Gene Therapy and Cardiovascular Diseases

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Abstract

Cardiovascular diseases (CVDs) are the leading causes of death globally and urgently require new novel therapeutic strategies. Gene therapy is the application of gene modulation technology to treat abnormal gene expression under disease conditions. Viral- and nonviral-based gene delivery systems are the foundation of gene modulation in target cells. Moreover, plasmid- or oligo-based gene modulation tools as well as new advancements in gene editing using CRISPR/Cas technology are currently being tested in a variety of clinical trials. Here, we summarized state-of-the-art gene therapy technologies as well as recent clinical trials and discuss the applications and lessons of gene therapy in CVDs.

Keywords

Gene therapy · RNA therapy · Cardiovascular diseases

1 Background

Genes, originating from segments of DNA or RNA, are the basic building blocks for the traits that make up organisms [1]. Phenotypic traits derive from a combination of our genetic material and the environment we stem from. This genetic material, including nuclear and mitochondrial DNA, is expressed through the transcription into RNA, which can act as functional molecules themselves or which can be translated into functional proteins [2, 3].

Importantly, gene sequences can exhibit dysfunctional behaviors which are known as mutations, and these mutations have the potential to lead to the development of diseases. These diseases caused by gene mutations are categorized as chromosomal diseases, gene disorders, or mitochondrial dysfunction [4]. In addition, certain infectious diseases such as acquired immune deficiency syndrome (AIDS) as well as some noncommunicable diseases like cancer, are known to be mediated by gene abnormalities. For example, mutations in the DNA repair gene, breast cancer gene 1 (BRCA1), are associated with an increased risk of a variety of cancers such as prostate,

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breast, and ovarian cancers owing to high loads of DNA damage and resulting in genomic instability [5, 6]. It is reported that people with a BRCA1 mutation have an extremely high risk for developing breast cancer (87%) and ovarian cancer (44%) compared to noncarriers of this mutation [7].

Several traditional treatments such as surgery, chemotherapy, and radiation as well as novel approaches such as hormone-based therapies, stem cell therapies, or immunotherapies, are widely used for cancer treatments and for targeting cardiovascular diseases (CVDs) [8–14]. Quite often, traditional therapies are not always successful at correcting the mechanism by which the disease occurs and rather treats the symptoms of the disease instead. Gene therapy, on the other hand, aims to target and potentially correct any genetic mutation causing a disease, providing a new treatment option which focuses on the initial source of any illness [15]. Initially, gene therapy was designed to introduce a new healthy copy where a gene was either mutated or absent in cells via a vector. The restoration of gene function following a therapeutic modification results in the correction of genetic abnormalities stemming from hereditary or environmental processes. With the advancement of gene therapy, however, also comes new techniques by which to manipulate the genome. New mechanisms involving gene editing and inactivation have emerged in recent years such as the CRISPR/Cas system and antisense strategies encompassing RNA-based therapeutics. While the disruption and silencing of genes through direct DNA and RNA editing tools are new and exciting developments in this field, we will primarily focus here on the state-of-the-art vehicle delivery approaches of introducing genes into cells [16].

2 History of Gene Therapy

The concept of gene therapy as a gene modification tool has been around since the 1970s. Despite the beneficial potential in reversing possibly life-threatening mutations, gene therapy also raised deep ethical concerns surrounding genetic modifications [17]. However, the field of gene therapy continued to grow since the 1980s, when the retroviral vector system was developed to efficiently deliver transgenes into mammalian cells and modify preexisting genes [18]. By the 1990s, the first approved gene therapy was applied to two children in the USA who suffered from adenosine deaminase deficiency-severe combined immunodeficiency (ADA-SCID). Two years after the gene therapy treatment, which was performed ex vivo after T cell apheresis using cell culture expansion and reinfusion into the patients after 9–12 days, the integrated vector-mediated ADA gene remained expressed in T cells [19]. This report was the first positive indicator that gene therapy could be an efficient and safe treatment option for patients suffering from immune deficient diseases [19]. Apart from genomic modifications, RNA interference (RNAi), in particular small interfering RNA (siRNA), has also been developed as a gene silencing therapy to block abnormal RNA or protein expression which may lead to disease [20, 21]. In 2003, siRNAs were first shown to mediate Fas cell surface death receptor (FAS) knockdown in vivo, which allowed for a reduced threat from fulminant hepatitis [22]. Notably, the first human trial targeting the vascular endothelial growth factor (VEGF) and kinesin spindle protein (KSP) used lipid nanoparticle (LNP) formulation of siRNAs. This technique was applied to cancer patients in 2013, providing both safe and pharmacokinetically sound evidence that siRNA-mediated gene therapy could be used effectively in humans [23]. In 2008, treatment of Leber's congenital amaurosis (a rare disease typically causing severe visual impairment) by recombinant adeno-associated virus 2 (rAAV2)-

