Chapter 1
Viral Vectors, Engineered Cells and the CRISPR Revolution

James E. DiCarlo, Anurag Deeconda, and Stephen H. Tsang

Abstract Over the past few decades the ability to edit human cells has revolutionized modern biology and medicine. With advances in genome editing methodologies, gene delivery and cell-based therapeutics targeted at treatment of genetic disease have become a reality that will become more and more essential in clinical practice. Modifying specific mutations in eukaryotic cells using CRISPR-Cas systems derived from prokaryotic immune systems has allowed for precision in correcting various disease mutations. Furthermore, delivery of genetic payloads by employing viral tropism has become a crucial and effective mechanism for delivering genes and gene editing systems into cells. Lastly, cells modified *ex vivo* have tremendous potential and have shown effective in studying and treating a myriad of diseases. This chapter seeks to highlight and review important progress in the realm of the editing of human cells using CRISPR-Cas systems, the use of viruses as vectors for gene therapy, and the application of engineered cells to study and treat disease.

Keywords CRISPR/Cas • Ophthalmology • Genome Surgery • Gene Therapy

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S.H. Tsang (ed.), *Precision Medicine, CRISPR, and Genome Engineering*,
Advances in Experimental Medicine and Biology 1016,
DOI 10.1007/978-3-319-63904-8_1

1.1 Introduction

Precise engineering of human cells using genetic tools has revolutionized biology. Frederick Griffith's observation of the transforming principle in *Pneumococci* almost 100 years ago was a foundational step that laid the ground work for the entire field of gene therapy and genome engineering [1]. At their core, these areas rely on the on the transforming principle. Avery et al. later discovered that DNA was responsible for the transforming principle, which allowed the field of modern molecular biology to take another groundbreaking step forward [2]. Experiments in DNA transfer into mammalian cells by Szybalska and Szybalski showed that genes could be transferred between cell lines to modify their phenotype [3]. Decades later, building on primary gene transfer experiments, genome editing tools such as CRISPR-Cas systems are revolutionizing how we modify human cells [4].

Coupled with the development of genome editing tools, controlled delivery of foreign DNA into human cells has been an ongoing challenge in biomedicine. Viruses represent an important and powerful tool that scientists have levied for foreign DNA delivery. In fact, one of the first viral gene therapy experiments occurred in nonhuman cell lines in 1964. Temin et al. showed that Rous sarcoma viral mutations could be passed on in chicken cells [5, 6]. The observation that viral sources could induce introduction of heritable DNA laid the foundation for viruses to become a crucial vector of genetic modification of eukaryotic cells. This chapter will cover important milestones in the use of three commonly used groups of viral vectors that have been successfully used to modify human cells in the laboratory and in patients: retroviral vectors, adenoviral vectors, and adeno-associated viral vectors. As of 2016, these three vectors make up more than half of all vectors used in gene therapy (Fig. 1.1).

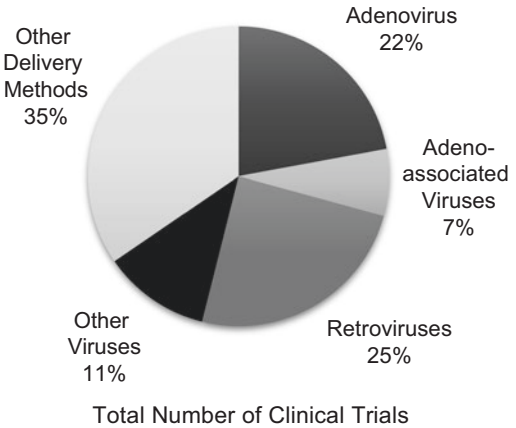

Fig. 1.1 Delivery methods used in gene therapy clinical trials as of August 2016. Viral vectors make up almost 65% of vectors used in gene therapy clinical trials, with a majority being composed of either adenoviral, adeno-associated viral, or retroviral vectors

Cellular manipulation has produced engineered cells with great therapeutic potential. Notably, the use of chimeric antigen receptor T-cells (CAR T-cells) and induced pluripotent stem cells (iPSCs) has been of keen interest in bridging the gap between genome editing *in vitro* using mouse models and eventually treatment of inherited human diseases, with promising efforts made in models of β-thalassemia and Duchenne muscular dystrophy. The use of CRISPR/Cas9 gene editing in conjunction with these methods has resulted in much more efficient correction of genetic abnormalities and restoration of function *in vivo.*

1.2 CRISPR-Cas Genome Manipulation

1.2.1 A Brief Overview of Genome Modification Using Endonucleases

Genomic incorporation of foreign DNA can occur by several means, most of which take advantage of protein recombination machinery, such as recombinases or integrases. Frequently, endogenous homologous recombination systems in eukaryotic genomes have been utilized by scientists to incorporate foreign DNA flanked by homologous sequences to the genomic locus of interest [7, 8]. Homologous recombination (HR) in eukaryotic cells is greatly stimulated after the introduction of a double-stranded break (DSB) in the host genome [7, 9]. If homologous recombination does not occur, an error prone process called non-homlogous end joining (NHEJ) can occur, resulting in mutations at the cut site [7]. Figure 1.2b diagrams the process of either non-homologous end joining or homologous recombination using a DNA donor, which could be supplied exogenously. A common method for introducing DSBs in host genomes is the use of site-specific endonucleases. These enzymes cleave DNA at sequence-specific regions [8]. The first implementation of site-specific endonucleases for eukaryotic genome modification was in mouse and plant cells using the meganuclease I-SceI, which has an 18-base pair recognition sequence [10, 11]. These meganucleases stimulated genome incorporation of foreign DNA by several orders of magnitude in mouse cells, putting a spotlight on an endonuclease approach for stimulating HR [11]. The downside of the I-SceI fixed 18-base pair recognition sequence moved scientists and engineers to design or discover reprogrammable site-specific endonucleases [8, 12].

