Antiviral Gene Therapy: Summary and Perspectives

12.1 VIRUSES: ESSENTIALS OF THEIR REPLICATION AND SUSCEPTIBILITY TO ANTIVIRAL GENE THERAPY

Viruses constitute the simplest and most abundant life form. Replication of viruses is largely dependent on delivery of their genomes to host cells and then usurping functions of target cells to produce proteins that are required to form viral progeny. The mechanisms by which viruses appropriate cellular functions predictably involve some disruption, which is responsible for induction of disease in virus-infected individuals. In addition to viral proteins, host factors are also required to facilitate replication of viruses. Typically, antiviral gene therapy aims to deploy DNA or RNA to achieve a therapeutic effect by inhibiting the expression of viral or host factor genes. A timeline of some of the significant developments and ongoing research efforts in gene therapy for viral infections is illustrated in Figure 12.1. Capacity of viruses to bypass dependence on host factors is highly constrained; therefore, inhibiting the function of host factors is useful to suppress emergence of escape by the pathogens. This has been useful in developing gene therapy for infections with HIV-1 [1] and hepatitis C virus (HCV) [2]. When using this approach, appropriate target selection is important. If targeted host factors have important cellular functions, then their silencing may cause toxicity. Another method of providing a high barrier to viral infection is through simultaneous inhibition of multiple viral targets using combinatorial approaches.

Types of diseases caused by viral infections are varied and depend on characteristics of the individual pathogens. For example, human immunodeficiency virus (HIV)-1 has a tropism for $CD4^+$ T cells of the immune system, and manifestation of the acquired immune deficiency syndrome results from insidious virus-induced depletion of the infected host T cells. By contrast, infection with Ebola virus follows a rapid and usually fulminant course. Although the prognosis of cases of Ebola virus infection is grave, the pathogen does not persist, and successful treatment is curative. This is different from the situation with HIV-1 infection, in which the stable proviral replication intermediate remains embedded in the genome of infected host cells and makes elimination of the virus very difficult.
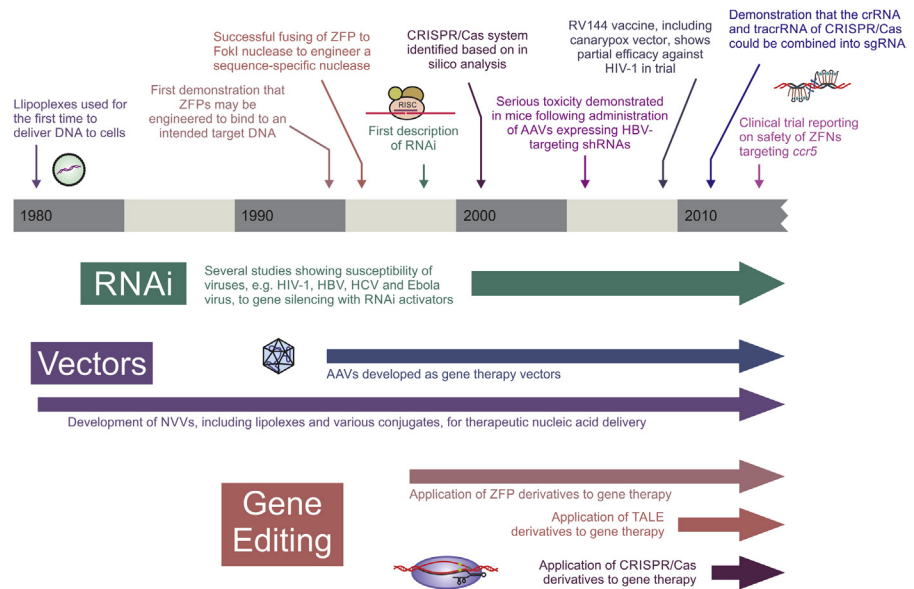
FIGURE 12.1 Some significant developments in the advancement of gene therapy for viral infections.
Milestones and major areas of ongoing technological development are indicated. AAV, adeno-associated virus; sgRNA, single guide RNA.