RPE65 became the first effective AAV-mediated gene therapy to show clinical efficacy and disease improvement in patients. Three parallel trials proved that patients who got a single subretinal injection of rAAV2-RPE65, to complement the causative mutation in the RPE65 gene, had long-term improvement in vision and light sensitivity [24–26]. Importantly, the follow-up studies showed persistent visual improvements in patients and did not raise any safety concerns [27–29]. Despite the successes seen with viral vectors in clinical trials, other gene editing techniques were advancing in parallel. With the development of the engineered clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) nuclease technology, the ability to manipulate DNA became increasingly simplified, efficient, and cost effective [30, 31]. The CRISPR-Cas technology improved the possibility of gene therapy mediated by engineered cells such as chimeric antigen receptor T cells (CAR-T) [32]. These engineered CAR-Ts were produced to recognize, target, and destroy cancerous cells in a more effective and localized manner [33]. After the first successful clinical trials using engineered CAR-T therapy for lung cancer in 2016, they have since been further utilized in active clinical trials for the treatment of leukemia, lymphoma, and solid tumors [34–38]. The existence of clinical trials using multiple gene therapeutic techniques brings to light the high demand and great advancements of multiple technologies that are being pursued to treat genetic abnormalities.

3 Material and Approach of Gene Therapy

Currently, the field of gene therapy has been broadly studied; however, it is still a therapeutic concept predominantly based in research laboratories with only a limited number of ongoing clinical trials [39]. The efficiency and specificity of gene delivery as well as gene regulation utilized in target cells are the two major obstacles that must be overcome to successfully achieve safe and effective genetic modifications [40]. In this part, we will summarize the viral and nonviral approaches that are currently utilized in gene therapy.

4 CRISPR-Cas-Mediated Gene Editing

Primarily, we see gene therapy as using a vector to replace a mutated or missing copy of a gene. Another form of treatment that is newer to the field of gene therapy involving editing and inactivation is the CRISPR/Cas system. This mechanism avoids the complications and risks associated with some viral vector delivery and the correlating toxicities and safety concerns that have been seen in several clinical trials over the years [41]. The CRISPR/Cas system was the most recent gene editing technique after the foundational mechanisms using transcription activator-like effector nucleases (TALENs) and first zinc finger nucleases (ZFN). This genome editing tool can be delivered to cells through AAV vectors [42], which have a safer history comparative to other viral vectors, as well as through other nonviral delivery strategies. CRISPR/Cas operates via the specific targeting of a segment of DNA in the genome by utilizing a particularly designed single-guide RNA (sgRNA) to identify only the region requiring intervention. Optimizing this specificity allowed by sgRNAs will continue to help reduce off-target effects currently seen by this gene editing technique [43, 44]. The Cas protein, an endonuclease allowing for breaks in DNA at the target site, can also be modified to reduce side effects. These modifications, however, do not make the CRISPR/Cas system superior or inferior to standard gene replacement therapy using viral vectors. Both methods have different advantages and flaws unique to their mode of action and delivery mechanism [41]. The mechanism by which the Cas protein cuts DNA inducing double-stranded breaks (DSBs) can also lead to the unintended activation of apoptosis pathways, such as triggering p53, instead of editing the DNA segment after the break [45]. One recent development with this technique is using the base editing (BE) system.

