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Engineering Gene Therapy: Advances and Barriers

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Currently, 33 gene-therapy drugs/products have been approved in the clinic. Over 3000 completed and ongoing clinical gene therapy trials have been reported worldwide. The development/maturation of tools for gene manipulation and gene delivery, as well as molecular advances in the diagnosis of genetic diseases, have played a central role in gene therapy, which have greatly revolutionized the field. Versatile and diverse genetic tools for gene manipulations and deliveries, with the possibility of short and long-term effects, are vital advantages of gene therapy tools, paving the road for the development of new therapies. However, efficacy and safety concerns, immune system responses, laborious approaches for developing and manufacturing, unknown gene-therapy drug interactions with the host and the high cost of drugs/products, are significant barriers in gene therapy. Here, the authors review the attempts for engineering of the gene manipulations that have been undertaken in the last three decades and used in clinical trials focusing on 1) gene-editing platforms, 2) viral gene delivery systems, and 3) nonviral gene tools. In this comprehensive review, the principles of these gene manipulation tools as well as advances and barriers for their application in modern therapies are discussed. Furthermore, trends and future directions in gene therapy are discussed.

1. Introduction

The human hereditary disorders are caused by small or large mutations in genes. Currently, more than 7000 rare genetic diseases have been reported, which affect about 300 million people worldwide. Although some treatments are based on conventional drugs, which reverse the symptoms, many do not repair the underlying genetic mutations. Human cancers are not only caused by genetic mutations, but also by nongenetic factors that contribute to carcinogenesis, cell invasion, and metastasis. Strategies for treating human cancers have always been considered one of the critical themes of clinical trials. However, most therapeutic approaches have been developed, based on conventional drugs depending on the cancer type and tumor stage. As gene mutations play a central role in the manifestation and progression of inherited diseases and cancers, therapies mediated by gene manipulations could be the best alternative treatment for these indications.[1–3]

Gene therapy is an intervention in which a defective gene is repaired in vivo, or a gene product is delivered to target cells by a clinically safe vehicle. The procedures of gene therapy aim to restore the function of a defective gene or replace a mutant gene with a paralogous gene.[4–6] Detecting the mutations and understanding the precise molecular mechanism of a genetic disease are the first steps for a gene therapy. The type of genetic modification and delivery method needs to be evaluated in pre-clinical surveys. At the clinical stage, the therapeutic drug or bioproduct is directly administered to the patient via the circulation or by injection into a target organ. Another form of gene therapy is the ex vivo therapy, where patients cells are genetically modified in the culture dish and then reinfused into the patients.[7] A schematic representation of gene therapy approaches and clinical translation is illustrated in **Figure 1**.

Over the last three decades of gene therapy, the field has been struggling with numerous challenges and barriers such as immune responses and nontarget effects. However, recent

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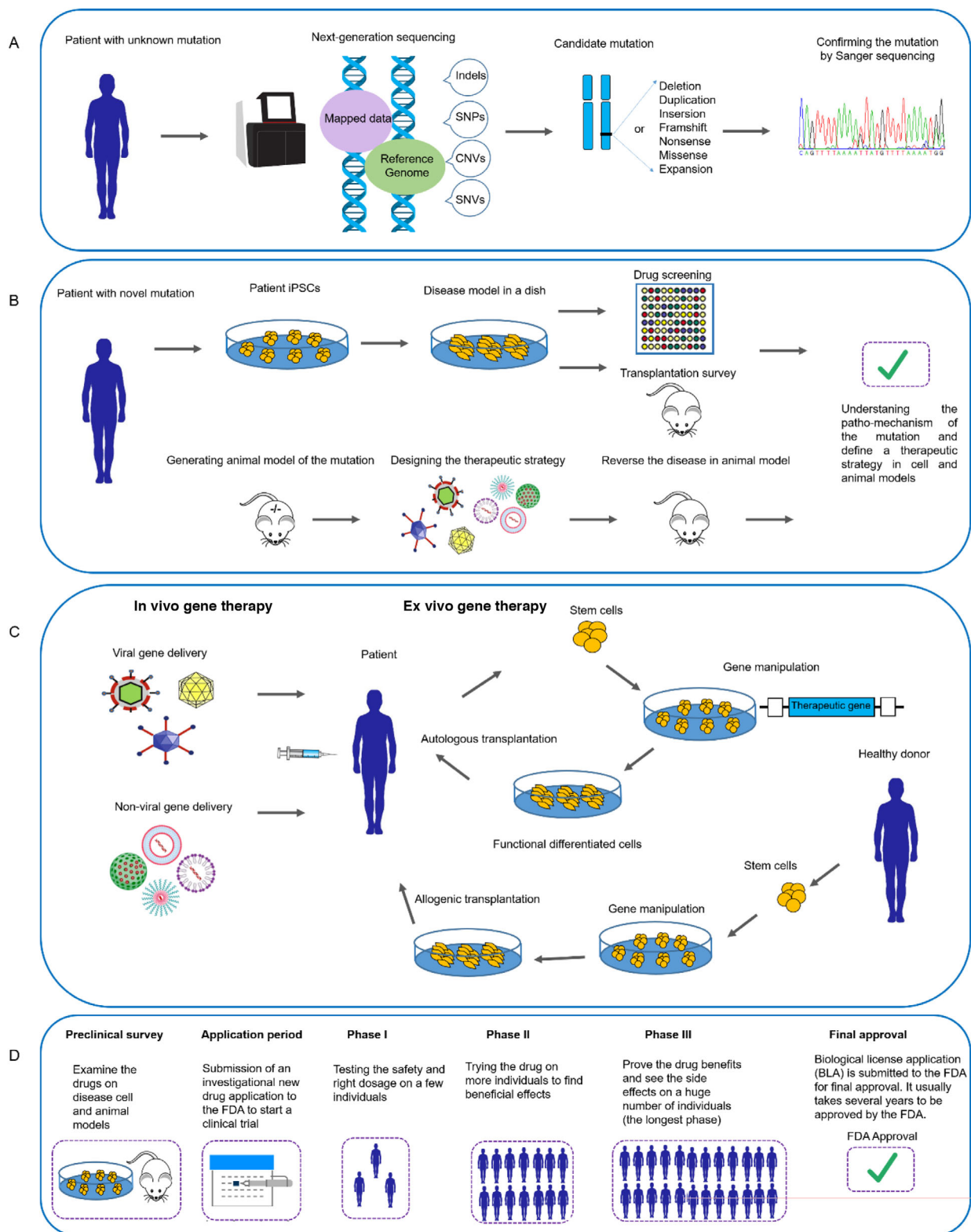Golestan University
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Figure 1. Schematic representation of a human gene therapy procedure. A) Detecting and confirming mutations causing genetic disorders by next-generation sequencing (NGS) and Sanger sequencing, respectively. B) Preclinical studies on cell and animal models of the genetic disorders to understand molecular mechanism of the disease as well as to develop a therapeutic strategy to reverse the disease status. C) Two principal procedures of gene therapy include in vivo administration of the gene therapy drugs and ex vivo genetically engineered stem cells. D) The processes of approval a gene therapy drug or product include preclinical, application period, phases I, II, III and final approval by relevant authorities.

numbers of successfully approved gene therapy products underline the advances of the field and its growing importance in future therapies.[8–10] This is also shown by the increasing numbers of gene manipulation and delivery tools that have been developed. The wide range of gene manipulations includes base substitution, gene replacement, gene deletion, or gene knock-in. Moreover, recent molecular diagnostic advances of genetic diseases have supported new designs of therapeutic strategies.[11–13]

Current genetic tools that are being used in clinical gene therapy trials include gene-editing systems, clustered regularly interspaced short palindromic repeats (CRISPR) Cas-associated nucleases, transcription activator-like effector nucleases (TALENs), zinc-finger nucleases (ZFNs), meganucleases (MNs)][14,15] and viral gene delivery vehicles (adenovirus, adeno-associated virus, lentivirus, retrovirus, and other common and noncommon viral vehicles).[16] Also included are nonviral systems (mRNA, naked DNA, RNA interference, DNA interference, aptamer, miRNA and other classes of therapeutic DNA or RNA oligonucleotides).[17,18] Indeed, these genetic tools have revolutionized the fields of gene therapy and personalized medicine, which was recently underlined by the chemistry Nobel prize for the pioneers of CRISPR gene editing, Jennifer Doudna and Emmanuelle Charpentier.

In this review, the principle of these gene therapy systems as well as their engineering advantages and obstacles in the clinic are discussed. In addition, we look at the applications of these genetic tools in current clinical gene therapy trials, as well as their trends and future directions in modern medicine.

2. Gene Editing Platforms

Gene or genome editing toolkits refer to engineered nucleases that can be applied for any desired DNA modifications, ranging from base substitutions to large DNA deletions or rearrangements. The most popular gene-editing tools are CRISPR/Cas, TALEN, ZFN, and MN systems.[19–25] All these programmable nucleases are exploited to produce a double-strand break (DSB) at a target DNA site. At the presence of an endogenous or exogenous template, DSB is repaired based on the template sequence by homology-directed repair (HDR). However, in absence of a template, it is randomly repaired by nonhomologous end joining (NHEJ). The concepts of HDR and NHEJ pathways are used for disease mutation correction and gene disruption by gene-editing systems (**Figure 2**A).[25–28]

In comparison to traditional methods of homologous recombination, these gene-editing devices are more specific, efficient, fast, and time-saving. These appliances can not only be used for a wide spread range of applications in generating genetically disease models and finding the causal link of a genotype–phenotype relation, but they also have high capacities for ex vivo and in vivo corrections of disease mutations and gene knock-out/knock-in.[29–32] Hence, the gene-editing systems raise great hope to treat human genetic disorders and cancers. Currently, the use of these toolkits is dramatically increasing in ex vivo clinical gene therapy trials. Somatic cells, such as hematopoietic stem cells (HSCs) and immune T cells are ideal candidates for such therapeutic trials, but also solid tissues and organs, such as the skin, can be targeted. The manipulation of the germline is dangerous, highly controversial, and unethical and there is widespread consensus in the scientific community, that this should not be attempted.

2.1. CRISPR/Cas Platform

CRISPR/Cas system originally belongs to the prokaryotic acquired immune system, which is involved in the degradation of invaded phage DNA. During the first phage infection, its DNA fragments are incorporated between short palindromic repeats into the CRISPR locus of the bacteria genome. In consequent infections, the fragments and a scaffold RNA are transcribed from the locus yielding RNAs known as CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA), respectively. The crRNA (20 nucleotides specific to phage DNA) and the tracrRNA form a complex, which functions as the guide RNA (gRNA) for the Cas enzymes. The Cas enzyme/gRNA complex binds to the invaded DNA and distinguishes a protospacer adjacent 3-nucleotide motif (PAM) and then generates a DSB in the phage DNA. This PAM is recognized by the PAM-interacting domain (PI domain). There are two domains in Cas9 known as HNH and RuvC that are responsible for the nuclease activity of the enzyme.[19,20,33,34]

For gene manipulations, tracrRNA along with crRNA is artificially synthesized into a single gRNA. The human codon-optimized Cas enzymes, particularly subtypes of Cas9 and Cas12a (Cpf1), are mostly used in research and clinical practices of human gene targeting.[35,36] RuvC and HNH domains are altered to abolish the endonuclease activity of the Cas9 enzyme. If one catalytic domain is mutated, the resulting protein is called nickase, which produces a single cut in one of the DNA strands. If both domains are mutated the enzyme is called dead Cas9 (dCas9) and is catalytically inactive. The dCas9 has widespread applications when fused with a modulator domain in gene activation or suppression and base editing. The most prominent feature of CRISPR/Cas, in comparison with other gene-editing platforms, is the easy cloning and multiplexity, meaning that it simultaneously edits different loci when providing several gRNAs. Taken together, the advantages of the CRISPR/Cas system are its simplicity, low cost, high efficiency, and versatile applications (Figure 2, **Table 1**).[37–41]

Over recent years, the basic version of CRISPR/Cas9 system has been improved for higher efficiency in gene editing and targeting applications. In addition, the gRNA and means of delivery have been further developed. The engineered CRISPR/Cas demonstrates maximized in vivo and ex vivo activities and delivery, minimized off-target effect, and regulated expression. For instance, the fusion of dCas9 to the activation-induced cytidine deaminase (AID) (called base editor, BE) is capable to convert C to T nucleotides without the necessity of a DNA template at the target site with NG PAM in human cells.[42–44] In addition, two engineered versions of Cas12a (cpf1) enzyme have been generated. The first version has a broader targeting range, high gene-editing activity and shows improved efficiency in multiplex gene targeting, endogenous gene activation, and C-to-T base conversion. The second engineered version of Cas12a enzyme has high-fidelity activity and minimized off-target effects[45,46] Interestingly, the next generations of CRISPR/Cas gene therapy as CRISPR-AID are emerging to edit DNA without a DSB. By covalently connecting a Cas core to a nucleotide deaminase enzyme,
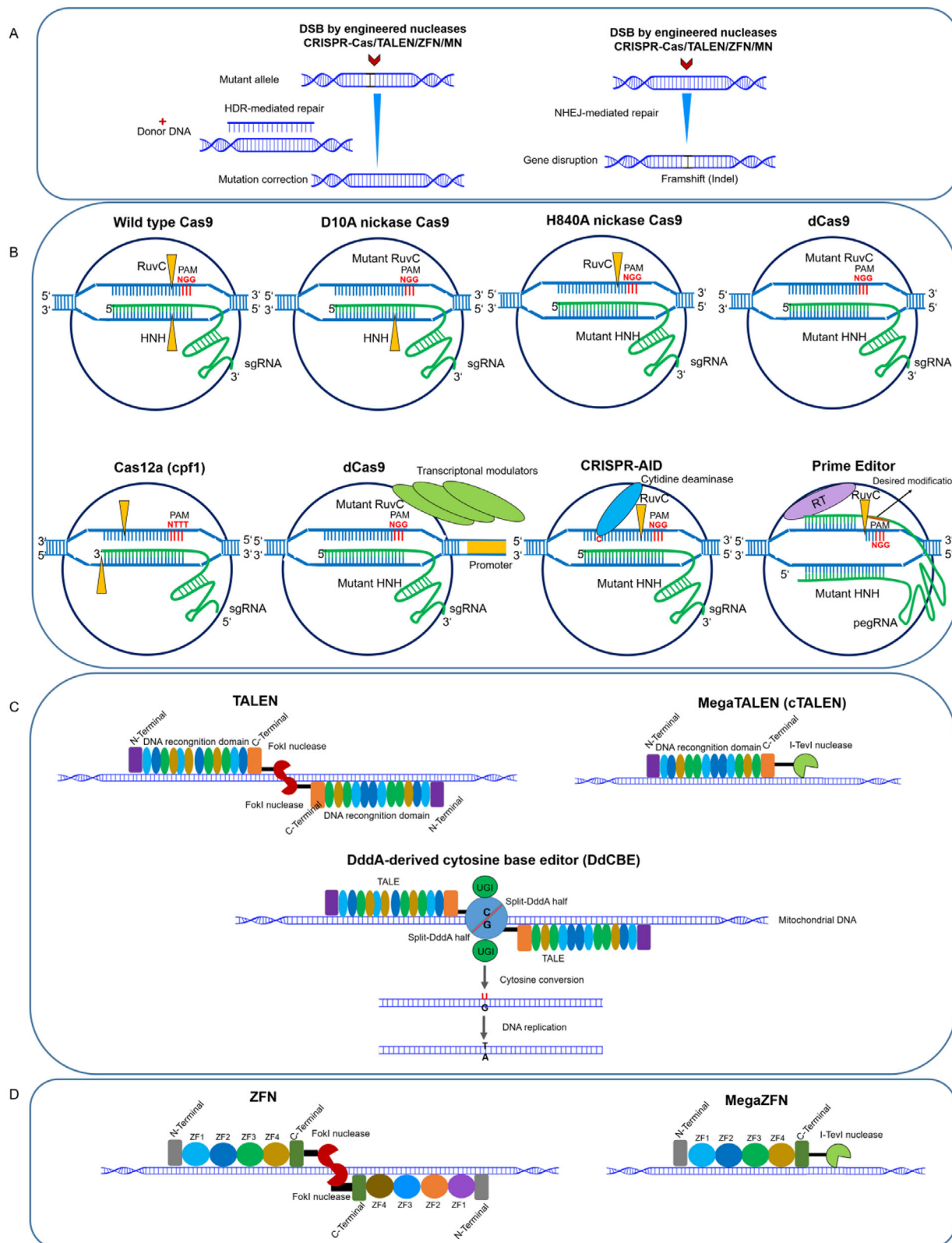
Figure 2. The principle and structure of gene-editing platforms. A) Targeted DSBs by engineered nucleases, Cas, TALEN, ZFN, and MN stimulate DNA repair pathways. Then, HDR and/or NHEJ pathways are recruited to repair the gap. These systems are being used for a wide spread knock-in and knock-out applications. B) The engineered version of CRISPR/Cas (humanized Cas9 and Cas12a) and its alternative (nickases, dCas9, CRISPR-AID, and prime editor) for clinical applications. C) The structure of TALEN, MegaTALEN and DdCBE. D) ZFN and MegaZFN gene-editing platforms.

Table 1. Marked features of gene-editing platforms.

Features	CRISPR/Cas	ZFN	TALEN	Meganuclease
Targeting principle	RNA/DNA interaction	Protein/DNA interaction	Protein/DNA interaction	Protein/DNA interaction
Design component	DNA	Protein	Protein	Protein
Essential components	sgRNA and Cas9	ZFA-FokI fusion protein	TALE-FokI fusion protein	Restriction enzyme
DNA binding element	sgRNA	Triple-confined ZFN	Single base recognition TALE	Meganuclease
ORF size	4.2 kb (+sgRNA)	2.1 kb	2.2 kb	Various (1.1-4 kb)
Functional mode	Single/pair	Single/pair	Single/pair	Single/pair
Multiplexing possibility	Highly feasible	Challenging	Challenging	Challenging
Target site	22bp followed immediately by PAM sequence (NGG for spCa9); up to 44 bp for double nicking	Typically, 18-36 bp per ZFN pair, GC rich region preferred	Typically, 30-40 bp per TALEN pair; 5' targeted nucleotide is T	Between 14 to 40 bp; central 4 bases intolerant to alter
Library construction	Highly feasible	Challenging	Challenging	Challenging
Methylation sensitive	Not sensitive	Sensitive	Sensitive	Sensitive
Time and affordability	Short (1–3 d) and highly affordable	Long (7–15 d) and affordable	Long (5–7 d) and affordable	Long and affordable
Off-target effect	High	Low	Low	Low
Ease of delivery	Easy, facile design of sgRNA and standard cloning techniques	Difficult due to extensive cloning needed to link zinc finger modules	Difficult due to extensive TALE repeat sequence	
Delivery for in vivo gene editing	AAV/lentivirus	AAV	AAV/lentivirus	AAV
Design availability	Simple	More complex	Complex	Complex
Cost	Low	High	High	High
Cytotoxicity	Variable to high	Low	Low	Low
Efficiency	High	High	High	High
Cleaving strand	Single-strand cleavage	Double-strand cleavage	Double-strand cleavage	Double-strand cleavage
Biasing repair event	NHEJ	NHEJ	HDR	HDR
Specificity	Positional and multiple consecutive mismatches tolerated	Small number of positional mismatches tolerated	Small number of positional mismatches tolerated	Small number of positional mismatches tolerated
Ease of engineering	Easily targets using routine cloning method and oligo synthesis	Hard, needs substantial protein engineering	Moderate, needs complex molecular cloning procedures	Hard, needs substantial protein engineering
Ease of in vivo delivery	As large size of Cas9, the ease of packaging is moderate	As small size of ZFN cassette, packaging in the various viral vector is relatively easy	As large size of each TALEN and unwanted recombination, the packaging is difficult into lentiviral vectors	As small size of meganuclease cassette, packaging in various viral vector is relatively easy
Ease of *ex vivo* delivery	Relatively easy through viral transduction and electroporation	Relatively easy through viral transduction and electroporation	Relatively easy through viral transduction and electroporation	Relatively easy through viral transduction and electroporation
Popular design servers	MIT Crispr tool, CRISPOR, CHOPCHOP	Zincfingertools, zincfingers	E-TALEN, CHOPCHOP	LAHEDES
Developed alternative versions	Cpf1, Cas9, nickase, dCas9, CRISPR-AID, Prime editor	AZP-SNase, OPEN ZF, AZP-scFokI, MegaZFN, mitoZFN	Tev-mTALEN, cTALEN, mTALENs, mitoTALEN, split-DddA$_{tox}$-TALE	MegaTEV, MegaTAL, ARCUS

conversion of C to T and A to G with no need for DSB repair, is possible. However, these technologies result in only base transitions, not base transversions.[47]

The efficient gene editing is hampered by the generation of DSB, donor DNA. To overcome these technical barriers, a new version of CRISPR system, termed prime editor (PE) has emerged. In PE, a Cas9 nickase is covalently linked to a reverse transcriptase (RT). The new system is able to perform any intended gene manipulation, including all base transitions and transversions (totaling 12 base to base conversions) and small insertion and deletion (indels; up to 80 bp). Similar to base editor,

prime editor does not need a DSB and a donor DNA required for gene editing. PEs exploited a prime editing guide RNA (pegRNA) encoding the intended DNA modification, which is incorporated into the host DNA after the reverse transcription.[48]

The results from the clinical trials of CRISPR/Cas gene editing confirmed the safety and feasibility of the technique for human gene therapy. In 2016, the first attempt of human clinical trial, mediated by CRISPR/Cas technology performed in China. The target group was patients affected by metastatic nonsmall cell lung cancer (NSCLC) and where chemotherapy and radiation therapy had failed. T cells from the site of the tumors were

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extracted and edited by CRISPR/Cas9 for ex vivo, disrupting the PD-1 gene. PD-1 encodes a receptor on the surface of the T cells and interferes with the recognition and disruption of the cancer cells. PD-1 knock-out T cells were reinjected into the patient's body.[49] By 2020, clinical trials databases have registered about 100 enrolled studies, that are using CRISPR/Cas system to edit or knock-out genes to treat human disorders ranging from monogenetic and complex diseases to infectious diseases caused by viruses. The prominent ongoing clinical gene therapy trials mediated by CRISPR/Cas technology have been listed in **Table 2**.

