (1996) National Academy of Sciences, USA

it. Additionally, strategies have been developed to improve the efficiency of the vector at transducing the target cells in order to administer lower, non-toxic doses.

Both sub-acute toxicity due to viral gene expression and chronic toxicity due to leaky viral gene expression arise weeks after the injection of early-generation adenoviral vectors but not after HDAd administrations [18, 19]. In contrast, systemic intravenous injections of high doses of either first-generation adenoviral or HDAd vectors can induce a potentially lethal, capsid-mediated, dose-dependent toxic response that occurs within minutes to hours after the injection [17, 20–23]. This acute inflammatory response by systemic adenoviral vectors has been observed in rodents, non-human primates, and humans, even though with different magnitudes depending on the host species, with mice being more tolerant to high vector doses [18, 21, 23]. This response is characterized by increased circulating

levels of pro-inflammatory cytokines and chemokines, a rapid hemodynamic response with hypotension, tissue edema, and vasocongestion [17, 24]. In humans, the death of a patient with partial ornithine transcarbamylase (OTC) deficiency that received a single intravenous injection of 6×10^{11} viral particles (vp)/kg of a second-generation (E1- and E4-deleted) adenoviral vector was attributed to this adenoviral vector-mediated toxicity [25].

Several mechanisms have been proposed to explain this acute inflammatory response, such as binding of adenoviral vector particles to antibodies [26–29], complement [26, 30, 31], innate immunity activation [32], and vector uptake and activation by reticuloendothelial system cells [21, 23]. The reader is referred to the chap. 3 by Dr. Byrnes of this book for a more detailed discussion of adenoviral vector toxicity and vector–host interactions.

Following intravenous injection of adenoviral vector particles, there is a nonlinear dose response to hepatic transduction, with low doses yielding very low to undetectable levels of transgene expression, but with higher doses resulting in disproportionately high levels of transgene expression. Unfortunately, high vector doses that result in systemic toxicity are required to achieve efficient hepatic transduction following systemic intravascular delivery. Kupffer cells laying on the wall of liver blood vessels directly contacting foreign material are responsible for this nonlinear dose response by avidly sequestering bloodborne adenoviral vector particles [28, 29]. Scavenger receptor A (SR-A) and scavenger receptor expressed on endothelial cells I (SREC-I) both expressed by Kupffer cells have been recognized as adenoviral vector particle receptors [33–35]. In addition, adenovirus-mediated hepatocyte transduction is hampered by the physical barrier of liver endothelial fenestrations [36–38]: Ad5 particles have a diameter of 93 nm with protruding fibers of 30 nm [37], whereas the diameter of human liver fenestration is ~ 107 nm, and thus, the relatively smaller size of liver fenestrations may be an obstacle for hepatocyte transduction in humans [38].

Once inside the infected cells, the HDAd genomes migrate to the nuclei where they are present as replication-deficient linear monomers both in cell culture and in mouse livers [39]. However, approximately 1–3% of nuclear HDAd genomes circularize and contain end-to-end joining of the adenoviral genome termini, at least in cell culture [40]. In the nuclei, HDAd genomes are assembled into chromatin through association with histones which promote efficient transgene expression [41, 42]. Although adenoviral vector genomes are episomal, integration into the host genome may occur. The frequency of HDAd genome integration in cell culture has been found to be $10^{-3} - 10^{-5}$ per cell depending on the experimental conditions [43–47]. Compared to culture cells, a lower in vivo integration frequency of 6.7×10^{-5} per cell was instead detected in mouse livers [48]. Based on this low frequency of integration, the risks of insertional carcinogenesis of HDAd vectors are likely very low.

Clinical Applications of HDAd Vectors

An HDAd vector has been used in an ex vivo phase I–II clinical trial to treat anemia of patients with end-stage renal disease [49, 50]. In this trial, dermal fibroblasts removed from the skin of patients were transduced ex vivo with an HDAd expressing erythropoietin (EPO) and then implanted autologously in the subcutaneous tissue under local anesthesia. A precise number of HDAd vector-transduced cells were implanted to obtain predetermined blood levels of EPO. There were no adverse events in this trial, and hemoglobin levels were sustained long term after a single treatment with the HDAd vector-transduced cells [49]. Besides EPO, this strategy has potential for applications in various clinical indications requiring sustained delivery of therapeutic proteins.

There has been a single case of in vivo intravascular administration of HDAd vector into a human patient. In this clinical trial, 4.3×10^{11} vp/kg of a HDAd vector expressing factor VIII (FVIII) was intravenously injected into a subject with hemophilia A [51]. This subject developed liver toxicity, increased IL-6, thrombocytopenia, and laboratory signs of disseminated intravascular coagulopathy. All these values returned to baseline by day 19 post-infusion. Unfortunately, no evidence of FVIII expression was detected [51] and several details about this trial remain unknown because they have not been published in a peer-reviewed journal.

Strategies to Improve HDAd Vector Therapeutic Index for Liver-Directed Gene Therapy

Given the potential for long-term transgene expression in vivo, various strategies to improve the therapeutic index of HDAd vectors and to overcome the obstacle of acute toxicity have been investigated. Because the severity of the acute response is dose-dependent and appears to correlate with vector systemic dissemination, the simplest approach was to preferentially deliver the vector to the target tissue, thereby allowing the use of lower, non-toxic doses.

For liver targeting, one strategy involved injection of HDAd vector directly into the surgically isolated livers of non-human primates that was shown to result in high efficiency of hepatic transduction with reduced systemic vector dissemination [52, 53]. However, this approach is invasive and consequently, a minimally invasive percutaneous balloon occlusion catheter-based method was developed to achieve preferential hepatocyte transduction. By this method, a balloon occlusion catheter was percutaneously positioned in the inferior vena cava (IVC) to occlude hepatic venous outflow and an HDAd vector was injected directly into the occluded liver via a catheter percutaneously placed into the hepatic artery (Fig. 2). This delivery method resulted in improvement of hepatic transduction compared to intravenous injection with negligible toxicity [54]. Moreover, this method allowed the delivery in rhesus macaques of a low dose of HDAd vector expressing the

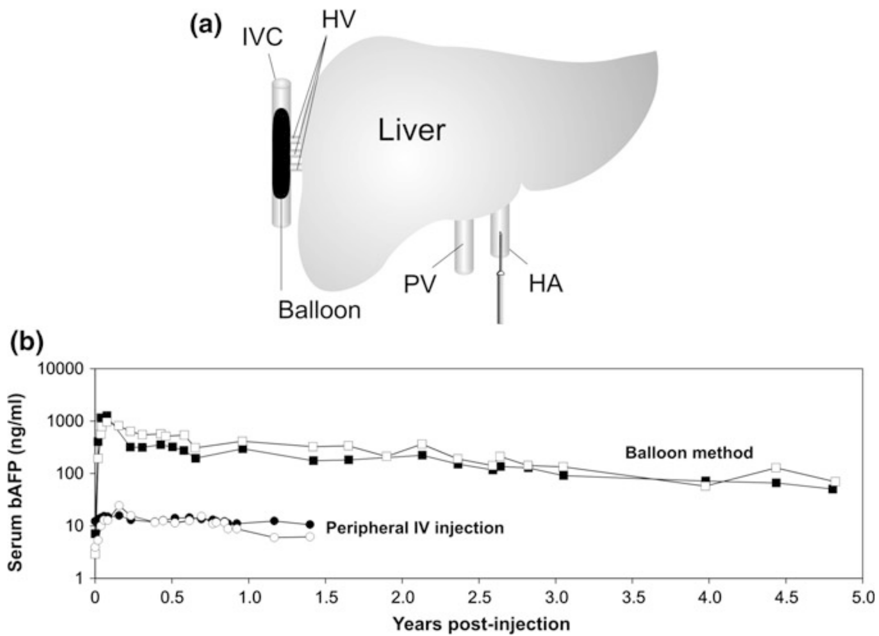

Fig. 2 **a** A sausage-shaped balloon catheter is percutaneously positioned in the inferior vena cava (IVC) under fluoroscopic guidance. Inflation of the balloon results in hepatic venous outflow occlusion from the hepatic veins (HV), and then the HDAd is injected through a catheter percutaneously positioned into the hepatic artery (HA). **b** Serum baboon alpha-fetoprotein (bAFP) levels following injections of HDAd expressing bAFP as secreted, non-toxic, non-immunogenic reporter gene by either the balloon method or by intravenous injection (adapted from [54])

human factor IX (FIX) to achieve sustained plasma FIX levels within the therapeutic range for hemophilia B [55].

This method was also used to deliver a HDAd vector expressing acid α-glucosidase (GAA) into baboons to investigate gene therapy for Pompe disease [56]. In this case, a single dose of HDAd also resulted in sustained liver expression and secretion of high levels of GAA. HDAd-driven hepatic GAA was detected in the heart, diaphragm, and skeletal muscles of the injected animals at levels that were predicted to correct the glycogen accumulation Pompe patients. Unexpectedly, a similar approach to deliver the low-density lipoprotein receptor (LDLR) gene into rhesus monkeys heterozygous for *LDLR* gene mutations resulted only in short-term reduction in hypercholesterolemia [57]. The reasons for such transient correction are unclear although differences in the transgene product and levels required to obtain reduction in hypercholesterolemia compared to those required to detect FIX or GAA might be involved.

Follow-up studies of different approaches for liver-directed gene transfer by HDAd vectors injected into baboons showed long-term transgene expression without adverse events for up to 7 years that corresponds to more than half of the

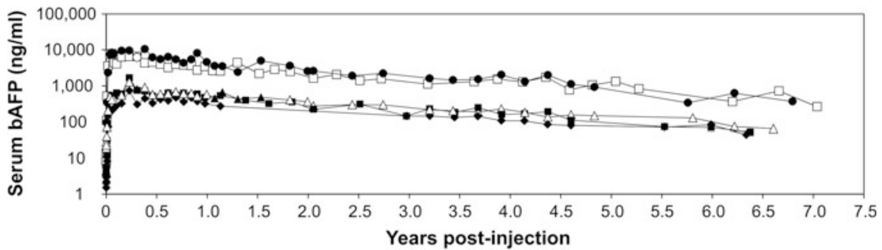

Fig. 3 Duration of transgene expression following administration of a HDAd expressing the bAFP in baboons (adapted from [59])

life span of most captive baboons [58]. Although they remained well above baseline values, the levels of transgene expression slowly declined over time to less than 10% of peak values by the end of the observation period (Fig. 3) [59]. The slow and steady decline in transgene expression is likely dependent upon the gradual loss of transduced hepatocytes due to physiologic hepatocyte turnover, loss of the extra-chromosomal vector genome, or a combination of both.

Intrahepatic injection of the vector is another approach to avoid vector systemic dissemination and can be effective in a limited number of disorders that can be treated by gene transfer restricted to few hepatocytes. Direct hepatic injections of adenoviral vectors have been performed by either laparotomy or ultrasound-guided percutaneous injections, are well tolerated in humans [60], and are similar to the procedure accomplished routinely for liver biopsies. This approach resulted in disease correction in a rat model of Crigler–Najjar syndrome, an inborn error of bilirubin metabolism [61].

Macrophages play a pivotal role in triggering the immune response to adenoviral vectors and have been a major target to dampen vector acute toxicity. Ablation of liver macrophages (i.e., Kupffer cells) by gadolinium chloride or clodronate liposomes prior to systemic vector injection resulted in increased hepatocyte transduction [29, 62]. However, the use of these compounds may be limited by their toxic effects [63–65]. Scavenger receptors on Kupffer cells bind adenoviral vector particles and remove them from the circulation, thus preventing hepatocyte transduction [66, 67]. Blocking the scavenger receptors can de-target HDAd vector particles from Kupffer cells to favor hepatocyte transduction [33, 66–68].

Polyethylene glycol (PEG) has been alternatively investigated to shield adenoviral capsids from binding to macrophages and blood factors. Coating of viral capsids with PEG resulted in prolonged circulating half-life, reduced Kupffer cell uptake, and increased hepatocyte transduction [35, 69, 70]. Moreover, it was found to improve vector safety, as shown by reduced activation of pro-inflammatory cytokines after systemic injection compared to uncoated vectors [71–73]. Nevertheless, PEG conjugation is non-specific, its interaction with capsid proteins is non-covalent, and it could abolish FX binding to vector, thus making viral capsids more accessible to complement binding that prevents liver transduction [74]. To address these issues, PEGylation has been directed to hexon hypervariable

region (HVR) to replace the natural FX shielding. These vector particles were protected from neutralization by natural antibodies and complement and retained efficient hepatocyte transduction in vivo [75]. Specific coupling of 5 K PEG or transferring to the hexon capsid protein of adenoviral vectors can also improve liver transduction, likely through evasion of Kupffer cells [69].

Another strategy to avoid Kupffer cell uptake is based on a chimeric vector in which the HVR of Ad5 is replaced with that of serotype 6. This Ad5/6 chimeric vector resulted in higher liver transduction and significantly lower hepatic toxicity compared to Ad5 vector in mice [76]. Although the above studies showed that HDAd-mediated hepatic transduction in mice was not compromised by PEGylation, this was not the case in non-human primates [77], emphasizing that caution should be taken in extrapolating results from rodents to larger animals and humans.

HDAd Vectors for Cystic Fibrosis Gene Therapy

Airway administration of early-generation adenoviral vectors resulted in lung inflammation due to expression of the viral genes in the vector backbone which are directly cytotoxic and result in an adaptive immune response against the transduced cells, ultimately leading to transient transgene expression [78–83]. In contrast, HDAd do not induce pulmonary inflammation and can drive long-term transgene expression [84–89]. Moreover, following administration of agents that open the tight junctions to gain access to the basolateral viral receptors, HDAd resulted in extensive and long-term transduction of proximal and distal airways from the trachea to bronchiolar epithelium and submucosal glands, which are targets for cystic fibrosis (CF) gene therapy [90, 91].

Aerosolization of HDAd formulated in 0.1% L-α-lysophosphatidylcholine (LPC) to open tight junctions into trachea and lungs of rabbits resulted in extensive transduction of the airway epithelium [92]. However, rabbits, including those given LPC only as controls, exhibited a transient decrease in dynamic lung compliance immediately following aerosol delivery. Aerosolization under bronchoscopic guidance of HDAd into the lungs of pigs also showed that transgene products were efficiently expressed in lung airway epithelial cells and submucosal glands [93]. Moreover, vector-encoded CFTR protein localized to the apical membrane of both ciliated and non-ciliated epithelial cells, mirroring the location of wild-type CFTR [93].

Taken together, these studies show high-efficiency transduction of the airway epithelium in large animal models and are promising for CF gene therapy. However, there are several issues that need to be addressed prior to clinical application of HDAd vector in CF gene therapy, such as the likely reduced efficiency of transduction in severely diseased CF airways, the requirement to transiently disrupt the tight junctions that raises safety concerns especially in CF patient airways colonized with several pathogens, and the high turnover of airway epithelial cells that might require multiple vector administrations.

Concluding Remarks

The results of several preclinical studies with small and large animal models have shown the potential of HDAd vectors for a wide variety of diseases. The ability to drive long-term transgene expression with no chronic toxicity, the large cloning capacity, and the low risks of insertional carcinogenesis are major strengths of HDAd vectors. However, the dose-dependent activation of the innate inflammatory response by viral capsids remains an important concern for those applications requiring systemic intravenous injections of high vector dose to achieve clinically relevant benefits. Although knowledge about adenovirus–host interactions occurring following systemic intravascular administrations has been largely gained, effective strategies still need to be developed to minimize, if not eliminate, the acute inflammatory response. Nevertheless, in vivo applications that require very low and/or localized vector doses or ex vivo gene transfer strategies that do not induce an innate inflammatory response hold potential for clinical translation. Physical method, such as balloon catheter-assisted delivery, for example, would result in more efficient and safer gene delivery at vector doses that are attractive for clinical applications.

To date, HDAd-transduced hepatocytes in small and large models, including non-human primates, are not eliminated by the immune system and have resulted in multi-year transgene expression. Whether this holds true for humans is not known at the present time, particularly in consideration of the outcomes of the AAV clinical trials for hemophilia B that resulted in a cytotoxic T lymphocyte (CTL) immune response against AAV-transduced hepatocytes [94, 95].

The safety of AAV has been challenged by few studies that documented hepatocellular carcinoma and vector genomic integration after AAV gene delivery in mice [96–98]. Moreover, a recent study reports that natural infections in humans with serotype 2 AAV resulted in chromosomal insertions activating proto-oncogenes in the liver, and it suggests that the AAV integrations cause cancer [99]. Although the risk of hepatocellular carcinoma development by AAV vectors remains to be fully understood, it is important to continue investigations on other vector systems, particularly those, such as HDAd vectors, that have a very low frequency of genomic integration.

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AAV Vector-Based Gene Therapy, Progress and Current Challenges

Klaudia Kuranda and Federico Mingozzi

Abstract Therapeutic efficacy of the adeno-associated virus (AAV) vector gene transfer has been shown by the large number of proof-of-concept studies in animal models. These preclinical studies established a rich pipeline of gene therapy drugs that could be brought to the clinic. Consequently, in recent years, the number of clinical trials in which AAV vectors were used for in vivo gene transfer increased significantly. The excellent safety profile and the high efficiency of transduction of a broad range of tissues promoted AAV vectors as the platform of choice for in vivo gene therapy and they have been successful in the clinic for a variety of indications including hemophilia B, choroideremia and other disorders. Aside from the evidence of clinical success, the recent market approval of the first AAV-based gene therapy drug in Europe represented another important milestone for the field of gene therapy, attracting the interest of investors after a long period of neglect. Nevertheless, clinical translation of novel therapies is a process that involves several bench-to-bedside iterations, during which possible issues of the novel technology may be identified and solved. For the AAV vector gene transfer technology, several hurdles have been highlighted in both preclinical studies and clinical trials; addressing these issues contributed to expand the number of indications in which clinical success was achieved. A lot more need to be carried out, for example to gather crucial information on the interactions between AAV-based therapeutics and

F. Mingozzi (✉)
Genethon, 1 rue de l'Internationale, 91000 Evry, France
e-mail: fmingozzi@genethon.fr

K. Kuranda · F. Mingozzi
University Pierre and Marie Curie-Paris 6, Paris, France

F. Mingozzi
INSERM UMR951, Evry, France

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the host immune system. In this book chapter, we will discuss some of the key approaches to design AAV-based gene therapy strategies and will present the main achievements and emerging issues of the field, using the liver as an example of target tissue.

Keywords Serotypes · Self-complementary · Deficiency · Hemophilia · Immune response · Humoral immunity · Chimeric capsid · Kozak sequence · Codon optimization · Hepatocyte · Genotoxicity

General Features of AAV Vectors

Wild-Type AAV

Adeno-associated virus (AAV) is a small (~25 nm) virus composed of a non-enveloped icosahedral capsid (protein shell) that contains a linear single-stranded DNA genome of about 4.7 Kb. AAV belongs to the family of *Parvoviridae*, genus *Dependovirus*, as it can replicate in the nucleus of target cells only in the presence of helper viruses such as adenovirus or herpes virus [1]. The AAV genome is flanked by two palindromic inverted terminal repeats (ITR, 145 bp) and includes two open reading frames, *rep* and *cap*. *Rep* encodes proteins involved in: (i) replication of the viral DNA; (ii) transcriptional control of viral genes; (iii) packaging of newly synthetized single-stranded AAV genomes into the capsid; and (iv) site-specific genome integration in the host cell DNA [1]. *Cap* encodes for the VP1, VP2 and VP3 proteins that form the capsid and for the assembly activating protein (AAP) that is required for the starting of capsid assembly in the nucleus [1]. AAV naturally infects humans; usually, exposure to the wild-type virus occurs at around one–three years of age [2–4] and is not associated with any known disease or illness [5]. Importantly, the timing of human exposure to AAVs determines the host immunological response to the recombinant AAV vectors. AAVs infect both dividing and non-dividing cells, integrate into specific chromosomic loci [adeno-associated virus integration sites (AAVS)] and remain latent in the host cell DNA unless a helper virus provides the functions for its replication [1]. Three integration sites for wild-type AAV serotype 2 have been identified: AAVS1-3, respectively, on chromosomes 19, 5 and 3. For AAVS1 the frequency of integration in human and rhesus macaque DNA is estimated to be about 0.5% [1]. More recently, a study on the integration of wild-type AAV in human liver established a link between the insertion of portions of the AAV genome the promoter regions of genes linked to cancer and hepatocellular carcinoma [6]. The relevance of this finding to AAV vectors has been disputed [7], and future studies as well as long-term monitoring of human subjects who received AAV vectors will help assessing the real genotoxicity risk associated with this vector platform.

Recombinant AAV Vector Structure and Production

In the genome of recombinant AAV vectors, the only viral sequences that are retained are the two ITRs (*cis* packaging signals) while the sequences encoding *rep* and *cap* are exchanged with the exogenous DNA of choice (that is flanked by the ITRs and it is referred to as the transgene expression cassette). *Rep* and *cap* are nonetheless required for the production of AAV vectors, and to this end they are provided in *trans* to the packaging cells together with the adenoviral helper functions [8, 9]. AAV vectors can be produced at high yields by transient triple transfection of mammalian cells [10] or infection of packaging eukaryotic [11] and insect cells [12]. The triple transfection method is one of the most commonly used for AAV vector production and it is based on the co-transfection into permissive cells (usually human embryonic kidney 293 cells) of three plasmids:

- one containing the transgene of interest flanked by the viral ITRs;
- a packaging plasmid encoding for the rep and cap proteins;
- a plasmid encoding for adenoviral helper genes [8, 13].

The purification of recombinant AAV vectors for preclinical and clinical applications is performed by either column chromatography or physical methods (CsCl-gradient centrifugation) [8]. Based on the purification method, the removal of both cellular debris contaminants and the AAV empty capsids may vary, and one important focus in the field is to continuously improve the AAV manufacturing processes to increase both vector yield and purity [8, 9, 14, 15].

Cells Transduction with AAV Vectors

Transduction of cells by AAV vectors occurs by a series of sequential events, including: interaction of the viral capsid with receptors on the surface of the target cell, internalization by endocytosis, intracellular trafficking through the endocytic/proteasomal compartment, endosomal escape, nuclear import via the nuclear port complexes [16], virion uncoating and viral DNA double-strand conversion leading to the transcription and expression of the transgene [17].

The conversion of the AAV genome from single-stranded to double-stranded DNA occurs by both de novo synthesis of the complementary DNA strand (second-strand synthesis) and base pairing of complementary single-stranded AAV genomes derived from separate AAVs co-infecting the same cell and carrying plus and minus genomes (strand annealing). The frequency and efficiency of strand annealing have been reported to increase proportionally by increasing the dose of AAV vector per cell [17].

Differently from the wild-type virus, the genome of the recombinant AAV vectors does not undergo site-specific integration in the host DNA but mainly remains episomal in the nucleus of transduced cells, while random integration events are observed with a low frequency (0.1–1% of transduction events) [5, 18, 19].

To date, 12 different AAV serotypes and 108 isolates (serovars) has been identified and classified [1, 20]. The versatility of the AAV production system allows to easily generate hybrid AAV vectors composed by the same transgene flanked by the ITRs from serotype 2 [21] (so far the most commonly used) and any of the available AAV capsid [1]. AAV vectors obtained through this pseudotyping method are often referred as to AAV2/n, where the first number refers to the ITRs and the second to the capsid. Since the capsid interacts with receptors on target cells and impact on the post-entry transduction steps, AAV vectors bearing different capsids have different transduction abilities (i.e., cell tropism and kinetic of transgene expression) and thus one can ideally choose the most appropriate to target the cell of interest [1, 22]. When screening AAV serotypes to identify the best suitable one to target human tissues, the choice of the preclinical model appears to be crucial for success in human trials [23]. This has been exemplified in a recent study in which, for example, mouse model repopulated with human hepatocytes appeared to be more predictive of the outcome of gene transfer in humans [24]; according to these findings, some AAV serotypes like AAV3B do not transduce efficiently mouse hepatocytes but outperform several other serotypes in transducing non-human primate livers [25].

AAV Vector Engineering

So far, AAV vectors have been generated from many naturally occurring serotypes [1, 22]. More recently, engineered AAV vectors have been generated carrying novel capsids derived from rational design or directed evolution, significantly expanding the AAV vector toolkit [22, 26, 27, 28]. In particular, the increasing knowledge of AAV capsid structure-function [29] allowed to modify specific capsid amino acid residues by rational design while the development of AAV capsid libraries and high-throughput screening methods allowed to generate a huge variety of novel capsids and to select the most efficient at transducing the desired cell type in vivo by directed evolution [26]. Another promising approach to capsid engineering recently proposed [30, 31] consists of reconstructing ancestral AAV capsids to obtain AAV vector variants with the desired tissue tropism.

Additional maneuvers to improve capsid characteristics consist of introducing point mutations that can result in enhanced trafficking of virions to the nucleus and decreased proteasomal degradation [32], leading to higher transduction efficiency and lower immunogenicity [33].

The development of novel "synthetic" AAV vectors responds to the need for improving transduction and efficacy while reducing immunogenicity, toxicity and off-targets. These second-generation vectors are currently being evaluated in several preclinical models and could possibly substitute in the future the vectors derived from the naturally occurring serotypes [22].

AAV engineering has not only involved the capsid but also the genome of the vector. These efforts have been aimed at overcoming some of the key limitations of AAV vectors, such as the slow onset of gene expression (due to the inefficient conversion of single-stranded to double-stranded AAV genome) and the limited DNA cargo capacity ($\sim$5 Kb). In particular, McCarty and colleagues showed that the second-strand synthesis step in AAV vector transduction can be circumvented by using self-complementary (sc) AAV vectors [34]. scAAV vectors are produced by mutagenizing one of the two ITRs flanking the transgene so that during the AAV vector production the rep protein cannot solve the replication intermediates [34]. This results in packaging of a "ready to express" complementary dsDNA DNA genome containing both plus and minus vector genome strands. However, since the self-complementary genome cannot exceed the normal AAV packaging capacity (4.7 kb) only transgenes up to $\sim$2400 base pair in length could be used to generate scAAVs, significantly limiting the number of applications of this platform [34]. Notably, scAAV vectors have been demonstrated to drive faster onset and higher levels of transgene expression in a variety of tissues in animal models [34, 35].

The small packaging capacity of AAV vectors (4.7 Kb) precludes the AAV-based delivery of a number of genes that exceed this length and/or the use of large physiological regulatory elements [36]. The AAV genome size limitation can be currently bypassed by using two main strategies: oversized AAV vectors and dual AAV vectors [36–38]. Oversized AAV vectors can be generated by using large (>5 Kb) ITR-flanked transgenes during AAV vector production, and this leads to the packaging of genomes of heterogeneous size, which are mostly truncated at around 5 Kb (the AAV packaging limit) [39–41]. Dual AAV vectors are instead generated by splitting a large transgene expression cassette in two separate halves (5′ and 3′ ends, or head and tail); each half of the cassette is packaged in a single AAV vector of regular size (<5 Kb) [37]. The re-assembly of the full-length transgene expression cassette is achieved upon co-infection of the same cell by both dual AAV vectors followed by: (i) homologous recombination between 5′ and 3′ genomes (dual AAV overlapping vectors); (ii) ITR-mediated tail-to-head concatemerization of 5′ and 3′ genomes (dual AAV trans-splicing vectors); or (iii) a combination of the two mechanisms (dual AAV hybrid vectors) [36]. Notably the use of both oversized and dual AAV vectors in vitro and in vivo results in the expression of full-length proteins and therapeutic efficacy in animal models; however, the efficiency of these systems is still low when compared to canonical single AAV vectors [36], thus requiring high vector doses to achieve therapeutic efficacy.

Additionally, it still needs to be clarified whether the expression of full-length proteins by oversized AAV vectors derives from the delivery of a minority of intact large genomes (>5 kb) or re-assembly of small fragmented genomes in infected cells [39]. The heterogeneous nature of the genomes contained in oversized AAV vectors [39] may constitute an additional challenge to the development of gene therapy products based on this platform. For dual AAV vectors, while promising results have been obtained in several animal models of diseases [37, 42, 43, 44, 45, 46], the need for high vector doses [45–47] and the fact that the efficiency of reconstitution of the full-length genome may vary based on the dual AAV vector system used and cell type (e.g., due to the inherent ability of the cell machinery to drive homologous recombination and/or other DNA repair mechanisms) [43] may represent important constraints to the broad use of this technology. Nevertheless, for some applications, like for gene transfer directed to confined tissues, like the eye, the use of the dual AAV vector platform may represent an efficient and viable gene transfer strategy for transgenes of >5 Kb in size [43, 48]. For other applications, like gene transfer for muscular diseases, promising results are being achieved [45–47]; nevertheless, further improvements of overall transduction efficiency are needed to support clinical development.

Clinical Applications of AAV Vectors

The first gene therapy was introduced in clinics about 20 years ago in order to treat adenosine deaminase deficiency (ADA-SCID) in children [49]. At that time, investigators used a retrovirus for gene delivery, but since then the vector toolkit available to investigators became more diversified. Currently, clinical trials using AAV represent about 6% of all gene therapy trials [50] and are recognized as the most promising in vivo gene delivery tool for treatment of multiple monogenic diseases (e.g., hemophilia, Duchenne muscular dystrophy) as well as by complex mix of genetic and environmental factors (e.g., rheumatoid arthritis, Parkinson's disease).

AAV-based gene therapy has been tested in over 60 clinical trials (http://www.gemcris.od.nih.gov/) showing the most outstanding results when immunoprivileged body sites, like the eye, are targeted [51]; nevertheless, overall safety profile of AAV appears to be impressive. Perhaps the most spectacular results were obtained in several trials in the context of Leber's congenital amaurosis (LCA), where blindness of treated subjects was successfully reversed [52–56]. None of those subjects had adverse effects nor developed antibodies against transgene (*RPE65*) or vector capsid. Another attractive target disease for gene therapy with AAV vectors is hemophilia B, in which stable expression of coagulation factor IX can correct the bleeding diathesis [57, 49]. Hemophilia B has always been considered as a relatively "easy" target, since a very small correction of factor IX

activity ($\sim$5% of normal circulating levels of the enzyme) significantly ameliorates symptoms of the disease. Differently from the *RPE65* deficiency trials, in the hemophilia trials conducted thus far, muscle [58, 59] or hepatocytes [57, 60, 61] were targeted with high doses of vector. This highlighted important interactions between AAV vectors and human immune system, which have not been predicted in preclinical studies.

Of note, in 2012 the European Commission has granted marketing authorization for the first gene therapy drug, Glybera® (http://www.unicure.com) [62]. This drug is an AAV1 vector encoding for lipoprotein lipase (LPL), injected intramuscularly, that has been shown to ameliorate the condition of patients with LPL deficiency (LPLD) [63–67]. Prior to development of this medicine, there was no cure available for LPLD and patients were obliged to follow a very restrictive low-fat diet and were subjected to frequent, life-threatening pancreatitis. Data from several clinical trials with the gene therapy drug suggest that injection of Glybera can ameliorate the disease phenotype and reduce the pancreatitis episodes.

In Table 1, we summarized the AAV-based clinical trials directed to diseases affecting various organ systems and using different routes of administration, including the lung via the airway, direct injection to muscle, brain and liver via hepatic artery. These examples illustrate well the wide range of possible applications of AAV vectors.

The Liver, a Versatile Platform for Multiple Gene Therapy Approaches

The liver is a particularly attractive organ for the development of gene-based therapeutic approaches for a number of reasons including: (1) It is one of the body's major biosynthetic organs; (2) studies in small and large animal models and in humans have demonstrated that it is possible to target hepatocytes with high efficiency using AAV vectors administered intravenously [57, 35, 61]; (3) despite the predominantly non-integrative nature of AAV vectors [19], multi-year transgene expression after gene transfer to the liver has been documented in large animals and humans [99, 100]; (4) expression of a transgene in hepatocytes induces antigen-specific tolerance mediated by regulatory T cells [101–104]; and (5) several preclinical studies demonstrate that it is possible to treat not only plasma protein deficiencies but also metabolic disorders with liver gene transfer, resulting in long-term cure for many of these disorders in small and large animal models (Table 2).