This allows for a single targeted base pair to be exchanged, for example, a C-G base pair can be exchanged for a T-A by cytidine base editors (CBEs) and the reverse mutation can also be corrected for A-T pairs exchanged for G-C using adenosine base editors (ABEs). This occurs by using a catalytically deactivated Cas9 endonuclease (dCas9) that does not induce DSBs, allowing for single base pair edits [46, 47]. RNA is also edited using CRISPR technology with the endonuclease Cas13 (Cas9 can be modified to target RNA instead of DNA as well; however, Cas13 exclusively targets RNA). Since this system does not require the protospacer adjacent motif (PAM) sequence that is necessary at the DNA editing sites, Cas13 can be more broadly used. There is an additional advantage, in that the Cas13 system does not permanently edit the genome since it is targeting RNA after transcription, resulting in nonpermanent changes (which could trigger immune reactions or lead to incorrect editing with DNA [48]). The method for base pair editing is similar to that of the dCas9 system, using dead Cas13 (dCas13) with the ADAR2 domain to edit adenosine to inosine in what is known as the REPAIR mechanism or with APOBEC1 for exchanging a cytidine to uridine using the RESCUE technique [41, 48–50].

The first CRISPR clinical trial utilized PD-1 edited T cells to treat non-small-cell lung cancer in China. Most patients had minimal side- and off-target effects, and a decrease in disease progression was also seen when edited T cells reached higher levels in patients [51, 52]. Edited PD-1 and CAR-T cells were also used as a combined treatment for the first CRISPR clinical trial to take place in the USA in 2018 to treat myeloma and sarcoma. The study was also deemed a success, in that it provided initial findings in combination with the first Chinese trial that CRISPR editing as a treatment for disease progression seemed to be relatively safe with acceptable side effects. Treatments in both trails did not produce an overwhelming immune response either, which was an early problem that was observed in some of the first clinical trials using gene replacement therapy [53]. Both off-target and on-target mutations were seen in both trials; however, while this safety concern is still valid and needs to be closely monitored in all future DNA editing trials, neither effects were detrimental to the patients and were found to take place primarily in noncoding segments of the genome.

Aside from cancers, CRISPR has also taken its first step in 2019 to treat a genetic disease, sickle cell disease (SCD), by increasing fetal hemoglobin levels in isolated and edited autologous blood stem cells [54]. These stem cells are reintroduced into the body of the patient and can then create a new population of hemoglobin-producing blood cells from the bone marrow. This technique is also quite specific as it involves ex vivo editing of the blood cells directly, which greatly reduces off- and on-target side effects seen with CRISPR editing through a delivery vector into the patient [55]. Overall, CRISPR editing to treat diseases of both genetic and acquired origins is still in its early stages. So far, the clinical trials that have taken place in the last few years have been used to primarily assess feasibility, toxicity, tolerability, and practicality before shifting the focus to successfully cure a disease [56].

5 Nonviral-Mediated Gene Therapy Methods

In the early 2000s, nonviral approaches were not a common tool for gene therapy due to low delivery efficiency and specificity [57]. In the past few years, production and modification of nonviral methods have greatly improved and led to a higher gene transfer efficiency while also allowing for long-term gene expression, not only in vitro but also in vivo. In addition, the low cost, ease of production, and reduced pathogenicity of nonviral applications have important manufactory and safety advantages over viral approaches [58]. Currently, siRNAs or RNA inhibitors, RNA mimics, modified mRNAs (modRNA), and other

oligonucleotide-based molecular products are gaining attention as potential therapeutic materials in the application for gene therapy [59].

6 siRNA and RNA Inhibitors

Since the first RNAi phenomenon was reported in 1990 [60] and the mechanisms behind it were clarified in 1998 [61], siRNAs have become a regular tool to perform gene inhibition in cell culture. SiRNAs are small RNA transcripts with a length of approximately 20–22 nucleotides and can disrupt protein translation by promoting the degradation of RNA transcripts through binding to the targeted mRNA [62]. Similar gene silencing can also be reached by antisense oligonucleotides (ASOs). These synthetic, single-stranded oligonucleotides prevent expression of a target protein by blocking the specific region of target RNA or DNA [63, 64]. For example, locked nucleic acid (LNA) is a kind of modified ASO with a bridged, bicyclic sugar moiety. LNA will bind to the target RNA forming a DNA-RNA hybrid, and RNase H-dependent degradation of the targeted RNA will then be activated [65].