The CRISPR/Cas9 system was applied to repair MYBPC3 gene mutations, which are associated with hypertrophic cardiomyopathy. The heterozygous mutation was corrected without a synthetic DNA template in human embryos. The edited embryos demonstrated no signs for mosaicism, off-target evidence, and any abnormality.[50] The base editor version of CRISPR/Cas9, yielded a targeting efficiency of about 90% in correcting FBN1 mutations of Marfan syndrome in heterozygous human embryos.[51] However, a less efficiency (about 25%) was observed in correcting A>G mutations of HBB-28 gene (hemoglobin subunit beta) in human embryos suffering from β-thalassemia.[52] Furthermore, correction of *GJB2* gene mutation which result in syndromic hearing loss, as well as skin diseases, is currently being performed using CRISPR/Cas9 in human embryo.[53] Despite these few attempts, there is a broad agreement among scientists for the safety of the method and ethical concerns for gene editing of human embryos. Germline gene editing is a red line that should not be crossed.

CRISPR/Cas system are also being used for rapid and optimized engineering of chimeric antigen receptor (CAR) T cell therapy technology.[54] CAR T cells specifically distinguish and delete antigen-expressing cells without the limitation of the major histocompatibility complex (MHC). The food and drug administration (FDA), approved CAR T cell therapy products of Kymriah and Yescarta revolutionizing cancer therapy of B-cell leukemia and lymphoma.[55–58] The next-generation CAR T cells that are safer and more controllable are being explored by CRISPR/Cas system. This is achieved by a number of gene manipulations, including knock-in of suicide and interleukins genes, knock-out of T cell receptor (TCR), MHCs, PD-1 and TGF-β receptor, insertion of the CAR cassette into the TRAC and TET2 loci, disruption of the genes involving in CAR T cells apoptosis.[59–61]

The first clinical trial to use CAR T cell therapy manipulated by CRISPR/Cas was reported in early 2018. Patients suffering from refractory cancers of melanoma, sarcoma, and multiple myeloma were enrolled in the study, which was finished in early 2020. In this trial, three endogenous TCR genes, TRAC, TRBC, and PDCD1 were knocked out in T lymphocytes to enhance antitumor immunity. Furthermore, NY-ESO-1 gene was introduced to recognize and/or target tumor cells. The results were promising and identified no safety concerns. The edited T cells revealed endurable engraftment in the patients for at least 9 months. The engineered T cells showed no clinical toxicities. Trafficking of edited T cells to the sites of tumor was observed in bone marrow as well as tumor biopsies.[62] Despite the effectiveness of CAR T-cell therapy in relapsed and refractory B-cell malignancies, cytotoxicity is still of concern. Recently, genome-scale CRISPR/Cas9 loss-of-function screens identified death receptor

signaling through FADD and TNFRSF10B as a main player of CAR T-cell cytotoxicity.[63]

The first clinical trial of an in vivo CRISPR/Cas9 gene therapy of a genetic disease was reported in March 2020. This technology was used to treat Leber's congenital amaurosis 10 (LCA10) causing childhood retinal degeneration and blindness, in which there is currently no routine cure. In this disorder, the gene of *Centrosomal Protein 290 (CEP290)* carries compound heterozygous or homozygous mutation involving c.2991+1655A>G in intron 26. The product of EDIT-101 is administered via subretinal injection of a single adeno-associated virus (AAV) to deliver the CRISPR/Cas gene-editing machinery to photoreceptor cells.[64]

Off-target activity, leading to mutagenesis and probably oncogene activation, remains a major barrier for in vivo gene-editing by CRISPR/Cas systems. The high expression of the system increases off-target activity.[65] Optimizing duration of CRISPR/Cas9 exposure, even by using the ribonucleoprotein approach or inducible systems, reduces off-target effects.[66,67] Furthermore, several other strategies have emerged to overcome this challenge. Using truncated gRNA, Cas9 paired nickase, dCas9 fused with *FokI*, dCas9 fused with deaminase for base editing and recently developed prime editor versions, markedly reduce the rate of off-target cleavage compared to the wild-type enzyme.[38,39,45,68,69]

The other challenge of the CRISPR system is targeted delivery. Proper delivery of CRISPR/Cas system to target tissues in vivo is ideal, particularly for cell types such as neural cells and it reduces unwanted tissue targeting. Adeno-associated virus (AAV) are useful and popular vectors for in vivo gene-editing. They are still limited to tissues in which transduction is clinically efficient, including the brain, eye, and muscles. Various serotypes of AAV allow high delivery efficiency to dividing and non-dividing cells in various tissue and cell types. Due to the limitation in AAV packaging, two AVV plasmids are mandatory for delivery of the CRISPR/Cas system. To circumvent safety challenges of a virus, a nonviral delivery vehicle is currently being developed for targeted gene delivery and to reduce potential safety risks.[70–73] For instance, for the clinical product of CTX001, electroporation method is used to deliver CRISPR/Cas9 in a ribonucleoprotein (RNP) form to HSCs. This product is now at the phase II clinical trial for the treatment of β-thalassemia.[74] The designation of priority medicines (PRIME) has recently been granted by the European Medicines Agency (EMA) for the CTX001 product.

The response of the immune system is another challenge of CRISPR/Cas-based gene therapies. In CAR T cells therapy mediated by the CRISPR system, the serum of some donors naturally contains antibodies against Cas9. Pre-existing T cell immune memory is observed in the donors having evaluated Cas9 antibody. Anti-Cas9 responses mediated by the immune system have a negative effect on the efficiency of gene-editing and could result in a serious immune/cytokine storm in individuals receiving the treatment. This immune response is triggered by CD8+ cytotoxic T lymphocytes (CTLs) and resulted in cell death of the Cas9-expressing cells, even when using noninflammatory AAV vectors as a delivery tool.[75–78] However, there are multiple strategies to evade from immunogenicity against Cas9 and minimizing the influence of CTLs. Masking of immunogenic Cas9 epitopes, changing antigen presentation of Cas9 epitopes, using Cas9 orthologs, exploiting immune privilege, and inducing

Table 2. Recruiting or completed clinical gene therapy trials based on gene-editing platforms.

Genetic tool	Product, target gene, and strategy	Type of cancer or genetics disease	Trial identifier
CRISPR/Cas9	Ex vivo gene manipulation, generating PD-1 knockout T cells	Advanced esophageal cancer	NCT03081715 (I)
		EBV-positive cancers	NCT03044743 (I)
		Mesothelin-positive multiple solid tumors	NCT03747965 (I)
	Ex vivo gene manipulation, generating CCR5 knockout CD34+ human hematopoietic stem/progenitor cells (hHSPCs)	HIV-1 infection with ALL	NCT03164135 (I)
	Ex vivo gene manipulation, generating CD19 knockout T cells	Relapsed or refractory CD19+ leukemia and lymphoma	NCT03166878 (I/II)
		Relapsed or refractory CD19$^+$ B-ALL or other B-cell lymphomas	NCT04037566 (I)
	Ex vivo gene manipulation, generating CD19 and CD20 or CD22 knockout T cells	Relapsed or refractory leukemia and lymphoma	NCT03398967 (I/II)
	Ex vivo gene manipulation for generating PD-1 and TCR genes knockout T cells (anti-mesothelin CAR-T cells)	Mesothelin-positive multiple solid tumors	NCT03545815 (I)
	Autologous CD34+ hHSPCs manipulation at the enhancer position of the *BCL11A* gene to increase fetal hemoglobin	β-thalassemia	NCT03655678 (I/II)
		Sickle cell disease	NCT03745287 (I/II)
	CD19-directed T-cell immunotherapy, allogeneic T cells genetically manipulated ex vivo	Relapsed or refractory non-Hodgkin's lymphoma	NCT04035434 (I/II)
	AGN-151587 (EDIT-101) is AAV vector expressing CRISPR/Cas9 system to correct CEP290 gene mutation that is responsible for LCA10	LCA	NCT03872479 (I/II)
TALEN	CD19-directed T-cell immunotherapy, allogeneic T cells	Refractory or relapsed CD19 positive large B-cell lymphoma or follicular lymphoma patients	NCT03939026 (I/II)
	Ex vivo gene manipulation, generating CD123 Knockout T cells	Patients with CD123 expressing in relapsed or refractory AML	NCT03190278 (I)
	B-cell maturation antigen (BCMA)-targeted ALLO-715 product, allogeneic CAR T cells	Patients with relapsed or refractory multiple myeloma (MM) refractory to at least three prior lines of MM therapy	NCT04093596 (I)
	Ex vivo gene manipulation, generating CD22 Knockout T cells	Patients with relapsed or refractory CD22+ B-ALL	NCT04150497 (I)
	Ex vivo gene manipulation, generating CS1 Knockout T cells	Patients with relapsed or refractory multiple myeloma (MM)	NCT04142619 (I)
ZFN	Autologous CD4+ T cells genetically modified at the CCR5 gene, by AAV delivery tool	HIV-1 infection	NCT00842634 (I) NCT01044654 (I) NCT01252641 (I/II) NCT02225665 (I/II) NCT02388594 (I) NCT03666871(I/II) NCT04201782 (I)
	Knockout of HPV16/18 E7 oncogene	HPV-induced cervical precancerous lesions; Epithelial cells DNA	NCT02800369 (I)
	Incorporating IDS gene under the control of albumin promoter in hepatocytes	Mucopolysaccharidosis II; MPS II (Hunter's syndrome) Hepatocytes	NCT03041324 (I/II)
	Incorporating IDUA under the control of albumin promoter in hepatocytes	MPS I (Hurler syndrome)	NCT02702115 (I/II)
	Inserting factor 9 gene under the control of albumin promoter in hepatocytes	Hemophilia B	NCT02695160 (I)
	Autologous CD8 + T cell genetically modified expressing IL13Ralpha2	Recurrent or refractory malignant glioma	NCT01082926 (I)
	Autologous CD34 + HSPCs genetically modified to disrupt the enhancer of the *BCL11A* gene	Sickle cell disease	NCT03653247(I/II)
		β-Thalassemia	NCT03432364 (I/II)

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immune tolerance are being used to reduce the immune responses. Furthermore, the choice of delivery vector and dosage, promoter and strategy design, target site, and administration path and finally, short-term or inducible expression of Cas9 could also be useful.[73,79–82]

2.2. TALEN Platform

TALEN is originally found in a plant pathogen of *Xanthomona* bacteria. The bacteria naturally use TALE to alter gene expression in host plant cells. The protein contains binding modules including, tandem repeats of about 34 amino acids. DNA recognition is conferred by two repeat variable diresidues (RVD) at amino acid positions 12 and 13 in each repeat. The rest of the amino acids of each repeat are structurally similar. RVD codes of ND binds to C base, NH to G base, HN to G or A bases, and NP to all four bases. In engineered TALENs, a DNA cleavage domain (*FokI*) nuclease fused to a DNA-binding domain to generate a breakage at a specific target site. However, TALEN gene-editing functions in pairs, to introduce the DSB. To efficiently generate a DSB, a proper spacing of 10–30 bp is essential between the two DNA binding sites (Figure 2, Table 1).[22,32,83,84] Then, these site-specific DSBs are used for TALEN-induced gene-editing applications.

The various configurations of amino acid sequences and DNA recognition of the TALE DNA binding domain provide valuable resources for engineering efficient TALE nucleases. Since each TALE module recognizes one base pair, TALEN can be adjusted to widespread selections for target sites.[85,86] In some DNA positions, the naturally RVDs reveal low diversity properties. Recent studies are identifying and characterizing new nonconventional RVDs to improve intrinsic targeting specificity and efficiency features. The affinity of the amino acid at position 13 of the nonconventional RVDs highly determines the base preference. By using this form of RVD, the discrimination between different nucleotides is improved and consequently, the specificity of TALEN tool is largely enhanced.[87,88]

The *FokI* nuclease needs self-dimerization to generate a DSB and this requires simultaneous expression of two distinct TALEs. Moreover, each monomer has the potential off-target effect as *FokI* cuts any DNA sequence. These barriers reduce the efficiency of TALEN for gene targeting and therapy.[89] To abolish *FokI*, several strategies were developed to generate monomeric TAL-based nuclease (mTALEN). mTALEN reduces the off-target activities by up to 50% when compared to TALENs. One strategy is the combination of TALEN with other molecular devices such as MN. The DNA binding domain of a TALE is combined with the cleavage domain of a MN. The hybrid nuclease called MegaTAL conferred highly efficient DNA binding activity of a TALE and specificity of a MN and it does not need dimerization.[90] Furthermore, the partially catalytic domain of I-TevI enzyme has been added to a TALE monomer to produce compact TALEN (cTALEN). I-TevI is a site-specific and sequence-tolerated MN. cTALEN functions as nickase and display a greater efficiency in targeted gene correction in comparison to the classic TALEN system.[91] The next-generation TALEN system is being generated based on monomeric activity and compact forms with high efficiency and specificity in gene targeting.

Mok et al. recently engineered an innovative base editing system termed DddA-derived cytosine base editor (DdCBE). The system is composed of the split interbacterial toxin DddAtox, TALE, and uracil glycosylase inhibitor (UGI) (Figure 2C). It forms a functional cytosine deaminase and efficiently converted C to T bases in double-stranded mitochondrial DNA (mtDNA). When cytidine deaminase was fused instead of TALE to the CRISPR/Cas system, it modified only single-stranded DNA (ssDNA), whereas the newly developed DdCBE system can directly edit double-stranded DNA (dsDNA). Thus, the dsDNA breaks of the CRISPR/Cas system, can cause loss of mtDNA copies. Furthermore, the delivery of sgRNA of the CRISPR/Cas system into the mitochondria is not effective. The CRISPR-free DdCBE system showed high target specificity with no toxic effect. In addition, this novel system does not affect mtDNA copies and reveals broad implications for the treatment of mitochondrial diseases.[92–94]

Cloning of TALEN arrays and repeat sequences is considered as the technical challenge of the tool. The low-efficiency and time-consuming vector preparations have decreased the wide applications of TALEN technology. However, to address this barrier, several techniques including the Golden-gate cloning strategy, the REAL (restriction enzyme and ligation) assembly method, the REAL-Fast assembly method, and the FLASH (fast ligation-based automatable solid-phase high-throughput) assembly technique have been established for fast TALE repeats assembling. All these methods efficiently assemble TALE repeat arrays on a single architectural framework.[95–97] Although TALEN gene editing has made huge progress over the years, a real in vivo gene therapy by using this technology is still limited by some issues for efficient gene editing and proper delivery, unknown immune responses, and off-target effects.

In 2015, the first TALEN-based genome editing for human use was reported in the USA. The case was an 11 month old girl fighting against high-risk CD19+ infant acute lymphoblastic leukemia (ALL, (t (11;19) rearrangement)). Her leukemia cancer cells were resistant to the alemtuzumab drug and were not detectable by the immune system. The donor T cells were successfully engineered by TALEN to express the chimeric antigen receptor against the B cell antigen CD19 (CAR19). After infusion of edited T cells, a very promising result was achieved and cancer cells were eliminated.[98] Over the last years, several gene therapy clinical trials and CAR T cell therapies are using the TALEN to reverse genetic disease status and cancer progression. In these ex vivo therapies, patients' T cells and/or HSCs are genetically modified and reinfused into the patients. They use viral and nonviral methods for proper delivery of TALEN system to stem cells. The completed or ongoing clinical gene therapy trials mediated by TALEN have been listed in Table 2.

2.3. ZFN Platform

In contrast to CRISPR/Cas and TALE systems that present in prokaryotes, zinc finger domains are naturally expressed in eukaryotic cells. They contain sequence-specific-DNA binding domains that regulate gene expression. Each zinc finger domain contains 3 to 6 zinc finger repeats recognizing between 9 and 18 bp. The repeat unit specifically recognizes 3 bp by the

interaction of α-helix residues with the major groove of the DNA. It consists of 30 amino acids and its finger-like structure is folded by interaction of Zn2+ and Cys$_2$His$_2$ residues. Engineered ZFNs are artificial restriction enzymes assembled by fusing a *FokI* nuclease to a DNA-binding zinc finger domain. Like TALEN, *FokI* dimerization is needed for DNA cleavage. Thus, the functional ZFNs have two distinct zinc finger domains with an intervening spacer site of 5-7 bases in which each monomer is coupled to a *FokI*. Hence, ZFN dimers selectively recognize 18-36 bp of DNA sequence at the DSB region (Figure 2, Table 1).[23,24,29] Generation of a DSB at the target DNA could be used for genome editing mediated by ZFN.

In comparison to TALENs, engineered ZFNs reveal lower specificity and efficiency. The amino acids composition of a finger, the number of fingers, and the interaction of the *FokI* nuclease are major players for target recognition and specificity of the system. Thus, structural and catalytical domains of ZFNs have been subjected to optimization and engineering. In a study, a new linker for intervening spacer of finger-finger and finger-*FokI* cleavage domain junctions was developed. The result dramatically improved ZFN configurations for efficient cleavage of target DNA.[99,100] As with TALEN, dimerization is a similar challenge for ZFN activity and efficiency. To resolve this issue, monomeric version of ZFNs has been generated. One solution is recruiting single-chain *FokI* (sc*FokI*) and align two *FokI* nuclease domains in one construct, called artificial zinc-finger protein sc*FokI* (AZP-sc*FokI*) platform, which efficiently edited targeted sites in comparison to basic ZFN.[101] Similar to TALEN, I-TevI nuclease domain is coupled with a zinc finger producing a monomeric ZFN that is used in genome editing.[102]

Alongside CRISPR/Cas and TALEN, ZFN is a prominent platform in the gene therapy field. ZFN is the first gene-editing system that was used for therapeutic applications. The first efforts of ZFNs were ex vivo gene therapy using own patients stem cells. Initial strategies include gene corrections of IL2Rγ and β-globin genes and also knock-out of CCR5 gene.[103,104] ZFN system is especially well known as a potential therapy for HIV/AIDS infection. As the CCR5 receptor gene was disrupted by this programmable nuclease in CD4+ human T-cells, yielding cells were resistant to viral entry. The *CCR5Δ32* allele produces a truncated protein that donates a complete protection against HIV-1 infection in homozygotes.[105] Currently, several independent clinical trials are using ZFN to target CCR5 receptor gene in T cells and hematopoietic stem and progenitor cells (HSPCs) for HIV-1 infection treatment.[106,107] Furthermore, the other gene-editing platforms including CRISPR/Cas and TALEN are being used for *CCR5* gene disablement in some clinical trials. The prominent completed and ongoing clinical gene therapy trials mediated by ZFN gene-editing have been listed in Table 2.

The ZFN is smaller than Cas9 and TALEN. Thus, it can be easily delivered using viral vehicles. As there are safety concerns of viral usage, the plasmid DNA encoding ZFN has the potential to overcome all the barriers associated with the viral therapeutic gene delivery.[107] ZFN delivery also depends on the human cell types. For instance, the mRNA electroporation method is efficient for the ZFN delivery to HSPCs. In addition, ZFN delivery mediated by AAV is efficient in CD4+ T cells when compared with in HSPCs. In a study, *BCL11A* gene disruption for expression induction of fetal hemoglobin in human CD34+ pro-genitor cells were targeted by the three gene-editing platforms of CRISPR/Cas9, ZFN, and TALEN. In comparison to the others, ZFNs revealed less off-target effect and more knock-out efficiency of the *BCL11A* gene, with increased levels of fetal hemoglobin in erythroid cells developed in vitro from the edited cells. These data demonstrated that different gene-editing platforms have variable off-target effects and cleavage efficiencies at different gene loci and in different human cell types.[108]

2.4. MN Platform

MNs are the most specific natural restriction enzymes that are present in archaebacteria, bacteria, yeasts, fungi, and a number of plants. They are called homing endonucleases that are divided into five classes based on sequence and structure motifs. The high specificity of MN gives them a high degree of precision and much lower cell toxicity than other naturally occurring restriction enzymes. As the most important family of MNs, LAGLIDADG nucleases are well known for gene targeting applications since the beginning of the genome-engineering era. They have all the essential elements and motifs to generate a DSB in the target DNA. They recognize a target sequence ranging from 14 to 40 base pairs in length (Figure 2, Table 1).[90,109,110] However, due to the emergence and development of efficient gene-editing toolboxes that were discussed above, MNs are currently less popular for clinical applications. As compared with MNs, the other gene-editing systems (CRISPR/Cas, TALEN, and ZFN) are easily engineered and manipulated.