Zinc-finger proteins were appealing first choices for the generation of engineerable endonucleases as these proteins contain specific nucleotide binding motifs that could be rearranged and then fine-tuned via selection for specific binding to a desired DNA sequence. When fused with an endonuclease domain, such as the FokI endonuclease, these proteins became some of the first engineered endonucleases, termed zinc finger nucleases (ZFNs) [13–15]. The average ZFN has an 18-base pair recognition sequence, which is constricted to the nucleotide triplets that zinc finger DNA binding motifs recognize via individual nucleotide binding domains.

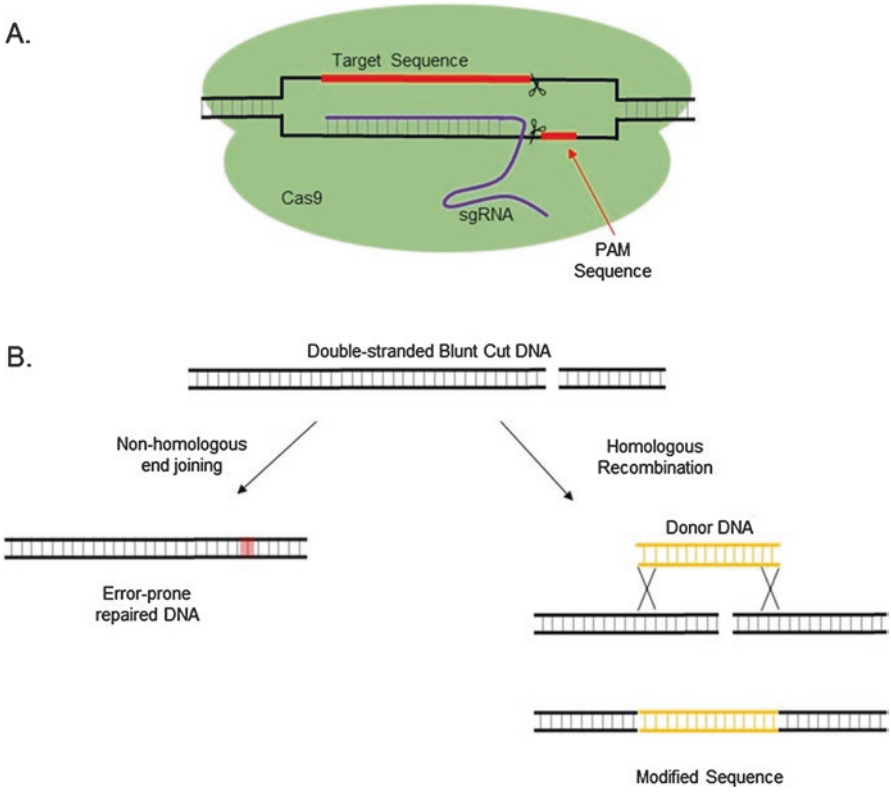

Fig. 1.2 Schematic of CRISPR-Cas9 DNA cleavage and DNA repair. (**a**) Cas9 complexes with the sgRNA to direct cleavage to region specified by the target sequence encoded on the sgRNA. (**b**) Repair of the double-stranded cut can occur via non-homologous end joining or homologous recombination. In the case of homologous recombination, if a donor DNA is supplied exogenously the region can be modified in a targeted fashion

These endonucleases are highly efficient and have been used in the modification of human cells as well as numerous other eukaryotic systems [16, 17]. However, rapid design and selection of ZFNs with novel binding sites that do not cleave off-target regions can still be a challenge [12, 18].

Inspired by the success of ZFNs, many groups turned their attention to DNA binding transcription activator-like (TAL) proteins of *Xanthomonas*, a plant pathogen that uses these domains for virulence factors in the nuclease of their host plant [19, 20]. TAL proteins, like zinc fingers, have a motif code for binding to DNA nucleotides, this time with a repeat motif recognizing one nucleotide instead of three, as in the case of zinc finger proteins [19, 20]. Fusion of an endonuclease, such as FokI, to these TAL proteins created TAL endonucleases or TALENs that offered another tool for engineering genomes [21, 22]. These proteins, while efficient at stimulating HR, have a downside of being large and contain repetitive regions, which can be problematic on the DNA level due to mutagenic recombination events [12, 23].

1.2.2 CRISPR-Cas Systems

While both ZFNs and TALENs are highly used in biomedicine, their individual disadvantages have led researchers to continue searching for easily engineerable and improved reprogrammable DNA cleaving enzymes. In the late 1980s, Ishino et al. noticed a group of repeated nucleotides in the E. coli genome while studying an unrelated enzyme [24, 25]. Later, groups found similar repeats in the genomes of other bacteria and archaea, leading to the name: clustered, regularly interspaced short palindromic repeats (CRISPR) [26–28]. The proteins often proximal to these repeat regions were given the name CRISPR-associated (Cas) proteins. Later groups would discover that CRISPR and Cas proteins were part of an immune system to protect from bacteriophage invaders [27, 29].

An important breakthrough in the study of the type II CRISPR-Cas system of *Streptococcus pyogenes* occurred in 2012 when the biochemical processing of DNA by this prokaryotic immune system was revealed [30]. In this three-component system, an endonuclease guided by two RNA molecules generates a DSB at a site determined by one of the RNA molecules. Cas9, the RNA-guided endonuclease, interacts with a CRISPR RNA (crRNA), which determines the location of cleavage, as well as a trans-activating RNA (tracRNA) to generate a protein-RNA complex capable of DNA cleavage [4, 30]. In the same paper, Jinek et al. fused the two RNA molecules to create a chimeric RNA called a single guide RNA (sgRNA), which was able to guide Cas9 to the desired cleavage site efficiently [30]. Within the sgRNA there is a ~20 base pair region that is important for sequence recognition with the cleavage site. This region must also be upstream of a canonical NGG triplet called a protospacer associated motif (PAM) in order for Cas9 to generate a blunt DSB [30–32]. Figure 1.2a represents Cas9 complexed with a sgRNA and the target DNA. In addition to this type II CRISPR-Cas system, the molecular mechanisms of the four other types of CRISPR-Cas systems have been elucidated to varying degrees [33].