Although infections with viruses remain significant global causes of public health problems, advances in their treatment have been particularly impressive during the past 25 years. Several improvements in antiviral therapy have been made, and many diseases are now effectively managed. Antivirals comprising small molecules have demonstrated particularly good efficacy against a range of pathogens. Prophylactic immunization and other preventative measures, such as behavioral modification to minimize risks of transmission of viruses, have also contributed to limiting the spread of viral infections. Despite these advances, current therapeutics have inadequate curative efficacy against certain viral infections, and it is likely that disease caused by the pathogens will remain problematic for many years. Mutations may change properties of existing viruses, and new pathogens are likely to emerge. Hence, flexible technology that allows for rapid development of antivirals based on rational design is particularly important to counter the serious health problems that may result. Gene therapy has the potential to be effective against a range of existing and emergent viruses. The technology may also complement and augment efficacy of current antiviral drugs. However, gene therapy is still faced with significant hurdles before it is widely implemented for treatment of viral infections. Particularly important are the specificity of the antiviral nucleic acids for their targets and concerns about efficiency and safety of delivery, costs, and manufacturing capabilities.

In addition to direct targeting of viral genes and host factors, gene transfer for immune-based treatment and prophylaxis has potential for countering many viruses (Chapter 11). Relative ease of generating sequences expressing appropriate antigens and ability to induce humoral and cell-mediated arms of the immune response are advantages over use of protein-based vaccines. Gene transfer using DNA transfection and recombinant viral vectors, such as derivatives of poxviruses, have become popular for eliciting immune responses, and efficacy of the approach is continually improving. Using the procedures to effect immunostimulation is generally easy to implement and has already been tested in clinical trials performed in resource-poor settings. Although complex, vectored immunoprophylaxis (VIP) [3] and modification of immune cells with engineered T cell receptors (TCRs) [4] or chimeric antigen receptors (CARs) [5] are exciting new developments that have potential antiviral utility.

Advances in antiviral gene therapy have been made through steady accumulation of knowledge and as a result of significant breakthrough discoveries (Figure 12.1). Two momentous advances in the field have been development of technologies based on gene editing (Chapter 3) and harnessing the naturally occurring RNA interference (RNAi) pathway (Chapter 2). Gene editing is a process by which specific target DNA sequences are altered to inhibit viral replication. Therapy based on this technology is applicable to direct inactivation of viral DNA replication intermediates, such as are produced during proliferation of hepatitis B virus (HBV), or through inhibition of host factors required for replication of a particular virus [6]. RNAi is an endogenous gene regulatory mechanism that typically causes post-transcriptional gene silencing. Artificial RNAi activators, which comprise mimics of intermediates of the pathway and contain homology to intended targets, have been used successfully to inhibit viral gene expression. Exploiting RNAi and gene editing have both generated considerable enthusiasm, and the technology has advanced to use in clinical trials for treatment of viral infections, which include those caused by HBV and HIV-1. Other effective methods of inhibiting viral gene expression included use of antisense oligonucleotides, aptamers and RNase P substrates.

12.1.1 Exploiting RNAi for Antiviral Gene Therapy

Both expressed and synthetic RNAi activators have been utilized successfully against various viral infections. Considerable effort has gone into ensuring good specificity and potency of the silencers against their viral cognates. Use of Pol III promoters to transcribe RNA that forms short hairpin RNA (shRNA) structures, which mimic precursor microRNAs (pre-miRs), has been particularly popular. However, demonstration that overexpression of HBV-targeting shRNAs could result in fatal toxicity in mice was an important caveat to researchers in the field [7]. Since this observation, various configurations of expressed RNAi activators have been engineered to improve control of production of the

antivirals. Using antiviral mimics of primary microRNAs (pri-miRs) has shown promise against HBV [8,9], HCV [10], and HIV-1 [11,12]. These RNAi activators may be transcribed from inducible and tissue-specific Pol II promoters. The artificial pri-miRs have been multimerized to result in combinatorial silencing that improves efficacy and provides a greater barrier to viral escape. As mimics of early intermediates of the RNAi pathway, functional coupling of processing of the pri-miR mimics to downstream steps of the pathway is thought to improve potency. Better silencing by antivirals allows for use of lower doses of the candidate therapeutics and diminished off-target effects. Developments with use of synthetic RNAi activators have also been impressive. Typically, synthetic RNAi activators comprise short complementary strands of RNA that constitute short interfering RNAs (siRNAs). Various chemical modifications have been used to augment silencing, diminish immunostimulation, and stabilize the gene silencers. Synthetic procedures are also adaptable for other purposes. One interesting application is the coupling of targeting aptamers to synthetic siRNAs, which enables specific delivery to virus-infected cells [13–16]. Availability of new methods for bioinformatic analysis has also been important to advance RNAi-based antiviral therapy. Because only the seed region of the guide strand of a potentially therapeutic siRNA is required to effect translational silencing [17], the likelihood of nonspecific inhibition of cellular targets may be high. However, availability of vast sequence databases and analytical algorithms have allowed for the design of antiviral RNAi activators that have minimal interaction with off-target sites.