As mentioned above, MNs are used to produce monomeric TALEN and ZFN. MegaTAL gene editing is a compound nuclease derived from the incorporation of TAL effector into a MN. This hybrid nuclease offers an extreme level of target specificity due to high DNA binding properties of a TAL effector, with high cleavage capacity of an MN. It has efficient delivery for therapy applications. For instance, megaTALs are expressed with a single $\approx$2 kb coding sequence (CDS), while the minimum CDSs for ZFN (2.2 kb), TALEN (2.5 kb), and Cas9 nuclease (4.1 kb) are significantly less in size. Thus, efficient packaging of megaTALs with DNA, mRNA, or with viral vehicles is easily possible. ZFN and TALEN require dimerization to function and generate a DSB while megaTAL, due to containing a TALE monomer, avoids from wrong heterodimerization. This is a prominent benefit for applications needing multiplex genes targeting.[90]

Modifying DNA recognition specificity of MNs is a big challenge. Recently, a next-generation of MN system known as AR-CUS platform was developed, with customization of the activity and specificity of the I-CreI (a MN enzyme). ARCUS is significantly smaller than other gene-editing systems (about 1.1 kb CDS). It is safer and more efficient than other gene-editing tools. Its delivery to specific target tissue is much easier than other gene-editing platforms. In contrast to some other gene-editing systems, ARCUS is capable to discriminate a target DNA with one different base pair. A potential gene therapy for retinitis pigmentosa by this platform is underway. ARCUS has been designed to distinguish the dominant C>A point mutation in the rhodopsin gene. One of the first therapeutic efforts by a meganuclease was carried out in an ex vivo method for corneal diseases. As corneal graft rejection is a major concern in patients affected
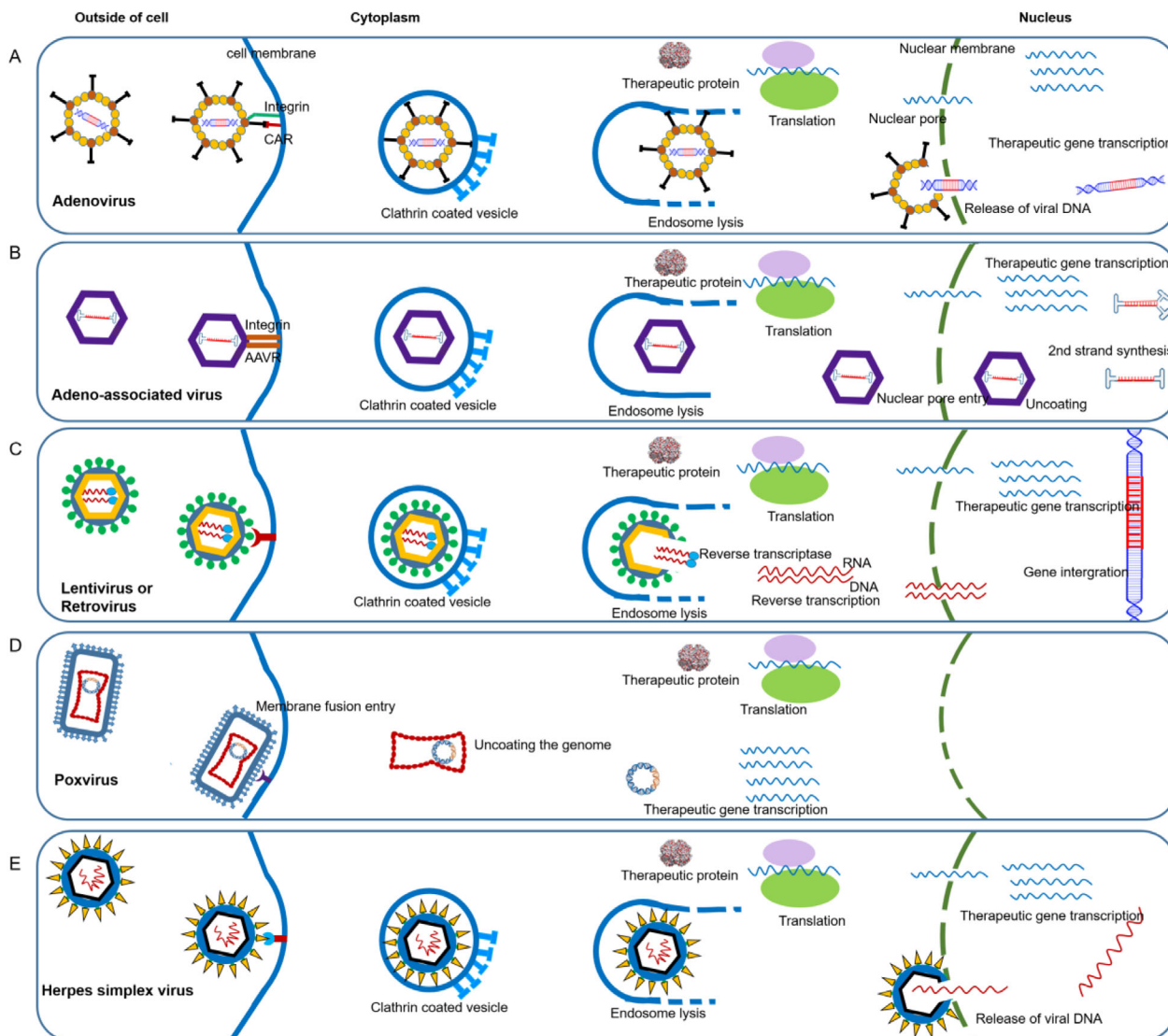
Figure 3. The transgene delivery mechanism is mediated by engineered viral vectors. A) Advs enter the cells by receptor-mediated endocytosis, unpacks in cytoplasm and inject its genome to the nucleus via nuclear pore. B) AAVs enter the cells by receptor-mediated endocytosis, and unpack in the nucleus. Then single strand genome is converted to the second strand and the transgene is transcribed. C) Lenti/retroviral delivery vectors enter the cells by two mechanisms, receptor-mediated endocytosis and membrane fusion. The reverse transcription occurs in the cytoplasm. Then their genome is integrated into the host genome by an integrase enzyme. D) Poxviruses enter the cells by membrane fusion, and unpack in cytoplasm. The therapeutic gene is transcribed in the cytoplasm. E) HSVs enter the cells by receptor-mediated endocytosis, unpack in cytoplasm and inject their genome into the nucleus via a nuclear pore.

by chronic herpetic keratitis (HK) with latent infection, an engineered meganuclease was used to target both latent and active forms of herpes simplex virus type 1 (HSV-1) in the endothelium and the stroma of the cornea. The method provided HSV-1-resistant corneas for transplantation. It was considered as a major achievement in gene therapy to reduce failure rates in cornea transplantation.[111]

3. Viral Gene Delivery Vehicles

Viral gene delivery systems have been used for in vivo gene therapy applications during the past three decades. They potentially infect and deliver therapeutic genes into the nucleus of the target cells. Common characteristics for all viral gene deliveries over non-viral ones are the well-organized events of infection and intracellular trafficking. These features include binding affinity of the capsid to the extracellular receptors, internalization and release to the cytosol, movement along the microtubules and entry of the viral DNA to the target nucleus through the nuclear pores. Over the last decade, viral vehicles have intensively been engineered for targeted gene delivery for transient as well as permanent gene expression.[16,112–114] The transgene delivery mechanisms mediated by common viral systems are shown in **Figure 3**. Furthermore, marked characteristics of these viral gene therapy vehicles are listed in **Table 3**.

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Table 3. Marked features of common viral gene delivery vectors.

Features	Adenovirus	Adeno-associated virus	γ-retrovirus	Lentivirus	Paxvirus	Herpesvirus
Family	*Adenoviridae*	*Parvoviridae*	*Retrovoridae*	*Retrovoridae*	*Paxviridae*	*Herpesviridae*
Genome (size)	dsDNA (34–43 kb)	ssDNA (4.7 kb)	ssRNA (+) (3–9 kb)	ssRNA (+) (9.75 kb)	dsDNA (200 kb)	dsDNA (150 kb)
Capsid and coat	Icosahedral, naked	Icosahedral, naked	Icosahedral, enveloped	Icosahedral, enveloped	Complex, enveloped	Icosahedral, enveloped
Virus size (nm)	70–90	18–26	80–130	80–130	170-200×300-450	150–200
Packaging capacity	7.5 kb	4.5 kb	9–12 kb	8 kb	25 kb	Up to 50 kb
Infection type	Dividing and nondividing cells	Dividing and nondividing cells	Dividing cells	Dividing cells	Dividing and nondividing cells	Dividing and nondividing cells
In/ex vivo applications	In vivo	In vivo	Ex vivo	Ex vivo	In vivo	In vivo
BBB-crossing	No	Yes	No	No	No	No
Transducing units mL^{-1}	1×10^{10}	5×10^{13}	1×10^{6}-10^{7}	1×10^{6}-10^{7}	1×10^{10}	1×10^{12}
Entry mechanism	Receptor mediated endocytosis	Receptor mediated endocytosis	Receptor biding, membrane fusion, and internalization	Receptor biding, membrane fusion, and internalization	Membrane fusion	Receptor-mediated endocytosis
Integration and transgene expression	Nonintegrating, transient	Integrating if Rep gene is included, potential long lasting	Integrating in active loci, long lasting	Integrating in active loci, long lasting	Nonintegrating, transient	Nonintegrating, transient
Approved products in the clinic and market	Gendicine Oncorine	Glybera, Luxturna Zolgensma Valrox	Rexin-G Strimvelis Zalmoxis Invossa Yescarta	Kymriah Zynteglo Abecma	–	Imlygic

3.1. Adenoviral Gene Delivery

Adenoviruses (Advs) are non-integrating dsDNA viruses with a 34–43 kb genome size. The icosahedral capsid is non-enveloped (naked) and the size of the virion ranges between 70–90 nm. Advs belong to DNA viruses of *adenoviridae* family. The genome is flanked by two noncoding inverted terminal repeats (ITR). It also contains ψ packaging sequences and eight transcription units including early units (E1, E2, E3, E4, and E5 genes), intermediate units (IX and IVa2 genes), and late units (L1, L2, L3, L4, and L5 genes) that are responsible for viral infection, replication and packaging.[115–117] By docking to the coxsackievirus-adenovirus receptor they infect host cells and cause simple infections of ocular, respiratory and gastrointestinal epithelium in human.[118] Advs are excellent shuttle vectors for transgenes or vaccine antigens deliveries to target cells and tissues in gene- and cancer therapy. Advs are genetically diverse DNA viruses with more than 50 various serotypes. Among them, Adenoviruses 5 and 8 are being used for gene therapy applications nowadays.[119]

Advs genome has been largely modified by the deletion of viral genes to produce safe and efficient gene delivery carriers. Three Advs generations have already been engineered with different capabilities of transgene delivery and the rate of infection. The first-generation has a partial deletion in E1 and E3 genes. That leaves the replication deficient and minimizes oncogenic properties. However, the strong immune response caused upon infection and the leakiness of viral genes expression remain to be addressed.[120] In second-generation Advs, E2A, E2B, and E4 genes were deleted but the obstacles of leaky viral gene expression and short-term transgene expression remained.[121] In the third generation of the vectors, all viral genes were deleted

except for the ψ and ITR segments. The capacity to carry larger therapeutic genes (more than 30 kb), the ability for stable transgene expression, and fewer contaminations with replicating virus particles were a considerable improvement. The newer Advs also showed to be less immunogenic than the previous generation of the vectors.[122]

Advs gene delivery vectors offer several advantages over other viral vectors. They deliver transgenes to a broad range of tissues and different cell types including both replicating and nonreplicating cell populations. New generations of Advs vectors can deliver multiple gene cassettes up to a size of 36 kb of DNA, which make them ideal for delivering large transgenes. They have a well-known genome, the possibility of a large-scale viral production and nonreplicative properties in target cells. Moreover, other advantages are the ability to induce robust transgene-specific T cell and antibody responses, simple gene manipulations and inherent adjuvant properties.[122–124] However, several concerns and barriers have been reported for Advs in clinical applications. Vaccine development and gene therapy trials are affected by a pre-existing immunity to Advs. Inflammatory events, immunogenicity, cytotoxicity, oncogenesis, and sequestering of viral particles by liver and spleen were major barriers of Advs in human gene therapy. Furthermore, the possibility of rereplication, nonspecificity, immunodominance of antigens over the antigen of vaccine transgene, immune modulation event and heterologous immunity with other viruses are concerns in Advs therapeutic applications.[125–131]

Advs were used for the first viral gene delivery in a clinical gene therapy trial. In 1992, the first engineered Adv was used in human gene therapy trials to treat alpha-1 antitrypsin deficiency. Advs delivered and expressed alpha-1 antitrypsin

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in hepatocytes.[132] Two years later, Advs were used for the treatment of cystic fibrosis. The engineered Adv delivered CFTR (cystic fibrosis transmembrane conductance regulator) gene to lung tissues of the patients.[133] Lately, it has been demonstrated that Advs ensured long-term expression of alanine-glyoxylate aminotransferase (AGT) in patients with primary hyperoxaluria type 1 (PH1).[134] Gendicine and Oncorine are two Advs-based gene therapy drugs for treatment of several cancers that were approved by the Chinese FDA in 2004 and 2005, respectively. E1 region of Adv5 has been replaced by *Tp53* gene in Gendicine. *Tp53* expression in cancer cells stimulates apoptotic pathways as well as suppressing DNA repair and survival pathways.[135] The *E1B-55 KD* gene, which inactivates Tp53, has been abolished in Oncorine. It selectively replicates in *TP53*-deficient cancer cells, while losing its replication ability in normal cells. Subsequently, Oncorine is released from the lysed cancer cell and induces cell death in neighboring cells.[136] In phase II of a recent clinical trial, engineered dendritic cells (DCs) by an Adv expressing wild-type *TP53* were administered to patients affected with advanced stage NSCLC after chemotherapy.[137] The recent engineered Advs which are being used in clinical trials for vaccine development and gene therapy applications are listed in **Table 4**.

Since 2019, the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) infection, also known as COVID-19, has resulted in one of the worst pandemics, causing health and economic challenges worldwide. Currently, several Advs based vaccines have been developed, termed Janssen, AstraZeneca, Sputnik-V (Gamaleya) and CanSino, harboring SARS-CoV-2 S spike gene codes for S protein. Advs allow to produce experimental S protein vaccines within 3–4 weeks. These vaccines were demonstrated to be safe in humans. The vaccines induced potent and sustained T-cell and B-cell responses to the S protein in young and aged volunteers. The efficiency of Advs vaccines examined to provide immunity against COVID-19 were 95%, 67%, 66%, and 65.7% for SputnikV, AstraZeneca, Janssen, and CanSino, respectively.[138,139]

3.2. Adeno-Associated Viral Gene Delivery

AAV is a small, helper-dependent, and ssDNA parvovirus. It has a 4.7 kb genome within a naked and icosahedral capsid. The genome harbors *Rep* and *Cap* genes flanked by ITRs that function in viral replication and packaging. The *Rep* gene encodes proteins of Rep78, Rep68, Rep52, and Rep40, while *Cap* gene encodes VP1, VP2, and VP3 proteins. Moreover, assembly-activating protein (AAP), which is essential for capsid assembly, is expressed from a different ORF within the *cap* gene. In recombinant AAV (rAAV), the therapeutic gene is replaced with 96% of the genome (rep and cap genes) and flanked by ITRs. Upon infection, the host cell integrates into the host genome in a site-specific manner to chromosome 19 (19q13.3q-ter, called AAVS1). Expression of integrated AAV remains quiescent unless the AAV lytic cycle is triggered by a helper virus. Having 13 different serotypes, AAV is a great gene delivery vector to a specific cell or tissue. AVV has unique biology and structure and has no disease associations. It is an ideal delivery vehicle for most in vivo gene therapy applications. Surprisingly, some AAV serotypes, particularly the AAV9 derived serotype, are able to cross the blood-brain barrier (BBB)

and are capable to transduce cells of the CNS. These properties hold huge promise in developing novel clinical therapies for neurological diseases. In comparison with Advs, AAV vectors provide a safer and well-tolerated gene therapy appliance for transduction given their naturally reduced pathogenicity and immunogenicity in humans.[140–145]

AAV-mediated gene deliveries are the most popular method among in vivo gene therapy-based-viral systems. It has been repeatedly engineered to improve successful gene delivery and therapy. AAV transduction efficiency has been increased by engineering the transgene cassette. For instance, AAV ITRs modifications have resulted in transgene expression without the need of a second-strand DNA synthesis. To optimize transgene expression and regulation in target cells, cell type specific or tissue-specific codon usage bias algorithms were performed.[146,147] The AAV capsid was modified to extend its tropism. The capsid and transgene were genetically engineered to avoid the host immune response. The AAV transgene cassette was modified to decrease the recognition of CpG sites by toll-like receptor 9 (TLR9) TLR9. These changes inhibit the induction of a TLR9-mediated innate immune response. One remaining challenge was that AAV evoked the CTL adaptive immune response in antigen-presenting cells. Using a tissue-specific promoter in front of the transgene restricts AAV transduction to a special set of target cells. Thus, transgene-mediated CTL response could be deleted. Furthermore, empty AAV has been used as a capsid decoy to overcome preexisting humoral immunity from recognizing the AAV-containing transgene.[148–154]

The rAAV was initially recruited in clinical gene therapy trials for monogenic disorders of cystic fibrosis and hemophilia. rAAV2-*CFTR* showed proper transgene transfer by airway delivery. Positive clinical effects were observed after two months in adult cystic fibrosis patients with mild lung disease.[155] rAAV2-Factor IX was applied intramuscular (IM) injection and hepatic delivery in patients with hemophilia B. Both delivery methods dramatically increased in vivo expression of *FIX* and partial correction of bleeding time.[156] Altogether, these initial trials identified a strongly consistent figure of rAAV based gene therapy in human. Glybera, Luxturna, Zolgensma, and Valrox are four gene therapy products based on AAV engineering that were approved for clinical and marketing use by relevant authorities in 2012, 2017, 2019, and 2020, respectively. Glybera is rAAV1 and contains a *LPL* gene variant LPL^{S447X} used for lipoprotein lipase deficiency. However, its production has ceased due to lack of demand caused by its high price. The luxturna drug is rAAV2 harboring a healthy copy of the *RPE65* gene applying for a retinal dystrophy. In addition, Zolgensma is a nonreplicating rAAV9 tool containing human *SMN1* gene and is used for spinal muscular atrophy (SMA) therapy.[157–159] Recently, valoctocogene roxaparvovec (BMN 270) with the trade name of "Valrox" was approved by FDA. Valrox is an AAV5-based gene therapy vector for treatment of severe hemophilia A encoding human B domain-deleted factor VIII (FVIII-SQ).[160]

3.3. Retroviral Gene Delivery

Retroviruses (RVs) are enveloped and nonicosahedral single-stranded RNA (ssRNA) viruses belong to the *retroviridae* family.

Table 4. Selected clinical gene therapy trials using viral gene delivery systems.

	Vector name	Indication	Transgene (mechanism of action)	Trial identifier	Phase
Adenovirus	ADV-TK	HCC	Adenovirus encoding *TK* transgene	NCT03313596	III
	CG0070	Bladder cancer	Adenovirus expressing *GM-CSF* gene	NCT01438112	III
	E10A	Head and neck neoplasms	Adenovirus encoding *Endostatin* gene	NCT02630264	III
	AdVEGF-D	Coronary artery disease (CAD)	Adenovirus encoding *VEGF-D* gene	NCT03039751	II
	Ad5.hAC6	Congestive heart failure	Adenovirus-5 encoding *human adenylyl cyclase type 6 (AC6) gene*	NCT00787059	II
	Ad5-yCD/mutTKSR39rep-ADP	Prostate cancer	Adenovirus encoding yeast CD fused to an improved TK (SR39) mutant and the adenovirus death protein (ADP)	NCT00583492	II
	LOAd703	Pancreatic adenocarcinoma/ ovarian cancer/ colorectal cancer	Adenovirus 5/35 encoding *TMZ-CD40L* and *41BBL* transgenes	NCT03225989	II
	ADV-TK/GCV	Glioblastoma	Adenovirus-mediated delivery of *TK* transgene	NCT00870181	II
	rAd-p53	NSCLC	Adenoviral encoding *TP53* gene	NCT01574729	II
	AdCD40L	Malignant melanoma	Adenoviral-5 encoding *CD40L* gene	NCT01455259	II
Adeno-associated virus	AAV2/6-FVIII	Hemophilia A	AAV2/6 encoding hFVIII	NCT04370054	III
	AAV8-RPGR	X-Linked retinitis pigmentosa	AAV8 encoding retinitis pigmentosa GTPase regulator (RPGR)	NCT03116113	II/III
	AAV2-REP1	Choroideremia	AAV2 encoding *rab escort protein 1 (REP1)* gene	NCT03496012	III
	AAV5-hFIXco-Padua	Hemophilia B	AAV5 encoding the Padua variant of FIX complementary	NCT03569891	III
	AAVrh10-h.SGSH	MPS IIIA	AAVrh10 encoding the N-sulfoglucosamine sulfohydrolase (SGSH)	NCT03612869	II/III
	AAV5-FVIII	Hemophilia A	AAV5 mediated gene transfer of hFVIII	NCT03392974	III
	rAAV2tYF-PR1.7-hCNGA3	Achromatopsia	rAAV2 expressing *CNGA3* gene	NCT02935517	II
	AAV9-GLB1	Type II GM1 gangliosidosis	AAV9 expressing *GLB1 (Beta-galactosidase)* gene	NCT03952637	II
	AAV2-hAADC	Aromatic amino acid decarboxylase deficiency (AADC)	AAV2 encoding *hAADC* gene	NCT02926066	II
	rAAV1-CMV-hGAA	Pompe disease	rAAV1 encoding acid alpha-glucosidase	NCT00976352	II
	rAAV2/5-hNAGLU	Sanfilippo syndrome B	rAAV2/5 encoding Alpha-N-acetylglucosaminidase	NCT03300453	II
	rAAV1.CMV.huFollistin344	DMD	rAAV1 encoding *Follistatin* gene	NCT02354781	II
	AAV2-REP1	Choroideremia	AAV2 encoding Rab-escort protein 1 (REP1)	NCT02553135	II
	AAV1/SERCA2a	Chronic heart failure	AAV1 encoding sarcoplasmic/endoplasmic reticulum calcium ATPase 2a (SERCA2a)	NCT00534703	II
	AAV2-GAD	Parkinson's disease	AAV2 encoding glutamic acid decarboxylase (GAD) gene	NCT00643890	II
	[6,6-2H2] glucose	Glycogen storage disease type IA	AAV8 encoding *glucose 6-phosphatase (G6PC)* gene	NCT04311307	II
	scAAV8OTC	Ornithine transcarbamylase (OTC) deficiency	AAV8 encoding *OTC* gene	NCT02991144	II
	ST-920	Fabry disease	rAAV2/6 encoding alpha galactosidase	NCT04046224	II

(Continued)

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Table 4. (Continued).