1.2.3 CRISPR Tools in Biology

The *S. pyogenes* CRISPR-Cas9 system was quickly adapted for use in human cells, which showed tremendous success for genomic introduction of foreign DNA [34, 35]. Application in human cells showed *S. pyogenes* Cas9 (SpCas9) could be used to correct pathogenic mutations across a variety of diseases, from Fanconi anemia to mutations involved in retinitis pigmentosa in patient-derived cells [36–38]. Delivery of CRISPR-Cas systems and foreign DNA to various cell lines using viral approaches will be discussed later in this chapter. A creative use of SpCas9's targeted cleavage is its use to eradicate proviruses within human cells, such as HIV and Herpes Simplex-1 [39, 40]. Similarly, Yang et al. used

Cas9 to remove all porcine endogenous retroviruses from a porcine epithelial cell line, with the ultimate future goal of safe porcine-to-human xenotransplantation [41, 42].

Currently, there is a veritable arms race to identify new CRISPR-Cas systems that could be used to engineer cells, with the same or improved genome engineering efficiencies as the systems currently in use [4, 43, 44]. Recently, Burstein et al. utilized metagenomic approaches to mine novel CRISPR-Cas systems from unculturable microbes [43]. This group identified and characterized two new systems, CRISPR-CasX and CRISPR-CasY, both of which are smaller than the CRISPR-Cas9 system, a benefit to gene targeting as size is a consideration in most gene delivery vectors [43]. Additionally, Kleinstiver et al. showed that by decreasing nonspecific interactions of SpCas9 with DNA, the off-targeting cleavage of SpCas9-HF (High Fidelity) was removed for 8/8 sgRNAs analyzed as compared to wild type SpCas9, which had off-targeting cleavage with 7/8 sgRNAs [45]. Other CRISPR-Cas systems such as the Cpf1 CRISPR system have been elucidated and used in human cells with good results, broadening the CRISPR toolbox for genome engineering [44]. Generation and identification of Cas9 proteins that contained altered PAM specificities have also expanded the diversity of CRISPR-Cas tools [46]. In addition to engineering cells for therapeutic applications, CRISPR-Cas systems have been used to make libraries of gene knockouts more efficiently than previous approaches such as small hairpin RNA knockdowns [47].

CRISPR-Cas systems have also been modified for a diverse group of applications. As off-target cleavage is a concern for wild-type Cas9, SpCas9 has been rationally engineered by several groups based on crystal structure data to increase its specificity and decrease the likelihood of off-target cleavage [32, 45, 48–50]. Another notable modification was the generation of a catalytically attenuated version of SpCas9, termed the SpCas9 nickase (SpCas9D10A). The SpCasD10A nickase can be used in pairs to increase the specificity of cleavage of a particular locus only if it is flanked by both sgRNA encoded sites [51]. Furthermore, a catalytically inactivated SpCas9 has been used for both targeted transcriptional repression and also as a chassis for fusion of genetic effector proteins such as activators, deaminases, and epigenetic modifiers [51–56].

1.3 Gene Therapy Using Viruses

1.3.1 Retroviral Vectors

Retroviruses are positive-sense RNA viruses that require reverse transcriptase to convert their RNA genome into DNA, and in turn integrate the DNA genome into the host genome [57, 58]. The canonical genome of a retrovirus contains four genes. The *pol* gene encodes a reverse transcriptase (which reverse transcribes the RNA genome to DNA), a RNase H (used to process RNA), and an integrase gene (which integrates the viral genome into the host genome) [59]. The *gag* gene encodes the

structural polyprotein, and the *env* gene encodes envelope proteins essential in binding to host cells and determining viral tropism [59]. Lastly, the *pro* gene encodes a protease that is required for maturation of the viral particle via proteolysis of immature polyproteins to functional components [59]. The first retroviral vectors used to transduce human cells were based on Moloney murine leukemia virus (MLV) and were capable of only transducing dividing cells efficiently [60]. Additionally, these vectors were refined to only integrate transgenes of interest and not viral genes, which was a crucial step as specific gene integration is essential for precise gene therapy and genome modification [60]. Further work in retroviral gene delivery led to the development of viral vectors derived from human immunodeficiency virus (HIV) and had the advantage of expanding viral tropism to non-dividing cells [61]. As HIV is in the genus of *Lentivirus* of the Retrovirus family, vectors based on HIV components are often referred to as lentiviral vectors and have an ability to transduce non-diving cells [59].

1.3.2 Modifications and Implementation of Retroviral Vectors

While integration of transgenes can be seen as a benefit in the sense of permanent modification of the host genome, it can also be deleterious if specific integration into safe-loci is not achieved. Early clinical trials aimed at correction of X-Linked Severe Combined Immunodeficiency (X-SCID) resulted in several patients developing T cell acute lymphoblastic leukemia due to vector insertion and activation of proto-oncogenes [62, 63]. Hence, mapping the insertion profile for a retroviral vector or the development of integrase-deficient lentiviral vectors (IDLVs) via mutations in the integrase gene are solutions to the potential danger of damaging integration [56, 61, 63]. Additional modifications of lentiviral envelope proteins allowed for broadening the cell tropism via a method termed pseudotyping [64]. For example, by employing envelope glycoprotein from rabies virus, a lentiviral vector can be pseudotyped to transduce neuronal cells [64]. Pseudotyped IDLVs have allowed for efficient targeting of numerous cell types. Additionally, lentiviruses and other retroviruses have had their tropism modified toward specific cell types using antibodies and small peptide ligands that bind to the target cell [65–67].