MiR-132 is a breakthrough example of RNA gene therapy and is the first antisense gene therapy to treat CVDs. Since 2011, miR-132 has been reported as a regulator of cardiac fibrosis [66, 67], cardiac hypertrophy, and cardiomyocyte autophagy [68]. A series of preclinical investigations further proved that the inhibitor of miR-132 (antimiR-132) could rescue cardiac hypertrophy and heart failure in mice and more importantly in pigs [68–70]. Notably, CDR132L, the miR-132 inhibitor applied in pigs, is a synthetic LNA-ASO modified with a fully phosphorylated backbone. In addition, large animal investigations reported a safe administration, high cardiac delivery efficiency, and clear reduction of miR-132 expression in the myocardium and plasma [69, 70]. With these promising preclinical results, CDR132L moved forward for the first-in-human study in 2019 involving 28 patients with stable chronic heart failure of ischemic origin (NCT04045405). Safety, pharmacokinetics, and heart failure relevant pharmacodynamic parameters are all intensively evaluated in this phase I clinical trial. After a 1-year follow-up, CDR132L has proved to be safe to administer to patients and can also be well tolerated without an apparent dose-limiting toxicity. Interesting, reductions of NT-proBNP, significant QRS narrowing, and positive trends for relevant cardiac fibrosis biomarkers were reported after CDR132L treatment in heart failure patients with the standard care of treatment [71].

Notably, several siRNA-mediated gene therapies have been approved and applied in the clinics [72]. For example, Alnylam's Onpattro (Patisiran) (NCT01960348) was approved by the Food and Drug Administration (FDA) as a novel RNA interference drug to treat hereditary transthyretin-mediated amyloidosis (hATTR), a rare disease characterized by extracellular amyloid protein deposition leading to multiple organ dysfunction [73]. In addition, several siRNA-mediated RNAi drugs are currently in clinical trials (phase II/III) such as SYL1001Sylentis (NCT 03108664) for dry eye disease or QPI-1007 Quark (NCT 02341560) for non-arteritic anterior ischemic optic neuropathy. Detailed siRNA and RNA inhibitor clinical trials are excellently summarized elsewhere [72, 74].

7 Nucleic Acid Drugs

The first successful genetic transfer in mice occurred in the 1990s with an overexpression of chloramphenicol acetyltransferase, luciferase, and β-galactosidase by an in vitro transcribed (IVT) RNA or DNA plasmid in the skeletal muscle [75]. In the following years, IVT mRNA was introduced for diverse applications, including protein substitution and vaccination approaches for cancer and infectious diseases [76–79]. IVT RNAs are synthesized RNAs that can be transcribed in vitro from DNA templates containing the sequence from either protein coding genes or noncoding RNA transcripts [80]. ModRNAs are IVT RNAs with modified nucleosides or synthetic nucleoside analogues which could reduce the innate immune response of the host cell and

improve tissue specificity. IVT RNAs have come into focus as novel drugs to revise abnormal genetic disorders, allowing for the overall improvement in the field of RNA pharmacology [81].

Although oligo nucleic drugs remain in the initial stages of preclinical or phase I/II clinical trials, some of the pilot investigations have broaden the potential applications of IVT RNA as the future of medicine. For example, in cancer immunotherapy, Melan-A, tyrosinase, gp100, Mage-A1, Mage-A3, and survivin IVT mRNA were utilized in metastatic melanoma patients in a phase I/II trial (NCT00204607) [82]. In addition, several phase I/II clinical trials using IVT mRNAs for the treatment of HIV infections demonstrated the safety of IVT mRNA vaccines and observed the induced responses of immunogens in CD8+ and CD4+ T cells [83–85]. Detailed IVT RNA clinical trials are well reviewed by Sahin et al. [86].

8 Viral-Based Approach for Gene Therapy

The advantage of viral vectors is their high infection efficiency in a broad spectrum of cells, ranging from prokaryotes to many eukaryotic cells. Therefore, recombinant viral vectors have the potential to package and deliver the transgene to the targeted cells. Viral vectors can be divided into genome-integrating vectors as well as non-integrating vectors, classified by whether the transgene can be continuously expressed in dividing cells [87].

Most RNA viruses with single-stranded RNA (ssRNA) or double-stranded RNA (dsRNA) are not able to integrate their genome into the host chromosome, with the exception of retroviruses. One of the best studied retroviruses is the human immunodeficiency virus type 1 (HIV-1) [88]. The first retroviral vectors which were used in human gene therapy trials [19] are derived from the Moloney murine leukemia virus (MLV) [89].