	Vector name	Indication	Transgene (mechanism of action)	Trial identifier	Phase
Retrovirus	Retroviral vector encoding ADA	Immunologic deficiency syndromes	Infusion of autologous CD34+ cells transduced with retroviral vector encoding *ADA* gene	NCT00598481	II
	γRV SF71pg91^{phox}	X-linked chronic granulomatous disease	CD34+ cells transduced by γRV SF71pg91^{phox} to express the gp91^{phox} subunit of the NADPH oxidase	NCT00927134	II
	γRV MDR1	Breast cancer	Retroviral transduction of the *MDR1* gene into peripheral blood progenitor cells	NCT00001493	II
	γRVL2SN-IDS	MPS II	T-lymphocytes, transduced with retrovirus L2SN expressing iduronate-2-sulfatase	NCT00004454	II
	γRV-1D3 HM CysTCR	Advanced melanoma	T cells will be transduced with a retroviral vector expressing the 1D3 HM CysTCR	NCT02654821	II
	Anti-MAGE-A3-DP4 TCR PBL	Cervical cancer Breast cancer Renal cancer	CD4 cells transduced with the retroviral vector expressing the anti-MAGE-A3-DP4 TCR	NCT02111850	II
	γRV-G-CSF	Fanconi anemia	CD34+ cells mobilization and collection after treatment with G-CSF	NCT02678533	II
	RV-TGFbDNRII	Solid tumors	TGFb DNRII-transduced autologous tumor infiltrating lymphocytes	NCT02650986	II
Lentivirus	OTL-200	Lysosomal storage diseases, metachromatic leukodystrophy	Autologous CD34+ cell enriched population that contains HSPC transduced ex vivo using a lentiviral vector encoding the human arylsulfatase A (ARSA) gene	NCT04283227	III
	CL20- 4i-EF1a-hyc-OPT	XSCID	Ex vivo transduction of the patient's autologous CD34+ HSC with lentivirus vector VSV-G pseudotyped CL20-4i-EF1a-hyc-OPT vector	NCT01306019	II
	OTL-101	SCID due to ADA deficiency	Autologous CD34+ cell enriched population that contains HSPCs transduced ex vivo using a lentiviral vector (LV) encoding the human adenosine deaminase (ADA) gene.	NCT04140539	III
	Lenti-D	Cerebral adrenoleukodystrophy (CALD)	Autologous CD34+ HSCs, transduced ex vivo with lenti-D vector encoding ABCD1	NCT03852498 NCT01896102	III
	OXB-102	Parkinson disease	Lentiviral vector expressing tyrosine hydroxylase (TH), aromatic L-amino acid decarboxylase (AADC) and GTP-cyclohydrolase (CH1)	NCT03720418	II
	βAS3-FB	Sickle cell disease	Autologous peripheral blood CD34+ Cells modified with the lenti/G-βAS3-FB vector	NCT02247843	II
	G1XCGD	X-linked granulomatous	Autologous CD34+ stem cells transduced by lentiviral expressing *GP91 (CYBB)* gene	NCT02234934	II
	AVR-RD-01	Fabry disease	Autologous CD34+ cell-enriched fraction that contains cells transduced with lentiviral vector/alpha-galactosidase A (AGA) expressing the human AGA	NCT03454893	II
	AVR-RD-02	Gaucher disease	Autologous CD34+ HSCs transduced with lentiviral expressing *GCase* gene	NCT04145037	II
	w1.6_hWASP_WPRE (VSVg)	WAS	Autologous CD34+ cells transduced with the w1.6_hWASP_WPRE (VSVg) lentiviral vector expressing *WAS* gene	NCT02333760	II
	RP-L201	Leukocyte adhesion defect – type I	CD34+ HSCs transduced ex vivo with lentiviral vector encoding the *ITGB2* gene	NCT03812263	II

(Continued)

Table 4. (Continued).

	Vector name	Indication	Transgene (mechanism of action)	Trial identifier	Phase
	LV-SGSH gene	MPS IIIA	Autologous CD34+ HSCs from MPS IIIA patients will be genetically modified *ex vivo* using CD11b.SGSH LV expressing the *SGSH* gene	NCT04201405	II
	LV-WAS	WAS	Autologous bone marrow derived CD34+ HSC transduced by lentiviral expressing *WAS* gene	NCT01410825	II
	RP-L102	Fanconi anemia	CD34+ cells transduced ex vivo with lentiviral vector carrying the FANCA gene	NCT04069533	II
Poxvirus	Pexa-Vec (JX-594)	HCC	Vaccinia GM CSF/TK-deactivated virus	NCT02562755	III
	Multipeptide CMV-MVA	BCR-ABL1 positive malignancies	The triplex vaccine is made up of 3 small pieces of synthetic CMV DNA placed into MVA	NCT04060277	II
	Pexa-Vec (JX-594)	Colorectal carcinoma	TK-deactivated vaccinia virus plus GM-CSF	NCT01329809 NCT03206073 NCT01394939	II
	Pexa-Vec (JX-594)	Colorectal carcinoma	TK-deactivated vaccinia virus plus GM-CSF	NCT01380600 NCT01469611	I
	MVA.HTI	HIV/AIDS	HIV-1 vaccines MVA.HTI and ChAdOx1.HTI With TLR7 agonist vesatolimod (GS-9620)	NCT04364035	II
	NYVAC-C	HIV/AIDS	NYVAC-C vaccination	NCT00490074	II
	ALVAC	Metastatic melanoma	TCR-engineered T cell therapy targeting NY-ESO-1 and melanoma differentiation antigens MART-1	NCT00610311 NCT00612222 NCT00670748	II
	ALVAC-CEA-B7.1	Colorectal cancer	ALVAC-CEA vaccine is a cancer vaccine containing a canary pox virus (ALVAC) combined with the carcinoembryonic antigen (CEA) human gene	NCT00027833	II
	GL-ONC1	Advanced cancers (solid tumors)	Engineered attenuated vaccinia virus prefer to locate, colonize, and destroy tumor cells	NCT00794131	I
	MVA/EBNA1-LMP2	Ppstein-Barr virus+ cancers	EBNA1 C-terminal/LMP2 chimeric protein-expressing recombinant MVA vaccine	NCT01147991	I
Herpes simplex virus	G207	Glioblastoma	Engineered HSV-1 kill tumor cells	NCT00028158	II
	Talimogene laherparepvec (T-VEC)	Breast cancer	T-VEC replicates in cancer cells induces antitumor immunity	NCT02779855	II
	OH2	Melanoma	HSV-1 encoding GM-CSF	NCT04386967	I
	HSV-1716	Recurrent high-grade glioma	HSV-1716 kills brain tumor cells and induces anti-tumor immunity	NCT02031965	I
	TBI-1401	Solid tumor	TBI-1401 targets tumor cells	NCT02428036	I
	T-VEC	Melanoma	T-VEC replicates in cancer cells induces anti-tumor immunity	NCT03747744	
	rQNestin34.5v.2	CNS cancers	Engineered HSV-1 replicates in CNS tumor cells	NCT03152318	I
	M032	CNS cancers	Engineered HSV-1 expressing IL-12	NCT02062827	I
	ONCR-177	Advanced solid tumor	Engineered HSV-1 kill tumor cells	NCT04348916	I
	C134	Glioblastoma multiform	IRS1-chimeric HSVl kill cancer cells	NCT03657576	I
Alphavirus	AVX701	Solid tumors	Vector encoding CEA immunogen	NCT00529984	II
	Vvax001	Cervical Cancer	Vector encoding HPV-derived tumor antigens	NCT03141463	I
	AVX901	HER2+ cancers	Immune system attacks HER2+ cancer cells	NCT01526473	I
	AVX701	Stage III of colon cancer	Vector encoding CEA immunogen	NCT01890213	I

(Continued)

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Table 4. (Continued).

	Vector name	Indication	Transgene (mechanism of action)	Trial identifier	Phase
	AVX101	HIV infections	vaccine replicon expresses the gag gene subtype C isolate of HIV-1	NCT00063778	I
Measles virus	MV-NIS	Multiple myeloma	Measles virus expresses human NIS gene	NCT02192775	II
	MV-NIS	Gynecologic cancer	Oncolytic measles virus encoding thyroidal sodium iodide symporter	NCT02364713	II
	TMV-018	Gastrointestinal cancer	Oncolytic measles virus encoding the prodrug converting enzyme super cytosine deaminase	NCT04195373	II
	Attenuvax	Advanced or metastatic NSCLC	An attenuated measles virus delivered sub cutem as a single agent to measles virus-positive NSCLC	NCT00828022	II
	MV-NIS	Recurrent or refractory plasma cell myeloma	Oncolytic measles virus encoding thyroidal sodium iodide symporter	NCT00450814	II
Reovirus	TOCA-511	Glioblastoma	TOCA-511 targets tumor cells	NCT04105374	III
	Pelareorep	Recurrent gynecologic carcinoma	Unmodified human reovirus targeting tumor cells	NCT01199263	II
	Wild type Reovirus	Advanced and recurrent pancreatic carcinoma	Reovirus targets tumor cells	NCT01280058	II
	Reolysin	NSCLC	Reolysin targets tumor cells	NCT00861627	II
	Reolysin	Metastatic melanoma	Reolysin targets tumor cells	NCT00984464	II
Coxsackieviruses	CVA21 (CAVATAK)	Malignant melanoma	Tumor cells oncolysis mediated by CAV21	NCT01227551	II
	CAV21	Advanced melanoma	Tumor cells oncolysis mediated by CAV21	NCT04152863	II
	CAV21	Melanoma	Tumor cells oncolysis mediated by CAV21	NCT01636882	II
	CAV21	Melanoma	Tumor cells oncolysis mediated by CAV21	NCT04303169	II
	CVA21	NSCLC	Tumor cells oncolysis mediated by CAV21	NCT02824965	I
Vesicular stomatitis virus	Voyager-V1 (VV1|)	Melanoma, HCC, NSCLC, endometrial cancer	VV1 also known as VSV-IFNβ-NIS, is an oncolytic virus therapy	NCT04291105	II
	VSV-IFNβ-NIS	Malignant solid tumor	VSV genetically engineered expressing NIS and human interferon Beta	NCT02923466	I
	VSV-IFNβ-NIS	solid tumor, HCC, NSCLC	VSV genetically engineered expressing NIS and human interferon Beta	NCT03647163	I
	VSV-hIFNbeta-NIS	Endometrial cancers and advanced stages of uterine corpus carcinoma	VSV genetically engineered expressing NIS and human interferon Beta	NCT03120624	I
	VSV-IFNβ-NIS	Refractory or recurrent leukemia and lymphoma	VSV genetically engineered expressing NIS and human interferon Beta	NCT03017820	I
Poliovirus	PVSRIPO	Malignant glioma	Oncolytic polio/rhinovirus has anti-tumor response and survival	NCT02986178	II
	PVSRIPO	Brain tumors	Oncolytic polio/rhinovirus has anti-tumor response and survival	NCT03043391	Ib
	PVSRIPO	Melanoma	Oncolytic polio/rhinovirus has anti-tumor response and survival	NCT03712358	I
	PVSRIPO	Glioblastoma	Oncolytic polio/rhinovirus has anti-tumor response and survival	NCT01491893	I
	PVSRIPO	Invasive breast cancer	PVSRIPO induces inflammation, innate and adaptive immune responses in tumor cells	NCT03564782	I

They have a diploid ssRNA genome encoding three genes of gag (structural proteins), pol (the enzymes of reverse transcriptase, integrase, and protease that are packaged with the RNA molecule), and env (the envelope proteins) are flanked on both sides by long terminal repeats (LTRs).[161] Gammaretroviruses and lentiviruses are subtypes of RVs, which contain the reverse transcriptase enzyme that converts the RNA genome into DNA in the transduced cell. These viruses are used in clinical gene therapy trials; however, the use of gammaretroviral vectors is more common. The virus entry mechanism into the target cell

is receptor dependent. Gammaretrovirus vectors that are used for gene therapy applications are derived from the murine the leukemia virus (MLV) by replacing the gag, pol, and env genes with a transgene. The ψ packaging sequence and LTRs are kept, as they are essential for vector production and inserting the viral genome into the target DNA, respectively. Thereafter, during virus production, a packaging cell line to produce recombinant viral particles is essential.[162] RVs efficiently infect dividing cells such as human stem cells during mitosis when the nuclear envelope is disassociated and incorporates into the target genome, demonstrating stable genetic expression/correction. Recombinant RV delivers a 9–12 kb therapeutic gene and produces high titers. RV shows the classic method for long-term gene therapies.[163,164]

Transgene expression of RV vectors was optimized along with progress in molecular cloning strategies. Generation of replication-defective RV vectors and capsid modified with the G protein from the viral envelope of the vesicular stomatitis virus (VSV-G) expanded its tropism and target cell types. The most important disadvantages of RV gene therapy vectors are the lack of cell specificity, as well as the possibility of insertional mutagenesis as it prefers to integrate the transgene into active transcription sites, CpG islands, and conserved noncoding DNA sequences. In spite of concerns related to oncogene activation or tumor suppressor deactivation, RV vectors are considered to be the best delivery systems for ex vivo gene therapy application.[165–168] Insertional mutagenesis and integration patterns near protooncogenes have increased the risk of leukemogenesis. This malignancy has been reported in the clinical trials that used RVs for severe combined immunodeficiency-X1 (SCID-X1), chronic granulomatous disease, and Wiskott-Aldrich syndrome therapies. To address this genotoxicity, a number of engineered RVs have been developed to ensure long-term gene expression/correction but reduce the risk of insertional mutagenesis.[169–172]

RV was initially used for adenosine deaminase-SCID (ADA-SCID) and X-SCID treatment.[173] Moreover, the first human gene therapy trial that used transplantation of autologous bone marrow stem cells was transduced by a RV vector.[174] Over 500 gene therapy clinical trials have been reported using RV to date. Recently, a nonintegrating RV-based CRISPR/Cas9 has been generated that caused no insertional mutagenesis.[175] During the last decade, five biological products based on engineering RV have been approved for the gene therapy market. Rexin-G, the only approved RV vector for in vivo application, contains cyclin G1 gene that was approved by FDA in 2010. It suppresses proliferation as well as promotes apoptosis in cancer cells. Exposed to collagenous proteins it also targets damaged tissues (e.g., inflamed lung, kidney, etc.). Thus, it is in the vicinity of activated T cells stimulated by COVID-19. The DeltaRex-G then enters the rapidly dividing T cells and induces cell death by arresting cell cycle at G1 phase, hence, reducing cytokine release and acute respiratory distress syndrome (ARDS) (NCT04378244). The other approved clinical products are genetically modified cells and are used as ex vivo procedure. Strimvelis is a gene therapy product, autologous CD34 hematopoietic stem cells (HSC) expressing ADA transduced by a RV vector. Zalmoxis are allogenic T cells expressing HSV-TK suicide gene transduced by a retroviral vector. Both Strimvelis and Zalmoxis were approved

by the EMA in 2016. Invossa are retrovirally transduced allogenic chondrocytes, that expresses TGF-β1 and it was approved in South Korea in 2017. Finally, Yescarta a CAR T cells product was approved by the FDA in 2017. It consists of autologous T cells targeting CD19 transduced by a RV vector.[176–180] Recently, a new gene therapy product engineering via a replication-competent RV was approved. The new product of Ecartus (also named brexucabtagene autoleucel) is a CD19-directed genetically modified autologous T cell immunotherapy. It is the first and only FDA-approved CAR T-cell therapy product for adult patients with relapsed or refractory mantle cell lymphoma (R/R MCL).[181]

3.4. Lentiviral Gene Delivery

Like RVs, lentiviruses (LVs) have a ssRNA genome with the size of 9.75 kb and integrate into the genome of their host cells. They contain three genes of gag, pol, and env and are derived from human immunodeficiency virus (HIV-1). In contrast to RVs, LVs are more complex and require more genes for their physiopathology. The engineered LVs are capable of efficient gene delivery to both dividing and nondividing target cells. They access the host genome by active transport through the nuclear pores. During the integration process, the virus prefers to insert within active transcriptional blocks. In the past two decades, LVs have been extensively optimized to ensure safe and efficient delivery by manipulation of viral elements to increase their transduction efficiency and minimize internal homologous recombination. LVs offer strong potential for ex vivo gene therapy applications. With their capacity of 8 kb transgene delivery, LVs offer strong potential for ex vivo gene therapy applications.[163,182,183]

The first-generation of LV-based gene therapy vectors contained the majority of the HIV-1 genome, including the gag, pol and other viral genes (accessory genes of vif, vpr, vpu, and nef, as well as the regulatory genes of tat and rev). It also contained VSV-G, encoded on a separate plasmid, which recognizes the low-density lipoprotein (LDL) receptor, thereby increasing LV tropism.[184,185] Accessory virulence genes of vif, vpr, vpu, and nef were removed in the second generation of LV vectors. However, the vector still was able to integrate in the host genome.[186] In the third generation of RV vectors, the tat gene was deleted and the gag/pol genes and rev/env genes were expressed from a separate plasmid. Deletions of the 3'-LTR of the viral genome resulted in self-inactivating (SIN) LV which removed the enhancer or promoter of the LTR.[187] Third-generation LVs have been used in clinical trials to deliver genes into nonproliferating or slowly proliferating cells such as HSCs, to treat hemoglobinopathies and primary immunodeficiency's. These also have been used for genetic manipulations of mature T cells to deliver chimeric antigen receptors and create tumor-specific cytotoxic T cells.[188,189] A new LV vector termed LTR1, is considered the fourth-generation of LV gene therapy vectors. It is more efficient and safer as well as delivers bigger transgenes in comparison with those LVs of previous generations. It was engineered by a reconfiguration of the genetic elements that control the complex replication event of the virus.[190]

The major concern of insertional mutagenesis with RV vectors remains the same with LV vectors, to potentially induce

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oncogenes during gene therapeutic applications.[191] However, the clinical data from the latest generation of LV vectors demonstrate a remarkably decrease of genotoxicity and leukemogenesis in patients.[192] In addition to ex vivo usages, LV vectors have also being applied for in vivo for gene therapies. A LV vector was engineered to express endostatin and angiostatin and an intraocular injected to the eye for the treatment of neovascular age-related macular degeneration (NVAMD).[193] In vivo applications of LVs harbor many barriers such as efficiency, tissue-specific promoters, and immunogenicity. Host cell membrane proteins including MHC molecules can be integrated into the LV envelope during the budding event. Subsequently, this leads to alloimmune reactivity and limits LV vector survival. Removing HLA class I genes from a packaging cell line, as well as gene manipulations of LV envelope proteins, greatly improve the stability of LV vectors in the serum.[194,195]

The first clinical trial that used LV vector was conducted in 2005. It delivered an antisense RNA, targeting the HIV envelope gene as well as transduced mature peripheral blood T cells for HIV infection treatment.[196] Later, genetically modified CD34+ HSCs that were targeted by LV vectors were reported in the clinical trials for treatment of several inherited disorders including X-linked adrenoleukodystrophy, β-thalassemia, Wiskott-Aldrich syndrome, and metachromatic leukodystrophy. Interestingly, using LV vectors revealed no adverse effects in the discussed trials.[192,197–199] CAR T-cell therapies using LVs have been successfully tested in patients with B-cell malignancies resulting in approval of the first genetically engineered cellular therapy using LVs. Kymriah is the first FDA-approved cell-based gene therapy product that is based on a RV which is being used in the clinic and market since 2017.[189] Initial results from two clinical trials using ex vivo autologous CD34+ HSCs and progenitor cell-based LV gene therapy for X-linked chronic granulomatous disease (X-CGD), show first promising results.[200] Zynteglo also known as Lentiglobin-BB305 is based on an ex vivo transplantation of HSCs genetically manipulated by LVs transferring HBB expression. The drug was approved by EMA in 2019.[201] Recently in 2020, two cell-based gene therapy products engineered by LVs, Liso-cel (Breyanzi) and OTL-200 (Libmeldy) were approved by FDA and EMA, respectively. Liso-cel also named as lisocabtagene maraleucel is a CAR T-cell therapy designed to target CD19-expressing cells in B-lymphomas. OTL-200 is an ex vivo, autologous, CD34+ hematopoietic stem cell-based gene therapy product designed to treat metachromatic leukodystrophy (MLD). Mutation in the arylsulfatase-A (ARSA) gene is responsible for the disease that results in sulfatides accumulation in the brain and other parts of the body. The cells are transduced ex vivo using a LV gene therapy vector encoding the ARSA gene.[194] In March 2021, Abecma (Idecabtagene vicleucel), a B-cell maturation antigen (BCMA)-directed CAR-T cell immunotherapy was approved by the US FDA for the treatment of adult individuals with relapsed or refractory multiple myeloma after four or more prior lines of therapy, including an immunomodulatory agent, a proteasome inhibitor, and an anti-CD38 monoclonal antibody. This product binds to BCMA, a surface protein expressed by myeloma cancer cells. The cells transduced ex vivo using an LV vector encoding the BCMA gene.[202]