IDLVs have been used as a method to deliver gene editing nucleases such as zinc-finger nucleases, transcription-activator like nucleases (TALENs), and CRISPR-Cas systems [56, 68, 69]. These programmable nucleases are used to cleave specific genomic regions and stimulate homologous recombination between the target locus and donor DNA. However, packaging gene editing components into IDLVs can be a challenge. In the case of zinc-finger nucleases, originally three distinct vectors were required, one for each zinc-finger nuclease (each of which cleaves one strand of genomic DNA) and a third for the donor DNA [69, 70]. More recently, fusing of each zinc-finger component to viral proteins has allowed for the generation of efficient singular IDLVs containing each zinc-finger nuclease and the donor DNA [69, 71]. For TALENs, the challenge has been that the repetitive nature of the DNA encoding

the nuclease can recombine during the reverse transcription of the viral genome [72]. However, using a similar approach as the zinc-finger nucleases, Cai et al. fused TALEN proteins to viral proteins to allow incorporation into a single vector [69]. Additionally, IDLVs have been modified further to inactivate the reverse transcriptase, allowing for vectors that can deliver TALEN mRNA into host genomes, avoiding the possibility of recombination by viral machinery [73]. CRISPR-Cas systems have also been delivered by IDLVs and integrating lentiviral vectors for a variety of experiments, ranging from library-on-library screening of CRISPR-Cas cleavage efficiency across the genome to removal of proviral DNA such as HIV-1 and Hepatitis B [68, 74–77]. In addition to nuclease delivery, an interesting application of IDLVs has been used to deliver nucleic acid modifying enzymes such as deaminases and epigenetic modifying enzymes such as histone deacetylases [56, 78]. Such *in situ* histone and DNA modification allows for genotypic or epigenetic change without the introduction of foreign DNA.

1.3.3 Translational and Clinical Progress Using Retroviral Vectors

Vectors based on retroviruses made an impact on treating human disease, and their use may increase as vectors become increasingly safe. A landmark study exemplified the success of a self-inactivating (SIN) γ-retrovirus vector to treat X-SCID [79]. This vector was an improvement on the previous generation Moloney murine leukemia virus vectors used to treat X-SCID and was shown to be less mutagenic due to a long terminal repeat (LTR) U3 enhancer deletion and the human elongation factor 1-α short promoter used to control the delivered gene [79–81]. Using this vector, the group showed that 8/9 treated patients exhibited improved immune function, with one patient dying due to a preexisting infection caused by an adenovirus [79]. Other recent trials have shown success in the use of integrating lentiviral vectors to reduce the autoimmune complications and microthrombocytopenia associated with Wiskott-Aldrich syndrome [82, 83]. One of the most promising uses of retroviral vectors is the modification of patient T-cells to target malignant cell populations by employing antigen receptors that bind to antigens specific to cancer cells. This method, called chimeric antigen receptor T-cell therapy, or CAR T-cell therapy, will be described in detail later in this chapter [84].

1.4 Adenoviral Vectors

Adenoviruses are non-enveloped double-stranded DNA viruses with ~35 kilobase pair genomes and are somewhat larger than many viruses commonly used in gene therapy [85]. In the adenoviral genome, several genes exist to regulate expression of

viral and host factors. These genes are often removed or manipulated to both make room for transgenes as well as decrease the ability of the virus to replicate after transduction into the host cell [86–88]. While there are over 50 serotypes of adenovirus, the most commonly used and best understood is serotype 5, often referred to as Human adenovirus serotype 5 (HAdV-5) [89]. These viruses, unlike retroviruses, have no endogenous integration machinery and do not incorporate into host genomes at high frequency, instead remaining as episomal elements [89]. Their episomal nature means that they have a much lower mutagenic potential than retroviruses. Naturally, the HAdV-5 vector has an affinity for transduction in hepatocytes, which is a benefit for delivery of transgenes to the liver, but a downside if other cell targets are desired [89, 90]. Additionally, adenoviral vectors have been shown to be highly immunogenic, due to natural exposure to adenoviral particles that most humans experience early on in life [89, 90].

1.4.1 Modifications and Implementation of Adenoviral Vectors

One of the first applications of HdAdV-5 for gene therapy was by Jaffe et al. who deleted the E1 and E3 viral genes to inhibit viral replication and make room for the human α1-antitrypsin gene and a β-galactosidase gene (as a marker of viral transduction). After intraportal injection into rats, the group found that α1-antitrypsin was detectable in serum for up to 4 weeks, demonstrating the power of modified adenoviral vectors for gene therapy [86, 91]. Shortly after, the same group showed the efficacious use of the HAdV-5 vector without E1/E3 genes to transfer human Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) genes into the respiratory epithelium of rats, demonstrating the potential for such vectors to treat Cystic Fibrosis [92].

Further removal of essential viral genes has produced vectors with transgenes flanked by inverted terminal repeats (ITRs, necessary for packaging the genome into the vector) referred to as gutless adenoviral vectors, with the viral genes needed for production supplied by the cell line used to manufacture the virus [93, 94]. "Gutless" vectors have been used to introduce DNA into human induced pluripotent stem cells (iPSCs) and embryonic stem cells (ESCs) by homologous recombination [88, 95, 96]. For example, such vectors have been used to repair genes involved in laminopathy, muscular dystrophy, and hemophilia B [96–98]. Given the large genome size of adenoviruses, these vectors are ideal delivery systems for genes that are too large for other viral vectors.