Lentiviruses are a complex subtype of retroviruses which can cause chronic and deadly diseases. Notably, the outstanding feature of lentiviruses is the high efficiency of infection and genomic integration in nondividing and terminally differentiated mammalian cells, including lymphocytes and macrophages. In addition, the ability to transport large genetic payloads as well as their stable long-term transgene expression makes them a very attractive tool for gene delivery [90, 91]. So far, three generations of lentiviral vectors have been developed for transgene modification [92]. First-generation lentiviral vectors originate from a significant portion of the HIV genome, including the gag and pol genes encoding for viral structural proteins and the viral RNA reverse transcriptase, respectively, as well as several additional viral proteins such as the envelope protein (VSV-G) [93]. VSV-G recognizes a ubiquitously expressed receptor such as low-density lipoprotein receptor (LDL-R) [94], which aids in a high transduction efficiency of the lentiviral vector in a wide range of cells [95]. The main improvements that were made to the second and third generations of lentiviral vectors were regarding safety. Second-generation lentiviral vectors were subsequently developed to remove accessory gene factors such as vif, vpr, vpu, and nef. Third-generation vectors split the viral genome into separate plasmids and removed the tat gene to further improve the safety of the vectors [96]. In 2003, the first lentiviral clinical application occurred by delivering a long antisense RNA sequence targeting the HIV-1 envelope gene for anti-HIV therapy [97]. It is important to note that eight years after the study, there was no apparent risk for serious adverse or long-term events occurring in this clinical trial [98].

The Sendai virus (SeV) is a member of the *Respirovirus* genus, a negative sense ssRNA virus from the Paramyxoviridae family. Due to the cytoplasmic gene expression of SeV, the absence of genomic integration is a unique feature of recombinant SeV vectors compared to a retroviral vector [99, 100]. SeV vectors have been used in clinical trials and tested in a live attenuated vaccine [101], in cancer [102], as well as in critical limb ischemia [103] for gene therapy.

A DNA viral vector is an additional virus system that employs double-stranded DNA (dsDNA) or single-stranded DNA (ssDNA) as its genomic materials. Adenoviruses (AdVs) are non-enveloped DNA viruses with a diameter of 70nm, a 36 kb dsDNA, and about 50 viral polypeptides [104]. So far, more than 50 different AdV serotypes have been characterized, and a majority of them can also be observed naturally in humans. In gene therapy, AdV types 2 and 5 were found to be good options for clinical trials due to the fact that they were not already associated with human diseases [105, 106]. In addition, adenoviral vectors have a packaging capacity of up to 8000 base pairs (bps) of foreign DNA, which is sufficient for the delivery of most therapeutic genes. Similar to lentiviruses, recombinant AdVs (rAdV) can also infect dividing and quiescent cells with equal transduction efficiency [107]. Notably, rAdVs can obtain a higher production yield (10^{10}–10^{11} infectious particles/ml) compared to other vector systems such as retroviral vectors [108]. However, rAdVs show no integration into the targeting cell genome, indicating short-term expression particularly in dividing cells. In addition, in vivo applications of adenoviral vectors could lead to cellular immunity and the generation of a humoral response, also reducing the expression or the effect of adenoviral therapy. Furthermore, the generation of neutralizing antibodies of rAdV could strongly reduce their utility, resulting in the difficulties of repetitive treatments [109]. In 1993, the first AdV-mediated gene therapy was performed to transfer cystic fibrosis transmembrane conductance regulator (CFTR) cDNA to treat cystic fibrosis in humans. The benefits of AdV treatment were observed, and no virus-associated adverse effect was detected, indicating that adenoviral vectors were effective at transferring genes to most organs in vivo [110]. However, immunogenicity still limits application of the AdVs in clinical trials [111].