3.5. Poxviral Gene Delivery

Poxviruses are complex, large, and enveloped viruses, which belong to the *poxviridae* family. They have a 190–200 kb linear dsDNA genome encoding more than 200 genes. Poxviruses are easily engineered and are capable to deliver therapeutic genes with the size of 25–40 kb. As they solely replicate in the cytoplasm of the target cells, there is no risk of viral DNA integration into the target cells genome. Poxviruses also have the potential to be prepared at high titers.[203–207] Two common poxvirus vectors that are used in gene delivery applications including vaccinia virus (VACV) and myxoma virus (MYXV). These two vectors are currently being used as anticancer therapy and oncolytic therapeutic vehicles. Additionally, MYXV has been applied in clinical trials of immunotherapies, cancer progenitor cells purging and graft-versus-host diseases treatments.[208,209] VACV is the best-characterized virus among all the poxviruses as it was initially utilized as a smallpox vaccine. The engineered VACV vector expresses various immunizing antigens used to target several cancer types. Rapid viral particle preparation, wide host range tropism, high capacity for transgene delivery and safe manipulations render poxvirus a proper viral tool for gene therapy application. In general, the major challenges with poxvirus vectors as oncolytic vehicles are the systemic delivery of the viral vectors and targeting metastatic tumor sites. For a successful therapy, high viral titers at tumor sites are essential but are hampered by the clearance of the intravenously injected virus.[210]

Four types of manipulations have been applied to poxviruses to improve their efficiency and safe gene delivery. First, gene modifications of viral genes produced more immunogenic vectors and thus induce antitumor immunity activity. For instance, viral genes of *A52, B15,* and *K7* coordinately suppress NF-κB pathway. Deletion of these genes activates NFκB, inducting proinflammatory cytokine/chemokines responses and attracting antigen-presenting cells at the infection sites. This consequently improves T cell responses against VACV-encoded antigens.[211,212] Moreover, the deletion of viral genes including thymidine kinase (TK) and vaccinia growth factors (VGF) leads to an increased tumor targeting by retaining viral replication in dividing tumor and cancer cells.[213,214] The second type of gene manipulations focused on the induction of antitumor immunity. Arming the VACV with genes encoding tumor-specific antigens to stimulate interferon genes, enhanced T cell-specific immunity.[215,216] The third type of manipulations was arming the virus to improve immunogenicity events. These include tumor-specific antigens and immune costimulatory molecules.[217–219] Finally, the fourth type of manipulations combined these vectors with alternative immunomodulatory regimens, such as immune checkpoint blockade.[220,221] Thereafter, safe and efficient VACVs have been engineered by coupling them with proapoptotic and immune-stimulatory genes. This could induce tumor cell death and enhanced recognition of tumor antigens by T cells.

Wild type of VACV was initially applied in a clinical trial for immunotherapy of patients with melanoma in 1995; however, the result was not promising. The first engineered VACV known as Pexa-Vec (pexastimogene devacirepvec) harboring the human granulocyte-macrophage colony-stimulating factor (GM-CSF) gene was used for melanoma patients in 1999.[222,223]

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Pexa-Vec is currently being evaluated in a phase III clinical trial for patients with advanced hepatocellular carcinoma (HCC).[224] Several engineered VACV vectors termed MVA, NYVAC, AL-VAC, MVA-5T4, rV-PSA, TG4010, and PANVAC have been reported in clinical trials for cancer therapy and vaccine development. As they are highly attenuated, host-restricted, poorly, or non-replicative. Furthermore, VACVs have been extensively engineered to express attenuated antigens for vaccine development against HIV, hepatitis B, herpesvirus, influenza, and other viruses.[203,225–227]

3.6. Herpes Simplex Viral Gene Delivery

Herpes simplex viruses (HSVs) are largely enveloped viruses with a dsDNA genome belong to the *Herpesviridae* family. The genome is 150 kb in size and contains 100–200 genes. There are two types of HSV, HSV-1, and HSV-2. Their genomes are complex and are divided into long and short unique parts (UL and US) which are flanked by inverted repeated sequences (TRL/IRL and TRS/IRS). HSV-1 has been widely explored in cancer treatment. Half of its genes are non-essential and are deleted in the engineered vector. Transcription, replication, and packaging of HSV-1 genome take place in the nucleus of the target cells as it needs the RNA polymerase II enzyme.[228,229] Engineered HSV has high capacity to deliver long transgenic DNA up to150 kb in size. The genetic complexity of the HSV genome is a unique feature as it is permitting to generate various types of attenuated viral gene delivery carriers. The engineered HSVs reveal wide oncolytic activities, strong long-term gene expression, and ability of gene delivery to the neurons.[230–232]

Amplicon, replication-defective and attenuated vectors, are three main groups of engineered HSV-1 for clinical applications. Amplicon vectors as the first-generation of engineered HSV-1, have similar structural, immunological features and a host-range comparable wild-type HSV-1 that contains a concatemeric DNA termed amplicon instead of the viral genome. It delivers multiple copies of a large transgene. Vector preparation is easy and toxicity is limited as viral coding genes were deleted.[230,233] Essential viral genes such as *IE* gene for in vivo and in vitro replication have been abolished in the second-generation of HSV-1 vectors, called replication-defective. Furthermore, deletion of *VP16* and *gH* genes inhibit replication or spread of HSV-1. These strategies produce safe, nontoxic and replicative-defective HSV-1 vectors that deliver antineoplastic genes, targeting cancer types such as malignant glioma.[234–236] The third-generation known as, attenuated HSV vectors, are a moderate form of HSV that includes the essential genes for in vitro replication; however, it could not replicate in vivo. It is utilized as a protective and therapeutic vaccine against cancer and other human diseases. Low immune response, transferring multiple genes, transducing variety of cells, low risk of side effects, delivering antiviral drugs and proliferating in tumor cells are advantages of this third generation of HSV based gene therapy systems. Furthermore, it is nonintegrative and has the capacity to deliver transgenes up to 50 kb in size. This is critical for therapies of some diseases that need large therapeutic gene bodies containing entire regulatory elements. Although, the major barrier in engineering HSV vectors for gene delivery to CNS is related to the establishment of a latent-like infection

that permits long-term therapeutic gene expression, without activation of viral genes that encode for cytopathic proteins or are essential for reactivation.[237–240]

Engineered oncolytic HSVs have been initially investigated as an oncolytic antitumor therapy against different types of tumors in glioblastoma, breast, colon, liver, prostate, pancreas, and head and neck carcinomas.[241–245] Imlygic is the only FDA-approved gene therapy product based on the engineering of HSV that is being used for the treatment of metastatic melanoma. It expresses human *GM-CSF* gene, selectively targets cancer cells, and induces antitumor immune responses.[246]

3.7. Other Viral Gene Delivery Tools

Six viral vectors discussed are quite common gene delivery vehicles for gene therapy applications. However, there are some other classes of viral systems that are less common. They include engineered or natural form of alphavirus, poliovirus, measles virus, reovirus, vesicular stomatitis virus, and coxsackievirus, and are being used as oncolytic viral vectors for cancer therapy applications. Their main features are listed in **Table 5**.

Alphaviruses are enveloped ssRNA virus with total genome size ranges between 11 and 12 kb. The virus recognizes cell surface receptors of laminin and heparin, which facilitates delivery of the RNA genome to the cytoplasm. Alphavirus-based gene therapy vectors have been largely engineered to provide the highest biosafety level for in vivo therapeutic applications. The engineered vectors are attenuated or replication-deficient with the wide host range infection. Three types of engineered alphavirus vectors known as naked RNA, recombinant virus, and DNA/RNA layered vectors, have been utilized preclinical and clinical trials particularly in oncology area. Alphavirus vectors delivering therapeutic or toxic genes administrated by intratumoral injections have revealed efficient tumor regression.[247–249] Some clinical gene therapy trials used alphavirus are listed in Table 4.

Polioviruses, ssRNA viruses are one of the most dangerous human viral pathogens and they have been robustly investigated over the last years. They have been considered as major viral vectors for the gene delivery and therapy of CNS-related indications as during the infection, the virus is almost exclusively restricted to neuron cells. Then, this natural neurotropism makes the PV vector a candidate to target CNS tumors, although targeting breast cancer, bone, and soft tissue sarcomas has been reported.[250,251] A number of clinical trials used poliovirus are listed in Table 4.

Measles viruses belong to *Paramyxoviridae* family. They are enveloped viruses containing nonsegmented ssRNA genome with about 16 kb in size. The measles viruses are well known by their tumor selectivity and oncolysis activities. High degree of tumor-suppressive potential, as well as proper safety reports as a viral vaccine, makes measles viruses a suitable delivery system for cancer therapy applications. Recent progresses in gene manipulation of measles viruses, resulted in generating recombinant viruses allowing noninvasive monitoring of viral replication/spread as well as immune-evasive that enhance its efficacy and efficiency.[252,253] A number of clinical cancer gene therapy trials which used measles viruses are listed in Table 4.

Table 5. Marked features of less-common viral gene delivery vectors.

Features	Alphavirus	Measles virus	Reovirus	Coxsackieviruses	Vesicular stomatitis virus	Poliovirus
Family	*Togaviridae*	*Paramyxoviridae*	*Reoviridae*	*Picornaviridae*	*Rhabdoviridae*	*Picornaviridae*
Genome (size)	dsRNA (11–12 kb)	ssRNA (16 kb)	dsRNA (16-27)	ssRNA (7.4 kb)	ssRNA (11 kb)	ssRNA (7.5)
Capsid and coat	Icosahedral and naked	Helical and enveloped	Icosahedral, nonenveloped	Icosahedral, nonenveloped	Helical and enveloped	Icosahedral, enveloped
Virus size (nm)	60–70	100–300	60	30	70	30
Packaging capacity	8 kb	6–8 kb	–	6 kb	4.5 kb	6 kb
Infection type	Dividing cells	Dividing cells	Dividing cells	Dividing cells	Dividing cells	Dividing cells
In/ex vivo applications	In vivo	In vivo	In vivo	In vivo	In vivo	In vivo
BBB-crossing	No	No	No	Yes	No	Yes
Transducing units mL^{-1}	1×10^8	0.5×10^8	1×10^{13}	1×10^9	1×10^6 to 10^8	1×10^9
Entry mechanism	Receptor interaction and low pH mediated endocytosis	SLAM dependent endocytosis	Dynamin dependent endocytosis	ICAM-1 dependent endocytosis	Clathrin dependent endocytosis	CD155 dependent membrane fusion
Integration and transgene expression	Nonintegrating, transient	Nonintegrating, transient	Nonintegrating	Nonintegrating, transient	Nonintegrating, transient	Nonintegrating, transient

Vesicular stomatitis viruses (VSVs) are enveloped ssRNA viruses, containing five genes, which prefer to replicate in malignant and cancer cells. They finally induce cell death in the cells. VSVs have several advantages as an oncolytic viral vehicle to target tumor cells. For instance, they are not gene attenuated which interferes with oncolytic antitumor activity. Moreover, VSVs replicate in different types of tumor cells and not in those cells harboring mutant tumor suppressors such as *Tp53* gene. Finally, engineered VSV vectors include immunomodulatory and/or suicide genes for further antitumor activity.[254,255] Some of the clinical trials which used VSVs are summarized in Table 4.

Reoviruses belonging to the *Reoviridae* family are nonenveloped human viruses with several segments of dsRNAs genome. Reovirus selectively infects cells with activated Ras and cMyc signaling pathways. The genes involving in these pathways are the most frequently mutated oncogenes in cancers. Furthermore, reovirus naturally has oncolytic activity in tumor cells that have an abnormality in *TP53*, *ATM*, and *RB* genes. Thus, reovirus directly targets and lyses cancer cells, interferes with tumor immunosuppressive mechanisms, introduces immune surveillance, and induces wide antitumor responses.[256–258] Some clinical trials for cancer treatment mediated by reoviruses are listed in Table 4.

Coxsackieviruses, enteroviruses belonging to the *Picornaviridae* family with an ssRNA genome are being used in several clinical trials. Their engineered form, CVA21 (CAVATAK) acts as oncolytic virus that showed promising results as an Imlygic gene therapy drug in the treatment of melanoma cancer and other solid tumors. CVA21 recruits decay-accelerating factor (DAF) to attach to the target cells and intercellular adhesion molecule 1 (ICAM-1) to enter the cells. Upregulation of ICAM-1 has been reported in many malignant tumor cells. Thus, this could trigger tumor cell oncolysis mediated by CVA21 viral gene delivery.[259–261] A few of the clinical trials that used CVA21 are summarized in Table 4.

4. Nonviral Gene Therapy Systems

Therapeutic nucleic acids administrated naked or coated with nanoparticles belong to nonviral gene therapy systems. Most nonviral delivery systems use biocompatible materials such as lipid, chromosomes, plasmid, cationic polymers, and conjugate complexes, so they are less immunogenic than viral vectors.[262,263] Therapeutic nucleic acids in nonviral gene therapy systems include plasmid DNA (pDNA) and messenger RNA (mRNA) as large therapeutic nucleic acids and RNA interference (RNAi), DNA interference (DNAi), antisense oligonucleotides (ASOs), microRNAs mimics, aptamers, and CpG oligodeoxynucleotides (CpG-ODNs) as small ones (**Figure 4**, **Table 6**).[264–268] A successful gene therapy needs a proper gene delivery system with low immunogenicity, high cell specificity, and high efficiency to deliver an adequate amount of transgene to cause the desired effect. In comparison to viral vectors, nonviral vectors are safer, of low cost and more reproducible, plus they also have no limitation in nucleic acid size for packing. However, the main limitation of non-viral vectors is their low transfection efficiency[18,269] and low cell specificity. The delivery procedures used for therapeutic nucleic acids are categorized into physical and chemical methods.[270–272]

Physical methods: The physical methods include needle injection, ballistic injection, sonoporation, photoporation, magnetofection, electroporation, and hydroporation that introduce genetic materials through the cell membranes. Whereas, needle injections bring the genetic materials directly into the cells, ballistic DNA injections shoot DNA particles coated with gold into the tissue. Electroporation, sonoporation, and photoporation are methods that respectively use electrical pulse, sound wave and laser pulse to create pores in a cell membrane to allow entry of therapeutic DNA or RNA. Magnetofection is a transfection method that uses magnetic fields to concentrate magnetic particles containing nucleic acid into the target cells. Finally,
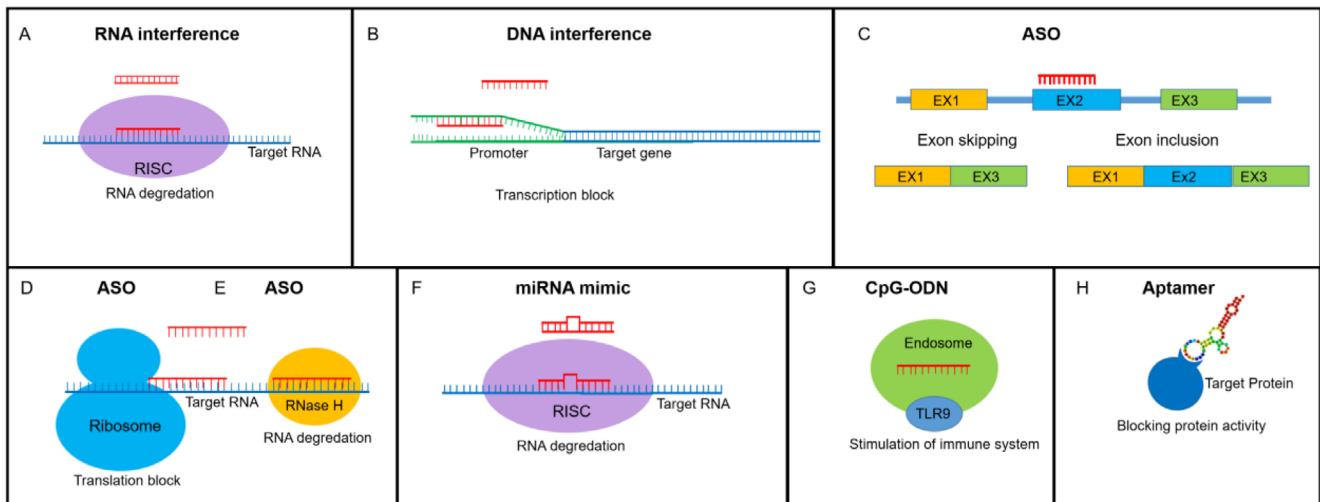
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Figure 4. Therapeutic oligonucleotides. A) RNAi induces RNA degradation by RISC. B) DNAi targets the promoter region of the target gene and interferes with its transcription. C–E) Three classes of antisense oligonucleotides cause exon skipping, block translation, and RNase H-mediated RNA degradation, respectively. F) miRNA mimic induces RNase H-mediated RNA degradation. G) CpG-ODNs function as an agonist of TLR9 and induce immune responses. H) Aptamer oligos interfere with functional domain(s) of the target proteins and blocking their activity.

hydroporation is a hydrodynamics-based transfection that manipulates cell permeability.[270]

Chemical methods: The chemical methods for gene delivery systems use synthetic or natural biodegradable particles, including cationic polymeric-based, cationic lipid-based, and peptide-based particles to facilitate the transfer of nucleic acids into the cells. Chemical vectors can interact with nucleic acids electrostatically and enter the cells by endocytosis.[18] Cationic polymeric-based and cationic lipid-based particles are the most common complexes that are being used in clinical applications. Cationic lipid-based delivery systems are classified into cationic liposomes, cationic emulsions, and solid lipid nanoparticles (LNPs). LNPs formulation typically consist of a cationic molecule, a linker and hydrophobic tails.[273] Furthermore, cationic polymeric-based systems include poly lactic-co-glycolicacid (PLGA), poly lactic acid (PLA), polyethylene imine (PEI), chitosan, dendrimers and polymethacrylates. In order to increase the stability and decrease the toxicity, phosphoethanolamine gylation (PEGylation) is incorporated into lipidic and polymeric nucleic acid nanocarriers.[274,275] Additionally, cationic lipid/nucleic acid complex (lipoplex), cationic polymer/nucleic acid complex (polyplexes) and cationic polymer/cationic lipid/nucleic acid (lipopolyplexes) are synthetic composites that condense therapeutic nucleic acids into small particles, protecting from enzymatic degradation and could improve proper delivery inside the target cells by interacting with the negatively charged cell membrane.[272,276,277] Altogether, these strategies achieve short-term effects, less immunogenicity, and safe clinical gene deliveries.

4.1. Naked Plasmid DNA

Using naked plasmid DNA (pDNA) or a naked PCR product are the simplest non-viral gene therapy methods. Naked pDNA and PCR product, without any carrier, can be administrated efficiently ex vivo and in vivo via electroporation, intravascular delivery, IM injection, and "gene gun" methods. Increasing the efficiency of these methods has made naked DNA gene delivery a safe procedure for gene therapy of genetic diseases.[278,279] Naked DNA is an attractive nonviral transgene expression device as it is easily generated via recombinant DNA techniques and conventional PCR methods. Naked DNA delivery efficiency is greatly inhibited by some obstacles including the size, shape, and polyanionic charge of the DNA. These affect the cell permeability of DNA as well as DNA survival from serum nucleases. However, coating the naked DNA by polycationic lipids, polymers and inorganic materials, significantly enhance the interaction with cell surface and cell uptake of DNA.

Direct gene delivery to skeletal muscle was one of the first gene therapy practices that was used in the clinic, as it particularly is an ideal target for local gene delivery. pDNA, naked or coated is considered moderately efficient for human gene therapy when locally injected. However, it is very inefficient and toxic in some cases, when administrated systemically. Naked DNA was initially used for the treatment of limb ischemia or peripheral arterial occlusive disease (PAOD), via IM injection of a pDNA encoding human vascular endothelial growth factor (VEGF). Patients revealed significant clinical improvement of vascular ankle-brachial index and electrophysiologic measures in the treated limb. Similar outcomes were observed in a clinical trial using IM injection of pDNA encoding FGF-1 gene in patients with lower leg ischemia. No change in FGF-1 serum levels was observed.[280,281]

In addition, "sleeping beauty" transposon is considered as a nonviral naked DNA gene delivery that is simple and cost-effective. Furthermore, it has acceptable safety parameters for human gene therapeutic applications. This delivery system is encoded by two pDNAs. One plasmid has a transposon structure containing ITRs that harbor a transgene and the other one encoding the transposase enzyme. Following the unset of the transposon expression, the transposase enzyme distinguishes the ITRs,

Table 6. Marked features of therapeutic oligonucleotides.