To date, liver gene transfer with AAV vectors has been tested in the clinic only for few indications, although the landmark results obtained in the context of liver gene transfer for hemophilia B [57, 61] paved the way to a number of clinical

Table 1 Current and completed clinical trials with AAV vectors

Disease		Transgene	Route of administration	AAV serotype	Reference
Familial lipoprotein lipase deficiency	Disorder that impairs breakdown of fatty acids	Lipoprotein lipase—LPL	Intramuscular	AAV1	[64, 65]
Cystic fibrosis	Lethal autosomal disease that affects mostly the lungs but also the pancreas, liver, kidneys and intestine	Cystic fibrosis transmembrane conductance regulator—CFTR	Lung, via aerosol	AAV2	[68–74]
Hemophilia B	Blood clotting disorder leads to an increased propensity for hemorrhage	Factor IX—FIX	Intravenous, intramuscular, hepatic	AAV8	[57, 60, 61]
Batten's disease	Fatal autosomal recessive neurodegenerative disorder that begins in childhood	Neuronal ceroid lipofuscinosis—CLN2	Intracranial	AAV2, AAV rh10	[75]
Canavan's disease	Most common degenerative cerebral diseases of infancy	Aspartoacylase—ASPA	Intracranial	AAV2	[76]
Parkinson's disease	Degenerative disorder of the central nervous system mainly affecting the motor system	Glial cell line-derived neurotrophic factor—GDNF or neurturin—NTN or aromatic L-amino acid decarboxylase—AADC	Intracranial	AAV2	[77–80]
Giant axonal neuropathy	Chronic neurodegenerative disease fatal by the third decade of life	Gigaxonin—GAN	Lumbar intrathecal injection	AAV9	–
Alzheimer's disease	Chronic neurodegenerative disease leading to short-term memory loss and ultimately to death	Beta nerve growth factor—β-NGF	Intracranial (stereotactic injection)	AAV2	[81]
Mucopolysaccharidosis	Lysosomal storage disease associated with developmental delay, severe hyperactivity, spasticity, motor dysfunction, death by the second decade	*N*-acetylglucosaminidase—NAGLU or heparan sulfamidase—SGSH		AAV9	–

(continued)

Table 1 (continued)

Disease		Transgene	Route of administration	AAV serotype	Reference
Galactosialidosis	Autosomal recessive lysosomal storage disorder with a broad spectrum of clinical manifestations	Protective protein cathepsin A—PPCA	Intravenous infusion	AAV8	–
Homozygous familial hypercholesterolemia	Severe elevation of serum LDL leading to premature and lethal coronary artery disease	Low-density lipoprotein (LDL) receptor LDLR	Hepatic artery	AAV8	–
Prader–Willi syndrome	Genetic disorder characterized by low muscle tone, short stature, cognitive disabilities and a chronic feeling of hunger that can lead to life-threatening obesity	Brain-derived neurotrophic factor—BDNF		AAV2	–
Heart failure	Wide range of abnormalities that impair normal cardiac function	Sarcoplasmic endoplasmic reticulum calcium ATPase—SERCA2a	Intracoronary	AAV1	[82, 83]
Becker muscular dystrophy	X-linked recessive inherited disorder characterized by slowly progressive muscle weakness of the legs and pelvis	Follistatin 344—FS344	Intramuscular	AAV1	[84]
Spinal muscular atrophy type 1	Autosomal recessive disease of early childhood leading to progressive muscle wasting and mobility impairment	Survival motor neuron protein—SMN	Intravenous peripheral limb vein	AAV9	[85]
Limb girdle muscular dystrophy type 2D	Progressive muscle wasting which affects predominantly hip and shoulder muscles	Alpha sarcoglycan—SGCA	Intramuscular or femoral artery limb perfusion	AAV1, AAV8	[86]
Duchenne muscular dystrophy	Recessive X-linked disease, which results in muscle degeneration and premature death	Mini-dystrophin or GalNAc transferase—GALGT2	Intramuscular	AAV2 rh74, AAV5	[87, 88]
Dysferlinopathy	Autosomal recessive neuromuscular disorder characterized by progressive muscle wasting	Dysferlin—DYSF	Intramuscular	AAV6	[89]

(continued)

Table 1 (continued)

Disease		Transgene	Route of administration	AAV serotype	Reference
Flexor tendon injury	Inability to bend fingers	Vascular endothelial growth factor—VEGF	Intramuscular	AAV2	[90]
Pompe disease	Glycogen storage disease, which damages muscle and nerve cells throughout the body	Acid alpha glucosidase—GAA	Intramuscular, diaphragm	AAV1	[91]
Alpha-1 antitrypsin deficiency	Genetic disorder provoking respiratory problems and impaired liver function	Alpha-1 antitrypsin—AAT	Isolated Limb Infusion; intramuscular	AAV1	[92, 93]
Leber congenital amaurosis	Autosomal recessive disorder associated with multiple genes and leading to severe vision loss or blindness	Retinal pigment epithelium-specific 65-kDa protein—RPE65	Subretinal	AAV2	[52–56]
Leber hereditary optic neuropathy	Mitochondrially inherited degeneration of retinal ganglion cells and their axons that leads to loss of central vision in young adult males	NADH ubiquinone oxidoreductase subunit 4—ND4	Intravitreal	AAV2	–
X-linked retinoschisis	Early onset retinal degenerative disease leading to juvenile macular degeneration in males	Retinoschisin—RS1	Intravitreal	AAV2tYF	–
Choroideremia	X-linked, recessive, degenerative disease of the retina leading eventually to blindness	Rab escort protein 1 REP1 or retinal pigment epithelium-specific 65-kDa protein—RPE65 or	Intraocular	AAV2	[94, 95, 62]
Neovascular age-related macular degeneration	Disease characterized by a growth of new blood vessels beneath the retina, which leakage causes damage to retinal cells and create blind spots in central vision	VEGF receptor—FLT01 or sFlt-1	Subretinal	AAV2	[96]
Congenital achromatopsia	Retinal disorder characterized absence of daylight vision from birth, photophobia and a slowly progressing loss of cone photoreceptors	Cyclic nucleotide gated channel beta—CNGB3	Subretinal	AAV2tYF	–
Rheumatoid arthritis	Long-lasting disorder that primarily affects joints. It typically results in warm, swollen and painful joints	TNFR-Fc	Intra-articular	AAV2	[97, 98]

Table 2 Examples of proof-of-concepts of therapeutic efficacy of AAV-based gene transfer in animal models

Disease	Transgene product	Route of administration	AAV serotype	Reference
Arginase deficiency	Arginase 1	Facial vein	rh10	[105]
Atherosclerosis	Apo-A1	IV (tail vein)	8	[106]
Citrullinemia type 1	ASS	IP, IP (in utero)	8, rh10	[107, 108]
Crigler–Najjar	UGT1A1	IP	8, 9	[109, 110]
Diabetic peripheral neuropathy	mIGF-1	IV (tail vein)	8	[111]
Ethylmalonic encephalopathy	Sulfur dioxygenase	Intracardiac	8	[112]
Fabry disease	Human α-galactosidase A	IV (intraportal and tail vein)	1, 2, 8	[113–115]
Familial amyloidotic polyneuropathy	Transthyretin T119M	IV (tail vein)	8	[116]
Familial hypercholesterolemia	Human LDL receptor	IV (intraportal)	2, 7, 8	[117]
Galactosialidosis	Cathepsin A	IV (tail vein)	8	[118]
Gaucher syndrome	Human glucocerebrosidase	IV (tail vein)	8	[119]
GH deficiency	Growth hormone	IP	8 (sc)	[120]
GSDIa	G6PC	Temporal vein, retro-orbital sinus	1, 2, 7, 8, 9 (sc)	[121–126]
GSDII	hGAA	Retro-orbital sinus	8	[127, 128]
Hemophilia A	m, c, hFVIII, cFVIIIdeleted, codop-hFVIII-V3	IV (intraportal, tail vein and cervical vein)	2, 5, 6, 8	[129–136]
Hemophilia B	c, hFIX, hFIX variants, hFIX-Padua	IV (intraportal and tail vein)	2, 5, 8 (sc)	[35, 137, 138, 139, 140]
Hereditary tyrosinemia	FAH	IV (facial vein, tail vein)	2,8	[141]
Homocystinuria	CBS	IV (intraportal) or IP	2	[142]
Inherited Apo-A1 deficiency	Apo-A1	IV (intraportal and tail vein)	8 (sc)	[143]
Liver fibrosis	HGF	IV (intraportal)	5	[144]

(continued)

Table 2 (continued)

Disease	Transgene product	Route of administration	AAV serotype	Reference
Mitochondrial neurogastrointestinal encephalomyopathy	Thymidine phosphorylase	IV (tail vein)	8	[145]
MPS VII	b-Glucuronidase	IV (tail vein)	2	[146]
MPSII	Iduronate 2-sulfatase	IV (tail vein)	8	[147]
MPSIIIA	Sulfamidase	IV (tail vein)	8, 9	[148, 149]
von Willebrand disease	von Willebrand factor	IV (tail vein)	8	[150]
OTC deficiency	OTC	IP, IV (intraportal and tail vein)	2, 7, 8, 9 (sc)	[151–153]
Phenylketonuria	PAH	IP, IV (intraportal)	5, 8 (sc)	[154–156]
Primary hyperoxaluria type I	AGT	IV (tail vein)	5, 8	[157]
Propionic acidemia	PCC	Intrahepatic injection, IV (tail vein)	8, rh10	[158, 159]
Renal fibrosis	Hepatocyte growth factor	IV (tail vein)	9	[160]
Sialidosis	Cathepsin A	IV (tail vein)	8 (sc)	[161]
Smith–Lemli–Opitz syndrome	DHCR7	IV (temporal and tail vein)	2, 8	[162, 163]
VLCAD deficiency	VLCAD	IV (tail vein)	8	[164]

studies of AAV liver gene transfer that are ongoing (e.g., Clinicaltrials.gov ID# NCT02396342; NCT02484092; NCT02618915; NCT00979238; NCT01687608) or about to start. Hemophilia B is a bleeding diathesis caused by mutations in the gene for blood coagulation factor IX (FIX). Initial results in the dog model of hemophilia B provided a strong rationale for targeting the liver to express the therapeutic FIX transgene [165]. In the first AAV-FIX liver trial, a single-stranded AAV2 vector carrying the human FIX transgene expressed under the control of a liver-specific promoter was administered through the hepatic artery [60]. This trial has been particularly important for the field of in vivo gene transfer, as it demonstrated for the first time that it was possible to transduce the human liver with AAV vectors, leading to therapeutic levels of transgene expression. Additionally, it allowed identifying important limitations of the approach related to vector immunogenicity [166] and preexisting immunity to AAV in humans. Following the results obtained in the AAV2-FIX trial, a second trial was initiated in which a self-complementary AAV8 vector encoding for a codon-optimized version of the FIX transgene was administered intravenously to target the liver of hemophilia B subjects [57, 61]. In this study, a short course of immunosuppression was used to block potentially detrimental immune responses triggered by the viral vector. This approach successfully demonstrated that it was possible to target the liver via the administration of an AAV8 vector delivered through a peripheral vein. Additionally, it showed that transient immunosuppression could be safely applied with gene transfer to avoid detrimental immune responses and leading to long-term expression of the transgene product.

Despite the small number of subjects enrolled in clinical trials conducted thus far, the experience with liver gene transfer with AAV in humans has resulted in important knowledge on the safety and efficacy of this approach and allowed testing of strategies to achieve the goal of safe and long-term correction for a number of genetic and metabolic diseases with liver gene transfer. In the context of hemophilia B, several gene transfer trials have been initiated or are about to start, with some preliminary results being released [167, 168] confirming the findings in the first trials.

Gene Therapy for Children—Question of AAV Persistence in the Developing Liver

Differently from vector administration in neonate animals, the transduction of post-mitotic or slowly replicating adult tissues is more efficient and stable. Long-term evaluation of the persistence of the AAV genome in adult dogs and primates indicates that vector expression persists for more than 10 years [99, 100]. In humans, long-term transgene expression for >5 years after the injection has been

demonstrated [61]; however, at present no human data exist on the persistence of AAV genomes in liver after treatment of pediatric subjects.

After birth the liver starts increasing its size depending on the metabolic requirements of the developing organism, reaching its limit before adulthood. In rodents, the better characterized model, most of the cells in the liver develop during the first 28 days of life, during which the number of hepatocytes increases proportionally with the size of the liver [169]. In adult mice, hepatocytes divide every 100–200 days, whereas in rats the division appears to be faster [169, 170], although the documented difference may be due to the different methods used for the evaluation of the hepatocyte turnover in the two species. In humans, data reported that the liver size increases during childhood and became stable approximately at 10–15 years of age [171]. Additional factors that may influence vector genome stability following AAV gene transfer to the liver are related to the intrinsic characteristics of the pathology, in particular whether gene transfer is performed in a fibrotic/cirrhotic liver [172] or in a liver with increase turnover [173].

The early demonstration that AAV vectors do not integrate in significant proportion in the host genome comes from experiments in which partial hepatectomy was performed after gene transfer, resulting in loss of transgene expression [174]. Similarly, in neonate mice the transduction of actively replicating cells with AAV leads to partial vector dilution over time, with reduction in transgene expression levels. This was recently described by Bortolussi and colleagues, who injected a neonate mouse model of Crigler–Najjar syndrome [109] with a therapeutic AAV vector at day 2 after birth; in these animals, partial loss of transgene expression was observed over time, resulting in lower levels of phenotype correction 17 months after gene transfer (measured by the levels of circulating total bilirubin in serum), which still remained within the therapeutic range [110]. Nevertheless, it is a known fact that proliferation of the neonate liver over time leads to dilution of the effect of gene transfer [175]. For some diseases like Crigler–Najjar syndrome or hemophilia, where the amount of transgene expression needed to rescue the diseased phenotype is low (about 5% of normal levels of enzyme activity is sufficient to convert both diseases from severe to mild [176, 177]), a single administration of an AAV vector at an appropriate dose may be sufficient to achieve lifelong correction of the disease phenotype also when gene transfer is performed in young pediatric subjects. For other diseases, requiring more robust transgene expression, vector re-administration is likely required [178]. Finally, it should be noted that important loss of transgene expression in mice is observed only when AAV vectors are given very early after birth (day 1 or 2). Vectors given at a later time point (>day 4) result in more persistent effect; similarly, higher vector doses result in better persistence of the therapeutic effect [175]. Thus, the issue of vector re-administration may be more relevant to those diseases with early lethality and no therapeutic options available [179].

To overcome the vector genome dilution following AAV treatment, several strategies have been developed in preclinical studies, all based on the enhancement of a stable integration of the viral genome in the host genome. For instance, Wang and colleagues [180] obtained 30-fold increase of the vector integration and

increased persistency in the mouse liver by inserting in the AAV genome a sequence derived from 28S ribosomal RNA present in multiple copies in the host.

Another method to stably integrate AAV genome is the utilization of nucleases, like meganucleases, zinc-finger nucleases or the most recent CRISPR/Cas9 system, that are able to cut a specific sequence in the host genome, thus inducing an homologous recombination with the vector genome. Examples of in vivo gene editing with AAV vectors exist [181, 182], in which robust expression of the transgene was achieved in neonate and adult animals throughout the liver development and even after partial hepatectomy. Another similar approach, relying on homologous recombination driven by homology arms flanking the transgene to be integrated in the genome, has been recently proposed, in which an AAV vector has been used to transfer a promoterless transgene downstream of the albumin gene [183]. However, all the approaches described at the moment suffer from the limitation of low efficiency, as logs higher doses of AAV vectors were needed (compared to "classic" gene addition strategies) to achieve detectable transgene expression levels, thus making translation of these results to the clinic challenging.

Nevertheless, the approaches described above demonstrate that the stable integration of a transgene into the liver DNA increases the persistence of expression in mice. There are still some limitations for the application of these methods to the clinics, and they are related in particular to (i) the high levels of transduction that are required to obtain the transgene integration and (ii) the possibility that off-target integration events could cause insertional mutagenesis. Notably, the issue of insertional mutagenesis is of particular relevance in a context of a rapidly dividing liver, like that of neonates.

Liver Mediates Induction of Tolerance to the Transgene Product

It has been shown that AAV vector-driven expression of human FIX (hFIX) in hepatocytes leads to tolerance to the transgene product [101], a phenomenon that is not entirely surprising, given the unique immunological environment of the liver, placed at the crossroads between the gut and the bloodstream [184]. The lack of responsiveness observed in liver-directed gene transfer with AAV vectors appears to be mediated by antigen-specific $CD4^{+}CD25^{+}FoxP3^{+}$ regulatory T cells (Tregs) [103], which play a central role in liver-mediated tolerance induction [102, 185]. Importantly, liver-mediated tolerance induction can be achieved for various transgenes [103, 186, 187] and can be used to eradicate ongoing antibody responses to antigens [188, 189] and ongoing autoimmune disease [190]. Thus far, preclinical results on the induction of tolerance mediated by gene transfer seem to be confirmed in clinical trials, in which, for example, in the case of the hemophilia B trials, no response to the FIX transfer transgene was noted, despite the fact some of them were carrier of null mutations in the FIX gene [57, 60, 61]. However, it should be

noted that thus far all subjects at risk of having a response to the transgene product have been systematically excluded from enrollment in gene therapy trials, thus underestimating transgene immunogenicity in gene therapy.

While animal models offer a valuable tool to estimate the risk of anti-transgene immune responses, some factors may complicate the evaluation of transgene immunogenicity in humans: (1) species specificity, as human transgenes may be highly immunogenic in lower mammalians [191, 192] but not in humans; (2) genetic background, which in case of models obtained by disrupting a gene, it may be associated with complete lack of tolerance to the expressed transgene [193, 194], thus not fully reflective of the heterogeneity of mutations found in humans; (3) some immunomodulatory drugs used in immunosuppression protocols for highly immunogenic transgenes [195] only work in non-human primates and humans (e.g., several monoclonal antibody-based immunosuppressive drugs).

The Interactions Between AAV Vectors and the Immune System

Cellular Immune Responses to AAV Vectors

AAV vectors are complex multi-component biological entities, composed by both a protein capsid and a nucleic acid. Each of these components may contribute to shaping the host immune response to gene transfer [196]. One key concept to keep in mind when studying immune responses to AAV vectors is that the viral capsid in AAV vectors is identical or nearly identical to the capsid of the wild-type virus, to which humans are exposed [3, 197, 198]. Thus, it is expected that the host immune responses triggered by vector administration will be similar to those associated with a natural infection with AAV, although high quantity of viral particles administered and their route of administration may contribute to the unique features on immune responses observed in gene transfer with AAV vectors [67, 196].

It is known that the DNA and capsid structure of AAV can be readily detected by the immune system, via Toll-like receptor (TLR)9 and TLR2, respectively, triggering adaptive immune responses to capsid and/or transgene product [193, 199, 200, 201, 202]. Recent findings also suggest that CpG sequences, a ligand for TLR-9, contribute to transgene immunogenicity [203]; although these observations are limited to preclinical animal models, factors influencing transgene immunogenicity should be carefully evaluated when approaching the clinic.

Cell-mediated immunity directed against the AAV capsid plays an important role in terms of both safety and efficacy of AAV gene transfer in humans. This was first evidenced in a clinical trial in which an AAV2 vector was introduced into the liver of severe hemophilia B subjects [60]. In this study, upon AAV gene transfer to liver, two subjects developed a transient and asymptomatic elevation of liver enzymes associated with loss of FIX transgene expression around week 4 after

vector delivery. These observations were associated with the expansion of capsid-specific CD8+ T cells, which likely were responsible for the immune rejection of transduced hepatocytes [166]. More recently, similar set of observations was made in the context of a clinical trial of AAV8 gene transfer to the liver of subjects affected by severe hemophilia B [57, 61]. This study showed that AAV8 vector administration in humans resulted in activation of capsid-specific CD8+ T cells and increased liver enzymes in 4 out of 6 subjects from the high-dose cohort, who received 2×10^{12} vg/kg of vector, ~7–9 weeks after vector delivery. In this study, timely intervention with oral corticosteroids was key to ablate the detrimental effect of the ongoing immune response on transgene expression, and even a short delay in the administration of immunosuppression resulted in a fast and significant loss of transduced hepatocytes [57].

Notably, results from both the AAV2 and AAV8 hemophilia B clinical trials highlight important differences in the biology of AAV vectors of different serotypes, as, for example, the timing of detection of the T cell responses differed in the two trials, which was significantly delayed for AAV8 versus AAV2 (weeks 6–9 vs. weeks 2–4, respectively).

As more data emerge from AAV gene transfer trials for hemophilia, the complexity of interactions between AAV vectors and the host seems to gain complexity. It is now evident that what appears to be an immune response to the AAV capsid can be triggered by many serotypes, including AAV2, AAV8, and AAV5 [57, 60, 61, 166, 167, 168]. However, what also is becoming evident is that other factors may concur in the determination of the outcome of gene transfer, as loss of transgene expression and increase in liver enzymes in some cases have not been associated with an immune response to the capsid [168]; furthermore, what complicates the overall picture is that in some instances intervention with steroids did not succeed in preventing/reversing loss of transgene expression [168].

Animal models fail to predict capsid immunogenicity in humans. One major limitation of all animal models (mice, rats, dogs, non-human primates, etc.) used to evaluate the safety and efficacy of gene transfer with AAV vectors is that they failed to predict the issue of T cell reactivity to the capsid in humans. Among multiple studies in mouse models [204–206], though some of them highlighted presentation of AAV antigens in vivo [207, 208], only one managed to recapitulate the human findings [57, 60, 61] showing that AAV8-transduced hepatocytes remain susceptible for CD8+ T cell-mediated lysis longer than those transduced with AAV2 vectors [33].

Despite difficulties, the efforts to develop a murine model continue in a hope to answer remaining questions about safety and efficacy of gene transfer. For instance, such model could help to evaluate the possible detrimental interactions of immunosuppressive regimens applied in the context of AAV gene therapy [102, 209].

Lessons learned from the clinic. The results in the AAV8 hemophilia trial [57, 61] represent an important stepping-stone in the management of unwanted immune responses in AAV gene therapy as they show that it is possible to monitor liver enzymes and administer transient immunosuppression with steroids only if required. However, it should be kept in mind that the ease of endpoint monitoring

characteristic of this trial (i.e., follow-up of liver enzymes and FIX expression levels to guide intervention with steroids) is unlikely to apply for all gene therapy scenarios. For example, for certain disease indications in which liver enzymes are constitutively elevated the use of immunosuppression "on demand" will not be feasible for the lack of endpoints to follow. One solution to the issue could be to administer an immunosuppression regimen upfront to all subjects. However, this is not an ideal solution to the problem, as not all individuals may have an immune response to the vector, the timing of immune responses may vary with the vector dose, serotype, etc. [57, 60, 61, 67], and immunosuppression may change the outcome of gene transfer by decreasing transduction efficiency [209] or triggering unwanted reactions to the donated transgene [102]. Finally, to date it is not entirely clear whether steroid administration will be effective in blocking T cell-mediated immunity to AAV in all gene transfer settings; in fact, it is becoming obvious that this intervention may not be effective in all cases [168].

Immune responses to AAV depend on the vector dose. One important aspect of T cell-mediated immune responses to AAV is that they seem to be detected in a dose-dependent fashion, a result consistent with published in vitro antigen presentation data [207, 208]. Above a certain threshold of capsid antigen load, activation of capsid-specific T cells may result in hepatotoxicity and loss of transgene expression; however, it is not clear at this point, what is the proportion of subjects that will mount a detrimental T cell response. Data from the AAV8 hemophilia B trial suggest that only a subset of subjects will require immunosuppression [57, 61], but the individual differences (HLA type, exposure to the wild-type virus, etc.) accounting for the different outcome of gene transfer between subjects remain unknown.

High purification of vector preparations decreases immune response. The influence of vector manufacturing on the immunogenicity of AAV vectors is currently being discussed. Important open questions include the role of empty capsids, which are found in variable proportions in vector preparations. While empty particles may act as decoys for anti-AAV antibodies [210], they may also contribute to the overall amount of capsid antigen being presented onto MHC class I [208]. The presence of contaminants deriving from the process used for AAV manufacturing (e.g., host cell DNA contaminants, plasmid DNA) is also a possible factor influencing the immunogenicity of AAV vectors.

Humoral Immunity Directed Against the AAV Capsid

The impact of neutralizing antibodies (NAbs) directed against AAV on vector transduction has been first evidenced in the AAV2-FIX liver gene transfer trial [60], in which one subject enrolled in the high vector dose cohort (2×10^{12} vg/kg) had a NAb titer to AAV2 of 1:2 and expressed peak levels of F.IX transgene of $\sim 11\%$ of normal, while another subject in the same dose cohort with a pretreatment NAb titer of 1:17 did not have any detectable circulating FIX following vector administration.

These results were also confirmed by experiments in non-human primates, a natural host for AAV8 [211], which showed that NAb titers as low as ~1:5 can completely block transduction of the liver following AAV8-FIX vector administration at doses of 5×10^{12} vg/kg [212].

After exposure to wild-type AAV, a significant proportion of individuals develop humoral immunity against the capsid, usually starting around 2 years of age [2–4]; however, maternal anti-AAV antibodies can be already found in newborns, disappearing a few months after birth before exposure to the virus later in life [2–4]. Thus, the window of time in which the majority of humans appears to be naïve to anti-AAV antibodies is narrow. Additionally, due to the high prevalence of anti-AAV antibodies in humans, and the cross-reactivity of these antibodies across AAV serotypes [2, 3, 4, 197, 213, 214], anti-AAV neutralizing antibodies can have a profound impact on the efficacy of gene transfer and should be carefully measured prior to enrollment of subjects in clinical trials.

Prevalence of anti-AAV antibodies in the target patient population should be carefully evaluated when designing a gene transfer clinical trial with AAV vectors, particularly when the vector is delivered intravenously. Aside from using highly sensitive assays to measure anti-AAV NAbs [215], preclinical studies should be used to assess the tolerance to anti-AAV antibodies based on the specific characteristics of the vector preparations (e.g., content of empty capsids [210] and doses administered (small vector doses are more prone to neutralization by NAbs). To this end, the use of in vivo models passively immunized with antibodies against AAV vectors [210, 216] can be helpful as it allows to consistently dosing animals with IgG to obtain the desired NAb titers.

When designing preclinical studies in preclinical animal models, it should be kept in mind that some preclinical animal models like humans are natural host for wild-type AAVs. For instance, anti-AAV NAb can be found in non-human primates [211] and have been documented in dogs and other species [217–219]; thus, prescreening of animals for anti-vector antibodies may be required for some animal species and AAV serotypes.

A comprehensive review of strategies to overcome presence of Nab is presented in [220].

Strategies to Improve Efficacy of AAV-Based Gene Therapy

The objective of gene therapy strategies based on gene transfer is to achieve long-term stable transgene expression at levels that are therapeutic. Based on this, one important lesson learned from the outcome of the two AAV clinical trials for hemophilia B targeting the liver is that therapeutic levels of transgene expression can be achieved in humans in a dose-dependent manner [99, 57, 60]. Unfortunately, vector doses positively correlate with unwanted anti-capsid immune responses that, if not counteracted, may decrease or even abolish transgene expression [99, 57, 60, 67, 166]. This issue is particularly relevant considering that therapeutic efficacy for

hemophilia B may be achieved by restoring only 1% of FIX activity while for other diseases (and transgenes) the threshold may be significantly higher. Despite the fact that therapeutic transgene expression levels vary depending on disease and the nature of the transgene product (e.g., intracellular vs. extracellular, with structural vs. enzymatic function), the goal of gene therapy strategies should be to maximize vector potency in order to decrease the vector dose and reduce the risk of immune response and toxicity. For example, vector potency for liver gene therapy can be increased by: (i) optimizing the design of the vector (capsid and/or genome) and of the transgene expression cassette (sequence and regulatory elements) and (ii) facilitating vector trafficking toward the nucleus.

Additional improvements in the efficacy profile of gene transfer can also be achieved by devising optimized vector delivery methods, as it has been shown for muscle [221]. In the case of liver, some studies have shown that catheterization of liver vasculature allows for more efficient delivery of AAV vectors in the presence of anti-capsid neutralizing antibodies [222]; however, clinically feasible and non-invasive delivery methods (e.g., intravenous infusion via peripheral vein) remain preferable to more invasive and potentially risky procedures.

Enhancement of Capsid Transduction Efficiency

During recent years the AAV serotype 8 has emerged as the most efficient natural AAV serotype for liver transduction upon systemic delivery in preclinical models [99, 20, 211, 223] and human hemophilia B trials [99, 57]. Recently, rational design of AAV capsid leads to the identification of novel capsid variants showing increased transduction efficiency as compared to their natural counterpart in animal models. In particular, the point mutations of specific tyrosine [32], serine, threonine and lysine [224] residues on the AAV2 capsids avoid the targeting of viral particles to the proteasome within the cells, thus increasing the vector load that reaches the nucleus [32, 224]. Interestingly, the intravenous delivery of these AAV2 capsid mutants to wild-type mice resulted in higher and more widespread liver transduction and also faster and higher transgene expression as compared to natural AAV2 [32, 224, 225]. Similar results have been reported by mutagenizing serine, threonine and lysine residues on AAV capsids 8, 5 and 1 [226]. However, a recent study reported minimal effect on the transduction efficiency of mouse liver upon systemic delivery of K137R mutant AAV capsids 7 and 9, in addition to the previously reported K137R AAV8 mutant [227]. Improved liver transduction and faster onset of transgene expression were also reported for the novel AAV2G9 capsid variant that has been generated by inserting the galactose-binding domain of AAV9 on the AAV2 capsid [228].

One of the limitations of testing the liver transduction ability of AAV vectors in mouse (and other small animal models) is that the results achieved may not be always extrapolated to the human liver. To overcome this problem, Lisowsky et al. [24] used a chimeric human-mouse liver model. In this model human hepatocytes

are transplanted in immune-deficient *Fah−/−* mice and repopulate the mouse liver having a selective advantage over the murine hepatocytes (5–40% repopulation efficiency). Using this model, Lisowsky and colleagues screened a library of novel capsid variants and identified one chimeric capsid, AAV-LK03, composed of five different natural AAV capsids, able to transduce human hepatocytes more efficiently than AAV2 and AAV8 [24].

One limitation of this humanized liver model is that the transduction of human hepatocytes is still tested in non-physiological conditions while the extracellular environment (e.g., extracellular matrix, blood composition, immune system) may impact on human liver transduction by AAV vectors in a real-life scenario.

Ultimately, the therapeutic advantage deriving from the use of these capsid mutants will have to be evaluated in the context of clinical trials.

Vector Enhancement at the Genome Level

The AAV genome size constraint is an important limit to the transfer of large transgenes to the liver and other tissues. As reported for tissues like the muscle and the retina, both oversized and dual AAV strategies may be used to express large (5 kb) transgenes in a given target tissue. A promising strategy is to use a truncated and engineered FVIII cDNA encoded by an expression cassette of 5.2 Kb that drives therapeutic expression of FVIII in animal models [229]. Interestingly, AAV8 vectors encoding for canine FVIII (5.8 Kb genome) were reported to correct the bleeding phenotype of HA dogs [36]. Then, subsequent studies in wild-type mice, using a reporter transgene, showed that oversized AAV2/8 vectors are about 25-fold less efficient than regular size AAV vectors [39]. Demonstration of transgene expression in mouse hepatocytes by systemic delivery of dual AAV trans-splicing vectors has also been reported [230], but it would be interesting, at present, to compare the efficiency of all the available dual AAV vector systems and to evaluate their possible side effects (e.g., related to the expression of the truncated proteins deriving from each individual vector injected) in the contest of liver gene transfer. Another possible approach to produce full-length FVIII in the liver is to express two separate FVIII-derived peptide chains (each encoded by regular AAV vector) that re-associate within the cells [231, 232].

Modifications to the vector genome, and in particular, the use of self-complementary (sc) AAV vectors [34] has shown to increase vector potency as compared to single-stranded (ss) AAV in the liver of small and large animal model upon systemic delivery [35, 233, 234, 235], suggesting that hepatocytes are, to some extent, inefficient in *de novo* second-strand synthesis [34]. Notably, scAAV vectors seem to provide faster and stronger transgene expression as compared to ssAAV, allowing reducing vector doses while maintaining efficacy [34]. Other studies have shown that transgene expression in mouse hepatocytes can be increased of more than tenfold by co-injection of scAAV2 vectors encoding for either the T cell protein tyrosine phosphatase (TC-PTP) or the protein

phosphatase-5 (PP5) with an ssAAV encoding for the transgene of interest [203]. If no toxicity would result from the expression of these phosphatases in the liver, this approach could be possibly useful to increase the expression of transgenes that are larger than 2 Kb and thus cannot be easily packaged in scAAV vectors [236].