The ultimate success of RNAi-based antiviral therapy is likely to be dependent on demonstrating superior efficiency when compared with existing therapies. In addition, adapting the silencing strategies to attain the inhibition that is required for a curative effect is important. These considerations are illustrated by the example of development of RNAi-based therapy for treatment of persistent HBV infection. A clinical trial is in progress for the use of nonviral formulations containing synthetic RNAi activators to treat HBV infection (http://www.arrowheadresearch.com/programs-overview). Suppression of viral replication that is better or as good as that of licensed drugs needs to be demonstrated. In addition, because the gene silencing mechanism is not permanent, ability of the treatment to achieve a durable effect that leads to curative elimination of all viral replication intermediates will be important. Transient RNAi-based silencing may be better suited to treatment of RNA viruses that do not have a stable DNA replication intermediate.

12.1.2 Advancing Gene Editing to Counter Viral Infections

Antiviral gene editing technology entails inactivation of DNA sequences; therefore, it is adaptable to disabling viral DNA replication intermediates and genes of host factors that are required for viral proliferation. To effect mutagenesis,

engineered sequence-specific DNA-targeting proteins have been coupled to nucleases. Repeated cleavage followed by cellular error-prone nonhomologous end joining results in mutagenesis at the intended target. Earliest studies showing feasibility of gene editing used derivatives of zinc finger proteins (ZFPs). Twenty years ago, the approach was shown to inhibit expression of the *bcr-abl* sequence that is generated after the chromosomal translocation that characterizes chronic myeloid leukemia [18]. The inhibitory effect resulted from binding of the engineered ZFPs to DNA and physical disruption of transcription of the *bcr-abl* sequence.

Engineered ZFPs comprise tandem arrangements of fingers that bind to triplets of nucleotides. Zinc finger nucleases (ZFNs) are typically generated by fusing DNA-binding domains with the nuclease from the type IIS restriction endonuclease, FokI, which cuts one strand of a DNA duplex. Therefore, complete ZFNs comprise dimers, and each of the units is designed to cleave complementary strands at an intended target. Although engineered ZFNs have been the subject of many years of study, application of the technology is complicated by variability in the efficiency of target binding and cleavage. Homing endonucleases (HEs), also known as meganucleases, may cause sequence-specific cleavage of viral targets, but engineering this class of nuclease is fraught with technical difficulties. Problems associated with the dual functions of target binding and DNA cleavage of domains of the HEs are constraining.

Use of derivatives of transcription activator-like effectors (TALEs), which include TALE nucleases (TALENs) or repressor TALEs, overcomes some of the problems of ZFNs. Monomers that make up TALEs do not show the same context dependence of the fingers of ZFPs, which circumvents the need to screen large panels of the engineered proteins to identify optimally acting editors. As an alternative to engineering nucleases, sequence-specific transcriptional repressors may well find favor for therapeutic inhibition of viral gene expression. The Krüppel-associated box (KRAB) is capable of inducing epigenetic changes at specific sequences of DNA to cause long-term transcriptional suppression. Substituting a repressor for a nuclease in the engineered proteins gets around the problem of possible mutagenic effects that may arise from nonspecific cleavage of host genomic DNA.

Recent discovery of the clustered regularly interspaced short palindromic repeats (CRISPR) with CRISPR-associated (Cas) systems has also generated enthusiasm among researchers interested in developing antiviral gene editing. Engineering these nucleases is convenient because site-specific cleavage of target DNA is based on interaction of a single-stranded RNA guide with the target DNA. Therefore, the approach obviates the more complicated methodologies that entail generation of sequences encoding proteins that bind to specific DNA targets.

All classes of gene editing technologies have been investigated for efficacy against viral infections. The early-stage clinical trial that assessed *ccr5*-targeting ZFNs is auspicious [1], and the potential for rendering of autologous CD4$^+$ T cells resistant to HIV-1 is an exciting prospect. TALENs have been generated that target HBV genes [19,20] and *ccr5* [21], and their potential for antiviral therapy is promising. Engineered CRISPR-Cas systems were also shown to be effective against replication of HIV-1 [22,23] and HBV [24] in cultured cells. As with RNAi against viral infections, efficient delivery and specificity of the effectors for their intended targets are important for clinical application of gene editing.