	RNAi	DNAi	SSO	ASO	Gapmer	Aptamer	miRNA mimic	CpG ODN
Structure	dsRNA	ssDNA	ssDNA	ssDNA	ssDNA	ssRNA/DNA	dsRNA	ssDNA
Size	20–25 mer	24 mer	13–50 mer	13–50 mer	16 mer	15–50 mer	17–27 mer	18–28 mer
Modification								
Mechanism of action	RISC dependent for mRNA degradation	Inhibition of gene expression	Exon skipping	RNase H-dependent degradation of RNA targets	RNase H-dependent degradation of RNA targets	Physically interacts with target protein and inhibits its function	RISC dependent for mRNA degradation or suppressing translation	Immunostimulatory TLR9 agonist
Administration	Intravenous infusion	Intravenous infusion	Intravenous infusion	Subcutaneous/Intravenous infusion	Intravenous infusion	Local injection	Local injection	Local injection
Approved drug	Patisiran Givosiran	–	Eteplirsen Casimersen Viltepso	Vitravene Spinraza Mipomersen Tegsedi Waylivra	–	Macugen	–	Defibrotide

cuts the transposon unit from the first plasmid and incorporates it into the genome, resulting in a permanent expression of the transgene. After several successful preclinical evaluations, it was eventually applied in the clinics to treat cancers and metabolic disorders. Recently, it is being used in CAR T-cell technology. In comparison to RV and LV vectors, the manufacturing of anti-tumor cell products based on the sleeping beauty transposon system is efficacious, safe, and cost-effective.[282–288]

Type 1 diabetes (T1D) is caused by intense inflammatory and autoimmune responses that damage pancreatic β cells. Proinsulin is a main target of the immune response and is often observed as the first adaptive immune response. Recently, the first serious clinical gene therapy for T1D, a naked plasmid (TOL-3021) for encoding proinsulin, has been used in a number of clinical trials that present promising results. TOL-3021 administered intramuscularly, induces immune tolerance and reduces the number of CD8+ T cells reactive to proinsulin, thereby reducing β-cell autoimmunity and improves insulin secretion.[289,290] Plasmid DNA encoding the 165-amino-acid isoform of human VEGF (pCMV – VEGF165), also named Neovasculgen (Pl-VEGF165) was approved by Russian Ministry of Healthcare in 2012 for treatment of atherosclerotic peripheral arterial disease (PAD).[291,292] In March 2019, collategene, the first gene therapy product in Japan, obtained Japan Ministry of Health, Labour and Welfare (MHLW) approval to treat critical limb ischemia. This naked plasmid encodes the human hepatocyte growth factor (HGF) as a potent angiogenic hormone to induce neoangiogenesis and improve ulcers in ischemic disease.[293,294]

4.2. mRNA

In vitro-transcribed (IVT) mRNA is found for in vivo transient protein expression to reverse a genetic disorder. The IVT mRNA has the same structures as endogenous mRNA, containing a 5′-cap, open reading frame (ORF), UTRs and a 3′-poly(A) tail. Over the past decade, mRNA-based drugs have been used for some cancers and infectious diseases vaccination, cancer immunotherapy as well as genetic diseases treatment.[295] IVT mRNA has several advantages compared with other therapeutics. It is relatively cost-effective, simple, and flexible. In contrast to plasmid and viral DNAs, IVT mRNA does not harbor the risk of insertional mutagenesis. It also does not need to enter the nucleus as it is translated in the cytoplasm. Furthermore, IVT mRNA is temporary active and completely degrades via cellular RNases.[295,296]

Short half-life, unfavorable effects on immunogenicity, instability, and poor in vivo expression have seriously hampered IVT mRNA for gene therapy applications.[295,297–299] However, these challenges have been basically solved by nucleosides modifications of mRNA as well as by improving the delivery materials and methods. 5′-cap, 3′-poly-A tail, and 5′/3′-UTRs structures have been modified in IVT mRNA. For instance, antireverse cap analog (ARCAs) improves the stability and translational efficiency of mRNA.[300,301] Moreover, nucleotides modifications that stabilize mRNA spatial structure improve translational efficiency.[302] It has reported that the U-rich RNA sequences activate immune cells and induce inflammation through the Toll-like receptors (TLR).[303,304] Reducing the U content of mRNA, lengthening the poly A tail, optimizing codons of GC-rich mRNAs and replacing

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uridine with 5-methyluridine (m5U) or pseudouridine, are effective methods to decrease or eliminate immunogenicity therapeutic RNA.[305–308] The delivery of large IVT mRNAs (1–15 kb) is more challenging than those small oligonucleotides.[298] Lipids and lipidoids are major components for mRNA delivery. Among these, DOTMA, lipid 5, LP01, C12-(2-3-2) and ATX-100 are used in clinical trials and DLin-MC3-DMA chemical was already approved by FDA.[309–314]

According to the data of current clinical trials, mRNA therapies are vastly used in cancer immunotherapy field. In 2001, the first clinical trial that used mRNA as a gene therapy drug was applied for dendritic cells (DCs). In *ex vivo* procedure, DC precursor cells were isolated, then exposed to tumor antigen encoding mRNA and transplanted into the individuals.[315] Cystic fibrosis (CF) is the first genetic disorder that was treated with mRNA-based gene therapy. The mRNA encoding CFTR was applied for pulmonary delivery.[316] Over the recent two decades, many mRNA-based gene therapies have been reported worldwide. These trials mainly focus on vaccine development for cancers as well as infectious diseases including influenza, rabies, HIV, cytomegalovirus and Zika virus, and COVID-19 infections (**Table 7**).[317]

Pfizer-BioNTech and Moderna vaccines are based on the mRNA technology that have been developed against COVID-19. The mRNA that encodes for the spike protein is encapsulated within lipid nanoparticles. These two vaccines are beneficial to provide immunity against SARS-CoV-2 infection with the efficiency of 95% and 94.5%, respectively. Both vaccines can cause various adverse effects, but these reactions are reported to be less frequent in the Pfizer/BioNTech vaccine compared to the Moderna COVID-19 vaccine. However, the Moderna vaccine compared to the Pfizer vaccine is easier to transport and store because it is less temperature sensitive.[318]

4.3. RNAi

RNAi pathway is a naturally gene silencing mechanism that degrades mRNA at post-transcriptional level in eukaryotic cells. The most important player of the pathway is a small (or short) interfering RNA (siRNA) that is a double-stranded RNA molecule with the size of 20–25 base pairs.[319] siRNA is generated from double-strand RNA precursors by Dicer endonuclease enzyme. It is loaded into the RNA-induced silencing complex (RISC) where the antisense strand (or guide strand) of siRNA, complementary to the target mRNA, is incorporated and the sense strand (or passenger strand) is degraded.[320–322] Following binding of the full-length sequence of antisense to the mRNA, argonaute-2 endoribonuclease, the catalytic core protein of the RISC, cleaves the target mRNA.[322] Hence, with the availability of transcriptome sequence, specific siRNAs can be designed and provide a powerful tool to treat genetic-based diseases and cancers.[323]

siRNAs have an easy design and synthesis and can target any gene transcript. Therapeutic siRNAs have been engineered for efficient and safe delivery to pass different extracellular and intracellular barriers to the target cells. Thus, siRNAs are encapsulated by nanovectors (lipid, polymer, or nucleotide nanocarriers) or chemically modified using conjugated ligands (lipids, carbohydrates, peptides, and antibodies).[324] The most common method for siRNA deliveries is LNPs includes two main groups

of stable nucleic acid lipid particles (SNALPs) and lipoplex.[325] PEGylation is also incorporated into lipidic- and polymeric-siRNA nanocarriers.[274] Moreover, a new conjugation method was developed in which N-Acetyl-D-galactosamine (GalNAc) covalently conjugates to siRNAs. These bioconjugates decreased siRNA hydrophilicity and increased its specific delivery to target cells.[326,327] Currently, GalNAc-siRNAs conjugates are being used in different phases of clinical trials for treatment of several genetic disorders.

Along with promising therapeutic applications of siRNA, it has still formidable challenges that need to be addressed for clinical usages. The main concerns are related to efficacy, cell-tissue selectivity, and the safety of the delivery system. In encapsulated siRNA, there are weak electrostatic interactions between the cationic nanoparticle and anionic siRNA. This reduces its efficacy.[328] Furthermore, PEGylation increases nanocarrier size as well as blood clearance.[329,330] Moreover, cationic charges of cationic lipid or polymer are the main cause of toxicity in nanoparticle encapsulated siRNAs. Design of siRNA-based drugs with defined degradable and metabolizable materials overcome this hurdle. However, materials with low toxicity and natural lipids such as cholesterol represent a safer delivery method for siRNAs.[331,332]

The first clinical trial using siRNAs was reported in 2010. Nanoparticles carrying siRNAs against the M2 subunit of ribonucleotide reductase (RRM2) were applied in patients with solid tumors.[333] In 2018, FDA approved the first siRNA-containing drug called Onpattro (also named Patisiran). This drug is a SNALP that delivers siRNA targeting transthyretin (TTR) mRNA to cure hereditary transthyretin-mediated amyloidosis.[334] In late 2019, the second siRNA drug termed Givlaari (Givosiran) was approved by FDA. Givlaari is a siRNA conjugated with GalNAc in which specifically targets aminolevulinate synthase 1 (ALAS1) mRNA in hepatocytes to treat acute hepatic porphyria.[335] Some ongoing or completed clinical trials of different siRNA-based drug candidates for various genetic diseases and cancers are summarized in Table 7.

4.4. DNAi

DNAi is observed in prokaryotes and plant cells. In RNAi pathway, argonaute protein has a profound role in gene silencing on the post-transcriptional level. The roles of prokaryotic and plant argonaute variants have remained elusive. However, recent evidence has suggested their involvement in gene regulation on the transcriptional level.[336,337] DNAi was initially applied as oligonucleotides targeting DNA, particularly regulatory regions containing CpG island in 1999.[338] Preclinical and initial clinical trials presented promising results and greatly support the development of DNAi technology as a cancer therapy tool. The first clinical trial, which used DNAi to target the Bcl-2 gene, was reported in 2012. In this trial, PNT2258, a Bcl-2-targeting liposomal formulation of synthetic DNA oligonucleotides called PNT100 were used. PNT100 are unmodified 24-mer DNA sequences that are complementary to the regulatory CpG region upstream of the *Bcl-2* gene. *Bcl-2* gene, which is considered an oncogene and has antiapoptotic activity as its misregulation induces several cancers, including lymphoma. Moreover, this gene plays a role

Table 7. Selected clinical trials used nonviral gene-tools.

Type of tool	Product	Indication	Mechanism	Trial identifier	Phase
Naked DNA	NL003 (HGF-X7)	Arterial occlusive disease, ischemia, ulcers, peripheral vascular disease	IM injections of *HGF-X7* gene into the calf expressing two variants of HGF known as HGF 728 and HGF 723	NCT04274049 NCT04275323	III
	XRP0038/NV1FGF	Peripheral vascular diseases	IM injections of XRP0038/NV1FGF plasmid that expresses FGF	NCT00566657	III
	AMG0001	Critical limb ischemia	IM injections of HGF plasmid in patients with critical limb ischemia	NCT02144610	III
	Vigil	Metastatic ewing's sarcoma family of tumors (ESFT)	Engineered autologous tumor cells by a plasmid encoding *GM-CSF* gene and a bifunctional, shRNA which specifically knocks down the expression of *furin* gene	NCT03495921	III
	Naked DNA-AMEP	Metastatic melanoma	IM electro-transferred pDNA encoding *AMEP* gene	NCT01764009	II
	PVX-2	HPV16+ atypical squamous cells	IM injecting the naked pDNA priming vaccine of pNGVL4a-Sig/E7(detox)/HSP70	NCT03911076	II
	Sleeping Beauty Transposed PBL	Glioblastoma, NSCLC, breast cancer, gastrointestinal/genitourinary cancer	Engineered autologous T-cells that expresses T-cell receptors reactive against mutated neoantigens in individuals with metastatic cancer	NCT04102436	II
	Sleeping beauty transposon system	MPS IH	Engineered autologous plasmablasts expressing α-L-iduronidase (IDUA)	NCT04284254	II
	JVS-100 (SDF-1)	Peripheral arterial disease	IM injections stromal cell-derived factor-1 plasmid in patients with critical limb ischemia	NCT02544204	II
	VGX-3100	HPV types 16 and 18 +anal neoplasm	IM injections followed by electroporation of plasmid encoding E6 and E7 proteins of HPV types 16 and 18	NCT03499795	II
	VM202	Critical limb ischemia	IM injections of HGF-X7 into the calf expressing two isoforms of HGF, HGF 728 and HGF 723	NCT01064440	II
	GX-188E	HPV types 16 and 18 +cervical intraepithelial neoplasia	A DNA-based vaccine, administered intramuscularly by electroporation	NCT02139267	II
	GTU-multHIV B/ LIPO-5	HIV-1 infection	IM injections of GTU-multHIV B and LIPO-5 vaccines that are respectively an HIV-DNA plasmid and a mixture of 5 HIV-lipopeptides	NCT01492985	II
	INO-5401/INO-9012	Urothelial carcinoma	IM injections of INO-5401 (mixture of 3 synthetic plasmids that target wilms' tumor gene-1 (WT1) antigen, prostate-specific membrane antigen (PSMA) and human telomerase reverse transcriptase (hTERT) antigen) and INO-9012 (a synthetic plasmid that expresses human interleukin-12 (IL-12)	NCT03502785	II
	SCIB1	Malignant melanoma	IM injections of SCIB1 plasmid encoding a modified antibody to stimulate the immune T cells	NCT01138410	II
	TOL-3021	Diabetes mellitus, type 1	A pDNA expressing the full-length sequence of human proinsulin	NCT03895437 NCT03794960 NCT03794973	II
mRNA	AGS-003	Advanced renal cell carcinoma	AGS-003 contains autologous DCs which are ex vivo transfected with patient-specific mRNA and synthetic human CD40 ligand RNA	NCT01582672	III
	DCs plus autologous tumor RNA	Uveal Melanoma	Ex vivo-transfected DCs loaded with autologous tumor RNA	NCT01983748	III

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Table 7. (Continued).

Type of tool	Product	Indication	Mechanism	Trial identifier	Phase
	mRNA 2416	Relapsed/refractory solid tumor malignancies or lymphoma, ovarian cancer	mRNA 2416 is a LNP encapsulated mRNA expressing OX40 ligand	NCT03323398	II
	MRT5005	Cystic fibrosis	MRT5005 restores CFTR function by transferring correct copies of CFTR mRNA to the lungs	NCT03375047	II
	GSK 692342	Tuberculosis (TB)	mRNA for development of TB vaccine	NCT01669096	II
	AZD-8601	Heart failure	Intracardiac injection of AZD-8601, encoding VEGF-A	NCT03370887	II
	CTX001	Beta-thalassemia	BCL11A modified CD34+ hHSPCs by CTX001 expressing CRISPR system	NCT03655678	I/II
	NCI-4650	Melanoma, colon cancer, gastrointestinal cancer, HCC	IM injection of an mRNA-based personalized cancer vaccine	NCT03480152	II
	iHIVARNA-01	HIV Infections	mRNA encoding a mixture of APC activation molecules (CD40L, an active variant of TLR4 and CD70) and the HIV target antigens contained in HIVACAT that injected by intranodal	NCT02888756 NCT02413645	II I
	CV7201 mRNA	Rabies virus infection	mRNA encoding the rabies virus glycoprotein	NCT02241135	II
	mRNA 1273	COVID-19 infection	LNP dispersion containing an mRNA encoding prefusion stabilized spike protein 2019-nCoV	NCT04283461	I
	ECI-006	Melanoma	DCs transfected *ex vivo* with TriMix. The drug is a combination of TriMix (mRNAs encoding for DC activating molecules [CD40L, CD70 and caTLR4]), and mRNAs encoding for melanoma-specific tumor-associated antigens (TAAs): tyrosinase, gp100, MAGE-A3, MAGE-C2, and PRAME	NCT03394937	I
	AZD8601	Male subjects with type II diabetes	Intradermal injection of AZD8601 (a manipulated VEGF-A RNA)	NCT02935712	I
	mRNA-1653	Human metapneumovirus and human parainfluenza infection	A combined human metapneumovirus (hMPV) and parainfluenza virus type 3 (PIV3) vaccine	NCT04144348 NCT03392389	I
RNAi	SYL1001	Dry eye disease	SYL1001 or tivanisiran is a chemically synthesized siRNA downregulating the transient receptor potential vanilloid-1 (TRPV1)	NCT03108664	III
	Inclisiran	Homozygous familial hypercholesterolemia	Inclisiran is a synthetic, chemically modified siRNA targeting proprotein convertase subtilisin kexin type 9 (PCSK9) RNA with a covalently attached triantennary N-acetylgalactosamine (GalNAc) ligand	NCT03851705	III
	Lumasiran	Primary hyperoxaluria type 1 (PH1)	Subcutaneously administered siRNA therapeutic targeting glycolate oxidase (GO)	NCT03681184 NCT03905694 NCT04152200	III
	Bevasiranib	Age related macular degeneration	siRNA drug inhibiting VEGF	NCT00557791	III
	DCR-PHXC	PH1, PH2, kidney diseases, urologic diseases	DCR-PHXC is a novel siRNA conjugated to N-acteylgalactosamine (GalNAc) downregulating the liver oxalate mRNA	NCT04042402	III
	Patisiran	Transthyretin amyloidosis (ATTR) with cardiomyopathy	Encapsulated siRNA downregulating the TTR mRNA	NCT03997383 NCT02510261 NCT01960348	III
	Fitusiran	Hemophilia A and Hemophilia B	siRNA downregulating antithrombin (AT) mRNA in the liver	NCT03417245 NCT03417102	III

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Table 7. (Continued).

Type of tool	Product	Indication	Mechanism	Trial identifier	Phase
	Givosiran (ALN-AS1)	Acute hepatic porphyrias (AHP)	A siRNA covalently linked to a hepatocyte ligand targeting aminolevulinate synthase 1 (ALAS1) in the liver	NCT03338816	III
	Inclisiran	Atherosclerotic cardiovascular disease	siRNA inhibiting hepatic synthesis of PCSK9	NCT03705234 NCT03400800	III
	Patisiran	Amyloidosis	Encapsulated siRNA downregulating the TTR mRNA	NCT02510261 NCT03759379	III
	Inclisiran	Heterozygous familial hypercholesterolemia	siRNA inhibiting hepatic synthesis of PCSK9	NCT03814187	III
	Bevasiranib (Cand5)	Diabetic macular edema	Cand5 targets the mRNA encoding for VEGF	NCT00306904	II
	DCR-MYC	HCC	A LNP encapsulated siRNA targeting MYC	NCT02314052	II
DNAi	PNT2258	Richter's syndrome	Synthetic oligo targeting BCL2 gene at transcriptional level by DNAi procedure	NCT02378038	II
	PNT2258	Lymphoma, diffuse large B-cell	Synthetic oligo targeting BCL2 gene at transcriptional level by DNAi procedure	NCT02226965	II
	PNT2258	Lymphoma, Non-Hodgkin's	Synthetic oligo targeting BCL2 gene at transcriptional level by DNAi procedure	NCT01733238	II
	PNT2258	Lymphoma, prostate cancer and melanoma	Synthetic oligo targeting BCL2 gene at transcriptional level by DNAi procedure	NCT01191775	I
Antisense	Alicaforsen	Crohn's disease	An antisense oligonucleotide drug that targets ICAM-1 RNA	NCT00048113	III
	G3139	Advanced malignant melanoma	Bcl-2 antisense oligonucleotide	NCT00016263	III
	G3139	Acute myeloid leukemia (AML)	Bcl-2 antisense oligonucleotide	NCT00085124	III
	Aprinocarsen	Metastatic NSCLC	A 20-mer antisense phosphorothioate oligonucleotide inhibiting PKCa	NCT00017407	III
	RO7234292	Huntington's disease	An antisense drug targeting huntingtin (HTT) RNA	NCT03761849	III
	Mipomersen	Atherosclerosis	An antisense oligonucleotide targeting apolipoprotein B RNA	NCT01598948	III
	Sepofarsen	LCA	Sepofarsen binds to mutated location in the pre-mRNA causing normal splicing of the CEP290 pre-mRNA	NCT03913143	III
	OGX-011	Prostate cancer	An antisense oligonucleotide inhibiting production of clusterin	NCT01083615	III
	Aganirsen (GS-101)	Neovascular glaucoma	An antisense oligonucleotide against insulin receptor substrate (IRS-1)	NCT02947867	III
	WVE-210201	DMD	An antisense oligonucleotide masking exon 51 in the DMD mRNA	NCT03907072	III
	AP 12009	Anaplastic astrocytoma, glioblastoma	A phosphorothioate antisense specific for the mRNA of human transforming growth factor beta 2 (TGF-beta-2)	NCT00761280	III
	AVI-4658	DMD	IM administered morpholino antisense oligomer targets exon 51 of DMD RNA	NCT00844597 NCT00159250	II
	SRP-5051	DMD	A phosphorodiamidate morpholino oligomer covalently linked to peptide directing the splicing of DMD mRNA	NCT04004065 NCT03675126	II
	BB-401	Head and neck squamous cell carcinoma (HNSCC)	An antisense DNA targeting EGFR	NCT03433027	II
	Antisense ODN CD40/CD80/CD86	Type 1 diabetes	An antisense DNA oligonucleotide targeting the pre-mRNA of cluster of differentiation antigen 40, 80 and 86 (CD40, CD80 and CD86)	NCT02354911	II

(Continued)

Table 7. (Continued).