Fine-Tuning of Transgene Expression Cassette

In addition to vector optimization, improvements in the design of the transgene expression cassette have also been widely reported to increase transgene expression and the therapeutic efficacy of AAV vectors [203]. To this aim, the design of various elements can be modified and improved such as transcriptional and post-transcriptional regulatory elements, GC content and codon usage. The regulatory elements include: promoter, enhancer, Kozak sequence, intron, UTRs, polyadenylation signal [237, 238]. So far, the promoters used in the liver of hemophilia B patients treated in the two AAV-based clinical trials are the hepatocyte-specific ApoE/hAAT promoter [consisting of human apolipoprotein E/C-I gene locus control region (HCR) combined with the human α1 antitrypsin promoter] [239] and the LP1 promoter (consisting of core liver-specific elements from the HCR and the hAAT promoter) [35]. While in preclinical settings, AAV-mediated transgene expression in the liver is achieved using both constitutive and tissue-specific promoters, it is important to highlight that the success of liver-targeted gene therapy is dependent so far on the selective expression of transgenes in hepatocytes. The restriction of transgene expression to hepatocytes is chosen to avoid the expression of the transgene product in antigen-presenting cells (that may boost anti-transgene immune responses) and to favor induction of immune tolerance to transgene products [101].

Recently, novel hepatocyte-specific transcriptional cis-regulatory modules (CRMs) have been identified containing evolutionary conserved clusters of binding sites for tissue-specific transcription factors. When the CRMs are used upstream of minimal liver promoters either strong (transthyretin, TTR) or weak (paralemmin, Palm), they enhance gene expression in mouse and NHP liver [240]. It is expected that these regulatory elements will potentiate transgene expression for liver-targeted gene therapy while, due to their small size, maintaining the overall transgene expression cassette within the packaging capacity of AAV vectors.

Codon optimization increases both mRNA stability and protein translation [241], and it has been successfully applied to increase expression of therapeutic hFIX and hFVIII proteins by the liver upon AAV-mediated gene transfer [35, 136, 234, 242, 243]. Ideally, the design of an optimal expression cassette for transgene expression should be performed before any experiment in animal models as the optimization of multiple elements in the transgene expression cassette can significantly increase the potency and efficacy of AAV vectors for liver gene transfer. This is exemplified by recent preclinical studies in which the optimization of multiple elements of the expression cassette has been combined with the use of a hyperactive hFIX protein

(obtained by introducing a gain-of-function mutation in the hFIX coding sequence (R338L; FIX Padua) [140, 244, 245]). Similarly, design of codon-optimized, engineered version of FVIII has allowed to achieve therapeutic levels of transgene expression at AAV vector doses that are safe and justify clinical development of a gene therapy for hemophilia A [135, 229, 243].

Based on the recent advances in vector design, it is expected that the development of optimized next-generation AAV vectors with higher potency for liver gene transfer will allow using lower and thus potentially safer vector doses while maintaining efficacy and will promote the clinical translation of the existing proof-of-concepts in animal models (Table 2).

Genotoxicity

One main advantage of AAV vectors, as gene therapy vehicles, consists in the low frequency of vector genome integration in the host DNA and the low risk of related genotoxicity [1]. Despite this, the issue of AAV-related genotoxicity is important in the context of gene therapy as random integration of vector genomes into the host DNA may lead to both loss- and gain-of-function mutations that may alter cell functionality and homeostasis leading to malignant transformation and tumorigenesis. Several studies showed that in the adult and neonatal mouse liver transduced by AAV vectors, the viral genomes remains mainly extra-chromosomal [239, 246] while a minority of them integrate into the host DNA [247] with a preference for sites that are close to active genes, ribosomal DNA and CpG sequences [246, 248, 249, 250, 251, 252]. The potential of AAV-induced genotoxicity in the context of systemic or liver-directed gene therapy has been investigated in the recent years. So far, insertional mutagenesis by AAV vectors has been reported in mouse after neonatal gene delivery [251, 253] but not after AAV administration to juvenile (6–8 weeks) or adult mice [19, 133, 254, 255, 256, 257]. In particular, two independent studies showed that systemic AAV administration to neonatal mice predisposes them to hepatocellular carcinoma (HCC) [251, 253] due to the insertion of viral genomes in the RNA imprinted and accumulated in nucleus (*Rian*) locus, encoding for many regulatory non-coding RNA (snoRNAs, microRNAs and lincRNAs). This integration leads to the misregulation of genes flanking the insertion site (*Rlt1* and various microRNAs), which promotes HCC [251]. Notably, in humans the upregulation of delta-like homolog 1–deiodinase type 3 (DLK1-DIO3), the orthologous genomic imprinted cluster of the *Rian* locus microRNA, has been also associated with poor survival in patients with HCC [251]. Interestingly, Chandler et al. also showed that the preference of viral genome insertion in specific loci (such as *Rian*, *albumin and α-fetoprotein*) is favored by their high transcriptional activity and positively correlates with AAV vector doses [251]. Chandler et al. [251] also reported that the upregulation of genes, which are close to the insertion site, depends on the strength of the promoter included in the transgene expression cassette. In particular, the strong chicken beta actin (CAG) and (thyroxin-binding

globulin) TBG promoters, but not the liver-specific hAAT promoter, induced the dis-regulation of gene expression leading to tumor formation [251]. Based on these findings, it becomes crucial to design and optimize the regulatory elements contained in the transgene expression cassette to find a balance among potency and possible genotoxic side effects. The tumor-initiating potential of scAAV vectors in the liver of adult mice and newborn rats has also been recently assessed [258, 259]. Surprisingly, no integration hot spots of scAAV genomes in the liver DNA and no increased frequency of tumors were found in adult and newborn-treated animals [259]. However, a side-by-side comparison of the profile and efficiency of viral genome integration in the liver upon administration of ssAAV and scAAV vectors is still missing.

It should be noted that while some of the studies conducted thus far in rodents on the insertional mutagenesis of AAV vector revealed their potential genotoxicity, studies in larger animal models such as dogs [100] and non-human primates [99], in which animals were followed for extended periods of time, raised no concerns over the genotoxicity risk of AAV vectors in liver. Similarly, studies in humans also support the safety of the approach, as no tumor formation has been documented ~5 years post-gene transfer [57]. However, numbers are still small, and long-term follow-up in more AAV-treated subjects over an extended period of time is needed. Additionally, follow-up of subjects treated with AAV at a pediatric age will help clarify the genotoxicity risk in this patient population.

Concluding Remarks

As the field of in vivo gene transfer with AAV vectors proceeds toward a more mature state, a growing number of applications of the technology are reaching the clinic. Today investigators are generally more familiar with the use of AAV vectors as therapeutic tools; however, a number of potential issues associated with the technology have yet to be addressed. These include in particular concerns related to the immunogenicity of AAV vectors and the ability to define the optimal combination of capsid and transgene to maximize levels and persistence of therapeutic efficacy. Additionally, unknowns related to the potential genotoxicity risk of AAV vectors and the issue of gene transfer in young individuals will have to be defined.

While human studies will ultimately answer most of the questions above, preclinical studies will remain crucial in guiding the design of clinical trials and testing the safety and efficacy of the future generation of gene therapeutics.

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Physical Methods of Gene Delivery

María José Herrero, Luis Sendra, Antonio Miguel and Salvador F. Aliño

Abstract Gene therapy can be defined as the use of nucleic acids (NAs) as medicines with the aim of correcting a deficient gene expression, introducing new functions in the cell, repairing mutations and modulating the gene expression. Two main classes of vectors, viral and nonviral, have been used for gene delivery in order to avoid the NAs hydrolysis by tissue nucleases and improve their cellular uptake. The ideal gene delivery vector should offer high transfection efficacy, cell specificity and low toxicity. However, the immunogenic and mutagenic side effects of viral vector as well as toxicity and low efficacy of nonviral carriers are limiting their application. In this respect, naked NAs delivery by physical methods could be the safest procedure for gene therapy strategies if the appropriate efficacy can be achieved. These procedures employ physical forces to permit the nucleic acid cross the cell membrane and reach the cell without any carrier agent. Although viral and nonviral chemical methods are widely employed in experimental research and clinical trials, the physical methods of DNA delivery are a strategy in increasing progress. In this chapter, the main physical procedures (microinjection, needle injection, needle-free jet injection, gene gun, electroporation, sonoporation,

M.J. Herrero · L. Sendra · A. Miguel · S.F. Aliño
Departamento de Farmacología, Facultad de Medicina,
Universidad de Valencia, Valencia, Spain
e-mail: Maria.jose.herrero@uv.es

L. Sendra
e-mail: Luis.sendra@uv.es

A. Miguel
e-mail: matasantonio@hotmail.com

M.J. Herrero
Instituto de Investigación en Salud La Fe, Valencia, Spain

S.F. Aliño (✉)
Unidad de Farmacología Clínica, Hospital Universitario y
Politécnico La Fe, Valencia, Spain
e-mail: alino@uv.es

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hydroporation, magnetofection and laser irradiation) for naked nucleic acids delivery are described, emphasizing their use justification, their development, the proposed mechanism of NAs transfer and their clinical use or potential application.

Keywords Microinjection · Needle injection · Needle-free jet injection · Gene gun · Electroporation · Sonoporation · Hydroporation · Magnetofection · Laser irradiation · Nucleic acids

Introduction

Gene therapy can be defined as the use of nucleic acids as medicines and the main objectives are to correct a deficient gene expression, to introduce new functions in the cell, to repair mutations and/or to modulate the gene expression at genomic or post-transcriptional level employing gene silencing strategies. However, the use of nucleic acids (NAs) as medicines is largely limited because of their rapid hydrolysis by the nucleases present in biological milieu and their low cellular uptake due to the high ionic charge of NAs and, in many cases, also to the high molecular weights. To avoid these issues, gene therapy vectors have been developed. Those can be classified as viral and nonviral vectors. However, in all cases, the ideal gene delivery vector should have high transfection efficacy, cell specificity and low toxicity. There is also evidence that naked DNA can be delivered to cells by physical methods, without any vehicle. The procedure employs physical forces to cross the cell membrane, delivering DNA into the cell without any carrier agent that could be cytotoxic or immunogenic, as observed when virus or chemical nonviral vectors are employed.

The ability of naked DNA to transfect the liver and skeletal muscle in small animals was early described [1, 2]. Then, several studies on different organs and tumors have confirmed this effect, although the efficacy observed is low when compared with other procedures employing viral vectors or chemical DNA complexes, which have been used for therapeutic purposes in clinical trials. Thus, in relation to therapeutic success, some parameters should be taken into consideration in order to evaluate the benefits of each of these gene transfer methods, such as: (a) hydrolysis protection; (b) efficacy of specific cell delivery; (c) cellular bioavailability of delivered NAs; (d) toxicity. Viral vectors offer, as the main advantage, the cell specificity of delivery due to their natural tropism, but they have limitations [3] mainly regarding immunogenicity and potential damage associated with random insertion of the gene. Nonviral chemical vectors provide lower immune effects than viral vectors, but endosomal escape must be improved in order to deliver NAs into cytoplasm with good bioavailability. Although viral and non-viral chemical methods are widely employed in experimental research and clinical trials, the physical methods of DNA delivery are a strategy in increasing progress (Table 1), since they are able to address the major challenges posed by chemical

Table 1 Characteristics of different physical methods for gene delivery

Method	Concept	Advantages	Disadvantages
Microinjection	Direct injection of nucleic acid into host cell through a glass capillary	Avoid natural barriers, precision	Delicate procedure, requires training, high time consume
Needle injection	Direct needle injection on a specific organ or tissue	The simplest and safest; targeted specific regions	Low efficiency; inflammation
Jet injection	Ultrafine stream of highly pressurized jet fluid	Needle-free device	Tolerable tissue damage
Biolistic	Particles propelled at high velocity	Gene gun device	Requires particles; the type and size define the toxicity and tissue penetration; cell damage
Electrofection	Electric field induced by voltage pulses	Simplicity; low cost; wide electrode variety; widely employed for in vitro, in vivo and clinic	Short-term pain, erythema, discomfort; tissue damage
Sonofection	High intensity ultrasound on cell membrane	Noninvasive procedure, efficacy (but lower than electrofection); combined with bubbles or nanocarriers increases the efficiency	Low precision; low reproducibility; cell damage by shear forces and increase temperature; tissue damage (but less than electrofection
Hydrofection	High pressure by high vascular flow injection	Single i.v. injection in small animals Catheter-guide in large animals	Hemodynamic changes; transitory increase in liver enzymes in plasma
Magnetofection	Magnetic field acting on magnetic particles	Noninvasive method; reagents are available; efficacy (but lower than electrofection)	Requires magnetic particles; particle aggregation;
Optofection	Laser pulses on single cell or small tissue area, combined with nucleic acid/complex or nanoparticles	Promote nucleic acid release from endosome	Inflammation; tissue damage, low irradiation area, low penetration capacity

and viral vectors and offer also great advantages, in relation to the adverse effects [4]. However, the direct comparison between viral and nonviral gene transfer is difficult because of the differences in experimental models and in the parameters used to evaluate the efficiency.

Microinjection

Microinjection is a successful method for nucleic acid delivery to the target cell. Gene transfer by pronuclear microinjection is the predominant method used to produce transgenic animals. The method (Fig. 1) was originally described by Gordon et al in 1980, and the protocol is often used as an important tool in biomedicine to create transgenic animals. It has also demonstrated the highest efficiency rate for nonviral livestock transgenesis [5]. Knockout mice can be generated by microinjecting humanized Cas9 (hCas9) mRNA and guide RNA (sgRNA) into fertilized eggs, in a single circular plasmid. The combination of both techniques allowed developing a rapid, easy and reproducible strategy for naked DNA delivery to target cells. However, microinjection requires precision and high accuracy for success. The procedure is delicate and requires computer control of micropipette movements and injection and presents both advantages and disadvantages. As advantage, it allows the delivery of the selected NAs, circumventing the natural barriers. As disadvantage, the procedure is highly time-consuming and several factors, such as the volume of injection, needle choice and place of injection [6], must be optimized in each case.

Cytoplasmic injections are faster and easier than pronuclear injection, but this procedure has limited success. Recently, it has been reported [7] a combined cytoplasmic delivery method termed "intracellular electroporetic nanoinjection." The procedure is unique since it manipulates transgenes using electrical forces. This microelectromechanical system uses electrostatic charge to physically pick up transgenes and place them within the cytoplasm. Then, the transgene is propelled through the cytoplasm and transferred into the pronuclei, electroporated with electrical pulses.

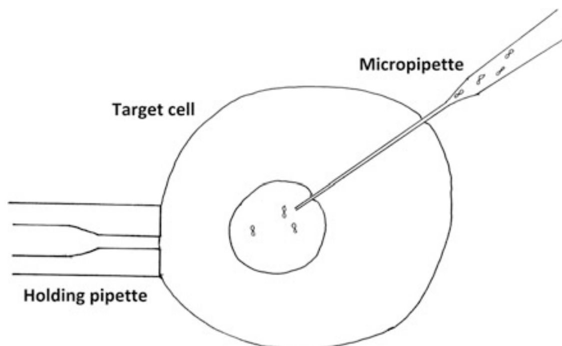

Fig. 1 Microinjection. Figure adapted by author from "Microinjection," Biocyclopedia (http://www.eplantscience.com/index/biotechnology/genes_genetic_engineering/techniques_of_genetic_engineering/biotech_microinjection.php; http://nptel.ac.in/courses/102103013/module5/lec3/4.html)

Microinjection has the capability for directly access the cell, but its application is limited because of the low rate of delivery. However, the use of automated systems [8, 9] has significantly increased its throughput, resulting in a technique with a renewed potential interest [10].

Needle Injection

The simplest and safest nonviral delivery system in vivo is the direct gene transfer employing naked DNA (Fig. 2). Wolf et al. [2], firstly reported in 1990 that intramuscularly injected naked DNA could be expressed in myofibers. Recently, an open-label clinical trial studying the efficacy and safety of naked DNA intramuscular injection concluded that gene therapy employing the human hepatocyte growth factor gene is safe and effective for treating critical limb ischemia. However, the procedure must be considered of low efficiency.

Myocardial gene delivery by direct injection is a simple method that offers the advantages of targeting specific cardiac regions, with high located DNA concentration. Several groups have demonstrated the feasibility of the technique [11],

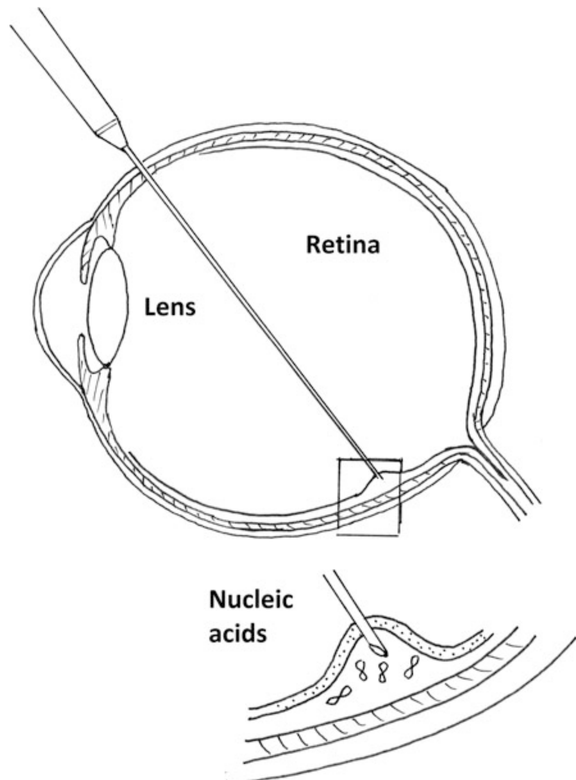

Fig. 2 Needle injection. Figure adapted by author from Kis et al. [119]

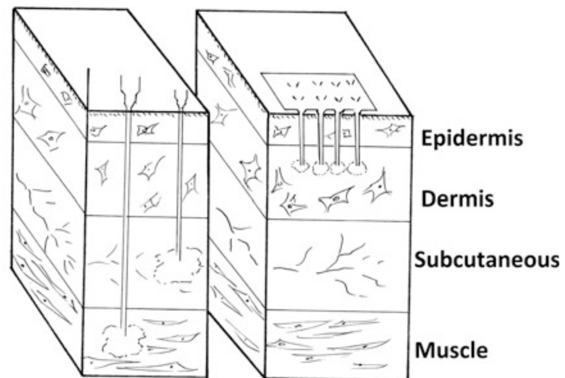

Fig. 3 Microneedle injection. Figure adapted by author from http://www.sciencedirect.com/science/article/pii/S0264410X11017877

but it has limitations due to the inflammatory effects induced by needle injection. The subsequent studies show that the efficiency of gene transfer mediated by direct injection is low, but the technique could be applicable for regional strategies, such as myocardial infarct. The potential interest of intracameral eye injection has been also explored [12] by small volume injection of an adenovirus encoding green fluorescent protein gene, with effective results.

Microneedle arrays (Fig. 3) are the next generation of needle injection procedure, consisting of a plurality of microneedles/projections generally ranging from 25 to 2000 μm in length, with ability for effectively enhancing the delivery of many therapeutic molecules, including DNA, to biological tissues [13]. Several microneedles designs have been developed for minimally invasive delivery of different compounds to tissue [14, 15]. The potential application for skin gene delivery, mainly for vaccination purposes was also investigated, employing a 4 × 4 array (260 μm in length), resulting in successful transfection of epidermal cells with detectable gene expression product in cutaneous tissue [16]. A great effort has been done in improving DNA vaccination, and several clinical trials involving combined procedures are currently being executed [17]. Microneedles have also demonstrated their efficiency to deliver siRNA across both the human and mouse skin, resulting in good tissue distribution with functional ability, as observed by the efficient silencing of reported gene expression in a transgenic fluorescent reporter mouse skin model [18].

Needle-Free Jet Injection

The liquid jet application is essentially a needle-free device concept that accelerates and disperses the therapeutic agent in a targeted organ or tissue site. The jet injection device delivers DNA as a solution by creating an ultrafine stream of high-pressure fluid that penetrates the tissue or organ (Fig. 4) and is distributed depending on the pressure exerted [19]. The pressure is important to efficiently

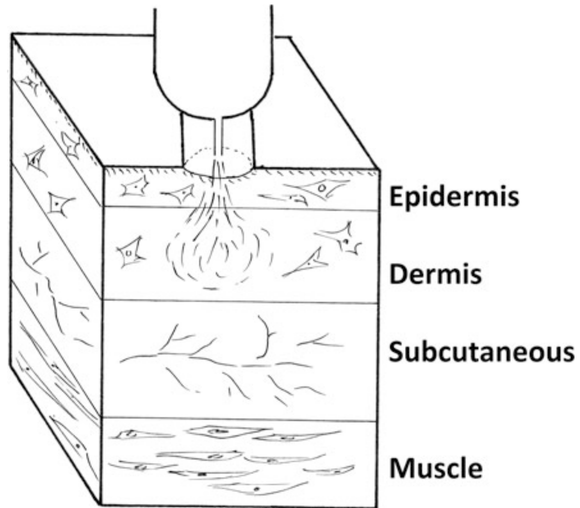

Fig. 4 Needle-free jet injection. Figure adapted by author from (2002). A.D.A.M., Inc. http://www.wakemed.org/adam/careguides/diabetes/diabetes_step6.html

transfect DNA into the tissue, overcoming the intraorgan hydrostatic pressure and preventing seepage [20]. The applied jet injection must lead to tolerable tissue damage in correlation with efficient gene transfer [21, 22]. Some of the conditions that meet these characteristics in cardiac gene transfer are reported (a) nozzle jet velocity, 110 m/s; (b) pressure range, 150–250 kPa; (c) distance range, 20–25 cm; (d) volume injection 100–500 μL [23]. Usually, there are two types of jet injection according to the volume employed: low volume (20–30 μL), which limits the area of transfection to 1 cm approximately; and high volume (>100 μL). In both cases, the range of DNA concentration is the same (0.1–1 μg/μL). The depth of tissue penetration and the efficiency of this method depend on the selected jet parameters.

The jet injection has been employed in a wide variety of clinical applications such as antigen immunization (vaccines), hormone delivery and local anesthesia. It has also been used for a wide variety of gene therapy strategies such as antitumor therapy by jet injection of DNA encoding suicide genes (cytosine deaminase) or genetic vaccines. Other applications involved genetic correction of skin diseases or the ABCB1 gene silencing with the aim of reversing P-glycoprotein-mediated multidrug resistance phenotype.

Gene Gun/Biolistic Delivery

Gene gun is a procedure in which particles bearing nucleic acids are propelled at high velocity with the aim of impacting on a biological target (Fig. 5) in order to penetrate deeply and transfect cells by delivery of cargo molecules. The procedure has been employed along for more than two decades to transfer DNA to a wide

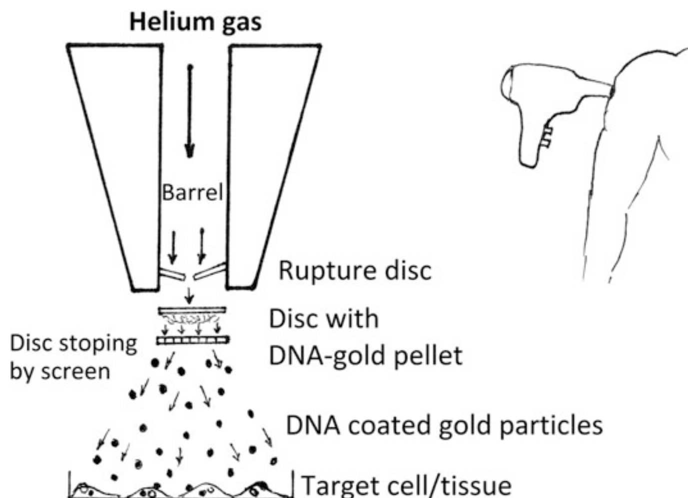

Fig. 5 Gene gun. Figure adapted by author from (2016). "Plant Transformation Using Particle Bombardment," African Biosafety Network of Expertise (ABNE) http://nepad-abne.net/biotechnology/process-of-developing-genetically-modified-gm-crops/plant-transformation-using-particle-bombardment/

variety of biological species such as bacteria, fungi, many types of plants, mammalian cells and organs. Currently, there are different gene gun devices commercially available (Powderject, PowderMed, Iaculor Injection) that can be used successfully for most of the purposes [24]. The most recommended material for preparing the particles employed with the gene gun procedure is gold [25] because of its high density, low toxicity and good immunotolerance. Tungsten particles have also been employed due to their lower cost, but their toxicity and non-biocompatibility limit their use. Stainless steel or polymer microparticles have also been recommended. More recently, titanium particles have been proposed [26] because they offer as advantages the low cost and low risk of cell damage due to their low density and good biocompatibility. On the other hand, the penetration ability of microparticles through tissue is largely dependent on their diameter size (0.5–100 μm). Thus, particles with large size are expected to follow an extracellular route, whereas smaller sizes use intracellular route. In this sense, for cell DNA delivery the smallest possible particles (0.6–6 μm diameter) are preferred [27].

The cell damage due to gas pressures employed for gene gun systems (from 20 to 60 bar), and particle impact on target tissue may be the major limitation of gene gun procedure. To minimize this adverse effect and increase the penetration depth of microparticles in the target, [28] it has been proposed to combine microneedles and gene gun procedures in a new concept of "microneedle-assisted microparticle delivery." Basically, the microparticles are compressed into a cylindrical pellet and placed on a sliding holder, which is accelerated by compressed inert gas (such as helium) along a cylinder. When the sliding holder reaches the end of the canyon,

the sediment is detached from the support, propelled at high speed and decomposed in the form of microparticles by impaction on an open mesh located at the end of the cylinder. Then, the separated microparticles penetrate into the desired target, having previously reduced the target/skin by using microneedles to create a number of holes through which the microparticles can enter, without the need for very high gas pressures.

Biolistic gene delivery has been used on several tissues and organs, including organotypic organ slice [29] to optimize the conditions of use with minimal damage. However, it has been mainly applied in DNA vaccines, where this method offers advantages with respect to other physical procedures. Gene gun DNA vaccines induce predominantly Th2 immune response against most antigens, but depending on them, it can also induce a balanced Th1/Th2 response [30]. Efficient cellular and humoral responses have been described against several virus antigens such as goose parvovirus [31], human papilloma virus [32] and human hepatitis C virus [33]. Although gene gun can offer advantages with respect to direct intramuscular injection, other reports show that gene gun mediates suboptimal response against neuroblastoma and papilloma [34, 32]. Clinical studies with DNA vaccines have also been driven for influenza virus. Although the results are promising, the immune response is not yet equivalent to standard vaccine delivery methods [35, 36].

Electroporation/Electrofection

Electroporation is a method employed to introduce a wide variety of non-permeable cargo molecules into cells, including drugs and genes. Electric pulses of different intensity and duration are applied according to the target cell, tissue or organ. The procedure has been well received for its use in vivo in the clinic due to its simplicity and the low adverse effects. The electroporation-mediated gene delivery (electrofection) has demonstrated to be an effective method for DNA vaccine and other gene therapy strategies based on transgene expression or gene silencing. Multiple types of electrodes have been designed [37] in order to adapt the procedure to the specific conditions of each target organ: plate electrodes, spoon electrodes to facilitate vascular electroporation, penetrating or nonpenetrating needles, electrodes mounted on a caliper, adapted pads of defibrillator, multielectrode arrays and several other customizations.

The cell membrane molecules are exposed to an electric pulse from dipoles oriented in the direction of the electric field, and then, they are distributed so that the area facing the cathode is depolarized, while the area facing the anode is hyperpolarized [38, 37]. Electric pulses above a certain threshold generate permeations and cause transient hydrophilic pores which permit passage of large molecules (Fig. 6), guided by the concentration gradient. Large pores that allow the entry of DNA into the cell are only created at the pole facing the anode [39–41]. Since the persistence of the pore is very short, the presence of DNA during the

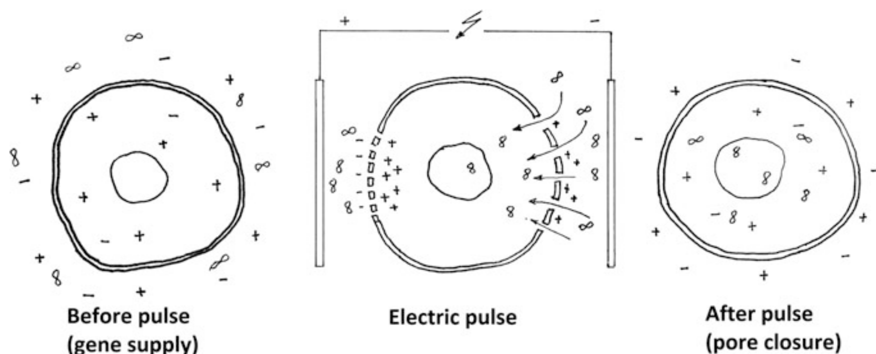

Fig. 6 Electroporation. Figure adapted by author from Mehta [120]

electric pulse is a necessary condition for efficient transfection. In addition, increased number and pulse duration result in greater functional pores. However, the exact mechanism by which DNA enters the cell remains unknown and although many experiments support that electroporation mediates direct DNA delivery into cytosol [42], some studies with intact cells [43] suggest that DNA could be trapped within lipid vesicles.

The in vitro electroporation is carried out on cells in suspension, introduced into a cuvette with two electrodes (connected to a power supply) located in two parallel faces. After the addition of DNA to the cell suspension, the programmed sequence of electric pulses is started. Electroporation in vivo requires the selection and insertion of special electrodes in target tissue or organ for transfection [44]. Two wave types have been used in electroporation, square wave and exponential decay, but for cultured mammalian cells and in vivo applications, the first yielded better gene transfer and expression with lesser tissue damage. On the other hand, a great variety of electrotransfer conditions have been employed: pulses from microseconds to milliseconds and the electric field from less than one hundred volts to more than one thousand volts. For each specific target tissue, the optimal conditions of gene transfer are largely dependent on the selected magnitude of the field, duration of pulse, number of pulses and their frequency. However, two strategies appear to have had success in the liver and skeletal muscle: (a) high intensity field/short pulses, such as 1–2 kV/cm and multiple short pulses <100 μs [45]; (b) low intensity field/long pulses, such as 200 V/cm with multiple longer pulses of 10–20 ms [46].

Electrotransfer has been assessed on many tissues showing encouraging results in many cases in DNA vaccines, cancer, and other diseases in liver, muscle, heart, lung and neurons. The DNA vaccines have been a major area of growth for gene electrotransfer. Many vaccines against infectious diseases have been evaluated in preclinical models, but the ones that have achieved the clinical trial phase are the antiviral vaccines [47], such as vaccines for human immunodeficiency virus [48], hepatitis virus B and C [49, 50], human papillomavirus and hantavirus causing hemorrhagic fever [51]. The primary tissue selected for electric pulses-mediated

DNA delivery was skin and deltoid muscle and its application mediated, as main adverse events, short-term pain, erythema or discomfort, but the procedure was well tolerated. With respect to cancer, we found (using the terms “gene electroporation AND tumor” on the Clinical Trials.gov Web database) the next trials: (a) advanced metastatic carcinoma (employing TGFβ2-antisense-GMCSF gene-modified tumor cell vaccine, in muscle); (b) leukemia (employing WT1 immunity via DNA fusion gene vaccination, in muscle); (c) malignant melanoma (employing hIL12 or antiangiogenic metargidin peptide, AMEP, intratumoral or intramuscular); (d) head and neck squamous cell carcinoma (hIL12, intratumoral); (e) breast cancer (mammaglobin-A DNA vaccine). Additional clinical trials in other organs are related to cancer/metastasis or infectious diseases (not specific pathologies for gene correction), and therefore, the electrotransfer strategies were performed as described above.