In addition to solely delivering DNA to replace or complement ineffective/mutated genes, groups have also delivered nucleases and recombinases that stimulate recombination between the donor DNA and the host genome [89, 99–101]. As discussed with lentiviral vectors, zinc-finger nucleases, TALENs, and CRISPR-Cas systems have been similarly delivered with adenoviral vectors [100, 101]. For instance, Perez et al. used zinc-finger nucleases encoded in a viral vector to dis-

rupt the CCR5 locus of CD4+ T cells with high frequency, making them resistant to HIV-1 infection [102]. However unlike lentiviral vectors, TALEN genes have been shown to be packaged stably with a lower spontaneous recombination frequency [23]. Also, multiple nucleases can be encoded in one efficacious vector due to the large genome size of adenoviral vectors [103]. Moreover, as in the lentiviral vectors discussed earlier, adenoviral vectors have modified to change cell type tropism. By modification of capsid components, the preference for liver tropism for adenoviral vectors has been decreased as well as re-targeted to other cell types, such as muscle cells [104, 105].

1.4.2 Translational and Clinical Progress Using Adenoviral Vectors

Adenoviral vectors were some of the first gene therapy tools used in clinical trials. A vector delivering a correct CFTR gene, which had been delivered successfully in rat epithelium, was trialed in humans in a landmark study [92, 106, 107]. While gene transfer did occur, with measurable transcription of transgene mRNA, functional replacement and symptomatic relief was not observed due to transient expression of mRNA and the decreased efficacy upon repeat admissions of the therapy (likely due to immunity associated against the vectors) [108, 109]. Notably, the first human death in a phase I viral gene therapy clinical trial occurred using adenoviral vectors attempting to correct a metabolic deficiency that leads to ammonia buildup. Jesse Gelsinger died shortly after administration of an adenoviral vector carrying an ornithine transcarbamylase gene after a severe reaction to the infusion [110]. While there are still several questions surrounding the exact reasons why Mr. Gelsinger had such a severe reaction, it has been proposed that his high ammonia level pre-infusion could have contributed [111]. Mr. Gelsinger's death was a tragic setback in the field of gene therapy but has highlighted the importance for stringency, informed consent, and quality practices in human gene therapy clinical trials [111, 112].

While the strong immune response that adenoviruses illicit in humans has been a challenge for gene delivery, it has also been used to the advantage of scientists and clinicians. By expressing viral or bacterial antigens proteins using adenoviral vectors, an immune response could be generated against the pathogen. Vaccination using adenoviral expressed antigens of *Mycobacterium tuberculosis* has shown stimulation of CD4+ and of CD8+ populations [113]. Similar approaches using HIV-1 antigens have yet to show significant immune protection [114]. To circumvent this, groups have used adenoviral vectors to express neutralizing antigens to HIV-1 intramuscularly, which have protected humanized mice from HIV-1 infection despite several high-titer exposures [115]. Another use for adenoviral vaccines has been to utilize them to help combat addiction to substances such as

cocaine and nicotine [116]. By covalently linking small molecule analogs of such addictive compounds, De et al. have used adenoviral vectors to illicit humoral immune responses to cocaine and nicotine in mice [116].

Using the potentially cytotoxic effect of viral infection, the use of adenoviral vectors as oncolytic viruses to target and kill cancer cells has shown great potential. To target transduction of adenoviral vectors to cancer cells, fiber modifications have been employed by several groups, for example by modification of the fiber capsid protein of HAdV-5 to contain an RGD-4C integrin binding motif, enhancing binding and transduction of ovarian and prostate cancer cells [117, 118]. In an animal model of ovarian cancer, this oncolytic vector significantly improved survival of treated diseased animals [117]. Additionally, multiple have modified adenoviral vectors such that they will replicate only in cancer cells by using cancer-cell specific promoters, such as prostate serum antigen promoter [119–122].

1.5 Adenoviral-Associated Viral Vectors

Discovered in 1965 in cell cultures co-infected with adenovirus, the adeno-associated virus (AAV) is a small non-enveloped parvo virus that is deficient in replication [123, 124]. The single-stranded AAV genome can integrate into the host genome after complementary strand synthesis or exist as an episomal element post-infection [124–126]. The AAV genome consists of approximately 4.7 kilobase pairs and is relatively refractory to size increases. This genome is composed of two open reading frames (ORF), *rep* and *cap*. Currently 13 AAV serotypes have been identified, many of which have different tissue/organ transduction profiles [127]. The capsid is composed of three subunits, VP1, VP2, and VP3, all of the *cap* ORF are expressed in the capsid with a stoichiometry of 1:1:10, respectively [128, 129]. The *rep* ORF is composed of four proteins that are essential for packaging, transcription, as well [130] integration into the viral genome into the AAVS1 locus on human chromosome 19 due to a Rep binding site at this locus [125, 130, 131]. The last gene encodes the assembly-activating protein (AAP), which is contained within the *cap* ORF in an alternate coding frame. This gene is used to assist in the assembly of VP1, VP2 and VP3 into the mature capsid [129, 130]. The entire genome is flanked by inverted terminal repeat (ITR) sequences, which cap either end of the genome with partially double stranded regions [125, 130].

AAVs utilize several cell surface receptors for host cell entry. The first discovered receptor was the heparin sulfate proteoglycan receptor, followed by discoveries of co-receptors including α5β1 integrin, CD9, the laminin receptor, and the hepatocyte growth factor receptor, all of which contribute to AAV tropism specification depending on the serotype [132–136]. Recently, an essential receptor for AAV host cell incorporation has been discovered via a genetic screen approach in a haploid cell line [137]. After tropism to the nucleus, AAVs stay latent unless a helper virus is present to assist in replication [125].