Type of tool	Product	Indication	Mechanism	Trial identifier	Phase
	Miravirsen	Chronic hepatitis C	Miravirsen is a β-d-oxy-locked nucleic acid-modified phosphorothioate antisense oligonucleotide targeting the liver-specific microRNA-122 (miR-122)	NCT01200420 NCT01872936 NCT01727934	II
	RG-012	Alport syndrome	A single stranded, chemically modified oligonucleotide inhibiting miR-21	NCT02855268	II
	MRG-106	Cutaneous T-cell lymphoma/mycosis fungoides	A locked nucleic acid-modified oligonucleotide inhibitor of miR-155	NCT03713320 NCT03837457	II
	DYN101	Centronuclear myopathy	A synthetically ethyl gapmer ASO designed against DNM2 pre-mRNA	NCT04033159	II
Aptamer	E10030 (Fovista)	Age-related macular degeneration (AMD)	Anti-PDGF pegylated aptamer	NCT00545870 NCT01944839 NCT01940900 NCT01940887	III
	REG1 (pegnivacogin)	CAD	Pegylated active RNA aptamer- 40 kDa PEG based factor IXa inhibitor	NCT01848106	III
	EYE001	Macular degeneration	Anti-VEGF pegylated aptamer	NCT00021736	III
	Zimura	AMD	Anti-C5 aptamer combined with anti-VEGF therapy	NCT04435366	III
	Zimura	Idiopathic polypoidal choroidal vasculopathy	Anti-C5 aptamer combined with anti-VEGF therapy	NCT02397954	II
	E10030	AMD	Anti-PDGF pegylated aptamer	NCT01089517	II
	Zimura	Geographic atrophy macular degeneration	Anti-C5 aptamer combined with anti-VEGF therapy	NCT02686658	II
	Zimura	Stargardt disease 1	Anti-C5 aptamer combined with anti-VEGF therapy	NCT03364153	II
	AS1411	AML	A G-rich DNA oligonucleotide aptamer targeting the protein nucleolin	NCT01034410 NCT00512083	II
	AS1411	Metastatic renal cell carcinoma	A G-rich DNA oligonucleotide aptamer targeting the protein nucleolin	NCT00740441	II
	Lexaptepid pegol (NOX-H94)	Anemia	A PEGylated L-stereoisomer RNA aptamer that targets and inhibits hepcidin	NCT02079896	II
	ARC1779	Three types of von-willebrand factor (VWF) related platelet disorders	An aptamer interfering binding of the VWF A1 domain to platelet GPIb receptors	NCT00632242	II
miRNA mimic	MRG-201	Keloid	A miR-29 mimic targeting the collagen mRNA and other genes playing a role in scar formation	NCT03601052	II
	MRX34	Melanoma	A liposomal miR-34a mimic that targets more than 30 different oncogenes as well as some gene involving in tumor immune evasion	NCT02862145	II
	MRX34	Liver cancer, SCLC, blood cancers, renal cell carcinoma, NSCLC	A liposomal miR-34a mimic that targets more than 30 different oncogenes as well as some gene involving in tumor immune evasion	NCT01829971	I
	MesomiR-1	Malignant pleural mesothelioma, NSCLC	A miR-16 mimic implicating as a tumor suppressor in a range of cancer types	NCT02369198	I
	MRG-201	Wound healing	A miR-29 mimic targeting the collagen mRNA and other genes playing a role in scar formation	NCT02603224	I
	TargomiRs	Malignant pleural mesothelioma, NSCLC	A miR-16 mimic implicating as a tumor suppressor in a range of cancer types	NCT02369198	I
CpG-ODN	IMO-2125	Metastatic melanoma	DNA-based immunomodulatory oligo and a TLR9 agonist is administrated into tumors	NCT03445533	III
	AV7909	Anthrax	The anthrax vaccine adsorbed (AVA) drug combined with CpG 7909 adjuvant	NCT03877926	III

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Table 7. (Continued).

Type of tool	Product	Indication	Mechanism	Trial identifier	Phase
	Tilsotolimod	Malignant melanoma	DNA-based immunomodulatory oligo and a TLR9 agonist is administrated into tumors	NCT04126876	II
	PF03512676	Metastatic breast cancer	A 24mer single stranded agonist of TLR9 expressed by B cells and plasmacytoid	NCT00824733	II
	TLR9 agonist	Malignant glioblastoma	Immunotherapy with CpG ODN	NCT00190424	II
	MGN1703	HIV infection	DNA-based immunomodulatory oligo and a TLR9 agonist is administrated into tumors	NCT02443935	II
	CPG 7909	Malaria	Immunostimulatory TLR9 agonist oligo	NCT00984763	II
	CPG 7909	HIV and hepatitis B	Immunostimulatory TLR9 agonist oligo	NCT00100633	II
	Tilsotolimod	Solid tumor	DNA-based immunomodulatory oligo and a TLR9 agonist is administrated into tumors	NCT03865082	II
	IMO-2125	Metastatic melanoma	Administration of the drug IMO-2125 in combination with ipilimumab or pembrolizumab	NCT02644967	II

in the development of resistance to chemotherapeutic drugs. Thus, some targeting procedures have been developed to inhibit Bcl-2 at the RNA and protein level. However, those methods are not sufficient for Bcl-2-related tumors such as non-Hodgkin lymphomas. PNT2258 strongly inhibits the *Bcl-2* promoter activity. Thus, it confirms the mechanism of action of the new DNAi approach. PNT100 oligos reveal safe and improved serum stability and it has acceptable pharmacokinetic features. Furthermore, it is well-tolerated and has antitumor activity in both in vivo and in vitro approaches. Particularly, PNT2258 demonstrates broad antitumor activities against Bcl-2-associated tumors of Richter's syndrome, lymphoma, diffuse large B-cell lymphoma, non-hodgkin's lymphoma and melanoma prostate cancer. As a DNA is directly targeted without breakage, the DNAi technology appears to be more effective in comparison to other approaches that target the gene at RNA or protein level.[339–342]

4.5. ASO

ASOs are small (12–25 nucleotides) single-stranded synthetic DNA or RNA molecules that specifically target pre-mRNA, mRNA, or ncRNAs through Watson–Crick base pairing. The binding results in degradation of their targets and downregulates gene expression.[343,344] In the last decade, this strategy has been applied for drug development to treat cancer, rare and common genetic diseases.[345–348] According to the target types and mechanisms of action, ASOs are classified into three main groups, gapmers, splice switching oligonucleotides (SSOs), and anti-miRNAs.

Gapmers are ASOs containing a central block of DNA flanked by chemically modified RNA bases. RNA bases enhance affinity to the complementary sequence of target mRNA, while DNA bases act as a substrate for the RNase H enzyme. When a gapmer binds to an mRNA target, RNase H degrades the RNA strand of RNA/DNA duplexes.[349] SSOs contain ribose or morpholino modifications to create an RNase H-resistant structure, which modifies its splicing by blocking the binding

of the spliceosome. Thus, SSOs are applied for correction of disease-causing splicing or expression induction of a therapeutic splice variant.[350] Synthetic ASOs, known as anti-miRNA oligonucleotides (AMO), anti-miRs or antagomiRs, bind to the complementary region in miRNAs and inhibit their function in pathological conditions.[351] miRNA-masking antisense oligonucleotides (miR-mask) block and cover the miRNA-binding sites in the 3UTR of the target mRNA.[352]

Drawbacks of ASOs are degradation by nucleases, poor cellular uptake, suboptimal pharmacokinetics/dynamics and low binding affinity to the target site.[344,353,354] To overcome such limitations for clinical applications of ASOs, three generations of modifications have been developed.[355] Phosphorothioate (PS) modifications are considered as the first generation of such modifications. Non-bridging oxygen in the phosphodiester bond is replaced by a sulfur group in PS. This improves the stability of ASO, as well as increases its half-life to several days. Furthermore, increasing ASO binding to serum proteins causes preserved ASOs in the blood circulation. This results in gradual uptake by the liver. Moreover, PS modification enhances RNase H activity.[356–358] However, the high toxicity and low specificity of PS-ASOs led to develop ribose alterations (as second generations of ASOs). Replacement of the 2-hydroxyl by 2"-O-methyl (2"-O-Me) or 2"-O-methoxyethyl (2-MOE) groups are most common ribose modifications. These improve ASOs stability and affinity to target sites. Furthermore, they are less toxic than PS-ASOs.[359] The third generation is a group of modifications to improve resistance to nucleases, binding-affinity, and pharmacokinetic properties.[360] The bridged nucleic acids (BNA) modification, links the 2" oxygen to the 4 carbon of the ribose through bridging carbons. This increases ASO hybridization to the target mRNA. The most common BNA includes locked nucleic acids (LNAs) corresponding to a methylene bridge connecting the 20-oxygen and 40-carbon of the ribose.[361] Another example of BNA is 2', 4'- constrained ethyl ((S)-cET) that is widely examined in the clinic.[348,362] Phosphorodiamidate morpholino oligomers (PMOs) and peptide nucleic acids (PNAs) belong to the first generation of ASOs. PMOs are stable, highly soluble, and nontoxic ASOs, where phosphodiester bond and ribose are

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replaced by a phosphorodiamidate bond and morpholine structure, respectively. In PNAs, ribose and phosphate moiety of natural nucleic acid are replaced by a polyamide backbone.[17] ASO-PMOs and ASO-PNAs are uncharged and have low affinity for binding to the plasma proteins. Thus, they are rapidly eliminated by urine.[363] Furthermore, the coupling of these structures to peptides increases ASOs tissue absorption.[364]

The ribose or morpholino modifications make ASOs more similar to RNA than DNA. Otherwise, RNase H does not target and degrade ASO-RNA duplex. Thus, this type of ASOs prevent translation by steric blocking mechanism. These modifications are widely used in ASOs that modulate alternative splicing by blocking the binding of splicing factors.[365,366] However, the RNase H activity could be restored in gapmer ASO by inserting a cleavage sensitive structure between a pair of non-RNase H-sensitive sequences.[367] Furthermore, fatty acids, peptides, or GalNAc are conjugated to the ASOs to improve their tissue uptakes.[368,369] APO(a)-LRX is a GalNAc3-conjugated ASO that is being currently applied in phase II of a clinical trial. It inhibits lipoprotein production in patients with cardiovascular disease.[370] Additionally, several anti-miR ASOs are being used in clinical trials to target dysregulated miRNAs molecules. For instance, miravirsen is an LNA PS-ASO that targets miR-122 in hepatitis C virus (HCV) infected individuals.[371] Moreover, the ASO RG-012 known as lademirsen, inhibits miR-21 in Alport syndrome patients.[372]

A modified tumor-infiltrating lymphocyte (TIL) oligomer is the first clinical ASO that is used for patients with an advanced form of melanoma.[373] In 1999, fomivirsen (also named Vitravene) was the first FDA-approved ASO, used for cytomegalovirus retinitis.[374] Fomivirsen is a PS-ASO that targets the *UL123* gene in cytomegalovirus. However, due to local inflammation and increased intraocular pressure, it was withdrawn from the gene therapy market. Mipomersen (also named Kynamro), a 2-MOE-PS-ASO was approved by the FDA for homozygous familial hypercholesterolemia. As a gapmer, mipomersen binds to the ApoB100 mRNA which leads to its cleavage and degradation by RNase H.[375] Eteplirsen (also named Exondys 51) is a PMO-SSO that was approved by FDA for the treatment of Duchenne muscular dystrophy (DMD).[366] Recently, golodirsen (Vyondys 53), another PMO-SSO drug for DMD, was approved by the FDA.[376] Eteplirsen and golodirsen bind to exon 51 and exon 53 splice sites of DMD pre-mRNA, respectively, both lead to the exon skipping. Viltolarsen (Viltepso) is a PMO to treat DMD that was approved by the FDA in August 2020. Similar to Golodirsen, Viltolarsen was designed to skip exon 53 on the dystrophin pre-mRNA in patients with a confirmed *DMD* gene mutation that is amenable by exon 53 skipping.[377] In February 2021, the FDA also approved Casimersen (Amondys 45) to treat DMD patients who have a confirmed mutation of the *dystrophin* gene that is amenable by exon 45 skipping. Casimersen binds to exon 45 of dystrophin mRNA precursor and excludes this exon during mRNA processing, resulting in a dystrophin protein that contains essential functional domains.[378] The FDA approved Nusinersen (spinraza), an SSO drug, in 2016 for treatment of children and adults with spinal muscular atrophy (SMA). Nusinersen is a 2′-O-Me-PS oligomer that is designed to correct SMN2 (survival of motor neuron 2) pre-mRNA splicing.[379] Inotersen (Tegsedi) is a 2′-MOE-gapmer approved by the FDA for the hereditary form of

transthyretin amyloidosis. The drug binds to the mutant form of *TTR* mRNA and inhibits its translation in the liver.[380] On May 2019, a new ASO named Volanesorsen (Waylivra), received its first global approval for the treatment of adult patients with familial chylomicronemia syndrome (FCS).[381] Volanesorsen inhibits apolipoprotein CIII mRNA and subsequently promotes triglyceride clearance.[382] There are several ongoing clinical trials to assess Volanesorsen utility in familial partial lipodystrophy, hypertriglyceridemia and partial lipodystrophy. In addition to these six approved ASOs drugs, several ongoing ASOs are currently being examined at different phases of clinical trials (Table 7).

4.6. Aptamers

Aptamers also termed "chemical antibodies" are short synthetic single-stranded RNA or DNA ligands (10-30 kDa) that interact with proteins. The interaction occurs through 3D complementarity (not base pairing) and inhibits protein activity. In the early 1990s, the first aptamers were generated through a technology known as systematic evolution of ligands by exponential enrichment (SELEX).[383,384] The principle of SELEX-based aptamer production relies on incubation of the target protein with a synthetic DNA or RNA oligo pool containing 10^{13-17} various sequences. In brief, the bound chains are recognized and thereafter, undergo several cycles of selection and amplification. Consequently, the resulting DNA or RNA oligonucleotides were identified by sequencing and introduced as aptamer for the target proteins.[385,386] To date, specific aptamers targeting peptides, proteins, viruses, bacteria, and whole cells could be produced by SELEX for both diagnostic and therapeutic applications. Additionally, aptamers are used as targeting vehicles for siRNAs, miRNAs, and conventional drugs.[387]

The aptamers as chemical antibodies have several advantages over protein antibodies including low production cost, nanoscale size, fast cell binding, versatile chemical modifications, lack of immunogenicity in vivo, and fewer side effects. However, the challenges include inherent physicochemical characteristics, entrapment in cellular organelles, off-target effects, and short in vivo half-life.[388,389] Thus, the aptamers were subject to chemical modifications improving their specificity, affinity, and half-life for clinical usages.[387] One of the most common modifications is those to increase the nuclease resistance aptamers and to overcome renal filtration. Replacing 2′-hydroxyl of RNA with a fluoro, amino or O-methyl groups or capping the 3′ end with inverted thymidine, increased both nuclease resistance and binding affinity of the aptamers.[390,391] L-RNA aptamers (also named Spiegelmer) are nonnatural L-ribose nucleotides, not affected by nucleases. In comparison to the natural d-form of RNA, L-RNA aptamers reveal high resistance to nuclease degradation and have great binding affinity.[392] Furthermore, conjugation of the aptamers with PEG, cholesterol, proteins, liposomes, nanomaterials, and aptamers multimerization could overcome renal filtration and improve half-life.[393,394] Additionally, aptamer–antibody hybrid complex improves in vivo pharmacokinetics of the aptamer.[395]

Pegaptanib (also named macugen) is the first aptamer that was approved and entered the clinic. The drug is a pegylated RNA aptamer that targets vascular endothelial growth factor (VEGF)

and is used for treatment of the neovascular (wet) form of age-related macular degeneration (AMD).[396,397] Furthermore, three more aptamers are being used in phase III clinical trials. These are Zimura and Fovista for the treatment of ocular diseases and REG1 (pegnivacogin) as an anticoagulation drug.[387] Currently, more than 30 aptamers with different modification are being used in the clinic for treatment of several disorders especially for ocular disorders, coagulation, inflammation and cancer (Table 7).

4.7. microRNA Mimics

MicroRNA (miRNAs) are endogenous small noncoding RNAs (18-25 nucleotides) involved in gene silencing at posttranscriptional level as siRNAs. Primary miRNA (pri-miRNA) transcript is produced by RNA polymerase II in the nucleus. It is cut by Drosha/DCGR8 complex resulting in a pre-miRNA. The pre-miRNA is subsequently transported to the cytoplasm where it is further processed by Dicer/TRBP complex into a loop-free structure. Then it loads into RISC, where the passenger strand is discarded. The mature single-stranded miRNA guides RISC to the 3′ untranslated region (3′ UTR) of the target mRNA, leading gene silencing via mRNA degradation or deadenylation.[398,399]

The gene silencing mechanisms of both miRNAs and siRNAs are associated with common complexes (e.g., RISC and Dicer). However, each miRNA might regulate the expression of many genes, while siRNAs inhibit the expression of one specific target. Moreover, close to 60% of protein-coding genes are under the control of miRNAs. The disruption of miRNAs function is associated with many genetic disorders.[400] Upregulated miRNAs are targeted by antimiRs ASOs, miRNA sponges, and miRNA masking. However, downregulated miRNAs can be restored through the small synthetic miRNAs termed miRNA mimics, or plasmid expressing the miRNAs. Therefore, miRNAs replacement therapy refers to synthetic miRNAs that restore the miRNA function and inhibit the translation process of targets genes.[267]

Similar to siRNAs, the development of therapeutic miRNA mimics face several hurdles including serum degradation, inefficient delivery as well as stimulation of the innate immune system. Chemical modification of the nucleotide backbone is a major strategy to overcome these barriers.[401] The most common modifications in the RNA duplex of miRNA mimics are locked and unlocked nucleic acids, methylation of the passenger strand, phosphorothioate (PS) and ribose 2′- OH group modifications, where ribose 2′-OH group is substituted with 2′-O-methyl (2′-O-Me), 2′-methoxyethyl (2′-O-MOE) and 2′-fluoro (2′-F) groups. These enhance serum stability and reduce immune activation of the miRNA mimics duplex.[267] Current available commercial miRNA mimics are subject to methylation of the sense strand. This could increase its serum stability.[402] Moreover, encapsulating miRNA mimics into the nanoparticles is an alternative strategy to increase the delivery efficiency. TargomiR is a current miRNA mimics delivery system used for cancer therapy in clinical trials.[403] The method consists of three components, nonliving bacterial nanocells, a miRNA mimic, and a surface-conjugated antibody as a targeting moiety.[404]

MRX34 is the first miRNA mimic that entered phase 1 clinical trial to treat individuals with liver cancer. Nanoliposomal encapsulated MRX34 restored the lost suppressor function of miR-34, which inhibits more than 24 known oncogenes.[405] A cholesterol-conjugated miR-29 mimic, remlarsen (MRG-201), is in Phase II clinical trial for the treatment of keloid scars. Moreover, a particular TargomiR, MesomiR1 (containing miR-16 mimics with epidermal growth factor receptor (EGFR)-specific antibodies) showed promising results for the treatment of non-small-cell lung cancer and malignant pleural mesothelioma.[403,406]

4.8. CpG-ODN

Synthetic oligodeoxynucleotides containing unmethylated cytosine-phosphate-guanosine motifs (also termed CpG-ODN) are immunostimulatory adjuvants stimulating the innate and adaptive immune responses.[407] CpG-ODNs mimic the structure of bacterial DNA as native ligand for TLR-9. Otherwise, CpG-ODNs attach to TLR-9 within phagoendosomes of antigen-presenting cells (APCs) including dendritic cells, plasmacytoid dendritic cells, and B lymphocytes.[408,409] TLRs recognize microbes and initiate immune and inflammatory responses to eliminate intruders. CpG-ODNs activate NF-kB pathway and initiate the production of proinflammatory cytokines and type I interferons in immune cells. Furthermore, they stimulate vaccine-specific cellular and humoral responses to treat infection, asthma, allergy, and cancer.[410,411] CpG-ODN becomes more effective in combination with an immunogen. They are used against the disorders causing a Th2/Th1 (helper T cell) shift or down-regulation of the Th2 immune response.[411,412] Furthermore, CpG-ODNs induce inflammatory immune responses and reduce tumor growth by stimulating the TLR9/p38 MAPK signaling in anti-tumor cells.[407,413]

Clinical applications of CpG-ODNs have major challenges. Naked CpG-ONDs have low cellular uptake due to electrostatic repulsion between negatively charge CpG and negative cell membrane. They are easily degraded by DNase enzyme. Moreover, CpG-ODNs need to be administrated at high doses, which cause side effects of the septic shock-like syndrome.[414–416] TLR9 is also expressed in various tumor cells including the brain, lung, breast, and gastric cancer cells.[417–419] Therefore, CpG-ODNs-based cancer therapies require careful consideration, as the stimulation of TLR9 receptor in some tumor cells could promote tumors growth and/or metastasis.[420,421] Optimizing the delivery systems and backbone modifications, partly overcome these limitations. Phosphorothioate modification of CpG-ODN backbone, improve stability, and avoid DNases-mediated degradation. Furthermore, conjugation with polycationic enhances APCs uptake, prolongs half-life, and prevents degradation of CpG-ODNs.[422]

Clinical applications of CpG-ODNs as adjuvants increased over the last decade. They are applied for cancers, infections, asthma, and allergic disorders. Initially, CpG-ODNs were examined for immunotherapy of malignant glioblastoma.[423] Today, they have been used in clinical trials for treatment of the cancers including malignant glioblastoma, melanoma, wilms tumor 1, basal cell carcinoma, germ cell tumor, B-cell chronic lymphocytic leukemia (B-CLL), colon, pancreatic, rectal, lung, cervical, epithelioid, papilla, breast, esophageal, prostate and ovarian cancers (Table 7).[414,422,424] Furthermore, about 20 CPG-ODN adjuvants combined with corresponding vaccines are in clinical trials for the treatment of infectious diseases including malaria,

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influenza, pneumococcus in HIV-infected adults, hookworm infections, cytomegalovirus infections, anthrax, and hepatitis B.[414]

5. Discussion and Future Directions

The first official clinical gene therapy trial was reported in 1990, in which a retroviral gene delivery vehicle was approved by the FDA. Later in 1995, an AAV gene delivery system was also officially approved by the FDA. More genetic tools emerged and were generated and tested in clinical gene therapy trials in the following years (**Figure 5**). Despite the efforts to launch new products over a period of three decades, only few gene therapy drugs have entered the clinics and the market. During this period, about thirty drugs/products have been approved by relevant authorities worldwide. These products are mainly used for monogenic human disorders and several forms of cancers and malignancies. Among these products, there are 17 engineered viral systems that deliver therapeutic genes to the target tissue. Gendicine and Oncorine drugs are adenoviral-based gene delivery vehicles. Glybera, Luxturna, Zolgensma, and Valrox drugs are four therapeutic AAVs. Imlygic and Rexin-G are therapeutic products based on HSV and retroviral gene delivery systems, respectively. Zynteglo, Kymriah, Lisa-cel, and OTL-200 are clinical cell products that depend on lentiviral delivery. Furthermore, Strimvelis, Zalmoxis, Yeskarta, Invossa, and the newly approved addition Ecartus are retroviral-based products. Furthermore, there are 12 approved drugs that are based on nonviral delivery systems. Among the nonviral products, neovasculgen and collategene are categorized as naked DNA. Furthermore, Patisiran and Givosiran are two therapeutic oligos based on RNAi technology. In addition, Eteplirsen and Spinraza are therapeutic oligos, but they modulate splicing events. Vitravene, Kynamro, Volanesorsen, and Tegsedi are antisense oligos that downregulate their target mRNAs by RNase H-mediated degradation. Finally, Macugen and Defitelio oligos act as aptamers that modulate protein activity.[8,10] A summary of approved gene therapy products and their mechanism of actions is shown in **Figure 6**.