With the exception of the smaller sequences encoding HEs, expression cassettes producing gene editors are typically larger than 3 kb in length. Moreover, in the case of ZFNs and TALENs, two cassettes are required to produce the two monomers that make up the dimeric nucleases. Likewise, typical engineered CRISPR/Cas systems comprise two cassettes: one encodes the nuclease enzyme and the other the guide RNA. Recombinant adeno-associated viral vectors, popular gene therapy vectors, have a limited capacity for accommodating transgenes. Therefore, complete dimeric nucleases may only be constituted by transduction of cells with two vectors, each expressing one of the monomers. An alternative to this complicated delivery approach is use of nonviral vectors (NVVs) to deliver mRNA encoding the gene editors' subunits. Formulation methods are not limited by the size of the mRNA that may be incorporated into the vectors, and the technique is now becoming more commonly used.

A useful feature of gene editing for viral gene therapy is that transient expression of the gene editor may be all that is required to effect potentially curative inhibition of viral replication. Once a target has been altered by introduction of mutations, a gene editing nuclease is no longer required. In fact, persistence of the effector is undesirable because the risk for off-target effects is increased. This feature is well suited to using NVVs to deliver nuclease-encoding mRNAs. Durability of gene editing that involves use of targeted transcriptional repressors, such as KRAB, to introduce epigenetic inhibitory effects is not yet well characterized. Transient expression of such repressors may be sufficient to cause the intended durable therapeutic inhibition. Avoiding unwanted effects on the host genome may be particularly useful for treatment of chronic HBV infection with transcriptional repressors. In individuals who are carriers of the virus, HBV DNA is often integrated into the hepatocyte genomes, and nuclease-mediated digestion may induce chromosomal translocations to cause unintended side effects.

12.1.3 Delivery of Antiviral Nucleic Acids to Target Cells

Optimizing delivery of nucleic acids is an objective that is common to advancing gene therapy for any disease. Location of the virus-infected cells is pertinent to devising the appropriate method of delivering the therapeutics. For example,

systemic administration of antivirals may be necessary to disable HBV genes in the liver. However, for treatment of respiratory infections, administration of the therapeutic formulations to the airways is usually more effective. The two main types of delivery vehicles, viral vectors and NVVs, have been discussed in detail in Chapters 4 and 5. Each has particular features that are suited to specific applications to antiviral gene therapy. Viral vectors are efficient carriers of sequences encoding expression cassettes, whereas delivery in vivo of expression cassettes with NVV formulations is usually modest. Because viruses essentially exist as nucleic acids parasites, they have evolved efficient mechanisms of cellular delivery of the nucleic acids, and this property has been exploited to generate viral vectors. Although recombinant viruses are highly capable vectors, they have drawbacks of high costs of production, difficulties with regulating the dose of therapeutics, induction of mutations in transduced cells, and eliciting neutralizing and potentially toxic immunostimulation. Durable expression, particularly by recombinant lentiviral vectors, is useful to achieve sustained antiviral effects, but utility of these vectors for antiviral application is narrow. RNA-based antivirals are growing in popularity, and NVVs are the preferred vectors for delivery of these nucleic acids. Because NVVs do not readily cross the nuclear membrane, the cytoplasmic site of action of antiviral RNAs, which include synthetic siRNAs and mRNAs, is a convenient feature. Various NVVs are under development, and these include lipoplex formulations and polymer conjugates. Because they are based on use of synthetic compounds, NVVs may be prepared on the large scale that is required for clinical application. Moreover, RNA-containing nonviral formulations are appealing because of their limited immunostimulation and avoidance of the mutagenic effects of DNA.

To attain adequate expression of an immunogenic protein, fewer cells need to be transduced than when delivering effectors that have a direct effect on viral replication. Moreover, the cells to be transduced may be readily accessible after intramuscular or intradermal delivery and are not necessarily the virus-infected cells. Recombinant adenoviruses, various poxviruses, protein coding sequences within NVVs, and electroporation of naked DNA together with adjuvants have all been used successfully to elicit antiviral immune responses.