Sonoporation/Sonofection

Similar to electric pulses, high intensity ultrasounds can permeabilize cell membrane to delivery cargo molecules by pore formation [52]. The procedure is known as sonoporation, and the size of the molecules that can cross the membrane is dependent on the intensity of the signal and the pore diameter, allowing the transfection of large DNA (sonofection) into cell cytosol. The physical effects of ultrasound (Fig. 7) on biological tissues include: cavitation (growth and collapse of microbubbles), radiation pressure (force in the irradiation field) and microstreaming (shear forces near the microbubbles) [53]. The mechanism of pore formation is

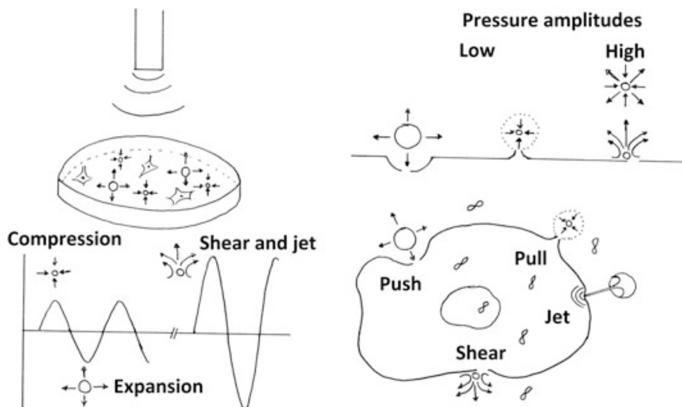

Fig. 7 Sonofection. Figure adapted by author from Tomizawa et al. [121]

unclear, but it seems that high intensity ultrasounds focused on tissue induce forces for mechanical movement of the extracellular fluid that facilitate cavitation or the collapse of air bubbles, impacting cell plasma membrane. Cavitation bubbles could be understood as the activity generated by ultrasound, which can happen in a liquid or a similar medium containing bubbles or pockets of gas or vapor [54]. Ultrasonic cavitation induced by low intensity pulses produces increased friction and shearing of the surrounding tissues. When the intensity is high, the amplitude of the oscillations of the bubble increases instantaneously, resulting in a transition of cavitation, which causes shock waves and microjets [55]. Microjets are powerful jets of liquid, caused by the asymmetric implosion of microbubbles, whose flow causes transient pores in the membrane of the cells and promotes the entry of drugs and genes into cells [56]. In summary, both pore formation and endocytosis have been described during sonoporation and recent results from FACS sorting and confocal microscopy support that the low uptake population showed endocytic uptake, whereas the high uptake was mediated via pores, induced by microbubbles propelled toward the cells [57]. These observations may be considered in order to select optimal ultrasound settings.

Sonoporation is widely used in clinic for several diagnostic (image) and therapeutic (relieve pain, cancer ablation, kidney stones) purposes. For gene transfer purposes, sonoporation seems to be more efficient in well-vascularized living tissue, causes less tissue damage than electroporation and can mediate good efficiencies in several tissues [58], such as muscle, brain, heart, kidney, lung and tumors. Interestingly, in rat myocardial infarct, the sonofection of human hepatocyte growth factor two hours after infarct induction resulted in a significant reduction in scar size three weeks later [59]. In addition, sonoporation-mediated tumor gene delivery under imaging guidance provides a promising option in cancer treatment with enhanced gene release, site specificity and reduced toxicity [60]. However, sonoporation has general limitations such as low efficacy, and mainly, the fact that the localization of the energy applied cannot be controlled with precision, which hinders the optimization of the method and its reproducibility, therefore showing lesser efficiency than electroporation. Nevertheless, sonoporation combined with microbubbles (commonly used as intravascular ultrasound imaging probe) and other nanocarriers such as polymeric nanoparticles, nanoemulsions, liposomes or micelles are contributing to increase the efficiency of sonoporation for gene delivery in vitro and in vivo [61]. Thus, ultrasound combined with DNA-bound bubbles have a renewed potential of sonofection because of the wide variety of cargo molecules (drug and genes) that could be delivered to targeted tissues by noninvasive methods and the higher efficiency as compared with administration of naked DNA alone [62, 63]. Moreover, microbubbles can also attach nanoparticles-bearing genes/drugs themselves, thus increasing even more their transport capacity [64].

Hydrodynamic Delivery: Hydroporation/Hydrofection

The hydrodynamic gene delivery via tail vein injection is a highly efficient procedure to deliver nucleic acids to liver in small animals. Early reports showed that DNA liver transfection could be achieved by portal vein injection of a large volume of a hypertonic solution [65]. However, the original concept of hydrodynamic delivery was not established until 1999 [66, [67] and was described as the rapid (5–7 s) mouse tail vein injection (27-gauge needle) of a saline solution containing a naked DNA plasmid in a volume equivalent to 1/10 of animal's body weight. That means, in a typical experiment employing mice weighing about 20 g, the injection in only five seconds of the wished amount of naked DNA (0.5–5 mg/kg) in 2 ml volume of saline solution. The procedure mediates high gene transfer mainly in the liver [68, 69], but also in other tissues such as kidney [70, 71], skeletal muscle [72, 73], cardiovascular tissue [74] and tumor [75].

The main adverse effects observed in mice after hydrodynamic injection are reversible changes in hemodynamic/electrocardiographic parameters and plasma increase in liver enzymes, and all of them normalized 90 min and three days after injection, respectively [73, 66]. In addition, no serum biochemistry sign of liver injury has been observed after eight accumulative doses (administered every 15 days), whereas efficacy of transgene expression increased accordingly, achieving long-term therapeutic plasma levels of the specific protein [76].

Although the complete mechanism of hydrofection by hydrodynamic injection has not been elucidated yet, significant advances have been achieved in the recent research observations in liver (Fig. 8), such as: (a) Early studies observed that hepatocytes located around the pericentral vein [77–79] and the transition area of zone-2 [80] is mainly transfected by hydrodynamic injection, suggesting that retrograde blood flow in the liver could be involved in the transfection mechanism. (b) Hydrodynamic injection greatly increases the pressure of inferior cava vein [81], suggesting that the normal blood flow in the liver, from portal vein to hepatic vein, must be committed. The inverted blood flow in hepatic sinusoidal territory was confirmed by intravital microscopy observations [76] and fluoroscope images [82]. (c) Gene transfer would partially involve the direct cytosolic delivery of pDNA through the cell membrane due to transiently increased permeability [83]. (d) Morphological liver changes induced by hydrodynamic injection have been observed by electron microscopy, and some researchers suggested the formation of small pores in the cell membrane during the injection [79]. However, the presence of pores has not been confirmed. The more evident hepatocyte change was the presence of a large number of cell membrane endocytic vesicles, without obvious membrane defects [76, 80, 84] but with the ability of fuse between them [85]. These observations suggested that plasmid DNA delivery to hepatocytes could involve a microfluidic uptake step. The penetration into cytoplasm should be mediated via an enforced diffusion process through transiently increased permeable sites in the cell membrane of endocytic vesicles. (e) A receptor-mediated mechanism for DNA uptake was also suggested by early reports [86], but further studies employing a

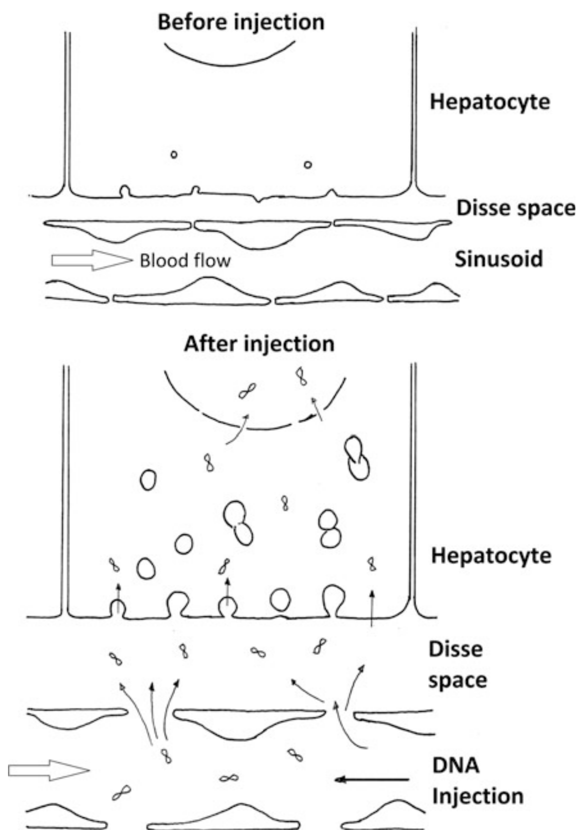

Fig. 8 Hydrofection. Figure adapted by author from Herrero and Alino [122]

wide variety of molecules (dyes, proteins, nucleic acids) with largely different molecular sizes (500 Da to >5 × 10^6 Da), and nanoparticles (5–15 nm diameter) did not support this concept [73, 87, 88]. In addition, whereas smaller nanoparticles have easy access to the hepatocyte cytoplasm of hydrofected human liver segments [89] and pigs in vivo [88], the large nanoparticles have a very limited access to hepatocytes and are usually located inside vesicle membranes of phagocytic cells.

To avoid the adverse hemodynamic effect of hydrodynamic gene transfer in large animals and humans, the main early strategy has been to reduce the injection volume by vascular direct injection into target organ. This strategy has reduced approximately tenfold the volumes required for liver delivery in rats [90], rabbits [91] and pigs [85, 92], achieving safe conditions, but the efficacy of gene expression was significantly lower when large animals were employed. To optimize the procedure in feasible human conditions, surgical and percutaneous catheter procedures (Rx-guide) have been explored in pig models. The anterograde injection (same direction of normal blood flow) via hepatic artery or portal vein requires a blockade of inferior vena cava to achieve sufficient vascular pressure [79], but gene transfer efficiency is not increased [93, 88]. However, higher hAAT gene transfer efficacy in

pig liver tissue has been observed two weeks after retrograde injection (200 μL of 20 μg/mL DNA at 20 mL/s speed) with balloon blockade of portal vein by catheterization [94]. On the other hand, a computer-controlled injection device for tissue-specific regional hydrodynamic delivery has been developed [95] and it has demonstrated to offer good efficiency, safety and reproducibility of reporter gene expression [93].

Although retrograde bile duct injection has been employed successfully in rats [96], the high frequency of endoscopic complications limits its interest. Thus, catheter-mediated retrograde hydrodynamic liver gene delivery is the most promising route for selective liver gene therapy, mainly since ex vivo human liver segments [89] have also been transfected by this procedure, achieving tissue protein expression.

Magnetoporation/Magnetofection

Magnetofection has been defined as the nucleic acid delivery under the influence of magnetic field acting on nucleic acid vectors associated with magnetic particles. Particles are prepared by association of conventional gene vectors (usually employing cationic polymers or lipids) with magnetic nanoparticles (typically iron oxide). An external magnetic gradient field pulls the magnetic particle-vector complexes bearing nucleic acids toward the cells to be transfected (Fig. 9). Early studies have shown the efficacy of this procedure in viral and nonviral gene delivery [97, 98], but no mechanistic difference of gene delivery was observed employing magnetofection or the analogous nonmagnetic vector. Despite this early conclusion, more recent studies support: (a) the evidence that a magnetic field can enhance the tissue penetration of magnetic particles [99, 100]; (b) that nucleic acid delivery

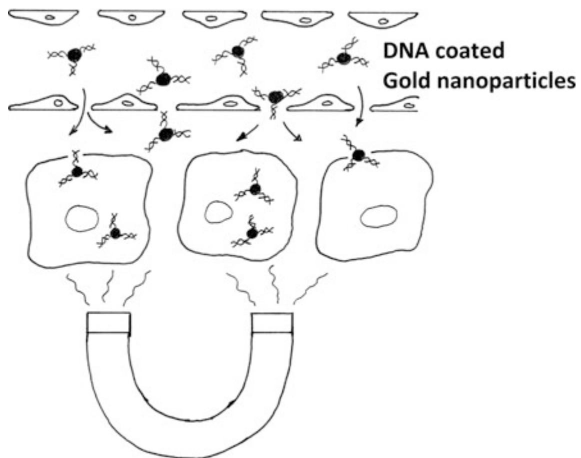

Fig. 9 Magnetofection. Figure adapted by author from Chemicell, Magnetofection™ (http://www.chemicell.com/products/Magnetofection/Magnetofection_separation.html)

under the influence of pulsating magnetic fields (0.5 to >2T) provides an additional interest [101, 102]; (c) that magnetic field can enhance contact and internalization of the magnetic vectors in the cells, resulting in high transfection/transduction efficiency [10].

Magnetofection reagents are commercially available (http://www.ozbiosciences.com, http://www.chemicell.com), and the optimal conditions of use as research tool can be found in the Web site of the commercial providers for both viral transduction and nonviral NAs transfection. Magnetofection has demonstrated its utility in in vitro models to deliver plasmids, small interfering siRNA, short hairpin RNA and antisense oligonucleotides to many cell lines, and a variety of primary cultures. However, the exact benefit of in vivo application of magnetofection remains unclear in some cases. From several studies reported on systemic delivery of nucleic acids to tumors, only in a few of them a magnetic field has been applied to enhance the accumulation [103, 104]. A successful experiment employing iron oxide nanoparticles to deliver an anti-metastatic gene (NM23-H1) in pulmonary metastasis resulted in tumor growth inhibition, but in this study, no magnetic targeting was involved [105]. However, significant efficacy of magnetofection has been observed for gene delivery to cardiac tissue [106]. Vaccine studies [107, 108] have demonstrated that magnetic DNA vaccines induce specific humoral and cellular immune responses, achieving several orders of magnitude higher transfection efficacy than naked DNA. Magnetofection has also been effective in the transfection of melanoma cells and tumors with a plasmid DNA encoding short hairpin RNA against an specific adhesion molecule, but less efficiently than gene electrotransfer in in vivo tumor gene therapy [109]. Thus, magnetofection is an easy and non-expensive procedure that offers safety and efficacy as potential advantages.

Laser or Photodynamic Delivery: Optoporation/Optofection

The optofection is a procedure to deliver nucleic acids (commonly complexed with lipids, polymers or nanoparticles) into cell cytoplasm, mediated by membrane permeabilization induced with laser pulses of high intensity and short duration on individualized cells. Early experiments demonstrated that laser transfection efficiency could be achieved with single and multiple pulses and small spot size range (0.3–2 μm), employing nanoseconds-pulsed laser Nd:YAG (neodymium-doped yttrium-aluminum garnet) [110]. The femtoseconds laser (titanium:sapphire) has been used for successful in vivo and in vitro transfection [111, 112]. Employing carbon nanoparticles activated by femtoseconds laser pulses, the laser beam could be expanded to cover centimeter-length area [113]. The use of laser-induced surface plasmons on metal nanoparticles also mediates the nucleic acid delivery into cells [114]. The throughput can be increased using gold nanoparticles, since laser light can be employed to illuminate a large amount of cells, at lower light intensity [115], resulting in tissue heating and formation of water vapor nanobubbles. Both heat and nanobubbles can permeate the cell membrane and deliver cell-impermeable compounds [116]. Other strategy is to

promote the release of nucleic acids from endosomes to cytoplasm using photosensitizers such as TPcS2a (disulfonated tetraphenyl chlorin) or AlPcS2a (aluminum phthalocyanine disulfonate), which co-localizes in endosomes with DNA complexes and after light pulse, the photosensitizer reacts with oxygen and induces the disruption of endosome membrane [117]. However, the mechanisms of molecular uptake and the influence of external parameters remain unknown. The main limitation of their use in vivo [112, 118] is related to the low penetration capacity, the tissue damage and the low irradiation area.

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Designer Effectors for Editing and Regulating Complex Genomes

Maximilian Müller, Tafadzwa Mlambo and Claudio Mussolino

Abstract Genome editing using customizable nucleases has developed tremendously in the last years providing new breadth in different branches of life science and system biology. In particular, the use of these technologies has been instrumental to shed light on the correlation between genotype and phenotype for a plethora of inherited diseases for which cellular or animal models were lacking. The possibility to combine specific targeting with transcriptome or epigenome regulators further expands the range of application of these customizable tools. The facile modification of complex genomes combined with the opportunity to quickly regulate gene expression promise to be major players in future research and medicine.

Keywords Genome editing · Designer nucleases · CRISPR-Cas9 · Gene therapy · TALENs · ZFNs · Transcription regulation

Introduction

The wealth of information generated in the last years with the use of high-throughput techniques to interrogate the genome, transcriptome and proteome has highlighted the importance of genetics in human health. In particular, this knowledge has led the field of gene therapy to progress immensely in the last 20 years. In general, gene therapy approaches to cure genetic disorders rely on two main strategies: (i) direct in vivo delivery of the gene therapeutic to the target organ

M. Müller · T. Mlambo · C. Mussolino (✉)
Institute for Cell and Gene Therapy and Center for Chronic Immunodeficiency,
University Medical Center Freiburg, Freiburg, Germany
e-mail: claudio.mussolino@uniklinik-freiburg.de

T. Mlambo
Spemann Graduate School of Biology and Medicine (SGBM),
University of Freiburg, Freiburg, Germany

M. Müller · T. Mlambo
Faculty of Biology, University of Freiburg, Freiburg, Germany

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and (ii) ex vivo modification of autologous cells that are transplanted back to the patient upon correction of the genetic defect (Fig. 1). In the first scenario, a replication incompetent viral vector is generally used to deliver its genetic cargo, usually consisting of a cDNA encoding for the gene that is mutated in the patient, in order to compensate for the missing function in the target cells. On the other hand, the second approach relies on the ability to isolate autologous cells from a patient, expand them in vitro and correct the genetic defect prior to their re-administration to the patient where they will establish a stable graft, thereby restoring the missing function. By applying these two principles, different institutions have shown clear clinical benefits in a variety of genetic and acquired disorders such as many primary immunodeficiencies (PID) [1–4], leber congenital amaurosis (LCA) [5], hemophilia [6], metachromatic leukodystrophy (MLD) [7], cancer [8] and HIV infection [9, 10]. Additionally, the modification of primate embryos [11, 12] and the controversial editing of human tripronuclear zygotes [13] certainly provide evidence that genome editing is developing faster than imagined.

However, these successes have gone through a series of events that seriously undermined the development of gene therapy in the late 90s. Indeed, in 1999 the first accidental death of an 18-year-old boy with a deficiency of ornithine transcarbamylase (OTC) deficiency who participated in a study conducted by Dr. J.M. Wilson at the

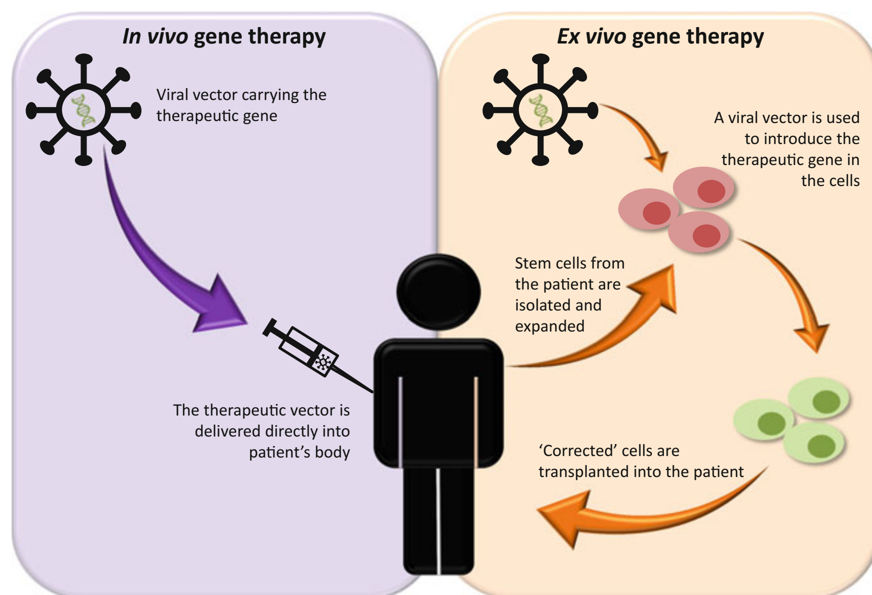

Fig. 1 Delivery strategies for human gene therapy. A replication incompetent viral vector carrying cDNA encoding the therapeutic gene is delivered in vivo (*left*) into the patient's body systemically or directly to the target organ via local injection. Alternatively, the genetic defect can be corrected by introducing the therapeutic gene ex vivo (*right*) in stem cells isolated from the patient. The corrected cells are subsequently transplanted back into the patient where they will establish a stable graft

University of Pennsylvania [14] raised serious concerns regarding the safety of gene therapy-based therapeutics applied to humans which led to the temporary shutdown of clinical research in many institutions [15]. Importantly, this unfortunate event had the consequence of improving the clinical protocols, the process of recruiting patients, the monitoring and oversight of clinical research with the aim of avoiding the problems encountered in the OTC deficiency clinical trial in the future.

To date, more than 1800 gene therapy clinical trials have been conducted for the treatment of different indications spanning from cancer to infectious and cardiovascular disease [16]. Substantial progress has been made in the gene therapy-based treatment of primary immunodeficiencies. Indeed, great knowledge and expertise has been acquired over the years to treat PIDs with allogeneic hematopoietic stem cell transplantation (HSCT) from a human leukocyte antigen (HLA)-matched donor which confers clinical benefit with high success rate [17]. However, for all those patients that lack a suitable donor, gene therapy offers an essential, and sometimes the sole, alternative by combining autologous HSCT with gene transfer methodology that, by exploiting different strategies, can complement the missing gene function in the patient-derived cells [18] prior to transplantation. In most cases, PIDs follow a simple Mendelian inheritance and theoretically can be cured by introducing a normal copy of the mutated gene in patient-derived stem cells; in addition, the corrected cells often possess a selective advantage over uncorrected cells and these two aspects strongly support the impressive results achieved in the clinics where almost all patients treated so far exhibited a clinical benefit [19]. However, the success of the first PID trials using γ-retroviral vectors to deliver the missing gene was strongly reshaped by the occurrence of severe adverse events, in particular T-cell acute lymphoblastic leukemia (T-ALL) [20–23] and myelodysplastic syndrome (MDS) [24, 25] as a result of insertional mutagenesis of the viral vector which led to the activation of LMO2, EVI 1, PRD1M16 and STBP1 oncogenes. Lessons learned from these trials have led to the development of safer vectors with the aim of reducing insertional mutagenesis. Self-inactivating (SIN) retroviruses in which the enhancer activity of the LTR has been deleted combined with insulators sequences and suicide genes have recently entered the clinics (NCT01129544) as well as lentiviral vectors that are less prone to integrate in 5′ regulatory regions compared to retroviruses [26, 27]. Although these trials are still ongoing and it is too early to draw conclusions, the first reports are encouraging [19] and suggest that clinical research to treat PIDs is turning in the right direction.

The different improvements discussed above to increase the safety of viral vectors, however, still do not overcome the risk associated with insertional mutagenesis and clonal dominance. Indeed, as shown in a recent gene therapy trial of β-thalassemia, a patient has become transfusion independent after lentiviral-based β-globin gene transfer but the therapeutic benefit has been shown to be mostly due to a benign dominant clone in which the expression of HMGA2 is upregulated of $\sim$10,000 folds due to the integrated vector [28]. Consequently, an approach that would eliminate rather than simply reduce the risk associated with the use of integrating viral vectors is certainly desirable. Genome engineering offers a

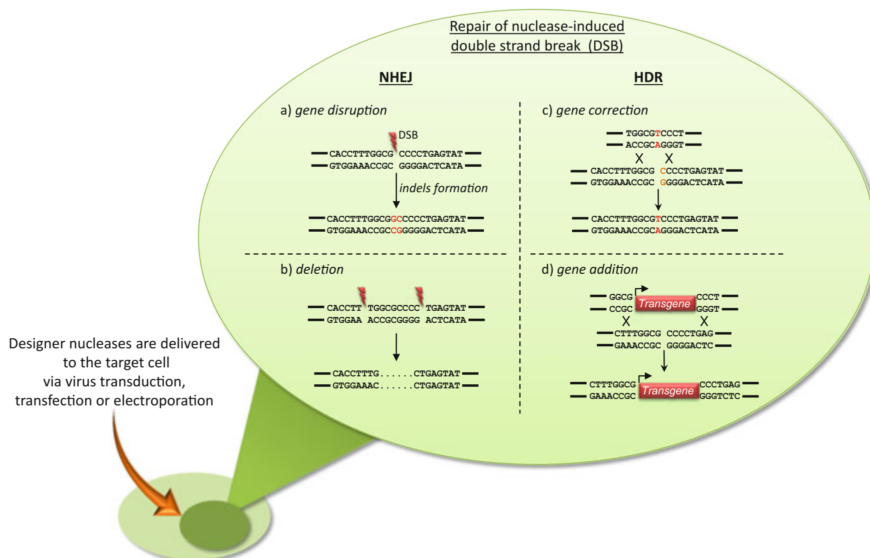

Fig. 2 Outcomes of double-strand break repair. Double-strand breaks (DSBs) introduced by designer nucleases can be repaired via the two main mechanisms of DNA repair: non-homologous end-joining (NHEJ, *left*) or homology-directed repair (HDR, *right*). NHEJ is 'error prone' and results in small insertion or deletion (indels) mutations at the break point. Thereby, it can be exploited in gene therapy for gene disruption (**a**) or to delete portion of a chromosome by introducing two DSBs in close proximity (**b**). HDR typically leads to the precise repair of the DNA break by using the sister chromatid or foreign DNA as a template. In this case, specific mutations can be corrected (**c**) or therapeutic cassettes can be inserted in specific genomic sites (**d**)

valuable alternative since it may allow for the in situ correction of the mutation underlying a genetic disorder or alternatively can be exploited to insert a normal copy of the mutated gene in specific 'safe' regions of the genome, the so-called safe harbors (Fig. 2) [29]. Here we discuss the different strategies and technology platforms that can be employed to modify the genome of target cells with the aim of repairing a genetic defect, highlighting advantages and pitfalls of a methodology that pledges to be a major player of tomorrow's clinical research and medicine for genetic disorders.

Genome Editing Strategies for Gene Therapy

Conventional medications can ameliorate the symptoms of a genetic disease but cannot be used to 'cure' its cause that is the genetic mutation. Thereby, alternative strategies should be considered in patients suffering from genetic disorders in order

to eliminate the cause of their illness. Genome editing allows for precise modifications in the DNA sequence of a cell and can be exploited in medicine to correct the genetic defect underlying a certain pathology. This can be achieved by introducing specific double-strand breaks (DSB) at the site where the DNA change is desired; in this scenario, the subsequent activation of the cellular DNA repair mechanisms can be exploited for therapeutic purposes (Fig. 2). Non-homologous end-joining (NHEJ) and homology-directed repair (HDR) are the main DNA repair pathways in eukaryotic cells. While the first has evolved to offer a rapid way to overcome the physiological DSBs that in daily life affect the genome of a cell (for example due to DNA replication, free radicals or ionizing radiations), the latter is only available during S phase when the sister chromatid offers an homologous template for repair [30, 31]. In mammalian cells, NHEJ is the most frequent mechanism of choice for DSB repair but, unlike HDR, it is 'error prone' and may lead to small insertion/deletion (indels) mutations at the DSB site which are needed to rejoin the non-compatible DNA ends. To achieve therapeutic benefit, NHEJ can be harnessed for example to inactivate a deleterious gene that harbors a gain-of-function or dominant negative mutation [32] or to introduce a protective mutation [33]. A similar effect can be obtained by inducing two independent DSB to achieve targeted chromosomal deletions [34]. However, activation of the HDR pathway is required to achieve correction of a deleterious mutation or to insert a therapeutic transgene in a specific region of the genome. This can be efficiently achieved by inducing a targeted DSB and contextually delivering an exogenous DNA template (plasmid or single-stranded oligonucleotide, ssODN) homologous to the target site. In this scenario, HDR frequency is increased by several orders of magnitude [35, 36] and can be exploited for gene correction [37] or targeted gene addition [38]. Therefore, the fundamental step that has to be undertaken in order to induce genomic modifications is the generation of a DSB in a specific site of the target genome. This can be achieved by using programmable nucleases which combine a tailored DNA-binding domain that defines the target site with a cleavage domain that introduces the lesion in the DNA double helix. So far, different sequence-specific designer nuclease platforms have been established which can be divided into two main groups based on their mode of interaction with the DNA. Meganucleases (MNs), zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) bind to their target through protein–DNA interaction while the clustered, regularly interspaced, short palindromic repeat (CRISPR)-CRISPR-associated 9 (Cas9) recognizes its target through base pairing between the DNA target and a guide RNA (gRNA) molecule. In the next section, we will describe the main characteristics of these genome editing tools.

Different Platforms, One Goal

The first step to obtain targeted modifications in a given genome is the introduction of a DSB in a specific location, and different tools have been developed in the last decades to provide researchers with a versatile platform to achieve this task. In this section, we will give an overview of the most widely used designer nucleases that have been used to achieve targeted genomic modifications in a variety of organisms.

Meganucleases (MNs)

Homing nucleases or meganucleases are enzymes identified for the first time in yeast [39] with members from the LAGLIDADG family which have been primarily used in gene-targeting applications [40]. These enzymes are relatively small and bind to long target sites of 14–40 nucleotides usually as monomers composed of subdomains binding to non-palindromic sequences or as homodimers binding to near-palindromic motifs. Re-targeting these enzymes to a DNA sequence of choice requires extensive protein engineering based on the combination of in silico modeling and in vitro directed evolution and selection of engineered MNs variants [41]. A proprietary engineering strategy has indeed provided MNs highly relevant for human gene therapy [42, 43], but the cumbersome protocols to generate tailored MNs have limited the widespread use of this technology.

Zinc Finger Nucleases (ZFNs)

The genome engineering field has been, however, dominated in the last 20 years by designer zinc finger nucleases (ZFNs). These chimeric enzymes combine the aspecific cleavage domain from the restriction endonuclease *Fok*I and a DNA-binding domain composed of an array of modules derived from eukaryotic Cys_2-His_2 zinc finger-based transcription factors. Each module within an array is composed of about 30 amino acids and is capable of binding to 3–4 nucleotides by interaction of key residues with the DNA [44]. Ideally, by exchanging these amino acids in the ZF-module one can easily redirect the module on a different DNA triplet. Increased interest in the 90s led to the exploitation of different strategies to identify ZF-based modules capable of binding to most of the 64 DNA triplets [45–49], thereby creating tailored DNA-binding domains by fusing them at will to target desired DNA sequences. Since then, tailored ZFNs have been employed to introduce genetic changes in a variety of systems such as primary human cells, including T cells [50], mesenchymal stem cells [51], hematopoietic stem cells [52, 53] and pluripotent stem cells [54]. However, over time it became evident that the DNA

recognition mediated by ZF-modules was not as simple as initially thought and most of the DNA-binding domains generated by simple modular assembly of predefined zinc fingers turned out to be not functional [55]. In addition, since the cleavage domain is functional only upon dimerization [56, 57], usually two distinct monomers have to be engineered in order to generate functional ZFNs, thereby making the entire process even more complex and less efficient. As a consequence, only a few academic laboratories were able to establish methods to consistently generate functional ZFNs with desired specificity, for example, by using platforms based on interrogating large libraries of ZF-modules pools in a bacterial-two-hybrid screen [58]. Alternatively, ZFNs could be purchased from Sigma-Aldrich, but the costs involved are significant (CompoZr® ZFNs).

Transcription Activator-like Effector Nucleases (TALENs)

An important revolution for genome editing came in 2009 when two independent groups reported on a novel DNA-binding domain identified in a plant pathogen of the genus *Xanthomonas* [59, 60]. These bacterial proteins, named transcription activator-like effector (TALE) proteins, are translocated by the pathogen via a type III secretion system [61] in the plant cells. Once in the cytoplasm, through a nuclear localization that resembles those of eukaryotes, they reach the nucleus to control the expression of host genes that support bacterial virulence [62]. The DNA binding is mediated by a central repeat domain composed of a tandem array of 15.5–19.5 modules [63] each composed of 34 nearly identical amino acids except for the last repeat that usually contains only 20 residues and is therefore referred to as a 'half-repeat.' The polymorphic amino acids in positions 12 and 13 within each module, usually referred to as 'repeat variable di-residues' (RVDs), determine its specificity for the DNA following an easy recognition mechanism in which one module specifically binds to one DNA nucleotide. According to this model, the four G, A, T, C nucleotides are specifically bound by the modules containing NN, NI, NG and HD RVDs, respectively, with additional RVDs capable of binding to more than one nucleotide [64]. Moreover, new RVDs have been characterized that allow more efficient and specific targeting [65, 66]. Even though at first sight the different modules within a TALE array do not suffer from context-dependent effects as is the case for ZF-based arrays, a more closer look has highlighted that affinity is strongly influenced by repeat composition and that specificity of the different modules can be altered by its context within the array [67, 68, 66]. An interesting aspect of TALE binding is the invariable presence of a thymine, immediately upstream of natural TALE target sites, which is probably bound by a repeat-like structure upstream of the TALE array [69]. This is an important constraint when screening DNA to search for a potential target of a designer TALE-based effector since the lack of the 5′T strongly reduces TALE binding [70]. However, by screening structural libraries, new TALEs which bind to target sites beginning with any nucleotide have evolved [71], thereby further expanding the targeting range of

designer TALE-based effectors. The straightforward 1:1 (protein/DNA) correspondence allows for the differential assembly of modules with desired specificity in order to generate DNA-binding domains tailored to any DNA sequence in a few steps. This can be done either by high-throughput methods that allow the generation of hundreds of TALE-based DBD in a short time [72, 73] or using common molecular cloning techniques as the 'Golden Gate' [74, 75]. For the *in house* generation of tailored TALE-based nucleases, many 'TALENs assembly kits' are available through Addgene, but for those that prefer to avoid establishing new techniques in their laboratories, TALENs can also be purchased from Life Technology. To take into account the different constraints that may affect TALEN efficacy, several tools have been developed that assist the researcher to find potential genomic targets that begin with a 5′T, have a correct spacer between TALEN monomers [70] and minimize the occurrence of predicted off-target sites [76]. Since 2009 when the DNA recognition modality of TALEs was cracked, the number of publications referring to this tool has steadily increased year by year (source: Web of Knowledge, http://wokinfo.com/) leading to more and more laboratories approaching genome editing for different purposes spanning from basic research to disease modeling and therapy. In the last 4 years in particular, the exploding activity around TALENs has led to their application in a variety of cellular and animal models including zebrafish [77], mouse [78], rat [79] and non-human primates [11]. The successful use of TALENs for disease modeling and therapy in induced pluripotent stem cells [80] and the recent success in modifying the genome of human primary cells [81, 82] certainly open new opportunities for the clinical translation of TALEN-based genome editing approaches to treat human disorders.