1.5.1 Modifications and Implementation of Adeno-associated Viral Vectors

AAV vectors have a long history of use in the field of gene therapy due to their effective tropism in different cell types and lower relative cytotoxicity. With such long history, they have also been modified in various aspects. The removal of the *rep* ORF is a major modification made in recombinant AAV (rAAV) vectors. Without this ORF, the propensity of the viral genome to integrate is decreased and the genome is more likely to exist in the cell as an extrachromosomal episome. This decreases the potential for insertional mutagenesis to the host genome [138]. Additionally, the Rep protein has toxic effects on the host cell and can reduce cell viability post-AAV infection [131]. Another modification made to some AAV vectors is the generation of self-complementary recombinant AAV (scAAV) genomes. By decreasing the genome size in half, the capsid can contain two complementary single stranded copies of the AAV genome. The major advantage of scAAVs is that they are much more efficient at transduction, increasing transduction by more than 140-fold in the original study by McCarty et al. [139]. The small size of AAVs and small packing capacity is an ongoing challenge for AAV vectors used in gene therapies. One solution to large cargoes is to split transgenes between two or more AAV vectors and co-infection, with the transgene transcript combined after transduction into the host cell [140]. Additionally, creating minimal versions of transgenes and regulatory elements have been proposed and attempted as a partial solution to the small DNA capacity of AAV vectors [140]. Gene delivery using AAV vectors (and other gene therapy vectors) falls in two broad categories: gene supplementation and gene replacement. Gene supplementation is useful when adding additional copies of a mutated or missing gene. Gene replacement can be used when the patient's ineffective allele must be inactivated or replaced for normal phenotype to be restored (as is in the case of dominant negative alleles). In the case of gene replacement, the delivery of engineerable nucleases (such as zinc-finger nuclease and CRISPR-Cas systems) to stimulate homologous recombination has been shown to be effective using AAV vectors in animal models [141–144].

The host immune response to AAV vectors is a major obstacle of varying severity depending on the method of delivery. For example, Brockstedt et al. showed that in mice antigen-induced immune reactions in intramuscularly delivered rAAV vectors encoding ovalbumin elicited a much reduced cytotoxic T-cell response to ovalbumin (however the a humoral response was still present) as compared to intraperitoneally, subcutaneously, or intravenously delivered vectors [145]. Additionally, neutralizing antibodies have the ability to inactivate systemically delivered AAV vectors, which can decrease transduction efficiencies in animal models and likely in human trials as well [146–148]. To combat neutralizing antibody effects on transduction into model organisms, Li et al. used *in vitro* directed evolution in the setting of human serum collected from a patient to identify regions in the AAV6 capsid crucial for evasion of neutralizing antibodies [149]. Using their results, this group generated chimeric AAV vectors capable of improved transduction in muscle tissue [149].

Additionally, much effort has been put into understanding the antigenic epitopes of the AAV capsid by many experimental and computational schemes [150]. Targeting AAV vectors to specific tissue types has largely been accomplished by identification of naturally occurring AAV serotypes that efficiently transduce the organ/tissue of interest [124]. The more scientists develop AAV vectors that utilize naturally occurring serotype transduction efficiencies, consider cell surface glycan interactions with AAV capsids, and engineer capsids through *in vitro* diversity generation and functional selection, the more they will be able to generate highly specific and targeted AAVs [124, 151–153].

1.5.2 Translational and Clinical Progress Using Adeno-associated Viral Vectors

With low toxicity, high transduction efficiencies across many tissue types, and facile manipulation, AAV vectors have become one of the most popular vectors for human gene therapy [124, 154]. As with adenoviral vectors, the first target for clinical trials using AAV vectors based on AAV2 was for the delivery of the CFTR gene in patients with cystic fibrosis [155]. Currently there are 173 recorded clinical trials involving AAVs as gene therapy vectors [156]. Recently there have been several successes using AAV vectors for gene therapy with effective therapeutic outcomes. One of these was an scAAV8 vector encoding human clotting factor IX for supplementation delivery to patients with Hemophilia B. Delivery of this vector to ten patients resulted in factor IX levels 1–6% of normal factor IX values. In patients who had a mean of 5.1 ± 1.7% of normal values of factor IX, there were 90% fewer bleeding events [157]. Another recent success involved AAV2 vectors encoding RPE65 to supplement mutated RPE65 genes in patients with Leber's congenital amaurosis as well as in a canine model of the disease. AAV2-RPE65 vector resulted in modest but temporary improvements in retinal sensitivity in patients and canine subjects [158]. Another important AAV vector therapy is the treatment of lipoprotein lipase deficient patients using AAV1-LPL^{S447X}, encoding a gain-of-function lipoprotein lipase which was shown to resolve chylomicronemia in lipoprotein lipase deficient mice. In a 2 year follow-up of a trial using this vector, Gaudet et al. showed that half of the treated patients showed a ≥40% reduction in fasting triglycerides, resulting in a clinical benefit to the patients involved [159]. This vector has been approved in Europe for clinical use, making it the first gene therapy ever approved in Europe or America. The vector, with the proprietary name of Glybera, costs nearly $1 million dollars per treatment, making payment for this treatment of a rare disease a serious consideration for patients and insurance companies [160, 161]. Looking forward, traditional AAV serotypes used for AAV vectors will likely be modified and tailored more specifically for the tissue targets. Recent success with AAV3-based engineered vectors suggests that they may be superior for *in vivo* AAV gene therapy as compared to many traditional AAV

Table 1.1 A summary of viral vector genome size and notable applications

Viral vector	Approximate genome size (kilobase pairs)	Notable applications
Adenovirus	36	In vitro gene delivery (highly immunogenic) [90, 126] Vaccination against pathogens and addictive compounds [87, 113, 116] Destruction of malignant cells [119, 121]
Adeno-associated virus	4.7	In vitro and in vivo gene delivery [140, 155] Relatively lower immunogenicity [145] Broad cell type specificity, depending on serotype [152]
Retrovirus (including lentivirus)	7–12	In vitro and in vivo gene delivery [57, 62, 68, 79] Modification of cells for ex vivo therapy [82]

serotypes used in clinical gene therapy applications (AAV5, AAV8, and AAV9) [162]. Table 1.1 summarizes some of the important features of each viral vector discussed previously.