In 1965, a number of "satellite viruses" were observed by electron microscopy (EM) from AdVs prepared in the lab [112, 113]. These small DNA viruses (20–25 nm in diameter) were dubbed AAVs due to their ability to replicate in the presence of AdVs [113]. Two years later, AAVs were first isolated from human tissue [114]. AAVs are one kind of *Dependoparvovirus* within the family Parvoviridae, and they have not only been found in humans but also in nonhuman primates. In addition, they are comprised of an icosahedral protein capsid of ~26 nm in diameter and a single-stranded DNA genome of ~4.7 kb [115]. The wild-type AAV capsid is composed of three types of subunits (VP1, VP2, and VP3). Two T-shaped inverted terminal repeats (ITRs) are located at the ends of the viral genome, and the viral replication and packaging signals are flanked between ITRs. Four rep gene-encoded proteins are the source of viral replication, and capsid subunits are alternatively spliced and translated by cap genes through different start codons [116]. The wild-type AAVs also have the ability to integrate into the human AAVS1 genomic locus [117]. In the early 1980s, the secondary structure of the AAV ITR region only allowed for a very limited number of plasmids cloned with AAV sequences [115]. Until 1984, engineered rAAV2 vectors were generated as a useful tool for gene transfer in mammalian cells and had become the foundation of AAV-mediated gene therapy [118]. rAAVs consist of the same capsid sequence and structure as wild-type AAVs. Importantly, removal of viral coding sequences enlarged the packaging capacity of rAAVs and reduced the genomic integration, immunogenicity, and cytotoxicity of AAVs. However, the gene packaging capacity of rAAVs is still under 5 kbs [119]. The best characterized and most widely applied AAV serotype is the naturally occurring AAV2. Notably, AAV9, a clade F AAV serotype isolated from human liver tissues, demonstrates the ability to bypass the blood-brain barrier [120]. Till now, 13 different human or nonhuman primate AAV serotypes have been classified [121]. However, rAAVs are a major type of AAVs which have been utilized in preclinical investigation and clinical trials. Since the early 1990s, clinical trials mediated by rAAV2 and rAAV1 vectors have been tested in several diseases including CF, hemophilia B, Canavan disease, and α1-

antitrypsin (AAT) deficiency [122–125]. These pilot phase I/II trials demonstrated a good gene expression duration of rAAV therapy as well as proved the safety of injection of rAAV.

9 Gene Therapy in CVDs

In the last three decades, several gene therapies have been tested for cardiovascular disorders including coronary or peripheral artery disease and heart failure [126]. After over 100 clinical trials, there has so far been no successful therapeutic effect reported for gene therapy in CVDs. In angiogenesis, therapeutic attempts are focused on the formation of new blood vessels driven by the production of cytokines which have so far been shown to recover some heart function in animal experiments [127]. For heart failure therapy, the modulation of Ca^{2+} in cardiomyocytes has become the main target of interventional therapy. Similar to therapies in angiogenesis, the beneficial effects of gene therapy in heart failure observed in animal studies did not translate to clinical trials in the last two decades [40]. Here, we will summarize the current clinical trials of gene therapy in CVDs.

To induce the formation of new capillaries or blood vessels, cytokines such as VEGF, FGF (basic fibroblast growth factor), and G-CSF (granulocyte colony-stimulating factor) have been tested in clinical trials as a form of gene therapy for CVDs [128, 129]. This technique has been used in over 20 clinical trials using naked plasmid DNA which carries the VEGF gene, injected into the myocardium of patients with severe coronary artery disease (CAD) in the late 1990s and early 2000s [130–135]. These randomized, double-blinded, placebo-controlled trials failed to show a beneficial effect on either the symptomatic or clinical outcome. One of possible reasons for this is the poor cardiac uptake of the naked DNA plasmid, thus limiting the biological activity in the human heart. Therefore, adenoviral-based cDNA delivery vehicles have also been tested for cardiac gene therapy in clinical trials. For example, AdGVVEGF121.10NH (commercial name: BIOBYPASS, adenoviral vector with a strong CMV enhancer/promoter, and VEGF-A121 cDNA) was used in a series of clinical trials to treat patients with CAD [136]. In preclinical animal studies, myocardium injection of an adeno-vector was able to improve myocardial angiogenesis, increase blood flow, and rescue heart function in the ischemic porcine heart [137–140]. A phase I clinical trial tested in patients with severe CAD also demonstrated that an intramyocardial injection of AdVEGF121 was well tolerated and provided some promising initial findings that showed a trend toward the reduction of myocardial ischemia injury [141, 142]. Interestingly, the Randomized Evaluation of VEGF for Angiogenesis (REVASC) trial reported that AdVEGF121 was associated with significantly improved symptoms and exercise capacity of CAD patients [143]. Unfortunately, other AdVEGF121 trials showed no difference of exercise capacity, time to ischemic threshold, or myocardial perfusion compared to the control patients [144]. Although these completed trials showed no significant beneficial effect in patients, there are still some ongoing clinical studies based on adenoviral vectors, such as three different VEGF-A isoforms in a phase I/II trial (NCT01757223) that had recently begun in 2020 to optimize the therapy.