12.2 PROSPECTS FOR ANTIVIRAL GENE THERAPY

Predicting the long-term role of gene therapy for viral infections is difficult. Apart from the many technical challenges, there are additional factors that will influence the future general use of antiviral gene therapy. When alternative treatment or prevention of a viral infection is effective and easily administered, the importance of antiviral gene therapy may be diminished. An example of such a case could play out with developments in the

treatment of HCV infection. Recent progress in HCV therapy using small molecules has been very impressive. The newly licensed combination drug, Harvoni™, produced by Gilead, is rapidly effective against HCV genotype 1 (http://www.gilead.com/news/press-releases/2014). Harvoni contains inhibitors of NS5A and NS5B proteins of HCV, and its administration to patients avoids the need for including interferon-α in the regimen. With successful implementation of the new HCV treatments, and if the management becomes widely affordable, the importance of gene therapy for HCV infection may diminish. Miravirsen, a modified oligonucleotide that is antisense to miR-122, has shown good efficacy in clinical trials [2]. Licensing and general use of this candidate host factor-disabling nucleic acid is likely to depend on its economical production and achieving antiviral efficacy that is at least as good as the recently licensed anti-HCV agents. Nevertheless, gene therapy will continue to be important for treating serious infections when cure using other approaches is not possible. Eliminating HBV and HIV-1 are examples where gene therapy is likely to play a role in curing the infections. In the case of chronic HBV infection, persistence of the stable covalently closed circular DNA is unaffected by existing therapies, and the gene editing approach potentially provides the means to disable this intractable viral transcription template. Likewise, use of gene editing to inactivate proviral sequences of HIV-1 or to render CD4+ T cells resistant to infection with the virus should be useful to cure HIV-1 infection.

A serious consideration for the long-term prospects of implementing gene therapy for treatment of viral infections relates to the practicalities of using the technology in resource-poor settings. Infection with viruses that are causes of serious health problems, such as HIV-1, HBV, and Ebola virus, often occurs in financially impoverished communities. Gene therapy formulations are complicated and expensive to prepare, and it is unlikely that the necessary financial resources are currently available in many of the countries where the infections have the most serious effects. Likewise, gene therapy-based methods may be difficult to execute and require a high level of expertise to implement. An example is the procedures involved with modification of autologous progenitors of CD4+ T cells ex vivo, their selection, and then re-infusion into patients to confer resistance to HIV-1 (Chapter 8). The technology is highly specialized, and a lack of expertise would limit access in some of the communities where treatment of HIV-1 infection is most needed. With improvements in methodologies and advancement of new technologies, it is expected that costs will diminish and procedures for performing gene therapy may become simpler. Ideally, this should result in future broader access of the technology to disadvantaged patients.

In the case of using gene transfer for immunostimulation to prevent or treat viral infections, the practical considerations are different. DNA-based vaccines and recombinant viral vectors that are used to induce immunity are generally administered using simpler procedures. Giving the vaccines to large numbers

of people is uncomplicated as long as the necessary cold chain requirements are met. However, immunotherapy using VIP and modified T cells that express engineered TCRs or CARs is more complex. As with gene therapy to target genes of viruses or host factors, implementation is likely to require simplification and improvement to make the technologies cost-effective for wider use.

A major advantage of gene therapy is the ability to develop the antiviral agents based on application of what are becoming simple rational design principles. This is the case for antiviral gene therapies that harness RNAi-based silencing, antisense oligonucleotides, and gene editing. On the basis of knowledge of the sequences of the targets, it is theoretically possible to develop generic methods that enable very rapid design of antiviral nucleic acids. Therefore, the speed with which gene therapy could be developed against an emerging pathogen is an advantage over use of small-molecule drugs to treat these viral infections. Identifying effective small molecule antivirals is also guided by rational design, but it typically requires detailed structural analysis of viral protein targets and empirical testing with optimization before use as therapy.

With advances in the broader fields of gene therapy, virology, synthetic chemistry, and molecular biology, a better understanding of factors that influence the safety, delivery efficiency, and antiviral actions of nucleic acids is likely to be reached. This will be important to facilitate implementation of more effective therapeutic measures to address the serious health challenges posed by existing and emergent pathogens. Availability of suitable tools will enable countermeasures to be put into place, which in turn will be important to deal with outbreaks of infections such as caused by the severe acute respiratory syndrome coronavirus during 2002/2003 and infection with Ebola virus. Gene therapy for viral infections is currently poised at an exciting phase. There have been significant successes, and realization of the full potential of the powerful technology is tantalizingly close. Significant goals are likely to be met in the near future, which in turn will influence the direction of the field.

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