1.6 *Ex Vivo* CRISPR Therapies

1.6.1 CAR T-Cell Therapy

Adoptive cell immunotherapy, or the transfer of lymphocytes to mediate effector function, is not a novel concept; in 1992, it was shown that a single infusion for CMV-specified CD8 CTLs could be used to treat disseminated CMV infection in post-allogeneic transplant patients [163]. In 2002, CD4 effector cells were shown to be efficiently transferred in HIV and elevated CD4 cell counts, and in 2005 it was shown that vaccine responses could be augmented in patients with myeloma using autologous T cells [164, 165]. It is generally believed that the beginnings of modern human immunooncology began with the approval of '1st Generation' Sipuleucel-T in 2010 and Ipilimumab, a CTLA-4 checkpoint inhibitor, in 2011 for treatment of castrase-resistance prostate cancer. '2nd Generation' agents included programmed cell death protein 1 (PD1) and PD1 ligand 1 (PD-L1) blocking antibodies as well as blinatumomab, a bi-specific antibody, an oncolytic GM-CSF-encoding herpes simplex virus known as talimogene laherparepvec or T-vec for metastatic melanoma, and CAR-T cells in 2014–2015 [166].

As previously mentioned, one of the most promising emerging uses of retroviral vectors to treat human disease, specifically cancer, is known as chimeric antigen receptor or CAR T-cell therapy. It involves the modification of patient T-cells to target malignant cell populations by employing antigen receptors that bind to cancer cell-specific antigens. CARs are fusion proteins that incorporate antigen recognition

variable region antibodies with T-cell activation domains. Unlike TCRs which recognize HLA-presenting peptides and are therefore restricted by the HLA-specific patients, CARs work by recognizing glycoproteins and intact cell-surface proteins and are HLA-independent. Originating from clinical trials of CAR-transduced T cells targeting α-folate receptor on ovarian cancer cells, many subsequent methods have been developed to insert CAR genes into T cells, including gammaretroviruses, lentiviruses, and transposon systems [167].

There are generally three different methods of adoptive cell therapy under investigation and reaching FDA approval: the use of tumor-infiltrating lymphocytes (TILs), chimeric antigen receptor (CAR) and T cell receptor (TCR) engineered T cells. TILs are produced after surgical excision and expansion of cells from a tumor biopsy and have been slow but progressive in development, with a recent phase 3 randomized trial (NCT02278887) underway for treating metastatic melanoma patients. In contrast, gene transfer-based methods that avoid the effects of immune tolerance are produced via peripheral blood lymphocytes and use viral or nonviral methods to engineer the cells and introduce the desired receptors. They involve the transfer of CARs made of antibody-binding domains fused to T cell signaling domains or alternatively TCR α/β heterodimers to promote the re-directing of T cells to target tissues. The first group of CARs was developed in 1991 as a fusion of the extracellular and transmembrane domains of CD8 to the cytoplasmic domain of the TCR ζ chain and shown to be sufficient to replicate TCR signaling; progressively more complicated designs have since been studied [168]. Most CARs currently in use are derived from mouse antibodies and have been shown in clinical trials to elicit both antibody and T cell responses; attempts to resolve this problem have focused on the use of humanized/fully human antibodies obtained from mice transgenic for human-Ig loci.

T cell costimulation experiments revealed the benefit of additional signaling moieties for CD19 CAR-T cell antigen-specific cytokine production and proliferation. Specifically, adding CD28 moieties and CD3 ζ domains to CD19 CAR-T cells enhanced rates of human leukemia cell eradication in mouse models. Other signaling domains, including TNF receptor super-family member 9 (4-IBB), have been shown to have a similar enhancement compared to CD19 alone [169–173]. Other approaches to enhance CD19 CAR-T cell activity include development of an Epstein-Barr virus (EBV) antigen recognized by CD19 CARs, central memory cells for genetic modification, and allogeneic cord blood T cell modification.

In 2016, the imposition of a clinical hold on Juno's JCAR015 in patients with relapsed or refractory B cell acute lymphoblastic leukemia (ALL) due to cerebral edema and death in two patients highlighted the need for skepticism in CAR-T cell therapy and further inquiry into the different modification and manufacturing processes employed by these candidates and the differential side effects that occur as a result. Supported by Phase II data and backed by FDA designations, companies are making the first steps to receiving regulatory approval for candidates to reprogram the immune system using CAR-T therapies; Kite has already filed submission for its KTE-C19 therapy for diffuse large B-cell lymphoma, and Novartis has plans to submit CTL019 for acute lymphoblastic leukemia in early 2017 [166].

The crossover of the CRISPR multiplex editing techniques to CAR-T therapy is a new and exciting area of active investigation. It has been shown that up to five genes can be simultaneously disrupted in mouse embryonic stem cells with high efficiency CRISPR-Cas9; specifically, CAR-T cells with either two or three gene disruptions (TRAC, B2M +/− PD-1) and analysis of *in vivo* and *in vitro* antitumor function. Using CAR-T cells targeting the B-cell antigen CD19, chosen for its expression by nearly all B-cell malignancies and restriction in normal tissues to expression in mature and precursor B cells, plasma cells, and follicular dendritic cells [11]. It was shown that anti-CD19 CARs were capable of activating T cells in a CD19-specific mechanism that could kill CD19+ primary leukemia cells *in vitro* [174, 175].

1.6.2 iPSCs

Reprogramming of somatic cells has allowed the creation of patient-specific induced pluripotent stem cells (iPSCs). They have the unique properties of self-renewal, large scale expansion, and ability to differentiate into endoderm, mesoderm, ectoderm, or even to hematopoietic stem cells (HSCs) in the presence of stromal cell co-culture or hematopoietic cytokines [176–178]. In as early as the 1960s, it was shown that a pluripotent state could be generated through the reprogramming of fully differentiated cells; essentially, it was demonstrated early on that totipotency could be achieved through alterations in the epigenetic profile [178]. Subsequent somatic nuclear transfer (SCNT), including the "Dolly" experiment, and cell fusion experiments revealed the presence of somatic cell-inducing cytoplasmic diffusible transacting factors in the oocyte/ESC in addition to the proof of reprogrammable terminally differentiated cells.