RNA-Guided Endonucleases (RGNs)

A real breakthrough in the field of genome engineering came in 2013 when the clustered, regularly interspaced, short palindromic repeat (CRISPR)-CRISPR-associated 9 (Cas9) system from the bacteria *Streptococcus pyogenes* (Sp) was reprogrammed to cleave desired loci in the human genome [83, 84]. Of the three known bacterial CRISPR-Cas systems [85], the type II is the most straightforward providing *bacteria* and *archea* with a flexible adaptive immune system against foreign DNA. This is accomplished by an endonuclease, the Cas9, which is instructed to cleave viral or plasmid DNA by an RNA molecule that is complementary to the invading DNA. While in nature the Cas9 nuclease forms a ribonucleoprotein complex with two RNA molecules (the trans-activating crRNA, tracrRNA, and the CRISPR RNA, crRNA), desired DNA cleavage can be obtained by combining the two RNA molecules in a single guide RNA (gRNA) [86] containing a 20 nucleotide sequence complementary to the genomic target site. In principle, these RNA-guided endonucleases (RGNs) can be tailored to any genomic site by simply exchanging the complementary target site of the gRNA molecule and

this has allowed researchers to even target multiple sites in a single run by delivering diverse gRNAs simultaneously targeting different genes [87]. The multiplexing capability of the CRISPR-Cas9 makes this system easier to engineer compared to the previously described genome editing tools and provides a potential promising approach to study polygenic disorders or complex pathways. The simplicity of using the CRISPR-Cas9 system has in the last years provided unprecedented advances in the field of biology, functional genomics and gene therapy since it allows facile genome modifications in any target genome. However, similar to ZFNs, not every target site can be targeted with this platform. Indeed, the Cas9 enzyme requires a specific nucleotide signature immediately following the DNA target called PAM (protospacer adjacent motive) which in the case of *S. pyogenes* Cas9 is an NGG dinucleotide. Only when this sequence requirement is fulfilled the Cas9 enzyme is able to induce a DSB, 3–4 nucleotides upstream of the PAM sequence. Moreover, the gRNA is usually transcribed from RNA polymerase III-dependent U6 promoter which requires an additional G nucleotide at the 5′ of the transcribed RNA. Taken together, these sequence constraints reduce the occurrence of potential target sites which are expected every 32 bp of random DNA sequence [88]. This targeting range is very good when compared to ZFNs for which a target is expected to occur every 50–500 bp depending on the assembling method [89, 90], but it is still significantly worse than TALENs; indeed, on average three TALEN pairs can be designed per base pair of random DNA [91]. Since the first reports in 2013, the CRISPR-Cas9 system has been adopted by laboratories worldwide to modify the genome of plants, cell lines and even of those organisms which were previously challenging to manipulate [88] including human tripronuclear zygotes [13]. With such a versatile tool in hand, genome editing has become more feasible today allowing more innovative applications in different branches of biology including disease modeling and human gene therapy.

Prospects and Challenges of Genome Editing for Human Gene Therapy

The nuclease platforms described earlier have been applied in a variety of disease models in proof-of-concept studies or at preclinical levels to evaluate the feasibility of genome editing strategies to treat genetic disorders and to pinpoint the risks associated with these therapeutics. Recently, ZFNs have been applied to humans to tackle HIV infection [92]. The rationale of this trial moved from the evidence that individuals harboring loss-of-function mutations in the *CCR5* gene (i.e., delta32 mutation), encoding for the major co-receptor for HIV entry, are resistant to HIV infection. This led researchers to use genome editing tools to specifically disrupt this gene ex vivo in patient-derived cells in order to render them HIV-resistant prior to re-transplanting them back to the patient as a potential alternative cure. Moreover, the evidence that such an approach could provide a benefit to the patient

was provided by the so-called Berlin Patient, an HIV-infected person that upon receiving an allogeneic hematopoietic stem cell transplantation from a donor homozygous for a loss-of-function *CCR5* mutation was cured of HIV infection [93]. Since the risks associated with allogeneic HSCT are still high, rendering the patient's own cells, either T cells or stem cells, resistant to HIV by inactivating the *CCR5* gene would represent a valuable source of autologous transplantable immune cells. Additionally, this approach is particularly promising because the edited cells, once back in the patient, possess an intrinsic selective advantage in the presence of the HIV virus which would allow them to expand and protect the patient from infections. Proof-of-concept studies in mice showed the promise of this approach either when applied to T cells [50] or to stem cells [94] and led to the first phase 1 clinical trial in which ZFNs have been used to knock out the *CCR5* gene in patient-derived T cells. Even though the patient cohort was small and the follow-up time short, the first results have clearly showed that genome editing technologies can be safely applied to humans providing a benefit that is directly linked to the efficiency of the knockout [92]. Indeed, the most prominent results were observed in one patient of the study that was discovered later to be heterozygous for the *CCR5* delta32 inactivating mutation highlighting that high frequency of gene knockout is essential to provide a clear therapeutic benefit in this setting. While the example provided relies on the occurrence of targeted indel mutations upon harnessing of the NHEJ repair pathway to inactivate the target gene, in most of cases, the objective is to achieve the specific correction of a genetic defect. This can be obtained by activating the HDR repair pathway to subsequently correct the desired mutation. This strategy has recently been shown in stem cells derived from an immunodeficient patient affected by SCID-X1 [52]. In this case, patient-derived hematopoietic stem cells were modified ex vivo by using ZFNs targeted to the *IL2RG* gene and an integrating defective lentivirus harboring a therapeutic gene cassette serving as a template for the HDR-mediated repair of the target locus. Although the efficiency of gene correction was in the range of 3–10% depending on the differentiation status of the cells, this was enough to restore hematopoiesis in gene-edited cells and facilitate their differentiation to functional lymphoid cells that possess a selective advantage over the uncorrected cells lacking the *IL2RG* gene product. The two examples reported here clearly demonstrate the potential of genome editing strategies for human gene therapy. Both represent an obvious breakthrough in the field of medicine since these kinds of strategies can be applied to most of the genetic disorders affecting the hematopoietic system or other organs. As mentioned, one of the challenges is to obtain enough corrected cells which can restore the disease phenotype once they are transplanted back into the patient. This is directly connected to the efficiency of genome editing; thereby, great effort is devoted to improve this frequency in clinically relevant cells by either regulating the cell cycle [95, 96] or by facilitating specific DNA repair pathways by using defined chemicals [97].

However, the major concern of using genome editing strategies for therapeutic purposes is their specificity. A number of studies have shown in the last five years that, besides their high efficacy in inducing targeted modification, most of the

designer nuclease platforms described suffer from cleavage at off-target sites [98, 99] that may lead to unexpected and deleterious genomic rearrangements [70]. While this aspect may be of limited danger when genome editing is performed in short-lived cells, as in T cells, off-target cleavage in highly proliferating hematopoietic stem cells may lead to their immortalization with devastating consequences for the patient [100]. Thereby, understanding and preventing designer nuclease-mediated off-target cleavage is of paramount importance especially in the view of clinical translation. The wealth of information on how designer nucleases recognize and cleave their target sites has led to major developments that have strongly enhanced the specificity signature of these genome editing tools. As we discussed previously, both ZFNs and TALENs rely on the aspecific cleavage domain derived from the restriction nuclease *Fok*I that upon dimerization is able to induce a DSB. However, *Fok*I dimerization can occur also between sites which are several megabases apart or even located on different chromosomes which may evoke off-target cleavage [101]. This was particularly evident using first-generation ZFNs which led to considerable cytotoxicity [102, 103] and was partially overcome by using obligate heterodimeric *Fok*I cleavage domains that reduce the formation of catalytically active ZFN dimers bound to off-target sites [104, 105]. To overcome this limitation by providing an additional level of specificity, several groups have used sequence-specific cleavage domains in the context of designer nucleases. As a result, the use of the restriction enzyme *Pvu*II was explored in the context of both ZFNs and TALENs [106, 107] to cleave genomic targets only if the designer nuclease binds in close proximity of a genomic *Pvu*II site. Similarly, fusions of TALE-based DNA-binding domains to meganucleases were also explored with the aim of reducing genotoxicity [108–110]. While these solutions have provided remarkable advantages in terms of specificity, they greatly reduce the occurrence of potential target sites in a given genome. Alternative strategies to reduce cleavage at off-target sites rely on inducing single nicks in the DNA helix rather than double-strand breaks. Designer nickases have been generated by inactivating one of the two *Fok*I domains within a designer nuclease pair and in the context of both ZFNs and TALENs have shown increased specificity [111, 112]. A similar solution has been adopted to reduce off-target cleavage mediated by the RGNs by inactivating one of the two cleavage domains of the Cas9 protein [113, 114]. However, the DNA–RNA pairing that dictates the targeting specificity of RGNs is highly flexible and tolerates mismatches including insertion and deletions which strongly expand the repertoire of cleavable off-target sites [115, 116]. Thereby, alternative strategies have been adopted to render RGNs more specific including the destabilization of the DNA–RNA duplex by shortening the guide sequence of the gRNA down to 17 nucleotides [117] and by creating dimeric RGNs by fusing the *Fok*I cleavage domain to the inactive Cas9 protein [118, 119]. Additionally, designer nuclease-mediated off-target cleavage can, in general, be improved by reducing their expression levels. This can be achieved by reducing the exposure of the genomic DNA to the active nucleases by delivering the cleaving enzymes as proteins [120–122].

Not Only Genome Editing

The genome editing tools described provide a valuable technology for the modification of genomes of different complexity. Additionally, the versatility of these platforms easily allows alterations of a cell at different levels such as the transcriptome or the epigenome. In this case, ZF- or TALE-derived DNA-binding domains, as well as catalytically inactive Cas9 (i.e., 'dead' Cas9 or dCas9) [123], can be fused to transcriptional regulator domains, histone or chromatin modifiers in order to create designer transcription factors or epigenetic modifiers. By using activator domains such as the herpes simplex virus-based VP64 and the p65 subunit of nuclear factor kappa B or repressor domains such as the Krüppel-associated box (KRAB) domain, several groups have provided evidence that target gene expression can be modulated at will [91, 124]. Importantly, gene regulation can be exploited not only to elucidate gene function or to engineer cellular pathways, but also to explore novel therapeutic opportunities for genetic defects [125, 126]. Particularly, interesting is the possibility to modulate endogenous gene expression to induce reprogramming of the target cell. Usually, reprogramming is achieved by ectopic expression of specific factors such as OCT4, SOX2, KLF4 and c-Myc in human fibroblasts [127]. However, the steps that result in reprogramming via over-expression of specific factors are complex and associated with aberrant activation of apoptotic program which strongly reduces its efficacy [128]. Consequently, there is a growing interest in exploring the use of tailored transcription factors to shed light on the reprogramming process and possibly make it more efficient [129]. One limitation of this approach is that multiple genes have to be regulated simultaneously, thereby relying on the delivery of multiple designer transcription factors which may be complicated if using TALE- or ZF-based DNA-binding domains. For those researchers particularly interested in simultaneous targeting, i.e., multiplexing, the CRISPR-Cas9 platform offers a valuable alternative. Indeed, the catalytically inactive dead-Cas9 previously described can be recruited at different target sites by simply co-delivering multiple gRNA molecules [130, 131]. Moreover, by targeting multiple positions within the same promoter, the effect on target gene expression can be substantially increased [88]. Additionally, orthogonal Cas9 from different bacterial species can be used to transcriptionally regulate and genetically edit multiple genes simultaneously [132]. One limitation of using designer transcription factors is that their effect is transient if they are not constantly expressed within the target cells. This can be achieved by using integrating lentiviral vectors which, besides the concerns associated with random mutagenesis discussed previously, are also not applicable for the delivery of TALE-based effectors [133]. Therefore, an alternative strategy to control gene expression is the use of designer epigenetic modifiers which, through transient expression, may lead to permanent epigenetic changes [134]. Transcriptional control holds great promise for the future especially in the field of gene therapy since it may allow for the understanding and treatment of complex polygenic disorders including neurological degenerations. Moreover, off targeting of designer transcription factors or epigenetic modifiers have not yet been reported highlighting their prominent safety compared to genome editing tools. Indeed, while any off-target binding of a designer

nuclease may induce a double-strand break leading to a consequent genomic rearrangement, off-target binding of a tailored transcription regulator can be silent if far from promoters or enhancers [123]. However, systematic studies using high-throughput techniques such as microarrays, RNA-seq or ChIP-seq should be conducted to further address this point.

Outlook

The technologies described allow the manipulation of cells at different levels spanning from the genome to the transcriptome and even the epigenome. Facile and targeted control of specific pathways will support researchers in all the fields of biology from basic research to system biology and particularly gene therapy. While optimism has risen after the great success of applying these tools in humans [92], many challenges have to be overcome particularly regarding their safety. It will certainly be interesting to monitor the growth and improvement of these technologies and the adaptations that will follow to establish designer nucleases and transcriptional regulators as novel players of tomorrow's medicine.

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siRNA Therapeutics to Treat Liver Disorders

Chengjung Lai, Cristina Martin-Higueras and Eduardo Salido

Abstract Deeper understanding of the molecular mechanisms of liver disease has prompted the investigation of a large number of strategies to manipulate the expression levels of specific genes involved in liver pathology. In addition to the search for small molecules that influence enzymatic activities, antibodies that bind and modulate specific liver proteins or gene therapy aimed at replacing a genetic deficit, numerous proof-of-concept studies and a significant number of clinical trials have used RNAi-mediated gene silencing as the methodology to treat liver conditions. In the last five years, we have witnessed a surge of attempts to manipulate liver-specific gene expression by using one of the four main approaches in the current RNAi landscape: shRNAi, dsRNAi, miRNA and siRNA. A major advantage of siRNA versus other antisense-based therapies is its highly effective and selective downregulation of gene expression. This means that an siRNA approach requires less material for administration into patients in order to achieve effective target suppression and avoids nonspecific off-target toxicity. Theoretically, siRNAs can be designed for targeting any disease-causing gene based on its target mRNA sequence alone. In the present review, we compile the main therapeutic applications of siRNAs to treat inborn errors of liver metabolism in the last decade and also touch on some of its applications in the fields of liver infectious diseases and cancer.

C. Lai
Dicerna Pharmaceuticals, 87 Cambridgepark Drive, Cambridge, MA 02140, USA
e-mail: clai@dicerna.com

C. Martin-Higueras · E. Salido (✉)
Instituto de Tecnologías Biomédicas (ITB), Centro de Investigaciones Biomédicas de Canarias (CIBICAN), Centre for Biomedical Research on Rare Diseases (CIBERER), Universidad La Laguna, Tenerife, Spain
e-mail: esalido@ull.es

E. Salido
Department of Pathology, Universidad de La Laguna School Medicine, 38320 Tenerife, Spain

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Introduction

RNAi Principles

During the last decade, a deeper understanding of the molecular mechanisms of liver disease has prompted the investigation of a large number of strategies to manipulate the expression levels of specific genes involved in liver disease. In addition to the search for small molecules that influence enzymatic activities, antibodies that bind and modulate specific liver proteins or gene therapy aimed at replacing a genetic deficit, numerous proof-of-concept studies and a significant number of clinical trials have used RNAi -mediated gene silencing as the methodology to treat liver conditions. In the last five years, we have witnessed a surge of attempts to manipulate liver-specific gene expression by using one of the four main approaches in the current RNAi landscape: shRNAi, dsRNAi, miRNA and siRNA (Fig. 1: RNAi world of possibilities, landscape). This review will focus primarily on the siRNA approach.

RNA interference (RNAi) is a mechanism to turn off gene expression. It was first uncovered by Fire and Mello in *Caenorhabditis elegans* [1]. Since then RNAi has

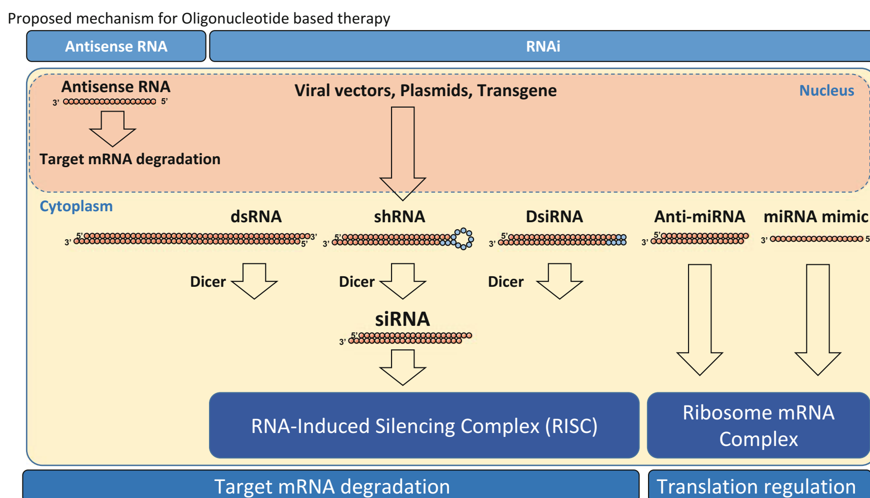

Fig. 1 Landscape of RNA interference strategies, ranging from older antisense RNA approaches to more modern RNAi therapeutics working through the RNA-induced silencing complex (*RISC*), and leading to target mRNA degradation, or the ribosome–mRNA complex, resulting in translation regulation

been observed in all evolutionary lineages, from plant to mammalian [2–4]. RNAi machinery begins with long double-stranded RNA molecules, which are processed within the nucleus by Drosha. Once in the cytoplasm, they are further processed by the endoribonuclease Dicer into short (21–22nt) double-stranded small interfering RNA (siRNA) duplexes and loaded onto Argonaute (Ago2) with the help of Dicer and the human immunodeficiency trans activating response RNA-binding protein (TRBP). Upon loading, Ago2 selects the siRNA guide strand based on 5' thermodynamic stability and subsequently cuts and ejects the passenger strand. This structure composed of guide RNA and protein, known as RNA-induced silencing complex (RISC), actively searches for mRNA with a guide strand-complementary sequence for degradation. The guide strand can remain bound to RISC and undergo several cycles of target mRNA recognition and cleavage which results in a long duration of siRNA activity. Today, synthetic short RNA duplexes (21–27nt) can be introduced systemically, be taken up by cells and subsequently engage RISC with or without Dicer and be used as a tool to effectively investigate gene functions and disease mechanisms (Fig. 2). Importantly, these approaches have shown impressive therapeutic potential that is expected to be fully realized in the near future [5–7].

RNAi therapeutic drug discovery usually starts with a large-scale screen to identify the most potent short RNA duplexes in cells that either endogenously or ectopically express gene targets of interest [8]. Computer-based algorithms developed from cumulative and empirical screen data have been demonstrated to successfully predict potent siRNA sequences [9–11]. Upon validation in cell-based test systems, identified potent siRNAs are further validated in animal models. In order to achieve their effects in animal models and patients, siRNA duplexes need to reach the cytoplasm after systemic administration through intravenous or subcutaneous injection. Further modification of siRNAs is essential so that they are resistant to multiple degradation enzymes found in serum, tissues and intracellular

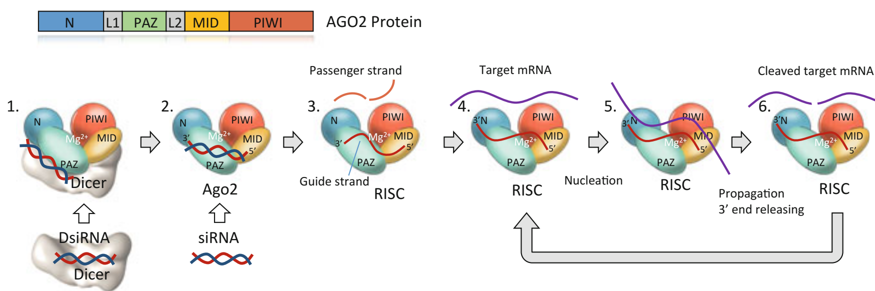

Fig. 2 Proposed mechanisms for small interfering RNA (*siRNA*) therapy. Once in the cytoplasm, double-stranded siRNA duplexes are loaded onto Argonaute (Ago2, shown with its various domains in different colors). Upon loading, Ago2 selects the siRNA guide strand and cuts the passenger strand, resulting in the RNA-induced silencing complex (*RISC*), which searches for mRNA complementary to the guide strand for degradation. The guide strand can remain bound to RISC and undergo several cycles of target mRNA recognition and cleavage which results in a long duration of siRNA activity

compartments. Chemical modifications have been widely applied to make naked siRNAs more stable, and less immunogenic, and to introduce better drug-like properties while simultaneously improving their ability to silence their targets [12–15]. Chemical modifications have been systematically investigated on every part of the siRNA molecules, from the backbone, to the sugars, to the bases, and on both guide and passenger strands. Theoretically, any modification has to be taken into consideration to maintain or even enhance efficiency of RISC loading and Ago2 slicing activities. Unfortunately, the exact molecular mechanism of how siRNA molecules dynamically load and achieve target silencing is not well understood. Crystal structures of complex of siRNA, target mRNA and Ago2 have been used as a guide to understand the potential effect of chemical modifications on RISC operation [16–20]. Nonetheless, the measurement of in vivo activities has been the most reliable and informative approach for identifying heavily modified, stable, active siRNA duplexes to empirically guide the evaluation of structure and activity relationships.

Frequently, the 2’ position of the sugar moiety of each nucleoside is modified from 2′-hydroxyl into 2′-deoxy, 2′-*o*-methyl, 2′-fluoro, 2′-*o*-methoxyethyl and locked nucleic acid (LNA). Some examples of backbone modifications are methylphosphonate, phosphorothioate, phosphorodithioate, thioester and other phosphate mimics [20–22]. Base modifications that alter Watson–Crick base pairings via changing of specific hydrogen bonding interactions have also been explored. Knowledge continues to accumulate on the role of chemical manipulations, not only on activity and pharmacodynamics but also on safety of synthetic oligonucleotides, which is important for the future development of siRNA therapeutics [14, 23–25].

Pros and Cons of RNAi-Based Therapies

A major advantage of siRNA versus other antisense-based approaches for therapeutic applications is that it utilizes cellular machinery that efficiently allows targeting of complementary transcripts, often resulting in highly effective downregulation of gene expression. This means that a siRNA approach requires less material for administration into patients in order to achieve effective target suppression and avoids nonspecific off-target toxicity. Theoretically, siRNAs can be designed for targeting any disease-causing or disease-associated genes based on its target mRNA sequence alone. Such unlimited potential has made siRNA a leading option when developing therapeutics for so-called undruggable targets which may not be inhibitable using conventional small molecule and antibody-based approaches. By selecting the unique sequences of genes targeted by siRNAs, highly specific target knockdown can be achieved while avoiding cross-reactivity to functional protein molecules of same family, which is a challenge for small molecule-based approaches. Theoretically, the high specificity may even allow

targeting of disease-specific alleles and spare the normal allele even when they differ only by one or a few nucleotide substitutions. However, careful design and selection of siRNA would be required in order to achieve allele-specific targeting as Ago2 does tolerate some mismatch between the guide strand of siRNA and target mRNAs [26–29].

The major hurdle in the development of siRNA therapies is the delivery of macromolecules to the desired cell type, tissue or organ. Oligonucleotides are rather large molecules as compared to traditional small molecule drugs, and their polyanionic and hydrophilic characters make them unable to freely cross the cellular membrane. These properties mean that siRNA therapeutics require delivery systems to correct their naturally negative pharmacokinetic characteristics. Nanocarrier (lipid nanoparticle, liposome and polymer-based) delivery and ligand conjugate-based (particularly, GalNAc direct conjugation) have proven to be successful for specific siRNA delivery in both preclinical models and clinical investigations [30–34]. However, clinically meaningful deliveries are currently limited to tumors and hepatocytes. Efforts have also been made to achieve delivery of oligonucleotides using a vitamin A-based liposome for hepatic stellate cell delivery [35]. Nanomaterial-based siRNA delivery has also been demonstrated to primarily target Tie-2 expressing lung endothelial cells or liver endothelial cells [36]. Phagocytic cells and certain cell types in the kidney have also been investigated as targets for siRNA delivery [37]. Practically, the current siRNA therapeutics can achieve profound target inhibition through intravenous and subcutaneous administration to reach hepatocytes and tumors. Oral administration is not possible with current technology. Delivery limitations represent one of the leading challenges for siRNA delivery as compared to small molecule therapeutics.

Hypothetically, siRNA therapeutics can be applied to cancer, autoimmune diseases, protein aggregation diseases and viral infections where undesirable disease-causing proteins can be corrected by targeting their transcripts. Genetic diseases with dominant negative mutations are another class of diseases that are appropriate for siRNA targeting. In addition, for some metabolic and genetic diseases, siRNA approaches can be designed to remove or reduce substrates of toxic metabolites to prevent or alleviate symptoms of those diseases. This substrate reduction strategy can serve as a potentially effective and safe way for treating diseases and will be further discussed in detailed in the next section (Section "RNAi for Substrate Reduction Therapy").

Multiple targets can be inhibited simultaneously without changing the therapeutic principles and fundamental physical composition of RNAi-based therapies. This point is particularly important for the treatment of cancers since combination treatment has been one of the main focuses for improving clinical response of cancer patients in the past two decades. Nanoparticles carrying more than one siRNA have been delivered to tumors in both preclinical and clinical studies [38, 34]. This demonstrates that siRNA-based therapeutics can be used to simultaneously inhibit multiple related gene targets to produce greater antitumor activity, without increasing off-target toxicity. Theoretically, multiple target inhibition can

also be applied to hereditary and metabolic disorders to achieve a synergistic effect acting on multiple points of the relevant pathway.

siRNA therapeutics have been demonstrated to have a long duration of activity in both preclinical and clinical studies. This is partially due to the aforementioned mechanism of RNAi machinery that utilizes stable and active RISC to continually target mRNA for a number of cycles within disease-relevant cells, even when excess siRNA drugs are completely removed from circulation by metabolism and excretion [39, 40]. This catalytic characteristic of RNAi is one of the most attractive features of RNAi-based therapeutics. This long duration of action may also be due to the additional time that diseased cells require to re-synthesize a sufficient amount of target mRNAs and proteins and return to its original diseased state after the RNAi machinery becomes ineffective.

Another advantage of siRNA therapeutics is that the drug discovery process can be relatively fast compared to traditional small molecule drug approaches. Chemical modifications, which give the siRNA therapeutics the main characteristics of a drug, can be readily applied to most sequences to achieve similar effects. Chemical modules for tissue-specific delivery and modifications for activity and stability can be reutilized and applied to other sequences of interest, readying them for in vivo investigation and optimization. Therefore, in vitro screens or algorithm predictions of duplexes with high potency can be followed immediately with the application of learned chemical modifications that are suitable for in vivo stability and tissue-specific delivery through conjugation or carrier-based delivery approaches. Hypothetically, since siRNAs have similar chemical structures, the class effect of oligonucleotide duplexes on pharmacokinetic and off-target toxic properties will be relatively predictable even for different siRNAs that are designed for different targets or diseases. However, the drug product synthesis and quality control process of siRNA therapeutics can be more complicated and challenging than for small molecule drugs.

Main Therapeutic Applications of RNAi in Liver Diseases

At the molecular level, liver disease can be simplified as being the result of either gain-of-function (GoF) of certain genes (including the addition of foreign genes in infectious diseases or the mutational activation of oncogenes in cancer) or loss-of-function (LoF) of any of a large number of genes involved in normal liver metabolism. Thus, RNAi applications are straight forward in GoF cases, while they involve manipulating the homeostatic balance of cellular pathways when LoF is the main mechanism of disease. Both approaches have been investigated with similar level of success in a number of liver diseases in recent years, and a good number of ongoing clinical trials underscore this trend. As far as GoF-related entities are concerned, we will concisely review the progress in the application of RNAi to infectious diseases and cancer in Section "Infectious and Neoplastic Diseases,"

while addressing in Section "Hereditary Liver Diseases" the hereditary liver diseases only.

The ability to turn siRNA into drugs has depended on chemical modifications that confer drug-like properties and facilitate safe and effective delivery to target organs [41, 42]. Formulations of lipid nanoparticles have emerged as agents to deliver siRNAs to hepatocytes and have resulted in a robust and durable reduction in genetic expression (called knockdown) of a variety of hepatocyte targets across multiple species [43–45]. Biodistribution of these parenterally administered lipid nanoparticles is predominantly to the liver, with a small fraction distributing to other organs that also have a fenestrated endothelium (e.g., spleen and kidney) [46, 47].

A key component of the liver hegemony in the application of siRNA to medical problems is the favorable position of this large target, with well-known surface receptors, such as the abundant asialoglycoprotein receptors (ASGPRs), which could be used as a port of entry for molecular therapeutics. Thus, favorable biodistribution has allowed remarkable success when liver is the target, resulting in around 50% of GalNAc-conjugated siRNA dose being picked-up by hepatocytes within 30 min of peripheral vein injection [33] (see Section "Recent Technological Developments in RNAi"). In addition, normal liver has slow cell turnover, which favors longer-term effects of RNAi-mediated hepatocyte manipulations. Thus, the first systemic clinical applications of RNAi therapeutics have had the liver as target organ. Several clinical trials are already in phase 3, and RNAi therapeutics targeted to the liver are likely to be approved by regulatory agencies within the next few years. And once a delivery technology is optimized and approved, it should speed up the rate at which new RNAi therapeutics become available.

Hereditary Liver Diseases

Treatment of Hereditary Diseases Due to Gain-of-Function Mutations

A handful of examples have recently documented the potential of RNAi as a therapeutic approach for inherited diseases caused by GoF mutations in a gene expressed in the liver. A deep knowledge of the metabolic consequences of such mutations has allowed important progress in preclinical and clinical investigations to treat conformational diseases such as some variants of alpha-1 antitrypsin deficiency and amyloidosis.

In alpha-1 antitrypsin deficiency (AATD), hepatocytes fail to produce sufficient AAT to prevent chronic obstructive pulmonary disease, which is particularly severe in smokers [48]. This autosomal recessive disease has a prevalence around 1:2000 among populations of Northern European ancestry and a carrier frequency of approximately 4% in the US population [49, 50]. One common pathologic allele, known as PiZ (p. E342K), also results in protein misfolding and aggregation within the hepatocyte endoplasmic reticulum, which in turn leads to liver disease in about 15% of PiZZ patients. These patients suffer hepatocellular damage, fibrosis and cirrhosis, with an increased risk for hepatocellular carcinoma over time.

Heterozygous individuals carrying a single copy of the PiZ allele show no apparent disease phenotype. Protein misfolding and liver damage can be viewed as the consequence of a GoF mutation, p.E342K, and a strategy to knock down the expression of this allele would make sense. How much reduction would be necessary to have a therapeutic effect has to be assessed, but the fact that heterozygous individuals lack liver disease is a hint that perhaps no more than 50% reduction would be needed in order to achieve significant benefits. In principle, RNAi technologies are well suited to achieve this goal. Proof-of-concept studies on this approach have been published using genetically modified mouse models overexpressing human Z-AAT [51]. These studies showed a decrease in Z-AAT accumulation in the liver by immunohistochemistry within 3 weeks after AATsiRNA-AAV8 vector injection. More recently, rAAV9 vectors overexpressing shRNA directed against AAT were also shown to significantly reduce the expression of human PiZ AAT with sustained efficacy both in vitro and in vivo [52]. Using integrative lentiviral vectors for a more permanent knockdown, this approach has been recently tested with shRNA directed against the PiZ variant of AAT in patient-derived induced pluripotent stem cells (iPSC) and their differentiated progeny [53].