The first official clinical trials using gene-editing systems of ZFN, TALEN, and CRISPR/Cas were approved in 2009, 2017, and 2018, respectively. Development and growth of these gene-editing platforms are improving the treatment strategies of genetic disorders. Among these systems, CRISPR/Cas is the most important and widespread platform that is currently being used for gene editing therapeutic applications. CRISPR/Cas9 is a natural and precise programmable gene-editing system that is simple and easy to implement and produce, in comparison with ZFNs and TALENs. Furthermore, it is developed for gene therapy with less byproducts. Importantly, its versatile applications, from base editing to large DNA replacement, have made it ideal for ex vivo gene manipulations of stem cells or immune regulatory cells. Novel gene therapies that exploit gene-editing systems are being developed for a wide range of human disorders, cancers, and monogenic diseases, mainly sickle cell anemia and β-thalassemia and LCA. Monogenic blood disorders are predestined for gene editing systems as the patient's bone marrow stem cells can be purified and the repressor gene of fetal hemoglobin is suppressed. This strategy induces the expression of fetal hemoglobin in adults and provides a compensation for mutant gene of β-globin.[10,74,98] Today, over 60 clinical trials using gene-editing systems are registered on ClinicalTrials.gov. However, little information on short-term toxicity, and no information on long-term toxicity of these systems is available yet. A survey on these clinical gene therapy trials demonstrates increasing demand of clinic for CRISPR/Cas9-mediated gene editing. In trials using gene-editing systems, 67% accounts for CRIRSR/Cas9, 24% for TALEN and 9% for ZFN (**Figure 7**).

In the next years, the gene-editing systems will become more important to treat genetic diseases. However, their future applicability and efficiency for the treatment of human cancer, monogenic and polygenic disorders are questionable. CRISPR/Cas system is expected to be the main method for gene editing among programmable nucleases until 2030. Less off-targeting mutations, high efficiency of gene editing, in vivo gene targeting, and clear ethical and regulatory guidelines are prerequisites for future gene-editing systems. Altogether, gene editing could efficiently improve the treatment and prevention strategies of human genetic disorders.

High efficiency of gene transduction, gene delivery to specific tissues, transient and permanent gene expression are benefits of viral gene delivery systems. The first FDA-approved clinical trial used an engineered retrovirus for ADA-SCID treatment in 1990. Here, retroviruses and lentiviruses were genetically manipulated for efficient gene delivery and specifically used for stable transgene expression in human cells. To date, they are applied for ex vivo gene manipulation of HSCs to treat monogenic metabolic and hematologic diseases. AdVs were initially used for gene therapy of genetic disorders; however, currently they are applied for short-term transgene expression in cancer gene therapy and vaccination development. AAVs are proper gene delivery systems for monogenic disease that facilitate gene delivery to a specific tissue or organ. AAVs are the only viral gene delivery vehicles to cross BBB, which makes them predestined to be used as a delivery system, treating genetic brain disorders. Different serotypes of AAVs can deliver therapeutic genes to a wide range of target tissues. Monogenic disorders, such as metabolic genetic diseases, hemoglobinopathies, hemophilia, Leber's congenital amaurosis, and spinal muscular atrophy are being treated in clinical trials with AAVs-based gene delivery systems. Other classes of viral gene delivery vehicles, mainly HSVs and poxviruses, are commonly used as oncolytic viruses that target and kill cancer cells. They are being developed to deliver suicide genes to cancer cells and have fewer side effects on other cell types.[192,425–428]

About two-thirds of gene therapy trials involve viral gene delivery vehicles. Currently, about 2400 completed or ongoing clinical gene therapy trials based on viral gene delivery systems have been reported worldwide (September 2020). Among these trials, 26% account for Advs, 12% for AAVs, 23% for retroviral, 15% for lentiviral, 14% for poxviral, 4% for HSVs and finally 6% for other viral gene delivery systems (Figure 7).

Nonviral systems that are based on small and large synthetic therapeutic DNA/RNAs are highly desirable as they are easier to produce and characterize. Furthermore, they have fewer safety concerns. However, they are nonintegrative and less efficient compared to other gene therapeutic toolkits. For the delivery of non-viral devices, both polymer-based and lipid-based carriers have been tested in clinical trials. Nonviral delivery vehicles have
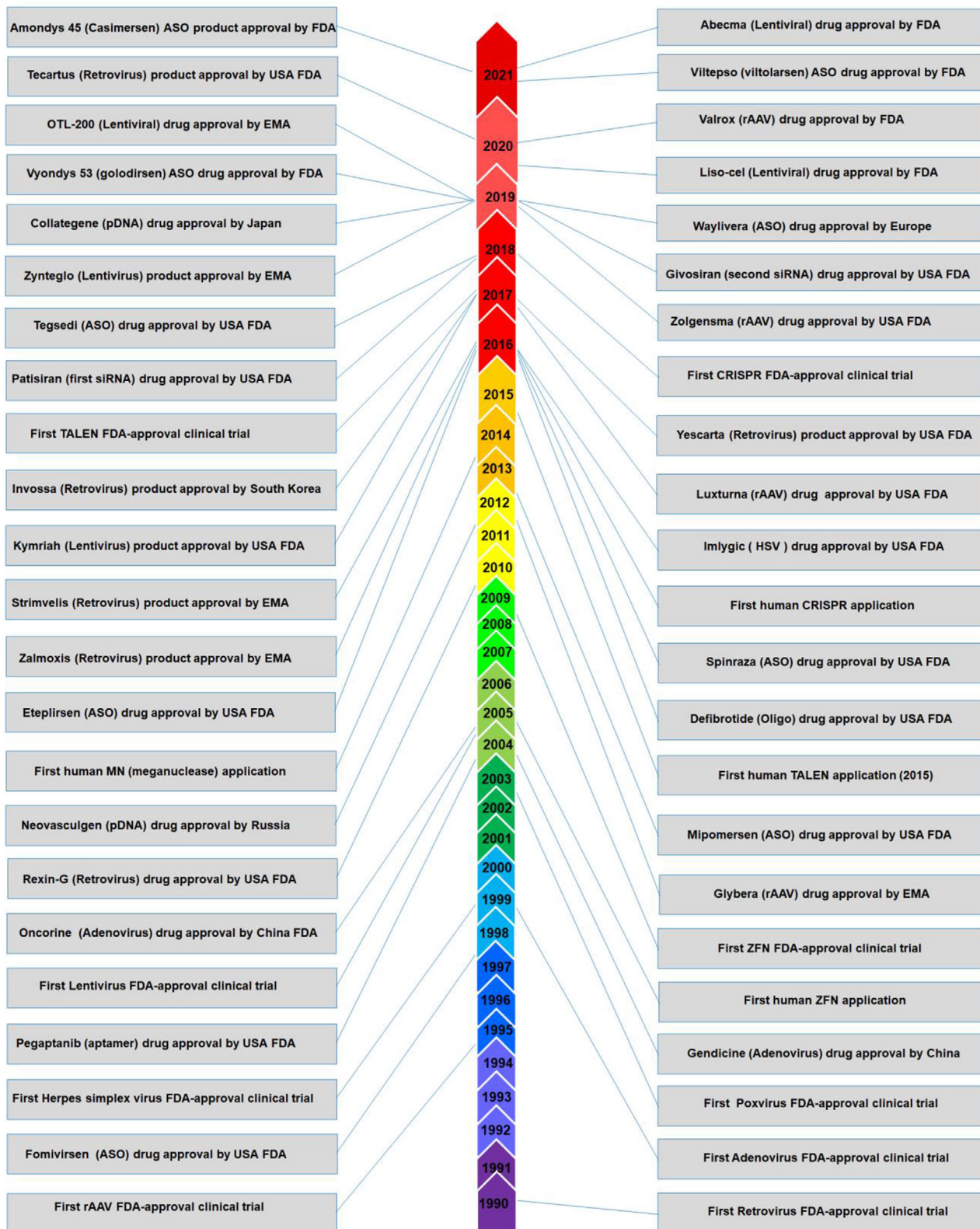
Amondys 45 (Casimersen) ASO product approval by FDA

Abecma (Lentiviral) drug approval by FDA

2021

Tecartus (Retrovirus) product approval by USA FDA

Viltepso (viltolarsen) ASO drug approval by FDA

OTL-200 (Lentiviral) drug approval by EMA

2020

Valrox (rAAV) drug approval by FDA

Vyondys 53 (golodirsen) ASO drug approval by FDA

2019

Liso-cel (Lentiviral) drug approval by FDA

Collategene (pDNA) drug approval by Japan

Waylivera (ASO) drug approval by Europe

2018

Zynteglo (Lentivirus) product approval by EMA

2017

Givosiran (second siRNA) drug approval by USA FDA

Tegsedi (ASO) drug approval by USA FDA

2016

Zolgensma (rAAV) drug approval by USA FDA

Patisiran (first siRNA) drug approval by USA FDA

2015

First CRISPR FDA-approval clinical trial

First TALEN FDA-approval clinical trial

2014

Yescarta (Retrovirus) product approval by USA FDA

2013

Invossa (Retrovirus) product approval by South Korea

2012

Luxturna (rAAV) drug approval by USA FDA

Kymriah (Lentivirus) product approval by USA FDA

2011

Imlygic (HSV) drug approval by USA FDA

2010

Strimvelis (Retrovirus) product approval by EMA

2009

First human CRISPR application

2008

Zalmoxis (Retrovirus) product approval by EMA

2007

Spinraza (ASO) drug approval by USA FDA

2006

Eteplirsen (ASO) drug approval by USA FDA

2005

Defibrotide (Oligo) drug approval by USA FDA

2004

First human MN (meganuclease) application

2003

First human TALEN application (2015)

2002

Neovasculgen (pDNA) drug approval by Russia

2001

Mipomersen (ASO) drug approval by USA FDA

2000

Rexin-G (Retrovirus) drug approval by USA FDA

1999

Glybera (rAAV) drug approval by EMA

Oncorine (Adenovirus) drug approval by China FDA

1998

First ZFN FDA-approval clinical trial

1997

First Lentivirus FDA-approval clinical trial

1996

First human ZFN application

Pegaptanib (aptamer) drug approval by USA FDA

1995

Gendicine (Adenovirus) drug approval by China

1994

First Herpes simplex virus FDA-approval clinical trial

1993

First Poxvirus FDA-approval clinical trial

1992

Fomivirsen (ASO) drug approval by USA FDA

1991

First Adenovirus FDA-approval clinical trial

First rAAV FDA-approval clinical trial

1990

First Retrovirus FDA-approval clinical trial

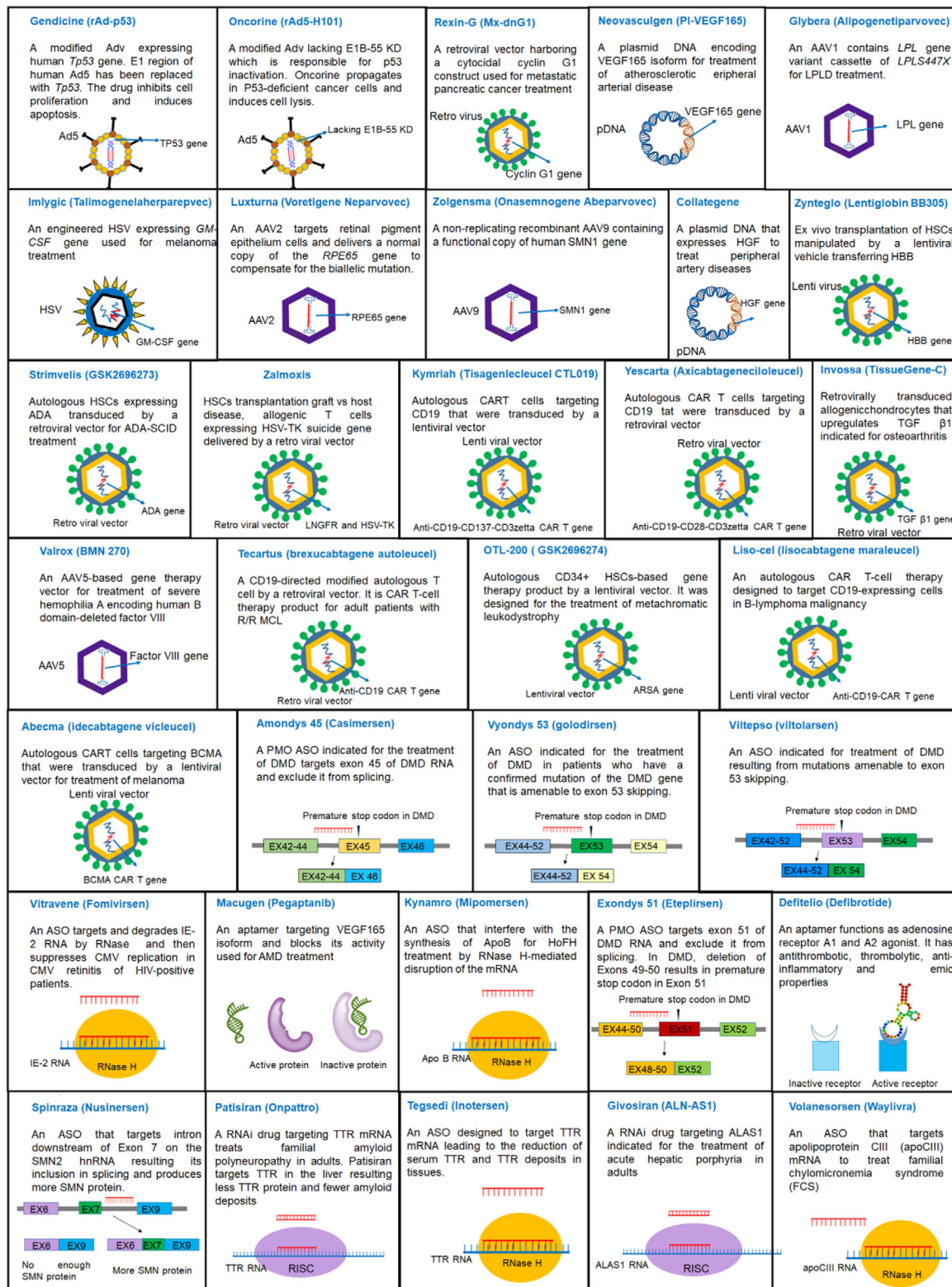**Figure 5.** Key events of three decades human gene therapy.

Figure 6. Schematic illustration and mechanism of action of 33 approved human gene therapy drugs or products. To date, two engineered Advs, four engineered AAVs, four engineered RVs, six engineered LVs, two naked DNAs or plasmid DNAs, and ten therapeutic oligo DNAs have been approved in the clinic and gene therapy market, mainly for the treatment of monogenic disorders and cancers.
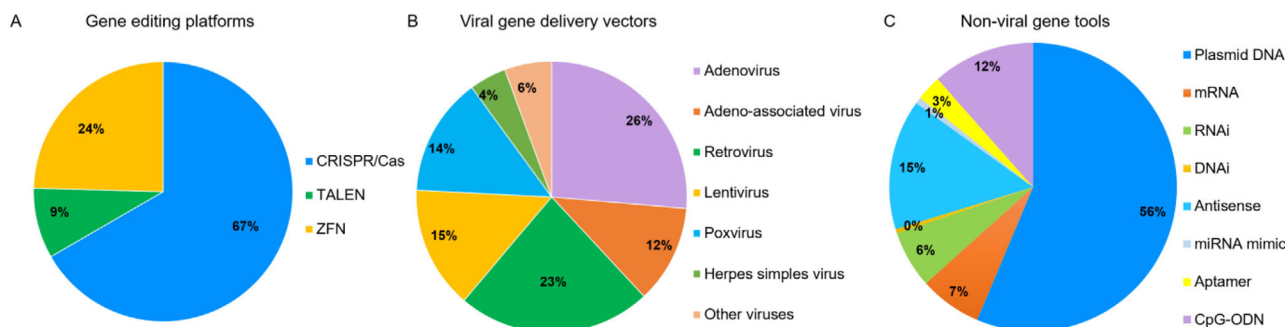
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Figure 7. Statistics of clinical gene therapy trials used genetic tools. A) Gene-editing systems. 38 (67%) trials used CRISPR/Cas, 5 (9%) trials used TALEN, and 14 (24%) trials used ZFN gene-editing system for gene therapy applications. B) Viral gene delivery systems. 609 (26%) trials used adenoviral, 270 (12%) trials used AAV, 535 (23%) trials used retroviral, 337 (15%) trials used lentiviral, 327 (14%) trials used poxviral, 102 (4%) trials used HSV, and 130 (6%) trials used other viruses for gene therapy applications. C) Non-viral systems. 474 (56%) trials used naked DNA or pDNA, 59 (7%) used therapeutic mRNA, 54 (6%) used RNAi, 4 (0.5%) used DNAi, 123 (15%) used antisense oligos, 6 (1%) used miRNA mimic, 24 (3%) used aptamer, 97 (12%) used CpG-ODN for gene therapy applications.

the potential to address many of the challenges of viral vectors, particularly concerning safety issues.[272,429]

Currently, the number of clinical gene therapy trials using nonviral systems is increasing. Less than one-third of gene therapy trials (about 900 completed or ongoing trials) use nonviral devices. Among these trials, 56% accounts for pDNA, 7% for therapeutic mRNA, 6% for RNAi technology, less than 1% for DNAi technology, 15% for ASOs, 1% for miRNA mimic, 3% for aptamers, and finally, 12% for CpG-ODN (Figure 7).

6. Conclusion

Gene therapies are transforming current treatment strategies for genetic disorders by using precise gene manipulation and gene delivery tools. Growth and maturation of these molecular tools have revolutionized the gene therapy field for promising therapies. On the other hand, the development of genetic tools is improving, bringing these innovations closer to the clinic. The spectrum of gene manipulations is expanding in current clinical trials. These include nucleotide substitution, gene replacement, gene knock-out, splicing modulation, and protein activity alteration. It is predicted that gene manipulation toolkits are vastly developed for efficient gene therapy during the next decade. Furthermore, they would be clinically safe, more effective, personalized and with less side effects.

Given the improvement and diversity of genetic toolkits and over 3000 clinical gene therapy trials, investments in the gene-therapy market, the next decade indeed looks bright and promising for gene therapies of human disorders. However, considerable technical concerns remain to be addressed. Currently, low efficiency and safety issues, immune system reactions, unclear gene manipulations and therapy guidelines, sophisticated approaches for drug development and manufacturing, mysterious gene–therapy drug interactions with the host and the high costs and prices of gene therapy drugs and products, are major challenges in gene therapy. Both in vivo and ex vivo therapeutic processes should be managed for developing safe gene-therapy products and appropriate gene expression and/or gene manipulation. Development of engineered viral strains, development of new viral vectors, and improvement of nonviral

gene delivery methods could overcome pre-existing immunity and enhance the efficiency of gene therapy practice. Furthermore, improving the systemic half-life of gene therapies would increase therapeutic durability.

Conflict of Interest

The authors declare no conflict of interest.

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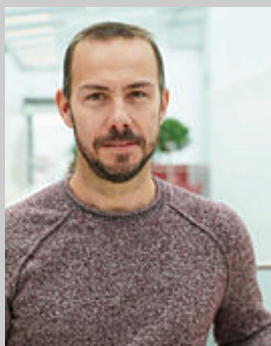
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