These results paved the way for one of the landmarks papers by Takahashi and Yamanaka in 2006, which showed the possibility of ectopic expression of a distinct and small set of transcription factors via retrovirus integration into differentiated cells. By identifying and serially reducing this set of genes into the minimal set of factors (Klf4, Sox2, Oct4, Myc) and demonstrating the retention of embryonic stem cell properties in these now 'induced pluripotent stem cells' (iPSCs), they set the stage for subsequent research on refining and implementing various methodologies to edit and induce functional pluripotency in a range of differentiated human cell types. The Yamanaka experiments additionally resolved and avoided the ethical debate around the use of stem cells sans human embryos [178]. Figure 1.3 demonstrates the process for *ex vivo* modification of somatic cells to iPSCs and ultimate correction of disease mutations by genome editing.

Studies using CRISPR/Cas9 editing in the transformation of iPSCs generated from somatic cells have demonstrated homologous recombination-based gene correction that could provide new avenues for treating certain genetic disorders, including β-thalassemia and Duchenne muscular dystrophy, as mentioned before [179].

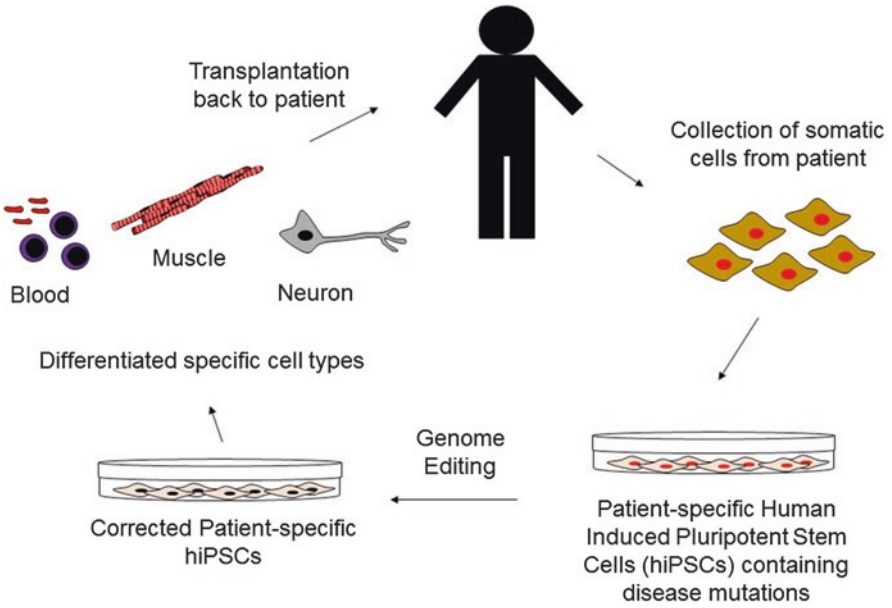

Fig. 1.3 Schematic of isolation of somatic cells from patients, generation of induced pluripotent stem cells, correction of disease causing traits, differentiation into specific cell type and transplantation back into the host

Cas9 methods were used to remove the premature stop codon in the DMD gene leading to Duchenne Muscular Dystrophy and resulted in partial restoration of protein function [180]. Additionally, patient-specific iPSCs generated from Hemophilia A patients were used in conjunction with Cas9-mediated editing to remedy the large-scale chromosomal inversions that underlie the disease process [181].

As for the future of iPSC and CRISPR therapy to treat human disease, many challenges remain. In the clinical setting, treatments generally rely on producing a defined gain of function at desired genes with high frequency; with this method, however, human cells prefer the imprecise pathway of non-homology end joining (NHEJ) repair of the double strand breaks in DNA as opposed to the homology-mediated editing [182]. Therefore, many approaches have been taken to shift the DSB repair pathway from the generation of NHEJ-mediated insertions and deletions to homology-mediated repair; these include cell-cycle dependent control of CRISPR/Cas9 delivery via small molecular NHEJ-inhibitors [183–186]. Additionally, the goal of generating complex hiPSCs with a wide variety of genetic alterations is hindered by the short conversion tracts of human cells and resulting limitation of either NHEJ or HDR mechanism to one side of the DSB [187]. This poses the biggest challenge of broadly applying iPSCs and CRISPR/Cas9 to editing the human genome as well as reveals the unrealized potential of the technology to produce tremendously helpful resources, such as condition human knockout iPSC libraries.

1.7 Conclusion

Genetic medicine has allowed for patient-specific treatment of disease. Progress in modification of patient-specific disease traits in cells, tissues, and whole organ systems has become closer to a reality thanks to multidisciplinary approaches to gene therapy. Modification of cells at the genetic level using CRISPR-Cas systems has revolutionized the ease and efficacy of cell modification, and delivery of genetic material using viral vectors has allowed a level of nuclear access previously unimaginable.

While these advances continue to progress, several key issues need to be solved. One of these issues is the targeting of gene delivery vectors to tissues and organs with spatiotemporal control. Often, genetic disease manifests in only a subset of tissues and organs, meaning that the delivered gene or cell must target that region specifically. Off-target effects of both gene delivery and cellular delivery can result in toxic outcomes and can lead to patient death, as was discussed in the history of viral vectors [62, 111]. Additionally, controlling the activity and timing of therapeutic gene expression or cellular activity may be crucial, as disease progression can be dynamic over time. An added layer of complexity is navigating the host immune system as it serves as a powerful barrier against both viral gene therapy as well as cellular approaches. Going forward, scientists and clinicians will continue to struggle with specificity and control in targeting precision gene therapies. However, the potential for the tools discussed in this chapter will continue to grow. In the coming decades, it is likely that most medicine will be practiced in a precise fashion with tailored cures for each patient's unique genome.

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