Preclinical studies in nonhuman primates (NHP) have shown that siRNA against AAT, prepared with unlocked nucleic acid (UNA) technology and delivered with a polymer targeting the endosomal pathway (an approach called Dynamic PolyConjugate—DPC, *Arrowhead Pharmaceuticals*), is able to reduce circulating AAT levels about 80% after 1.5 months of treatment. Moving closer to clinical application of this therapeutic approach, a phase 1a/1b, single dose-escalation study [54] to determine the safety, tolerability and effect of siRNA (ARC-AAT) on circulating alpha-1 antitrypsin levels in healthy volunteers and patients has been undertaken by Arrowhead Pharmaceuticals. siRNA directly conjugated to GalNAc has also been tested in a phase 1/2 trial by Alnylam Pharmaceuticals [55].

A number of mutations in the transthyretin (*TTR*) gene, which codes for a liver-produced serum protein that binds and transports thyroxine and retinol, result in amyloidosis involving peripheral nerves, gastrointestinal tract, heart and kidneys. Amyloidosis has a poor prognosis, with life expectancy normally below 15 years after diagnosis and few therapeutic options (liver transplant and pharmacochaperones). More than 100 mutant forms of TTR are prone to misfolding and aggregation into amyloid fibrils that accumulate in tissues and induce organ failure in conditions known as familial amyloidotic polyneuropathy (FAP) [56–58] and familial amyloidotic cardiomyopathy (FAC) [59–61]. The most common mutation associated with FAP is p.V30M, while the predominant FAC-associated mutation is p.V122I. These diseases are inherited in an autosomal dominant manner, such that most patients are heterozygous for the *TTR* mutations, and the amyloid deposits consist of mutant and nonmutant transthyretin [62, 63]. Using siRNA-containing nanoparticles, preclinical studies in NHP and two consecutive clinical trials showed significant dose-dependent lowering of transthyretin levels for approximately one month [30]. Phase 2 studies in FAP patients receiving intravenous injections of *TTR* siRNA encapsulated in lipid nanoparticles (LNP, Alnylam

Pharmaceuticals) resulted in 80% *TTR* knockdown over 9 months and significant clinical improvement, without severe adverse effects after one year [30]. Phase 3 studies [64] aimed to evaluate the safety and efficacy of these nanoparticles, at 0.3 mg/kg, every three weeks for 1.5 years are expected to end in July 2017, with the primary goal of improving neuropathy severity, compared with placebo.

Similarly, *TTR* siRNA has been developed by Alnylam Pharmaceuticals to treat FAC, a type of amyloidosis for which no current treatment seems to significantly change the fatal outcome (less than 5 years after diagnosis). Safety of the LNP-encapsulated *TTR* siRNA in healthy volunteers was evaluated in a phase 1 trial [65]. Phase 2 studies showed a remarkable 87% reduction in serum TTR, with just one case of severe adverse effect (hepatocyte injury, with transaminase elevation), but no changes were observed in clinical evaluation of the cardiomyopathy [66, 67]. In a large cohort of about 200 patients, efficacy and safety of a GalNAc-conjugated *TTR* siRNA is being evaluated in a phase 3 trial [68] with initial subcutaneous administration of 500 mg during 5 days, followed by weekly doses for 1.5 years, with the primary goal of improving cardiovascular parameters, hospitalization and mortality, compared with placebo.

Treatment of Hereditary Diseases Due to Loss-of-Function Mutations

RNAi for Substrate Reduction Therapy

A large number of inborn errors of metabolism are due to LoF mutations involving key enzymes in a variety of pathways mainly active in the liver, resulting in the harmful accumulation of substrates. Substrate reduction therapy (SRT) is a strategy successfully used in some inborn errors of metabolism to reduce the level of the substrate to a point where residual degradative activity might be sufficient to prevent or diminish substrate accumulation to levels that can be well tolerated by the patient. Detailed knowledge of the rate-limiting steps and regulatory feedback mechanisms central to each disease is needed in order to identify the best candidate as a target for an RNAi approach.

Acute liver porphyrias are good examples of hereditary liver diseases where specific enzymes have been successfully targeted by siRNA to reduce the production of toxic porphyrins. Both dominant (acute intermittent porphyria—AIP; hereditary coproporphyria—HCP; and variegated porphyria—VP) and recessive (aminolevulinic acid dehydratase porphyria—ADP) variants result in overproduction of the neurotoxic porphyrin precursors 5-aminolevulinic acid (ALA) [69]. The neurotoxic effect of accumulating porphyrins accounts for the acute neurovisceral attacks of severe abdominal pain, constipation, hypertension, tachycardia, seizures and paralysis [70]. The attacks are precipitated by any circumstance increasing the expression of liver 5-aminolevulinic acid synthase (ALAS), the first and rate-limiting enzyme of the heme synthesis pathway [71–73]. The resulting overload of the pathway reveals the reduced enzymatic activity downstream, and ultimately, less heme is produced. This reduction in the free heme pool results, in turn, in a release of the feedback inhibition of *ALAS1*

and further upregulation of hepatic *ALAS1*, which makes the metabolic unbalance worse. Heme replacement, with negative side effects such as iron overload, has been about the only available treatment for years. A better alternative would be to prevent the upregulation of *ALAS1*. Thus, *ALAS1* has been proposed as a good target for RNAi therapeutics in acute hepatic porphyrias to reduce the production of neurotoxic porphyrins. Preclinical studies in NHP have shown that subcutaneous administration of *ALAS1* siRNA targeted via the hepatocyte ASGRs (ESC-GalNAc-*ALAS-1* siRNA) results in a dramatic inhibition of heme intermediates [74]. Phase 1 trials to assess the safety of subcutaneous administration of ESC-GalNAc-*ALAS-1* siRNA will be followed by trials to explore the effects of such therapy on hematin levels and the frequency/severity of porphyria attacks.

Primary hyperoxaluria type 1 (PH1) is a genetic disease due to a deficit of alanine–glyoxylate aminotransferase (AGT) activity in hepatocyte's peroxisomes. The majority of PH1 alleles are missense mutations that result in severe reductions of AGT enzymatic activity in the peroxisome, with a wide range of residual activity, depending on the mutations present in both alleles. This enzyme metabolizes glyoxylate to glycine, and LoF mutations in *AGXT* gene result in the oxidation of glyoxylate to oxalate, which can only be excreted in the urine. High oxalate levels lead to calcium oxalate (CaOx) stone formation and renal parenchyma damage, with progressive deterioration of renal function and, eventually, end-stage renal disease (ESRD). Combined renal and liver transplantation is needed in many PH1 patients to avoid the life-threatening systemic accumulation of oxalate that takes place after ESRD [75, 76].

Endogenous glyoxylate production occurs mainly in the peroxisomes and mitochondria, being glycolate an important precursor of glyoxylate in humans [77]. Due to the high affinity of glyoxylate reductase (GRHPR) to convert glyoxylate into glycolate, important sources of glyoxylate such as hydroxyproline are also metabolized into glycolate [78]. Peroxisomal glyoxylate can result from the activity of either D-amino acid oxidase (DAO) on glycine or glycolate oxidase (GO) on glycolate. GO, encoded by *HAO1* gene, is an FMN-dependent α-hydroxyacid oxidase, which transforms glycolate into glyoxylate. Peroxisomal glyoxylate is normally detoxified by AGT into pyruvate and glycine by transamination with alanine. Excess glyoxylate in peroxisomes is converted to oxalate by GO or is metabolized in the cytoplasm, either reduced to glycolate by GRHPR or oxidized to oxalate by lactate dehydrogenase (LDH).

Using genetically modified animals, we demonstrated that GO is an effective target for treating PH1 [79]. First, we generated a GO-deficient mouse ($Hao1^{-/-}$) that presented high urine glycolate levels but no additional phenotype, indicating that GO is a safe target for inhibition, something that can also be inferred from the finding of a child lacking GO without subsequent related phenotype, other than high urine glycolate [80]. Next, we produced double KO mice ($Agxt1^{-/-}$ $Hao1^{-/-}$) that showed low levels of oxalate excretion compared with hyperoxaluric mice model ($Agxt1^{-/-}$). We have also shown that siRNA against *Hao1*, formulated in LNP by Dicerna Pharmaceuticals, can be safely administered to mice by i.v. injection. This treatment results in massive and specific hepatocyte targeting, with

durably reduced GO expression (Fig. 3), which translates into decreased urinary oxalate levels and lack of CaOx deposition in induced hyperoxaluric animals (Fig. 4) [81]. Similar results were also reported with siRNA duplexes formulated in LNP [82] or through GalNAc conjugation [83] by Alnylam. Both companies are now conducting clinical trials with this approach.

RNAi for Homeostasis Balance

RNAi also has a place in another strategy aimed at re-establishing a homeostatic balance after a LoF mutation has resulted in a weakened arm of such homeostasis. The coagulation and complement cascades have been recently manipulated by RNAi as a therapeutic strategy.

Hemophilia is a prime example of this approach. Various forms of hemophilia are the result of mutations in genes such as the ones coding for factor VIII or IX [84, 85], yielding poor clotting of blood due to low thrombin production. Thus, RNAi can be applied to inhibit the production of antithrombin, restoring the coagulation homeostasis [86]. Phase 1 clinical trials have shown good tolerability in healthy volunteers, with no severe adverse effects observed over 70 days [87]. A single subcutaneous dose of 0.03 mg/kg resulted in a 30% decrease in antithrombin, with increased thrombin production. In hemophilic patients, a remarkable 70% decrease in antithrombin and a threefold increase in thrombin production were reported.

Spontaneous activation of the complement cascade is the basis for some serious hereditary diseases such as paroxysmal nocturnal hemoglobinuria (PNH) [88] and atypical hemolytic-uremic syndrome (aHUS) [89–91]. PNH results from somatic mutations in the X-linked gene coding for phosphatidylinositol glycan complementation group A (PIGA), an enzyme essential for the synthesis of certain membrane-associated complement regulatory proteins such as CD55, C8 binding protein and CD59. Due to the phenomenon of X-chromosome inactivation, the red blood cells arising from the mutant clone lack these GPI-linked proteins and are very sensitive to lysis by activated complement. The genetic defects responsible for aHUS involve one of the ten genes known to regulate complement activation.

As a consequence, complement-mediated red blood cell destruction and renal damage are significant in these patients. Remarkable improvements in the control of these diseases have been recently achieved with anti-C5 monoclonal antibodies [92]. An alternative therapeutic approach is the knockdown of the C5 component of complement by RNAi. Preclinical studies in rats and nonhuman primates have shown 90% inhibition of the complement pathway after decreasing C5 levels to less than 3% with anti-C5 siRNA. A phase 1 clinical trial [93] with GalNAc attached to the siRNA is underway.

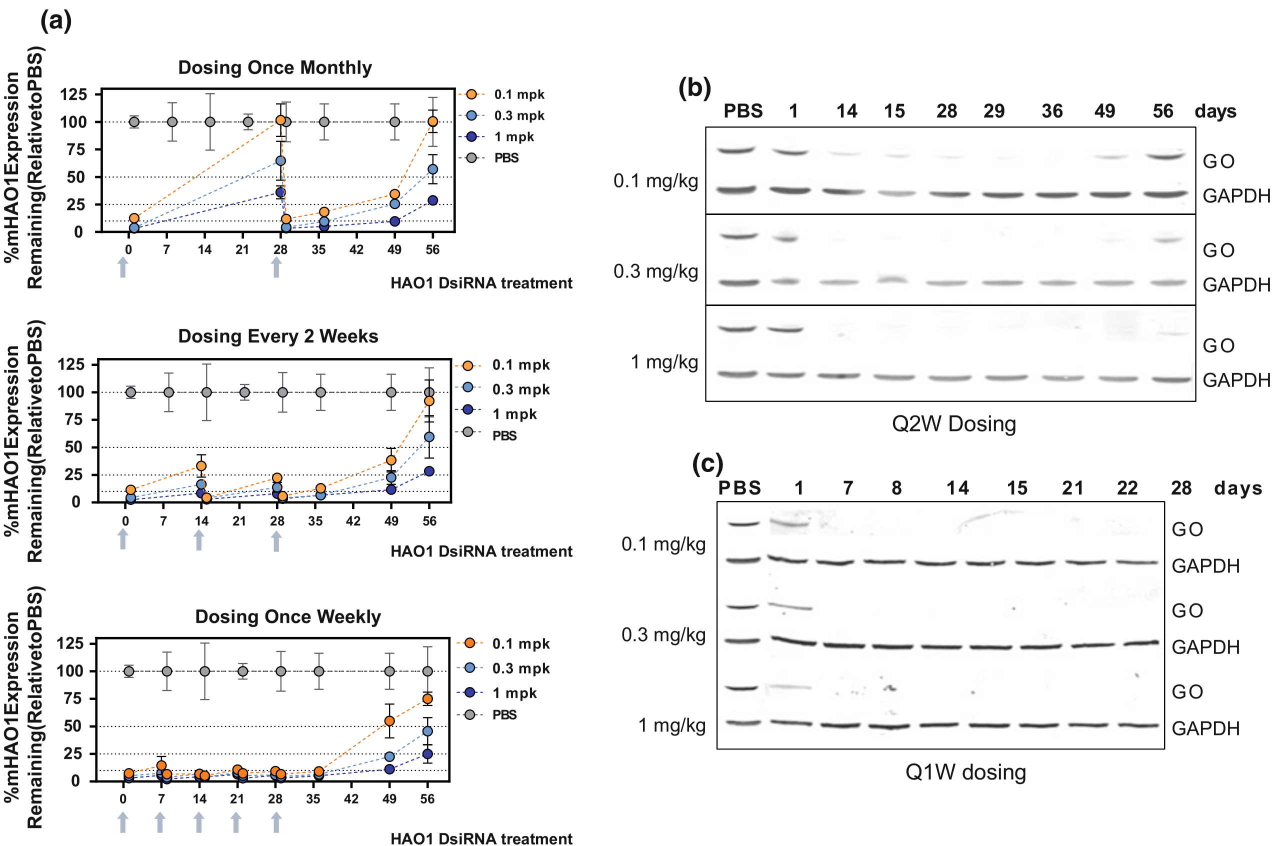

Fig. 3 Knockdown of *Hao1* mRNA (*left panels*) and protein (glycolate oxidase, GO, *right panels*) achieved with various *Hao1* DsiRNA doses and administration schemes

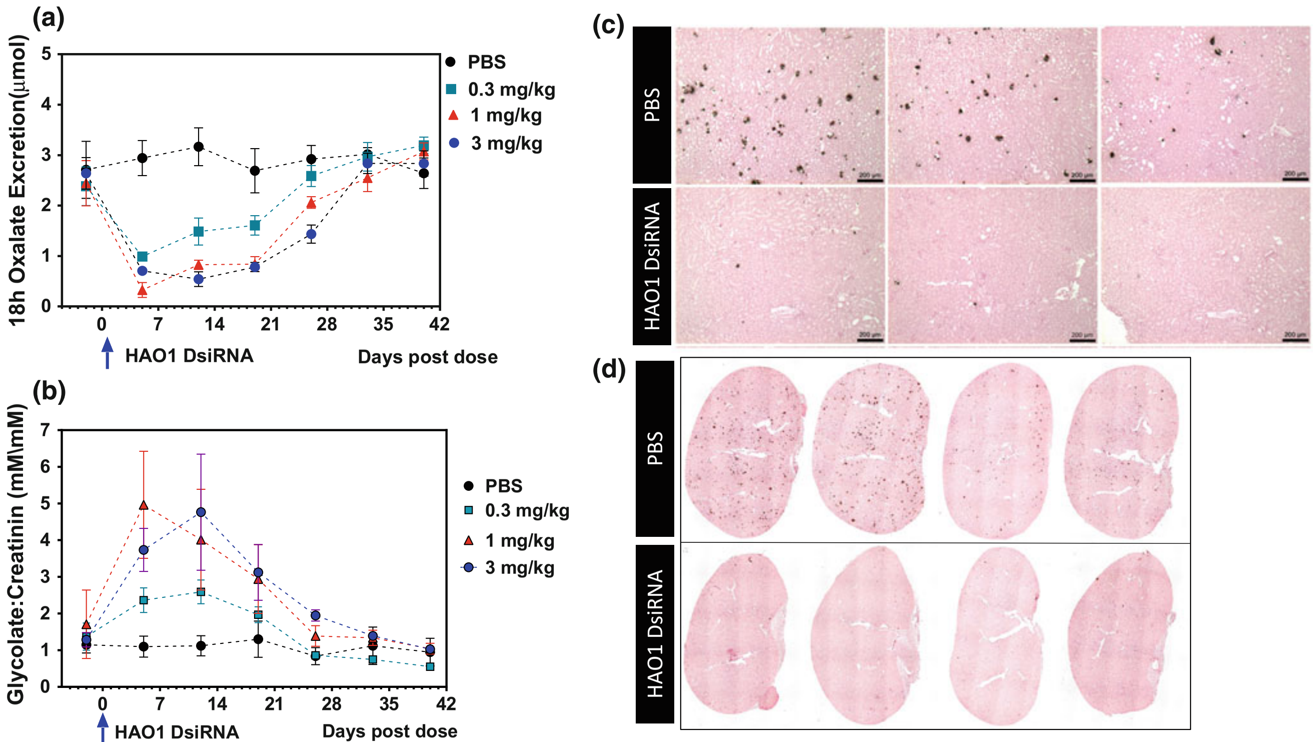

Fig. 4 Significant reduction in urine oxalate excretion (**a**), with a parallel increase in urine glycolate/creatinine ratio (**b**) achieved by various doses of Hao1 DsiRNA administration to *Agxt*$^{-/-}$ mice. The *right panel* (**c**) and (**d**, low magnification) shows kidney sections of *Agxt*$^{-/-}$ mice after metabolic overload with ethylene glycol. Mice injected with *Hao1* DsiRNA do not develop nephrocalcinosis (calcium oxalate deposits, shown as *black precipitates*), while animals treated with PBS show extensive calcium oxalate deposits in the renal parenchyma

Common Metabolic Liver Disorders

Liver cholesterol metabolism is the center of a significant effort by the pharmaceutical industry. In addition to some of the most prevalent hereditary diseases, such as familial hypercholesterolemia (FH) [95], elevated blood LDL cholesterol (LDL-c) is a major cause of morbidity and mortality in developed countries, as a multifactorial disease of complex genetic determinism. In FH, very high levels of LDL-c cause atherosclerosis and increased risk of coronary artery disease at an early age, as a result of a mutation in one of three genes (*LDLR, APOB* and *PCSK9*) known to account for 60–80% of FH. Deep molecular understanding of LDL synthesis in the hepatocytes has allowed the identification of PCSK9 as a key regulator of this pathway [96]. *PCSK9* downregulation has been proposed as a strategy to treat familial hypercholesterolemia [97]. Preclinical studies in NHP have demonstrated up to 80% knockdown of *PSCK9*, with subsequent reduction in LDL cholesterol levels of approximately 60%. In a phase 1 dose-escalation study in healthy adult volunteers with serum LDL-c of 3.0 mmol/L or higher, a single intravenous injection of *PSCK9* siRNA resulted in 70% reduction in circulating PCSK9 plasma protein and 40% reduction in LDL-c from baseline relative to placebo [32]. Using GalNAc conjugation to enhance liver targeting allows subcutaneous administration, which together with other improvements in nucleic acid chemistry, such as dual phosphorothioate siRNA or the “enhanced stabilization chemistry” (ESC), is being used in clinical trials aiming to achieve sustained reduction in LDL-c for more than three months after a single dose of these siRNA-based treatments [98].

Liver fibrosis is a common denominator in many chronic liver diseases. Infectious, toxic and autoimmune liver diseases result in hepatocyte injury that, when sustained over time, usually triggers the accumulation of collagen and other extracellular matrix proteins that lead to progressive liver damage that can be severe enough to distort liver architecture and vascular network in an irreversible path to liver cirrhosis and chronic organ failure [98]. Regardless of the origin of liver damage, medical intervention to dampen the path to liver fibrosis is a high-value goal. Detailed molecular understanding of the scar deposition in the liver has identified the hepatic stellate cells as the main actors of fibrogenesis [99]. These cells, located in the Disse space between the endothelium and the hepatocyte, are normally quiescent and function as lipid (vitamin A)-storing cells. A number of cytokines and chemokines that mediate chronic inflammation have been shown to activate stellate cells, inducing their proliferation and differentiation into highly fibrogenic myofibroblasts. Disruption of the extracellular matrix and direct stimulation of stellate cells by toxins can also induce this phenotypic change and contribute to fibrogenesis. Some of these changes are mediated by miRNAs [100]. Thus, feed-forward mechanisms are involved in liver fibrosis and collagen production by stellate cells plays a central role. Vitamin A-coupled liposomes can be used to specifically target stellate cells and knockdown important genes for fibrogenesis [101]. Preclinical studies have shown 90% suppression of procollagen α1(I)

expression, reduction of septa formation and 40–60% decrease of collagen deposition in mice with progressive and advanced liver fibrosis treated with siRNA to the procollagen α1(I) gene [102].

Altered expression of miR-29b has been suggested to affect the pathogenesis and progression of liver fibrosis by downregulating the expression of HSP47 and lysyl oxidase [103, 104]. HSP47 is an endoplasmic reticulum molecular chaperone that plays a central role in procollagen processing by myofibroblasts. After the synthesis of polypeptide chains, HSP47 assists the correct folding and stabilization of triple-helical procollagen molecules, a process crucial for subsequent secretion, cleavage and fibril formation of collagen [105]. Subsequently, siRNA directed to HSP47 in stellate cells was shown to be safe in phase 1 clinical trials in healthy volunteers and phase 1b/2 trials are assessing safety in patients with liver fibrosis [106, 107].

Infectious and Neoplastic Diseases

A thorough review of the use of RNAi in infectious and neoplastic diseases is beyond the scope of this chapter, but it should be kept in mind that a lot of the progress achieved in the application of RNAi to inborn errors of metabolism has been only possible using practical knowledge obtained in the numerous preclinical and clinical studies dealing with infectious and neoplastic diseases.

One of the main challenges in the fight against certain viral infections derives from the genomic instability of the infectious agent, which can mutate quickly to bypass the immune response built against it. Hepatitis C virus (HCV) is one of the most prevalent liver infectious diseases, it spreads by blood contact, and chronic infection can lead to cirrhosis and liver cancer [108]. To fight HCV, a set of three shRNAs are being used in clinical trials to reduce the chances of viral escape and prevent reinfection [109, 110].

Another circumstance in which RNAi has been quickly adapted to fight viral diseases has been the Ebola epidemic. Ebola is a lethal virus, which infects many cell types, including liver, endothelial cells and macrophages, and it is transmitted by contact with body fluids, although not every contact is infectious. siRNA to Ebola virus has been successful in preclinical studies with NHP to suppress infection that could be the result of exposure to an infected needle [111]. Safety studies were conducted in humans, but efficacy has been tested only in NHP due to ethical concerns. Nevertheless, during the 2014 Ebola outbreak, the siRNA product produced by Arbutus was used to treat patients intravenously in emergency circumstances. Initial administration induced treatable complications, but dose-escalating studies caused high cytokine levels, probably due to lipid-amplified innate immune stimulation [112]. Administration of TKM-130803 at a dose of 0.3 mg/kg/day by intravenous infusion to 14 adult patients with severe Ebola virus disease [113] was not shown to improve survival when compared to historic controls [114]. Nonintravenous forms of administration, pharmacological suppression

of TLR-mediated activation of the innate immune response and dose adjustment may improve the clinical outcome in the future.

The use of RNAi in cancer has followed various strategies. The most straight-forward approach, the suppression of dominant mutated oncogenes, has been the focus of recent clinical trials such as *K-RAS* in pancreatic adenocarcinoma [115] and *MYC* in hepatocellular carcinoma [116, 117]. Dicer substrate-based siRNA against *MYC* is being delivered with proprietary lipid nanoparticle delivery technology (EnCore, Dicerna Pharmaceuticals) that is in a phase 1b/2 trial now.

The cancer vaccine approach in oncology patients can also be improved by the use of RNAi [118]. The idea of loading dendritic cells with cancer antigens to promote anticancer immune response can be improved by simultaneously knocking down the expression of genes such as *CBLB*, favoring T cell activation, cytokine production and immune cell proliferation [119].

Since some miRNAs, such as miR-34 in liver cancer, have been known to function as tumor suppressors, miRNA mimetics have been proposed as targeted therapies [120]. miR-34 is known to be a direct target of p53, and its induced expression promotes cell cycle arrest, apoptosis and senescence. It also decreases cancer stem cell performance and metastasis. Preclinical studies in mice showed that systemic delivery of miR-34 reduced the size of hepatocellular carcinoma and improved survival [121]. The miRNA mimetic MRX34 is being tested in a clinical trial [122].

Recent Technological Developments in RNAi

In the past two decades, more than 60 systemically or topically delivered siRNA therapeutics have been clinically investigated (Table 1). More than 15 diseases and 25 targets have been studied. This review will continue to focus on the discussion of siRNA reagents systemically delivered by nanocarriers (such as lipid nanoparticles), liposome, polymers or conjugation approaches.

The advancement of delivery methods for siRNA continues, as researchers aim to maximize the specificity of siRNA delivery while minimizing toxicity and degradation effects that compromise drug efficacy. Technologies to develop direct conjugation to deliver siRNA to target hepatic cells have matured and provide some potential advantage over synthetic carrier and LNP delivery methods. These advantages include predictability of toxicity, simplicity of Chemistry, Manufacturing and Controls (CMC) and the flexibility of route of administrations (both subcutaneous and intravenous injection) [123]. This delivery technology utilizes a small molecule ligand, called N-acetylgalactosamine (GalNAc), which chemically attaches to siRNAs to mediate the delivery of nucleic acid payloads to hepatocytes that express high levels of ASGRs. This delivery technology has proven to be successful in both preclinical animal model and clinical studies for liver-specific disease [30–33]. Unfortunately, this ligand-mediated approach has been challenging for delivery to tumors since no ligand-receptor system has been

Table 1 List of clinical trials involving RNAi

Indication	Drug	Delivery	Pathology	Target	Status	Sponsor	NLM identifier
Amyloidosis	ALN-TTR01	LNP, IV	ATTR	*TTR*	Phase 1, completed on Feb 2012	Alnylam	NCT01148953
Amyloidosis	ALN-TTR02	LNP, IV	NHV	*TTR*	Phase 1, completed on Jun 2014	Alnylam	NCT02053454
Amyloidosis	ALN-TTR02	LNP, IV	ATTR	*TTR*	Phase 2, completed on Jan 2014	Alnylam	NCT01617967
Amyloidosis	ALN-TTR02	LNP, IV	NHV	*TTR*	Phase 2, completed on Nov 2012	Alnylam	NCT01559077
Amyloidosis	ALN-TTR02	LNP, IV	ATTR	*TTR*	Phase 2, ongoing, start date, Oct 2013	Alnylam	NCT02053454
Amyloidosis	ALN-TTR02	LNP, IV	ATTR	*TTR*	Phase 3, invitation, start date, Jul 2015	Alnylam	NCT02510261
Amyloidosis	ALN-TTR02	LNP, IV	ATTR	*TTR*	Phase 3, ongoing, start date, Nov 2013	Alnylam	NCT01960348
Amyloidosis	ALN-TTRSC	GalNAc conjugates, SC	NHV	*TTR*	Phase 1, completed on May 2015	Alnylam	NCT01814839
Amyloidosis	ALN-TTRSC	GalNAc conjugates, SC	ATTR	*TTR*	Phase 1, ongoing, start date, Oct 2014	Alnylam	NCT02292186
Amyloidosis	ALN-TTRSC	GalNAc conjugates, SC	ATTR	*TTR*	Phase 2, ongoing, start date, Jan 2015	Alnylam	NCT01981837
Amyloidosis	ALN-TTRSC	GalNAc conjugates, SC	ATTR	*TTR*	Phase 2, recruiting, start date, Oct 2016	Alnylam	NCT02595983
Amyloidosis	ALN-TTRSC	GalNAc conjugates, SC	ATTR FAC	*TTR*	Phase 3, recruiting, start date, Dec 2014	Alnylam	NCT02319005
Genetic disorders	ALN-AS1	GalNAc conjugates, SC	Acute intermittent porphyria	*ALAS1*	Phase 1, recruiting, start date, May 2015	Alnylam	NCT02452372

(continued)

Table 1 (continued)

Indication	Drug	Delivery	Pathology	Target	Status	Sponsor	NLM identifier
Genetic disorders	ALN-AT3SC	GalNAc conjugates, SC	NHV and hemophilia A, B	*SERPINC1*	Phase 1, recruiting, start date, Jan 2014	Alnylam	NCT02035605
Genetic disorders	ALN-AT3SC	GalNAc conjugates, SC	Hemophilia	*SERPINC1*	Phase 1/2, recruiting, start date, Sep 2015	Alnylam	NCT02554773
Genetic disorders	ALN-CC5	GalNAc conjugates, SC	PNH	*C5*	Phase 1/2, recruiting, start date, Jan 2015	Alnylam	NCT02352493
Genetic disorders	DCR-PH1	LNP, IV	NHV and PH patients	*HAO1*	Phase 1, recruiting, start date, Dec 2015	Dicerna	To be assigned
Genetic disorders	ALN-GO1	GalNAc conjugates, SC	NHV and PH patients	*HAO1*	Phase 1/2, recruiting, start date, Mar 2016	Alnylam	NCT02706886
Genetic disorders	ALN-AAT	GalNAc conjugates, SC	A1ATD	*SERPINA1*	Phase 1/2, recruiting, start date, Jul 2015	Alnylam	NCT02503683
Genetic disorders	ARC-AAT	Polymer, IV	NHVs and A1ATD patients	*SERPINA1*	Phase 1, recruiting, start date, Feb 2016	Arrowhead	NCT02363946
Genetic disorders	TD101	Naked siRNA, direct injection	Pachyonychia congenita	Mutant K6a	Phase 1, completed on Aug 2008	Transdem	NCT00716014
Liver fibrosis	ND-L02-S0201	VitA-LNP, IV	NHV	*HSP47*	Phase 1, completed on Feb 2014	Nitto Denko	NCT01858935
Liver fibrosis	ND-L02-S0201	VitA-LNP, IV	Hepatic fibrosis	*HSP47*	Phase 1, ongoing, start date, Oct 2014	Nitto Denko	NCT02227459
Metabolic disorders	PRO-040201	LNP, IV	Hypercholesterolemia	*APOB*	Phase 1, terminated on Jan 2010	Arbutus	NCT00927459
Metabolic disorders	ALN-PCS02	LNP, IV	CVD	*PCSK9*	Phase 1 completed on Sep 2012	Alnylam	NCT01437059
Metabolic disorders	ALN-PCSSC	GalNAc conjugates, SC	CVD	*PCSK9*	Phase 1, completed on Nov 2015	Alnylam	NCT02314442

(continued)

Table 1 (continued)

Indication	Drug	Delivery	Pathology	Target	Status	Sponsor	NLM identifier
Metabolic disorders	ALN-PCSSC	GalNAc conjugates, SC	CVD	*PCSK9*	Phase 2, recruiting, start date, Jan 2016	Alnylam	NCT02597127
Oncology	siRNA-EphA2-DOPC	Liposome, IV	Advanced cancers	*EphA2*	Phase 1, recruiting, start date, Jul 2015	M.D. Anderson Cancer Center	NCT01591356
Oncology	siG12D LODER	Implant into tumor	Pancreatic ductal adenocarcinoma	*KRASG12D*	Phase 1, completed on July 2013	Silenseed Ltd	NCT01188785
Oncology	siG12D LODER	Implant into tumor	Advanced pancreatic cancer	*KRASG12D*	Phase 2, start date, Jun 2016	Silenseed Ltd	NCT01676259
Oncology	ALN-VSP02	LNP, IV	Advanced solid tumors with liver involvement	*KSP, VEGF*	Phase 1 completed on Aug 2011	Alnylam	NCT00882180
Oncology	ALN-VSP02	LNP, IV	Cancer	*KSP, VEGF*	Phase 1 completed on Sep 2012	Alnylam	NCT01158079
Oncology	Atu027	Liposome, IV	Advanced solid tumors	Protein kinase N3	Phase 1, completed on Sep 2012	Silence	NCT00938574
Oncology	Atu027	Liposome, IV	Metastatic pancreatic cancer	Protein kinase N3	Phase 1/2, completed on Jan 2016	Silence	NCT01808638
Oncology	CALAA-01	Nanoparticles, IV	Solid tumor	*RRM2*	Phase 1, terminated on Sep 2012	Calando	NCT00689065
Oncology	DCR-MYC	LNP, IV	Solid tumors, MM, NHL, PNET	*MYC*	Phase 1, recruiting, start date, Dec 2014	Dicerna	NCT02110563
Oncology	DCR-MYC	LNP, IV	HCC	*MYC*	Phase 1b/2, recruiting, start date, Dec 2014	Dicerna	NCT02314052
Oncology	TKM-080301	LNP, IV	NET and ACC	*PLK1*	Phase 1/2, completed on Aug 2008	Arbutus	NCT01262235
Oncology	TKM-080301	LNP, IV	Hepatic metastatic cancers	*PLK1*	Phase 1, completed on Jun 2012	NCI	NCT01437007

(continued)

Table 1 (continued)

Indication	Drug	Delivery	Pathology	Target	Status	Sponsor	NLM identifier
Oncology	TKM-080301	LNP, IV	Advanced HCC	*PLK1*	Phase 1, start date, Jun 2014	Arbutus	NCT02191878
Ophthalmology	SYL040012	Naked siRNA, eye drop	High intraocular pressure	*ADRB2*	Phase 1/2, completed on Sep 2012	Sylentis	NCT01739244
Ophthalmology	SYL040012	Naked siRNA, eye drop	Ocular hypertension open-angle glaucoma	*ADRB2*	Phase 2, completed on May 2013	Sylentis	NCT01739244
Ophthalmology	SYL040012	Naked siRNA, eye drop	Open-angle glaucoma	*ADRB2*	Phase 2, completed on Jan 2016	Sylentis	NCT02250612
Ophthalmology	QPI-1007	Naked siRNA, intravitreal	Optic atrophy	Caspase 2	Phase 1, completed on Apr 2013	Quark	NCT01064505
Ophthalmology	PF-04523655	Naked siRNA, intravitreal	Diabetic macular edema	*RTP801*	Phase 2, completed on Nov 2013	Quark	NCT01445899
Ophthalmology	AGN211745	Naked siRNA, intravitreal	AMD	*VEGFR1*	Phase 1, terminated on Feb 2009	Allergan/siRNA	NCT00363714, NCT00395057
Ophthalmology	Bevasiranib (Cand5)	Naked siRNA, intravitreal	Diabetic macular edema	*VEGF*	Phase 2, completed on Dec 2007	OPKO Health	NCT00306904
Ophthalmology	Bevasiranib (Cand5)	Naked siRNA, intravitreal	Wet age-related macular degeneration	*VEGF*	Phase 2, completed on Dec 2007	OPKO Health	NCT00259753
Ophthalmology	Bevasiranib (Cand5)	Naked siRNA, intravitreal	Postpterygium excision management	*VEGF*	Phase 4, completed on Apr 2012	Arrowhead Regional Medical Center	NCT01736449
Ophthalmology	SYL1001	Naked siRNA, eye drop	NHV	*TRPV1*	Phase 1, completed on Jun 2012	Sylentis	NCT01438281
Ophthalmology	SYL1001	Naked siRNA, eye drop	Ocular pain	*TRPV1*	Phase 1/2, completed on Apr 2015	Sylentis	NCT01776658
Viral infection	TKM-100201	LNP, IV	NHVs	Ebola	Phase 1, completed on Jul, 2012	Arbutus	NCT01518881

(continued)

Table 1 (continued)

Indication	Drug	Delivery	Pathology	Target	Status	Sponsor	NLM identifier
Viral infection	ARB-001467	LNP, IV	HBV	HBV	Phase 2, recruiting, start date, Dec 2015	Arbutus	NCT02631096
Viral infection	ARC-520	Polymer, IV	NHVs, infusion rate study	HBV	Phase 1, recruiting, start date, Jan 2016	Arrowhead	NCT02535416
Viral infection	ARC-520	Polymer, IV	HBV	HBV	Phase 2, recruiting, start date, MAR 2014	Arrowhead	NCT02065336
Viral infection	ARC-520	Polymer, IV	NHVs	HBV	Phase 1, recruiting, start date, Nov 2014	Arrowhead	NCT01872065
Viral infection	ARC-520	Polymer, IV	e antigen-positive HBV	HBV	Phase 2, recruiting, start date, May 2015	Arrowhead	NCT02452528
Viral infection	ARC-520	Polymer, IV	e antigen-positive HBV	HBV	Phase 2, recruiting, start date, Oct 2015	Arrowhead	NCT02604212
Viral infection	ARC-520	Polymer, IV	e antigen-negative HBV	HBV	Phase 2, recruiting, start date, Nov 2015	Arrowhead	NCT02604199
Viral infection	ARC-520	Polymer, IV	HBV	HBV	Phase 2, recruiting, start date, Dec 2015	Arrowhead	NCT02577029
Viral infection	ARC-520	Polymer, IV	HBV	HBV	Phase 2, recruiting, start date, Apr 2016	Arrowhead	NCT02738008
Viral infection	ARC-520	Polymer, IV	HBV	HBV	Phase 2, recruiting, start date, Apr 2016	Arrowhead	NCT02738008
Viral infection	ALN-RSV01	LNP, IV	Respiratory syncytial virus	RSV	Phase 1, completed on Nov 2007	Alnylam	NCT00496821
Viral infection	ALN-RSV01	LNP, IV	Respiratory syncytial virus	RSV	Phase 2, completed on Nov 2007	Alnylam	NCT02035605
Viral infection	ALN-RSV01	LNP, IV	Respiratory syncytial virus	RSV	Phase 2, completed on Jun 2009	Alnylam	NCT00658086

(continued)

Table 1 (continued)

Indication	Drug	Delivery	Pathology	Target	Status	Sponsor	NLM identifier
Viral infection	ALN-RSV01	LNP, IV	Respiratory syncytial virus	RSV	Phase 2b, completed on May 2012	Alnylam	NCT01065935
Miscellaneous	QPI-1002	IV	Patients undergoing major cardiovascular surgery	*p53*	Phase 1, completed on Nov 2010	Quark	NCT00554359
Miscellaneous	QPI-1002	IV	Prophylaxis for kidney transplantation	*p53*	Phase 1/2, completed on May 2013	Quark	NCT01064505
Miscellaneous	RXI109	Intradermal	Dermal scarring after surgery	*CTGF*	Phase 2, ongoing, start date, Mar 2014	RXi	NCT02079168
Miscellaneous	RXI109	Intradermal	Dermal scarring after surgery	*CTGF*	Phase 2, recruiting, start date, Jul 2014	RXi	NCT02246465
Miscellaneous	RXI109	Intradermal	Dermal scarring after surgery	*CTGF*	Phase 2, ongoing, start date, Nov 2013	RXi	NCT02030275
Miscellaneous	RXI109	Intradermal	Dermal scarring after surgery	*CTGF*	Phase 1, completed on Nov 2014	RXi	NCT01780077
Miscellaneous	RXI109	Intradermal	Dermal scarring after surgery	*CTGF*	Phase 1, completed on May 2014	RXi	NCT01640912
Miscellaneous	RXI109	Intradermal	Neovascular AMD	*CTGF*	Phase 1/2, recruiting, start date, Nov 2015	RXi	NCT02599064

identified that is suitable for this purpose. On the other hand, by taking advantage of the enhanced permeability and retention of the leaky vasculature of most solid tumors, nanoparticle carriers were designed and screened to achieve efficient delivery to tumor tissues, while avoiding nonmalignant tissues. To date, a small number of phase 1 clinical trials of RNAi therapeutics that utilized nanoparticle-based delivery have been completed in patients with solid tumors. Encouraging results from these pioneering clinical studies show that RNAi therapeutics can successfully and safely inhibit targeted gene products in patients with cancer [116, 124, 34]. However, there were also limited clinically relevant pharmacokinetic and pharmacodynamic data, making it difficult to fully understand the potential of nanoparticle-based delivery systems for cancers of different tissue origins, genetic backgrounds and surrounding microenvironments. In preclinical studies that used xenograft models, human cancer cells displayed a great degree of diversity in their uptake of lipid nanoparticle-based delivery system [125]. Since we do not completely understand the uptake mechanism of these nanoparticles in cancer cells, future clinical results will be very helpful for investigating the specificity and mechanism of nanoparticle delivery methods.

Several nanocarrier-based RNAi therapeutics aimed at treating metabolic and genetic disorders have been completed or are ongoing (Table 1). However, the recent breakthroughs in applying direct conjugation to naked siRNA duplexes have offered potential advantages over nanocarrier-based delivery system as mentioned above. Encouraging results from clinical studies show that direct conjugation of siRNA therapeutics can successfully and safely inhibit targeted gene products in patients with multiple genetic and metabolic disorders. Notably, this siRNA conjugation approach would not have reached its current stage of success without the progression of the accumulated knowledge on generating fully stable antisense oligonucleotide molecule by utilizing chemical modifications [14, 126, 127, 128]. Improvement of oligonucleotide stability makes it possible to achieve effective suppression of gene expression with treatment at low dose levels and infrequent dosing of siRNA therapies. The current technical advancements on applying chemical modifications to generate extremely potent and stable siRNA duplexes also make it clinically possible to deliver enough drug materials in one small syringe that is sufficient to achieve an efficacious and durable biological effect through a single injection.

Future Challenges in RNAi Therapeutics, Including Safety Issues

With a great potential to become the next generation of medicines, siRNA therapeutics have yet to gain market approval. Even so, considering that RNAi was only discovered in 1988, and considering the limited knowledge on molecular mechanisms of Ago2, siRNA and target mRNA interaction, the pace of development of

RNAi therapeutics has been relatively rapid. As mentioned above, one of the reasons for this rapid progress is that chemists were able to take advantage of knowledge gained from antisense oligonucleotide therapeutics on molecule stabilization achieved through non-natural chemical modifications. The first siRNA drug approval could occur in the next several years, yet there remain challenges to be overcome in order to make this class of therapy the new generation of medicines. It will take time to screen for any potential the long-term toxic effects of siRNA-based therapies once they have been broadly applied in patients of different diseases and ages. There is also currently no clinically acceptable method to deliver siRNA to nonhepatic tissues and organs.

The criteria for target selection for siRNA therapeutics are very similar to the traditional small molecule drug discovery. Good understanding of the therapeutic hypothesis and predicted on-target toxicity is important to guide the entire drug development process. Taking information from human genetics, direct toxicity can be predicted by investing whether humans with null or hypomorphic mutations on proposed targets are asymptomatic. However, for certain targets where expression is not restricted to liver, specific symptoms from null or hypomorphic mutation might be due to lack of expression beyond the liver, and additional experimental investigation may be necessary to fully understand the effects of liver-specific target knockdown. This specific inhibition of hepatocyte functions by siRNAs due to preferable hepatic delivery might become advantageous when toxicity is a problem with systemic inhibition of the same targets with small molecule drugs. However, this is also a potential limitation for siRNA therapeutics, which are not currently able to modify nonhepatic expression, in entities where the expression of the target gene in nonhepatic tissues is an important part of the mechanism of disease.

It has been shown that the duration of gene silencing is a function of the cell doubling time, which is presumably due to dilution of active RISCs in the daughter cells. Gene silencing in nondividing cells can be on the order of a month or more in vivo, but as the time between cell division decreases (as is the case with cancer cells), the silencing time decreases. In vivo studies, including clinical investigations, indicate that siRNAs need to be dosed more frequently for oncology applications. As mentioned above, the only efficient way to deliver siRNA to tumor cells is through nanocarriers and this delivery is limited to solid tumors, as evidenced in the preclinical models. The molecular mechanism of LNP uptake into tumor cells is largely unknown. Understanding this mechanism is going to help the design of successful clinical studies and future patient selection. Investigation of the delivery efficiency of specific nanocarriers on large numbers of human cancer cell lines that have publicly accessible gene and protein expression data [129] should be a rational approach to initiate this effort. However, the effect of cancer cells on their surrounding microenvironment and vasculature which might also affect nanocarrier uptake will not be resolved with this type of approach.

siRNA therapeutics can cause on-target toxicity owing to irreplaceable functions in the normal physiology of their targets. Like small molecule drugs, RNAi therapies have potential off-target effects that could cause unintended toxicity unrelated to carriers. These potential off-target effects include downregulations of genes

through mRNA degradation triggered by RISCs or translational inhibition mediated by a miRNA-like mechanism due to sequence complementarity. This miRNA-like off-target effect only requires partial sequence complementary at the seed region (nucleotide position 2–8 of guide strand) to target mRNA [130, 131]. Chemical modification of guide stand of siRNAs, in particular the 2′-OMe substitution of position 2 of guide strand, has been demonstrated to significantly reduce off-target effects [132]. Another initial concern of siRNA toxicity was their potential genotoxicity. However, it is now believed that siRNA therapeutics are unlikely to be genotoxic based on their properties and the results of oligonucleotides tested so far. However, genotoxicity testing is recommended for oligonucleotides that contain non-natural modifications and the use of the complete drug product is suggested to provide the most clinically relevant assessment [133].

siRNAs can also trigger an innate immune response activating interferon and inflammatory pathways through the activation of Toll-like receptors (TLRs) 3 and 7 [134–136]. Injection site reactions have been observed sporadically in several clinical trials. Currently, the most clinically advanced siRNA conjugate therapeutic against a liver gene is ALN-TTRSC, for the treatment of TTR amyloidosis (ATTR). A phase 3 clinical study was initiated to evaluate the safety and efficacy of ALN-TTRSC in patients with TTR-mediated FAC. This multicenter, multinational, randomized, double-blind, placebo-controlled study is scheduled to be finished around the end of 2018 [68]. Results of this study should provide more insight on the long-term opportunities and practical applications of siRNA therapeutics in general. Lessons learned from this and other trials will also be helpful to further understand whether siRNA therapeutics are safe and can be used to treat chronic liver disorders.

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Oligonucleotide Therapy

Michela Alessandra Denti and Giuseppina Covello

Abstract Oligonucleotides (ONs), the focus of the chapter herein, are mostly known for their utility to selectively manipulate RNA processing by increasing or decreasing target gene levels, in particular by inducing enzymatic RNA degradation, blocking or mimicking miRNAs, inhibiting mRNA translation or modulating pre-mRNA splicing. Since these mechanisms of action are based on the Watson–Crick base pairing to target sequences, ONs are highly specific compounds. The possibility of a large scale and standardized production of these compounds makes them attractive for the therapy of inherited disorders. To date, four ONs have received marketing authorization and more than 100 have been, or are, under clinical trials. Several different oligonucleotide chemistries have been explored, each with its own delivery hurdles and toxicology patterns. Only a limited knowledge is available concerning the cellular and subcellular mechanisms of ONs uptake, transport and metabolism, presently making the improvement of ONs' delivery and toxicology a challenging task. The purpose of this chapter is to review the state-of-the-art advances on ONs for applications in inherited disorders and give an overview of what is known regarding their delivery and safety, based on pre-clinical and clinical studies.

Keywords Antisense oligonucleotides · Delivery · Exon skipping · miRNAS · Preclinical models · Safety · Toxicity · RNAse H

M.A. Denti (✉) · G. Covello
RNA Biology and Biotechnology Laboratory, Centre for Integrative Biology,
University of Trento, Trento, Italy
e-mail: michela.denti@unitn.it

G. Covello
e-mail: giuseppina.covello@unitn.it

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Introduction

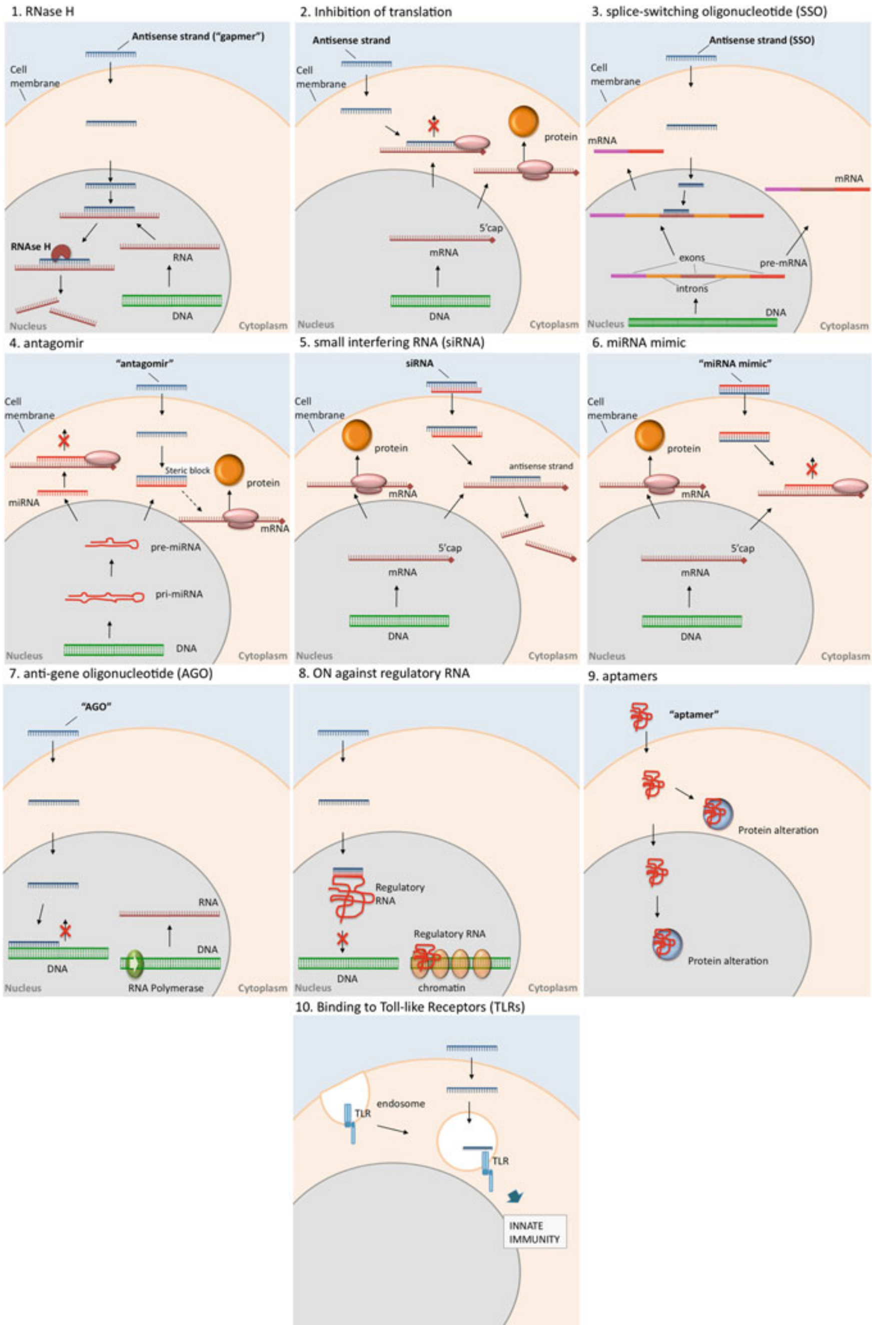
The increasing knowledge of RNA biology and chemistry is stimulating renewed efforts to target the RNA itself or the splicing and translational machinery as entry points for therapeutic intervention. These improvements have helped to develop several strategies to modify the splicing pattern of a mutant pre-mRNA or eliminate or block viral or gain-of-function RNAs, mRNAs coding for unwanted proteins or mRNAs that bear disease-causing mutations, miRNAs, to achieve therapy.

Oligonucleotides (ONs) are short synthetic nucleic acids designed to specifically bind RNA (or DNA) via Watson–Crick base pairing. In the current chapter, an overview is given on how ONs can be employed to manipulate or block splicing, inhibit mRNA translation, block microRNA function, or induce mRNA degradation through an RNAse H-mediated mechanism. We will review the current state-of-the-art ONs' therapeutic applications, with a special focus on our current understanding of ONs' pharmacokinetics and toxicology, in animal models and in human.

Mechanisms of Action

Two classes of ON actions can be distinguished: (a) mechanisms based on Watson–Crick base pairing between the ON and its target nucleic acids (Fig. 1, mechanisms 1–8) and (b) modes of action implicating the ON binding to cellular proteins (Fig. 1, mechanisms 9 and 10).

1. **Antisense oligonucleotides directing RNAse H to the target RNA**. The majority of the ON drugs investigated in the clinic function via an RNase H-dependent mechanism (Fig. 1) [1]. Two out of four US Food and Drug Administration (FDA)-approved ONs work via RNase H-mediated degradation: mipomersen [2] and fomivirsen [3–5], both developed by Isis Pharmaceuticals (now Ionis Pharmaceuticals). These antisense oligonucleotides (AONs) stimulate mRNA cleavage through the recruitment of RNase H, an endogenous endonuclease involved in the DNA replication process [6]. RNase H cleaves the RNA strand of a DNA–RNA hybrid duplex and releases the intact AON upon cleavage [7]. RNase H is extremely sequence-specific, so three or more mismatches result in a complete loss of activity [8]. In dominant diseases, allele-specific RNAse H degradation could be obtained by designing the AONs to target the point mutation in the mutated mRNA [9] or a single nucleotide polymorphism unique to the mutant RNA [10]. To protect the AON from degradation and increase its affinity to the target RNA, several nucleotides at each end of the AON are usually chemically modified, to generate what has been called a "gapmer": efficient induction of RNAse H degradation is obtained with a gap of six to eight unmodified DNA nucleotides [1].


1. RNase H
Antisense strand ("gapmer")
Cell membrane
RNAse H
RNA
DNA
Nucleus
Cytoplasm
2. Inhibition of translation
Antisense strand
Cell membrane
protein
5'cap
mRNA
DNA
Nucleus
Cytoplasm
3. splice-switching oligonucleotide (SSO)
Antisense strand (SSO)
Cell membrane
mRNA
mRNA
exons
pre-mRNA
introns
DNA
Nucleus
Cytoplasm
4. antagomir
"antagomir"
Cell membrane
Steric block
protein
miRNA
mRNA
pre-miRNA
pri-miRNA
DNA
Nucleus
Cytoplasm
5. small interfering RNA (siRNA)
siRNA
Cell membrane
protein
antisense strand
mRNA
5'cap
mRNA
DNA
Nucleus
Cytoplasm
6. miRNA mimic
"miRNA mimic"
Cell membrane
protein
mRNA
5'cap
mRNA
DNA
Nucleus
Cytoplasm
7. anti-gene oligonucleotide (AGO)
"AGO"
RNA
DNA
DNA
Nucleus
RNA Polymerase
Cytoplasm
8. ON against regulatory RNA
Regulatory RNA
Regulatory RNA
DNA
Nucleus
chromatin
Cytoplasm
9. aptamers
"aptamer"
Protein alteration
Protein alteration
10. Binding to Toll-like Receptors (TLRs)
endosome
TLR
TLR
INNATE IMMUNITY

◂**Fig. 1** Schematic representation of different mechanisms of oligonucleotide action. *1* Gapmer AON, inducing RNAse H degradation. *2* Steric inhibition of translation. *3* Splice-switching ON (SSO). *4* Antagomir, blocking endogenous miRNAs. *5* Small interfering RNA (siRNA). *6* microRNA (miRNA) mimic. *7* Anti-gene ONs. *8* ONs directed against regulatory RNA species. *9* Aptamer, whose binding alters protein surface. *10* Binding to Toll-like receptors (TLRs) in the endosome

2. **Antisense oligonucleotides blocking mRNA translation via steric occupancy**. AONs functioning as translation inhibitors (Fig. 1) are targeted against the 5′ untranslated region (5′ UTR) of a mRNA and prevent the movement of ribosomes down the transcript and/or inhibit the physical assembly of the 40s and 60s ribosomal subunits onto the mRNA sequence [11–13].
3. **Antisense oligonucleotides modulating pre-mRNA splicing in the nucleus**.

Splicing of an exon in a pre-mRNA is a multistep nuclear process which, beside the essential and highly conserved 5′ and 3′ splice sites, involves the binding of several proteins to additional sequences in both exons and introns, which regulate exon inclusion (splicing enhancers) or exclusion (splicing silencers). Indeed, it is possible to modulate pre-mRNA splicing by splice-switching oligonucleotides (SSOs, Fig. 1) hybridizing to the 5′ splice site, the 3′ splice site or splicing enhancers or inhibitors. SSOs are single-stranded nucleic acids (typically 15–25 nt in length) whose chemistry has been modified to ablate RNAse H -activity, as they are intended to operate in the nucleus via an occupancy-only mechanism. SSOs were first used to redirect cryptic splicing in Beta-globin pre-mRNAs bearing intronic mutations that introduced aberrant splice sites [14], but the interest in their use for the treatment of a number of genetic diseases has been increasing as they can force exon exclusion (“exon skipping”) or exon inclusion, restore a malfunctioning splicing pattern, shift the ratio between existing splice isoforms as well as modulate polyadenylation so to inhibit or promote expression of a target mRNA (reviewed in Veltrop and Aarstma-Rus [15]). Notably, SSOs have been used to induce the skipping of one or more additional exons, to restore the transcript reading frame [16]. Paradigmatically, SSOs have been employed in the therapy of Duchenne muscular dystrophy (DMD), in which the deletion of portions of the dystrophin gene produce transcripts that, by lacking several exons, lose the correct reading frame. By skipping additional exons, dystrophin-reading frame is restored, and the severe DMD phenotype is converted in a milder Becker distrophy reviewed in [17]. While SSO-based therapies are undergoing clinical trials for DMD [18–20]; [21, 22] and spinal muscular atrophy (SMA) [23, 24], similar splice-modulating strategies are being devised for the therapy of several other genetic diseases reviewed in: [25–28]. In 2016, the FDA approved the first SSO drug: Exondys 51 (eteplirsen), an AON inducing skipping of dystrophin exon 51, developed by Sarepta Therapeutics. SSOs can also be used to induce reading frame disruption and protein knockdown [29].

4. **Oligonucleotides to inhibit microRNAs ('antagomirs')**.

MicroRNAs (miRNAs) are tiny RNA molecules, 21–22 nucleotides in length, which regulate gene expression by binding to mRNAs and repressing their translation and/or inducing their degradation (Fig. 1). miRNAs are transcribed as primary miRNAs (pri-miRNAs) in the nucleus, subsequently cleaved into precursor miRNAs (pre-miRNAs) which are then exported to the cytoplasm and further processed into mature miRNAs. By taking part in a complex network of gene expression regulation, miRNAs can have several roles, from cell proliferation to cell death, from cell specification to cell differentiation. miRNAs can take part in pathological processes as well, and the deregulation of several miRNAs has been implicated in cancers, neurodegenerative diseases and other pathologies. In particular, in cancers, miRNAs have been described, which act as tumor suppressors, by inhibiting the expression of oncogenes. On the other hand, onco-miRs are found overexpressed in cancers, and inhibit the translation of tumor suppressor genes. In the latter case, a possible treatment can be to block the production of the overexpressed miRNA, or its function, with an antagonizing AON ('antagomir') complementary to the precursor or the mature miRNA [30]. The first antagomir drug (Miravirsen, against miR-122) entered Phase II clinical trials in 2010 for the treatment of hepatitis C virus infection [31, 32]. Antagomirs have been designed as potential drugs for the treatment of several other diseases as, for example, breast cancer, glioma and brain tumor, obesity, and Alzheimer's disease.

5. **Small interfering RNAs (siRNAs)**.

SiRNAs are short 20- to 24-bp dsRNA ONs that bind target RNA via base complementarity and mediate its degradation by eliciting the RNA interference pathway (Fig. 1). Since a chapter in this book is entirely dedicated to siRNAs, we will only shortly mention them here, to note how, only few years after siRNAs were first developed [33], a VEGF-targeting siRNA for the treatment of wet age-related macular degeneration was investigated in clinical trials (bevasiranib, developed by OPKO Health) [34, 35]. Although the study identified no safety issue, it was terminated because the trial was unlikely to meet its primary endpoint. Subsequent studies have suggested that VEGF-targeted siRNAs might prevent neovascularization via an alternative mechanism, through toll-like receptor 3 (TLR3, see later) [36, 37]. Since then, several siRNAs have entered into clinical trials with promising results [38].

6. **microRNA mimics**.

In those diseases in which the overexpression of a miRNA can be beneficial, to recover a pathologically downregulated miRNA or to suppress a detrimental gene, double-stranded RNA (dsRNA) oligonucleotide mimics have been designed to exogenously provide miRNAs (Fig. 1) [39]. The first Phase I clinical trial involving miR mimics (miR-34 mimic; MRX34) was initiated by Mirna Therapeutics in 2013 and is focused on replacing miR-34a expression in patients with various advanced

solid tumors [40]. Phase II studies are anticipated in 2017. Moreover, results were recently reported for a Phase I Study with miR-16 mimics for patients with malignant pleural mesothelioma (MPM) and advanced non-small cell lung cancer (NSCLC) [41].

7. **Anti-gene oligonucleotides (AGOs)**.

Anti-gene oligonucleotides (AGOs) act in the nucleus by binding to the genomic double-stranded DNA (dsDNA) in a sequence-specific fashion (Fig. 1). They can either form a triple helix or invade into the dsDNA and displace one of the strands, by binding to the other strand via Watson–Crick base pairs [42]. AGOs can block the binding of transcription factors or act by stalling RNA polymerases [43, 44]. AGO conjugates have also been used to drive targeted gene repair [45, 46]. As compared with AON approaches targeting RNA, accessing dsDNA has proven considerably more challenging: Although some preclinical data on their efficacy are available [47], further optimization is needed before AGOs could enter into clinical trials.

8. **Oligonucleotides against regulatory RNAs**.

As increasingly more noncoding RNAs are being discovered as regulators of gene expression [48], attempts are being made to therapeutically exploit their functions [30]. AONs complementary to a regulatory RNA can act as antagonists and thus increase the expression of genes repressed by the regulatory RNA itself (Fig. 1).

9. **Aptamers**.

Aptamers are different from the ONs described so far, in that they do not rely on base complementarity but rather function via their tertiary structures, by recognizing and strongly binding their targets (both small molecules and proteins). Aptamers typically bind to cellular or extracellular proteins, affecting the functionality of the downstream effectors (Fig. 1). For example, the first FDA-approved aptamer, pegaptanib (Macugen, developed by OSI Pharmaceuticals and Pfizer for the treatment of wet age-related macular degeneration), acts by blocking VEGF from binding to its receptor on the cell surface, which in turn inhibits intracellular signaling and blocks neovascularization [49, 50]. Other therapeutic aptamers are currently in clinical trials for various disorders: pegnivacogin, an RNA aptamer inhibitor of coagulation factor IXa, coupled to its controlling complementary sequence oligonucleotide anivamersen (REG1; [51, 52]); Fovista, a platelet-derived growth factor (PDGF) antagonist, and Zimura, an anti-Complement C5 aptamer (Ophthotech) [53, 54]. Aptamers have also been used for the targeted delivery of siRNAs, SSOs miRNA mimics and antagomirs [55].

10. **Oligonucleotides binding to Toll-like receptors (TLRs)**.

As seen above in the case of VEGF mRNA-targeting siRNAs, ONs can trigger inflammatory responses via interactions with receptors as membrane-bound Toll-like receptors (TLRs) or cytosolic RIG-I family receptors [56]. The elicitation of innate immunity represents a major problem for the use of AONs and siRNAs in therapeutics. However, the other side of the coin is that ONs can be used as agonists or antagonists of TLRs or RIG-I, to modulate the innate immune system [36, 57, 58, 37]. In particular, DNA or DNA analogues containing CpG motifs have been shown to be bound by TLR9 and elicit pro-inflammatory responses, and is therefore being used as a potent vaccine adjuvant [59].

Chemical Modifications of Oligonucleotides

The currently used ONs are rarely regular RNA or DNA oligonucleotides. Unmodified ONs are rapidly susceptible to degradation by endo- and exonucleases in biological fluids and their overall charge prevents them from penetrating through the cell membrane. Alternative chemistries have been therefore developed to improve affinity and potency, boost nuclease resistance and stability in the circulation and in target cells, reduce toxicity and enhance cell penetration and accumulation [60–63].

Phosphorothioates. The first and most important modification introduced in an oligonucleotide has been the replacement of one of the non-bridging oxygen atoms in the phosphodiester bond by a sulfur atom (Fig. 2; [64]). The phosphorothioate (PS) backbone is compatible with RNase H-mediated activity, and both FDA-approved RNAse H-based drugs mipomersen [65, 2] and fomivirsen [3–5] bear a PS backbone. PS backbone has also been used in SSOs. Although it creates a modest reduction in binding affinity, the modification has the advantage to improve resistance to nucleases. PS modification also promotes protein binding, therefore retarding renal clearance of systemically administered ONs, by favouring the interaction with albumin and other blood proteins. However, the downside of this increased binding to proteins is a higher toxicity [64].

2′ modifications. To improve nuclease resistance and increase binding affinity to RNA, second-generation ONs were developed to incorporate modifications at the 2′ sugar position. The two most widely studied 2′ modifications are 2′-*O*-Methyl (2′ OMe) and 2′-*O*-Methoxyethyl (2‘OMOE) (Fig. 2; [66]). Fully 2′ modified ONs do not support RNAse H -activity, and can therefore be used as SSOs in the nucleus, avoiding the cleavage of the hybrid between the pre-mRNA and the ON by RNAse H. RNAse H-activity can be, however, gained by the use of ‘gapmers’ that contain a central region of about seven nucleotides not modified in their 2′ positions, flanked by 2′ modified regions. Mipomersen [2] is a 2′OMOE gapmer. Modifications of the 2′ position of the sugar (2′-*O*-Me and 2′-F) have also been introduced in siRNAs,

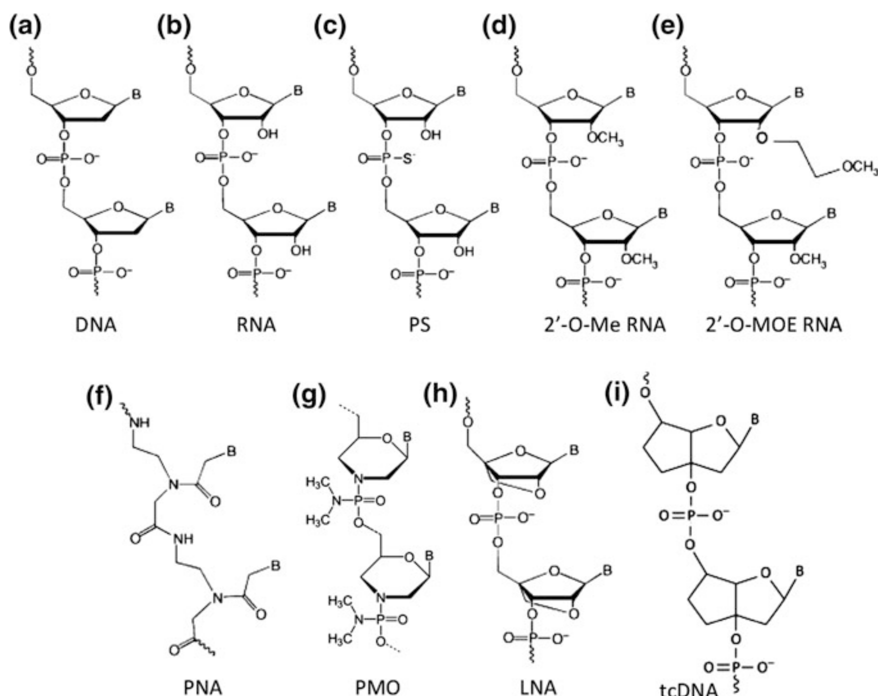


Fig. 2 Chemical modifications of oligonucleotides. **a** DNA. **b** RNA. **c** Phosphorothioate (PS). **d** 2′-*O*-methyl (2′-OMe). **e** 2′-*O*-methoxyethyl (2′-*O*-MOE). **f** Peptide nucleic acid (PNA). **g** Phosphorodiamidate morpholino oligomer (PMO). **h** Locked nucleic acid (LNA). **i** tricyclo-DNA (tc-DNA)

where they reduce immunostimulatory effects due to the binding to TLRs [56], and off-target effects (OTEs) [67].

Several SSOs under development have both the 2′ and the PS modification: for example drisapersen for the treatment of DMD is a 2′OMe-PS [18–20] while nusinersen for the treatment of SMA is a 2‘OMOE-PS [23, 24, 68].

Neutral backbones. In addition to the negatively charged ONs described above, two more chemistries have been used: peptide nucleic acids (PNAs) and phosphorodiamidate morpholino oligomers (PMOs). Both these modifications provide neutral backbones and high resistance to nucleases; however, they do not support RNase H-activity. Therefore, these chemistries have mainly been used in SSOs [69].

PNA is a synthetic nucleic acid analogue that contains an *N*-(2-aminoethyl) glycine backbone linked to nucleobases via methylene carbonyl (Fig. 2; [70, 71, 42]). This neutral peptide-based backbone provides high affinity to complementary nucleic acids [72]. To get good water solubility and to improve cell entry by facilitating the binding to the negatively charged surface of cells, cationic lysine residues are commonly incorporated in the PNA ONs [61].

PMOs are non-charged ONs in which the sugar is replaced by a six-membered morpholino ring, and the phosphodiester bond is replaced by an uncharged phosphoramidate linkage (Fig. 2; [73, 74]). Generally, PMOs used are longer than the corresponding 2O′Me-PS AONs, due to their slightly lower affinity. Moreover, being neutral, they do not form complexes with cationic lipids or other commonly used cationic delivery reagents, so they are difficult to transfect. Eteplirsen, the recently approved SSO for the skipping of dystrophin exon 51, is a PMO [21, 22].

Bridged rings. Modifications involving bridging of the sugar ring have provided several new ON chemistries (Fig. 2): the locked nucleic acid (LNA) containing a 2′-*O*, 4′-*C*-methylene bridge in the *β*-D-ribofuranosyl configuration [75], the constrained ethyl (cEt; [76]), and the tricyclo-DNA (tc-DNA; [77]). These modifications dramatically increase nuclease resistance and binding affinity. They do not support RNase H-activity, but can be used in antisense gapmers. LNA and tc-DNA have been effectively used in SSOs in preclinical studies [78, 79]. Due to the very high affinity of the LNA to its target RNA, the LNA SSOs also showed reduced specificity [78]. In the applications that use ONs as steric inhibitors, the specificity issues associated with LNA have been solved by using a mixmer of LNA and DNA backbone sequence [80] or LNA and 2′ OMe backbone [81, 82].

Delivery

Two main problems are encountered in the development of ON-based therapeutic approaches: to accomplish the delivery of the active ON in the right intracellular compartment (nucleus or cytoplasm, depending on the mode of action) and to reach the tissue of therapeutic interest, while minimizing exposure of other tissues. Several barriers oppose to the movement of ONs in the body, and their relative importance depends on the ON chemistry and on its formulation.

Tissue barriers in ON delivery. The kidney and the liver are the primary tissues of distribution for systemically administered ONs, accumulating up to 40 and 50% of the administered ON, respectively [83]. A vascular endothelium, made of tightly joined cells, surrounds the capillary lumen. It allows the passage of molecules the size of naked ONs into many tissues, but limits the passage of nanoparticles, except in tissues such as the liver and the spleen, where gaps or fenestrations are present between endothelial cells [84]. The phagocytic cells of the reticuloendothelial system (RES) play an important role in the biodistribution of both naked and nanoparticulate oligonucleotides [85]: The Kupffer cells of the liver sinusoids, as well as splenic macrophages, usually take up ONs incorporated in liposomes and other nanoparticles [86], despite many attempts to evade uptake by these cells by modifying nanoparticle surface with polyethylene glycol (PEG) or other inert polymers [87, 88]. Mononuclear phagocytes, on the other hand, express a number of cell surface receptors, such as scavenger receptors and integrins, which can be

involved in the uptake and clearance of free ONs [89, 90]. Scavenger receptors have been implicated in the uptake of PMOs conjugated with cell-penetrating peptides (CPPs) [91], while their role in uptake of free PS ONs in vivo is still controversial [92]. Even if the ONs accumulate in the liver, they end up in Kupffer cells and other hepatic cells, rather than in hepatocytes [93]. Therefore, when targeting ONs to hepatocytes, delivery reagents are needed [94]. Renal clearance plays a major role in ON pharmacokinetics and biodistribution. Several types of ONs are in the range of 3–6 nm or less and are therefore ultrafiltered by the kidney [95]. siRNAs and uncharged backbone ONs do not bind to plasma proteins [96, 97] and are therefore rapidly excreted by the renal route. PS ONs, on the other hand, bind to plasma proteins and have a slower renal clearance, accumulating at higher levels in other tissues [98]. However, the kidney is also the primary route of excretion of PS ONs, which involves nuclease degradation products. PMOs, on the other hand, are rapidly cleared by the kidney as intact molecules [96]. The systemic delivery of ONs to the Central Nervous System (CNS) is hampered by the blood–brain barrier (BBB), made of tightly linked endothelial cells supported by a network of pericytes and astrocytes processes [99]. Several studies have attempted the use of nanoparticles for the delivery of drugs across the BBB, but with limited success [100]. The most promising approach to the problem involves the use of PMOs conjugated with CPPs [101], and several studies have reported systemically administered CPP-PMOs successfully reaching the brain [102, 99]. However, concerns remain about the possible systemic and CNS toxicity of polycationic CPPs. Also, systemically delivered tc-DNA SSOs have been recently reported to have an effect in the brain [79].

Cellular barriers in ON delivery. The pharmacology of ONs depends on their route of endocytosis and trafficking [103, 104]: Upon reaching the cell surface, all ONs (either free, or conjugated or nanoparticulate) enter cells via endocytic pathways depending on clathrin, caveolin, or dynamin and then traffic through several intracellular compartments (late endosomes, trans-Golgi, lysosomes), thus remaining separated from the cytosol and the nucleus by membrane barriers [105]. The endosome escape barrier has been recognized as the most important impediment to the effective therapeutic use of ONs. ONs within endomembrane compartments are pharmacologically inhert, but a small portion can spontaneously escape to the cytosol. Once PS ONs reach the cytosol, they shuttle to the nucleus [106]. As our understanding of the machinery of intracellular trafficking improves, several attempts are being made to breach the endosomal barrier, by silencing key proteins [107] or by using cell lines with defects in trafficking processes [108, 107]. In a recent paper, for example, silencing of endosomal sorting complex required for transport-I (ESCRT-I) led to the dramatic increase of a miR-21 antagomir effectiveness [107].

Delivery strategies. A widely used strategy to deliver anionic ONs has been to complex them with cationic lipids, thus forming lipid nanoparticles (LNPs). This strategy has been implemented in particular for the therapeutic use of siRNAs (while SSOs and other single-stranded ONs are often delivered as free ONs). LNPs are typically 100–200 nm in size and PEG-coated to minimize phagocytosis by

RES cells and protein binding. Once in the endosome, the cationic lipids of the LNPs interact with anionic membrane lipids leading to dissolution of the LNP and release of the ON in the cytosol [109]. However, the interaction of cationic lipids with cellular membranes is possibly also the reason for the toxicity of LNPs [110, 111]. Progresses in the optimization of the cationic lipids has led to increased ON effectiveness, and reduced toxicity [112]. Because of their size, LNPs can only exit the circulation at sites where the endothelial barrier is fenestrated (liver, spleen). This has led therapeutic approaches to mainly focus on liver-based diseases. However, efforts are being made to target LNPs to other tissues, such as lung [113, 110, 111]. In an interesting ongoing clinical trial, mimic RNAs against miRNA-16 (TargoMiRs) are being delivered via bacterial nanocells targeted to lung cancer by an anti-EGFR antibody [41]. Various types of polymeric nanocarriers are also being explored, as an alternative to LNPs, albeit they are somewhat lagging behind LNPs in the clinical use. Conjugates of cell-penetrating peptides (CPPs) with PMOs or PNAs have also shown promise as delivery vehicles [101]. While toxicity has been observed in mice treated with moderate doses of the peptide conjugates [114, 115], CPP-PMOs have been used in vivo for effective oligo delivery at doses below those causing observed toxicity [116]. The most studied conjugates involve a variety of CPPs containing arginine residues interrupted by short hydrophobic sequences, linked to PMO SSOs designed to induce exon skipping in dystrophin pre-mRNA. In the *mdx* mouse model of DMD, strong splice modulation was observed in skeletal muscle, with several conjugates. Recent versions have also increased dystrohin expression in the heart. Although the mechanism of CPP-PMOs delivery is not entirely clear, a recent paper has described a correlation between the in vivo efficacy and differential uptake and nuclear delivery of these CPP-PMOs in cultures of skeletal muscle cells versus cardiomyocites [117]. Moreover, CPP-PMOs, as well as tc-DNA ONs, were found to spontaneously form nanoparticles, whose uptake is then mediated by scavenger receptors [91]. A recent development is the incorporation of positive piperazine residues directly in the PMO backbone, which has shown impressive results in preclinical trials against Marburg virus in monkeys and was well tolerated in a phase I trial in man [118]. In a different approach, a non-peptidic cell-penetrating moiety, consisting in an octaguanidinium dendrimer, has been covalently linked to the 3′ end of a PMO [119]. Such PMOs, called "vivo-morpholinos," (VMOs) are efficiently uptaken in cells [119] and have been successfully injected systemically in *mdx* mice showing good activity in exon skipping and dystrophin production and no signs of toxicity at the injected doses. However, several groups recently observed lethargic behavior in mice immediately after intravenous injection, and high mortality rates [94]. Alteration in the clotting system inducing cardiac arrest was indicated as the possible cause of death [120, 121]. In the last couple of years conjugates have been also developed to target ONs to a specific tissue [122]. Particularly promising are glycoconjugates involving delivery through the asialoglycoprotein receptor (ASGR). Researchers at Alnylam Pharmaceuticals developed multivalent *N*-acetylgalactosamine (GalNac) conjugated siRNA for the targeted delivery to the liver [123] and several clinical trials are now ongoing, hopefully soon providing data on the effectiveness and toxicity of

these conjugates. GalNac based conjugates have also been used to successfully deliver PS and other modified gapmer ONs to hepatocytes in mice [124–126]. Also peptide–oligonucleotide conjugates have been designed for the targeted delivery. Although an early study demonstrated the feasibility of using cyclic Arg-Gly-Asp (RGD) and other integrin ligands to increase uptake and effectiveness of a 2′ OMe-PS SSO [114], clinical studies on RGD-ON conjugates lag far behind those on glycoconjugates. Aptamers also represent a powerful strategy for the targeted delivery of ONs, and their potential has been widely studied in cell culture, mainly with aptamer-siRNA chimeras in cancers [127]. However, aptamer-ON conjugates have not been explored in preclinical trials nor progressed to clinical trials so far.

Delivery Routes and Pharmacokinetics

The most used delivery route of ONs has been parenteral injection, either intravenous (IV) infusion or subcutaneous (SC) injection. ONs pharmacokinetic properties are similar across genders and species [98]. Following SC administration, PS ONs are rapidly absorbed from the injection site into the circulation with peak plasma concentrations reached within 3–4 h [98, 97, 128]. Nearly complete absolute bioavailability has been observed after SC administration in monkeys [98]. Following either IV or SC administration, plasma concentrations rapidly decrease from peak concentrations in a multiexponential way: PS ON transfers from blood to tissues in minutes or a few hours, followed by a much slower terminal elimination phase (half-life of up to several weeks) consistent with the slow elimination of ONs from tissues [98]. PS ON, in fact, are extensively bound to plasma proteins ($\geq 85\%$), albumin in particular, across all species [97, 128]. In contrast, PNA, PMO and unmodified free ONs have a more rapid clearance from circulation, primarily due to either excretion in urine or metabolism in blood [73]. Most clinical trials have followed the systemic IV route which results in the ON distributing to liver, kidney, bone marrow, lymph nodes and a minor part accumulating in adipocytes [97]. Increasing the doses to deliver sufficient ON amounts to the target cells has the disadvantage of increasing toxicity. Delivery routes different from IV infusion have been explored to obtain tissue-specific delivery of sufficient amounts of the ON to achieve a therapeutic effect with minimal toxicity. The local administration has also an advantage in limiting ON degradation due to nucleases. In the case of muscular diseases, skeletal muscle is the target tissue. Local intramuscular injections might not be convenient to reach several different muscles, but have proven successful in delivering free PMO (eteplirsen) or 2′OMe (drisapersen) SSOs targeting dystrohin exon 51 and in restoring dystrophin expression in pivotal proof-of-concept clinical trials for DMD [129, 19]. Delivery of ONs to the CNS has faced a renewed interest in view of several diseases which could benefit of ON therapeutics [99, 28, 130]. In this context it is worth to mention that while the BBB represent an obstacle to the systemic delivery of ONs to the CNS, it might also have the beneficial effect to

confine ONs in an encapsulated organ, minimizing the risk of toxicity by preventing the transport of ONs to the peripheral circulation and allowing relatively decreased dosing. Once in the CNS, ONs benefit of an efficient uptake mechanism in both neurons and glial cells [130]. Intracerebroventricular (ICV) and intratecal (IT) injections have been used for the direct delivery of ONs to the CNS. Two phase I clinical trials have reported the delivery of ONs using IT infusion with promising results: in amyotrophic lateral sclerosis (ALS) [131] and in spinal muscular atrophy (SMA) patients [23]. Future trials will hopefully determine whether the ONs reach therapeutic concentrations throughout the entire brain. Moreover, the delivery through repeated IT injections has the disadvantage of requiring specialist expertise and is a relatively expensive method of administration. An attractive delivery approach might in the future be intranasal (IN) administration [132]: molecules can be transported along the olfactory and trigeminal nerve pathways and the rostral migratory stream. The eye is a small, enclosed and easy compartment to access and an immune-privileged organ [133] which is also becoming increasingly important as a target tissue for ON therapies [25, 53]. This is also indicated by the existence of several FDA approved RNAse H-based ONs targeting the eye (mipomersen and fomivirsen) or aptamers (pegaptanib), and several siRNAs and aptamers (Zimura, Fovista) in advanced clinical trials [38]. Different intraocular delivery routes are in use: intravitreal, subretinal, or suprachoroidal injection [134] have been used. Indeed, fomivirsen, a 21-nt PS ON, has been used since 1998 for the intravitreal treatment of cytomegalovirus-associated retinitis, in immunocompromised patients, including those with AIDS [3–5]. Preclinical studies indicated that in the retina, the concentration of intact PS ON increased during the first days after administration, reaching the maximum observed concentration five days after intravitreal injection [135]. Intraocular delivery, however, is rather invasive and might lead to complications such as retinal detachment. The topical and periocular routes are less invasive and are promising alternatives on which research is focusing. Recently, a phase III study on Aganirsen, a topical inhibitor of corneal angiogenesis, showed that eye drops containing the 25-mer 2′deoxy PS AON significantly inhibited corneal neovascularization in patients with keratitis [136, 137]. However, after instillation, nucleic acids are retained by the superficial tissues but do not significantly penetrate intraocularly [138, 139]. Due to their negative charge, ONs are potential candidates to be delivered into the eye by iontophoresis [140, 141].

Toxicology and Safety

Building on the preclinical and clinical data available so far, it is possible to summarize some general patterns in the toxicology of ON drugs. Each ON chemistry has stereotypic toxicity profiles, and while non-charged ONs do not interact with cellular proteins and tend to have fewer systemic toxicities as compared to other ONs, LNAs have higher potential for hepatotoxicity and some other

toxic effects [142]. Vivo-morpholinos (VMOs), however, have been observed to induce clotting and cardiac arrest in mice, possibly as a result of the interaction with proteins due to the added octaguanidine dendrimer [120, 121]. It has to be noted, however, that in general many of the preclinical toxicity risks identified in preclinical studies on animal models, where not noted in subsequent clinical trials in humans. There are two broad classes of potential toxicities for ONs: (1) hybridization-dependent toxicities, due to the binding of the ON to the target nucleic acid or, alternatively, to off-target nucleic acid due to complete or partial complementarity [143]; (2) hybridization-independent toxicities, not due to Watson–Crick base pairing between the ON and a nucleic acid. The latter class of toxicity might at times exhibit some sequence dependency, due to the chemical composition of the ON.

Hybridization-dependent toxicity. Binding of an ON to an unintended target RNA with very similar complementary sequence and one or two mismatches is expecially possible with chemistries with high affinity such as LNA, where shorter ON sequences are designed. Moreover, siRNAs or mimic miRNAs might potentially elicit miRNA-like effects on mRNAs with as few as 7 nucleotide matches to the seed region. In contrast, because inhibition of translation and splice-switching depend on the position of the target sequence on the RNA, the involvement of these mechanisms in off-target effects (OTEs) is less likely. There is a legitimate concern that hybridization-dependent OTEs might not show up in preclinical studies, due to the sequence differences between species. As the monky is most analagous to man, the use of nonhuman primate in toxicity testing has been suggested. However, it has been noted that toxic responses due to hybridization-dependent OTEs are not commonly observed [142]. The reason might be four: (1) since they depend on sequence complementarity, and differently from what happens with other kinds of drugs, potential OTEs can be predicted and anticipated by bioinformatic approaches; (2) not all sites on an RNA might be accessible; (3) not all off-target RNA are in tissues that receive pharmacological concentrations of an ON; (4) due to synergistic pathways of threshold effects, not all off-target genes produce toxic effects when they are knocked down.

Hybridization-independent toxicity falls in three categories: accumulation effects, pro-inflammatory mechanisms and binding to proteins. Cytoplasmic basophilic granules have been observed to accumulate in kidney or liver epithelium, and less commonly in other tissue types, and are considered to reflect accumulation of ONs. At high doses, the prominence of the granules correlates with the severity of degeneration in the kidney and the liver. In rats, ON accumulation in the kidney has been associated with an increase in progressive nephropathy. Toxicity is likely a result of lysosomal breakdown, or in the liver also due to cytokine release by activated KCs. However, for 2′OMe-PS ONs, the histological changes seen in animal studies do not correlate with data from several clinical trials, which indicates no effect on renal function [144, 145]. Other chemistries may result in renal toxicity, as for LNA in clinical studies [145]. Additionally, cytokines-containing granular/vacuolated macrophages have been observed in tissues and this probably reflects the pro-inflammatory properties of many of the ON drugs tested.

Table 1 Clinical studies on ON-based therapeutic agents

ON class	ON chemistry	Therapeutic agent	Indication	Target	Approach	Sponsor	Clinicaltrials.gov identifier	Reference
RNAse H	2′OMOE-PS gapmer	Mipomersen (Kynamro)	HoFH	Liver	SC injection	Ionis Pharmaceuticals	NCT00607373 approved	[2]
RNAse H	PS	Fomivirsen (Vitravene)	CMV retinitis	Eye	Intravitreal injection	Ionis Pharmaceuticals	NCT00002356 approved	[3]
AON	PS	Aganirsen (GS-101)	CN, iCRVO	Eye	Topical	Gene Signal	NCT02947867	[137]
SSO	2′OMe-PS	Drisapersen (Kyndrisa)	DMD	Muscles, heart	SC injection	BioMarin Pharmaceuticals	NCT01480245	[20]
SSO	PMO	Eteplirsen (AVI-4658)	DMD	Muscles, heart	IV injection	Sarepta Therapeutics	NCT02255552 approved	[22]
SSO	2′OMOE-PS	Nusinersen (IONIS-SMNRx)	SMA	CNS (motor neurons)	IT injection	Ionis Pharmaceuticals	NCT02193074	[23]
Antagomir	PS LNA	Miravirsen	Hepatitis C	Liver	IV or SC injection	Santaris Pharma	NCT01200420	[32]
miRNA mimic	Liposome-encapsulated dsRNA	MRX34	Primary or metastatic liver cancer	Liver	IV injection	Mirna Therapeutics	NCT01829971	[40]
miRNA mimic	EGFR-targeting nanocell dsRNA	TargoMiRs	MPM and NSCLC	Lung	IV injection	Asbestos Diseases Research Foundation	NCT02369198	[41]
Aptamer	PEGylated 2′OMe RNA	Pegaptanib (Macugen)	AMD	Eye	Intravitreal injection	OSI Pharmaceuticals, Pfizer	NCT00021736 approved	[49]

(continued)

Table 1 (continued)

ON class	ON chemistry	Therapeutic agent	Indication	Target	Approach	Sponsor	Clinicaltrials.gov identifier	Reference
Aptamer	PEGylated 2′OMe, 2′F RNA	REG1 (Revolixys)	PCI, ACS, CAD	Blood	IV injection	Regado	NCT01848106 terminated	[51, 52]
Aptamer	PEGylated 2′OMe, 2′F DNA	Fovista	AMD	Eye	Intravitreal injection	Ophthotech	NCT01944839 NCT01940887	[53]
Aptamer	PEGylated 2′OMe RNA	Zimura	Dry AMD	Eye	Intravitreal injection	Ophthotech	NCT02686658	[54]

2′OMe 2′-*O*-methyl; *2′OMEO* 2′-*O*-methoxyethyl; *ACS* acute coronary syndrome; *AMD* age-related macular degeneration; *AON* antisense oligonucleotide; *CAD* coronary artery disease; *CMV* cytomegalovirus; *CN* corneal neovascularization; *CNS* central nervous system; *DMD* Duchenne muscular dystrophy; *dsRNA* double-stranded RNA; *HoFH* homozygous familial hypercholesterolemia; *iCRVO* ischemic central retinal vein occlusion; *IT* intrathecal; *IV* intravenous; *MPM* malignant pleural mesothelioma; *NSCLC* non-small cell lung cancer; *PCI* percutaneous coronary intervention; *PS* phosphorothioate; *SC* subcutaneous; *SMA* spinal muscular atrophy; *SSO* splice-switching oligonucleotide

Immunostimulatory effects have been associated with many siRNAs and ONs, especially those with PS chemistries, in several preclinical studies [142]. Base pairing is also a factor in inducing pro-inflammatory effects, and the shorter base pair length of most LNA gapmers seems to reduce their pro-inflammatory potential. In rodents, the main mechanism for the immunostimulatory activity has been identified in TLR receptors stimulation and cytokines release, which give rise to lymphoid hyperplasia and lymphohistiocytic cell infiltrates. In monkeys, and mainly for PS chemistry, ON-mediated inflammation is mainly due to the activation of the alternative pathway of complement. In these animals, lymphoid hyperplasia has been observed in lymph nodes, spleen, lymphohistiocytic infiltrates have been observed in several organs. This pattern in monkeys is accompanied by gromerulonephritis and vasculitis, due to complement activation and initial injury to the endothelium, although TLR stimulation might also have a part in the vascular injury. In human clinical trials, however, vasculitis does not appear to be a significant problem, as monkeys seem to be much more sensitive to ON-mediated complement activation than humans. However, since other pro-inflammatory effects have been noted in clinical trials, such as flu-like symptoms and reactions at injection sites in case of SC or intramuscular injections, particular attention is placed to these unwanted effects in ongoing clinical trials. Unexpected hepatic toxicity has been reported in mice, and to a lesser extent in rats, cynomolgus monkey and human, at doses well below those generally accepted to result in accumulation-related hepatic effects. The chemistry most commonly associated with hepatic toxicity is LNA, but some siRNAs and 2′-MOE PS ONs have also presented toxicity in the liver. Several mechanisms have been hold responsible for this adverse effect in mice: modulation of transcriptional pathways, DNA damage, clathrin-mediated endocytosis for specific LNA ONs [146] and inactivation of critical cellular processes or activation of hepatocellular antiviral responses for other LNAs [147]. While most renal toxicities are considered to be due to accumulation of ONs within lysosomes of the proximal tubule, some candidate molecules have demonstrated renal toxicity at doses lower than those at which accumulation-related effects are shown. A case of acute tubular injury and some cases of transient tubular disfunction were reported in an LNA clinical trial [148] and a case of acute tubular necrosis in a long-term treatment with a 2′-MOE PS ON [149]. Glomerulonephritis has been described associated mostly to PS ONs, in a small percentage of studies in rodents and monkey lasting more than 3 months. The mechanism was considered to be related to immune pathogenesis and local inflammatory activity in the kidney. ON-associated glomerulonephritis is rarely observed in clinical trials, but there have been some reports, including with mipomersen and drisapersen. Mild thrombocytopenia has been observed in mice, rats, and monkeys, and occasionally in clinical studies, with approximately 10% of 2′-OMOE-PS ONs.

Current Oligonucleotide Therapies in Human Diseases

The absolute majority of registered clinical trials using antisense or siRNAs are for treatment of different forms of cancer. Beside this, the most common target organs are the liver and the eye [150]. In Table 1 we provide an overview of the ON drugs (with the exception of siRNAs) currently approved by FDA, and of some of the most promising currently in clinical trials. Table 1 does not include CpG ONs which have been tested in several clinical trials as vaccine adjuvants or immunotherapeutics for allergy, cancer and infectious diseases. For a thorough review of CpG ONs we refer the reader to [59].

Concluding Remarks

We are living through very exciting times for the filed of oligonucleotide therapeutics as four ON drugs have been approved and many are in clinical trials. A range of potential new clinical applications and new approaches have been described. In this promising landscape, delivery of ONs to the right tissue and in the intracellular sites where they function is much needed to improve the so far low effectiveness of ON drugs. However, delivery remains a key obstacle [94]. Modifications and conjugation of ONs have been studied to overcome this obstacle, but these features also increase the possibility of toxicity of the drug, and the difficulties in its scale-up and production. Similarly, small molecules being studied to improve ONs endocytosis and intracellular trafficking might be toxic and present unwanted effects.

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Index

A
Adaptive immunity, 45, 48, 50
Adenovirus vector, 37–41, 43–46, 48–52
Animal models, 38, 39, 41, 42, 46, 47, 51, 52
Antisense oligonucleotides, 192, 194

B
Biodistribution, 38–41, 49

C
Chimeric capsid, 97
Clonal dominance, 9, 18, 19, 27
Codon optimization, 98
Common integration sites, 15
CRISPR/Cas9, 145, 148

D
Deficiency, 82–84, 86–88
Delivery, 196, 199–203, 208
Designer nucleases, 142, 147, 149

E
Electroporation, 121–124
Encapsidation, 61, 62
European Medicines Agency (EMA), 2
Exon skipping, 194, 201

G
Gene addition, 2, 3
Gene gun, 115, 119–121
Gene repair, 2
Gene reprogramming, 2
Gene supply, 2
Gene therapy, 37–39, 47, 48, 50, 137–140, 142, 145, 146, 148, 149
Genome editing, 138, 140, 141, 143–147, 148
Genotoxicity, 9, 16, 19–29, 78, 99, 100

H
Hematopoietic stem cell gene therapy, 9, 10
Hemophilia, 77, 82, 83, 89–96, 98, 99
Hepatocyte, 80, 83, 90, 91, 93, 97, 98
Hepatotoxicity, 42, 44, 45, 47, 49, 51, 52
Hereditary diseases, 165, 167, 169, 172
Humoral immunity, 94, 95
Hydroporation, 125

I
Immune response, 89, 92–96, 98
Immunodeficiency, 2
Inborn errors, 167, 173
Innate immunity, 44
Insertional mutagenesis, 9, 13–15, 20, 24, 29
Insertional oncogenesis, 6
Intrahepatic injection, 67

K
Kozak sequence, 98
Kupffer cells, 64, 67, 68

L
Laser irradiation, 129
Liver, 160, 163–168, 172–174, 182, 183

M
Magnetofection, 127, 128
Metabolism, 164, 167, 172, 173
Microinjection, 116, 117
MiRNAs, 192, 194, 195, 204
Monogenic, 1
Multifactorial, 1

N
Needle-free jet injection, 118, 119
Needle injection, 117, 118
Nucleic acids, 114, 119, 125–129

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P
Preclinical models, 204

R
Replication, 61–63
Retroviral vectors, 9, 10, 12, 19, 23, 26, 27
RNA, 1–4
RNAi, 160, 162, 164–169, 173, 174, 181, 182
RNase H, 192, 194, 197–199, 203

S
Safety, 195, 203
Self-complementary, 81, 89, 97
Serotypes, 80, 81, 93, 95
Shock, 39–42, 47, 52
SiRNA, 160–169, 172–174, 181–183
Sonoporation, 123, 124
Sub-acute toxicity, 63
Systemic dissemination, 65, 67

T
TALENs, 141, 143–145, 147
Thrombocytopenia, 40, 42–44, 47, 50–52
Toxicity, 2, 4–6, 197, 200–204, 207, 208
Transcription regulation, 148, 149

V
Vectors, 3–6
Viral integration, 13, 15, 16

Z
ZFNs, 141–143, 145–147