Another interesting study of gene therapy in CVDs occurred by targeting Ca^{2+} ATPse and SERCA2a, a key factor for Ca^{2+} reuptake by the sarcoplasmic reticulum [145]. Since the early 1970s, the sarcoplasmic Ca^{2+} ATPase was found to be an important molecule for heart function in animal models and was also found to be reduced in different CVDs [146–149]. The idea to restore levels of SERCA2a has been an extremely popular strategy for gene therapeutics in heart failure [150]. In 2007, patients with advanced heart failure were treated with an AAV1 containing the SERCA2a gene to restore protein expression (known as the CUPID trial, calcium upregulation by percutaneous administration of gene therapy in patients with CVDs; NCT00454818) [151, 152]. It was the first phase I clinical trial to use an AAV gene therapy for heart failure and

simultaneously verified the safety and feasibility of the treatment [153]. Unfortunately, the blinded, randomized, placebo-controlled, multicenter study failed to demonstrate positive clinical outcomes. The AAV1- SERCA2a treatment does not improve heart function in patients with heart failure and severely reduced ejection fraction, ischemic and nonischemic etiology (NCT01643330), or left ventricular assist devices (NCT01966887) [154, 155]. Although these current attempts did not show positive therapeutic results, several other approaches are currently ongoing to improve gene therapy in heart failure. For example, a phase I clinical study (NCT04179643) that commenced in 2020 is testing BNP116.sc-CMV.I1c, a chimeric AAV2/AAV8 capsid with a high specificity for cardiac and skeletal muscles with less off-target effects in the liver and lungs [156], in patients with class III heart failure.

10 Future Perspectives of Gene Therapy in CVDs

Critical problems of gene therapy in CVDs involve the insufficient gene transduction into heart tissue or cells [157]. Currently, heart-specific gene delivery technology still limits the application of gene therapy in CVDs. Notably, naked plasmid transfection as well as viral-mediated gene delivery did not cause major safety concerns in most phase I/II trials (summarized above). Transfection of the naked plasmid showed a short-term expression time when compared to the AAV systems which could prolong gene expression [158]. However, the neutralizing antibodies of AAVs reduce the vector transduction efficiency and lead to a big obstacle of AAV application in the clinics [159]. In addition, the high cost of AAV manufacturing for clinical applications is still a challenge for normal patients. Thus, improving cardiac cell specificity, reducing the innate immune response, and reducing production price as well as long-term gene expression and stability are the main goals for the next generation of AAVs used in gene therapy.

11 AAV Engineering for Heart-Specific Therapy

To overcome low specificity in the heart, or more specifically cardiomyocytes, capsid engineering of AAVs tries to improve cell-type tropism. Several AAV serotypes have now been identified since the first AAV was observed, and they have been seen to share similar structures such as genome size and genetic organization. However, the differences are in the amino acid composition of the capsid proteins. Thus, it is possible to obtain chimeric viral particles by AAV engineering through transencapsidation [160]. The capsid reengineering can help to optimize receptor binding and transduction efficiency and more importantly tissue target selectivity of rAAV. Currently, capsid chimera libraries are derived from a variety of AAV serotypes or the random mutation of the capsid region and are a good platform for heart-specific peptide selection [161, 162].

In addition, engineered or random capsid mutagenesis, DNA shuffling, and direct selection are the most commonly used techniques to generate new rAAV variants [163–165]. For example, AAV2i8 and AAV-SASTG, two AAV2 chimeras, achieved a higher cardiac and skeletal muscle transduction efficiency with a lower off-target phenotype seen in the liver [166, 167]. In addition, Pulicherla and colleagues generated engineered liver-detargeted AAV9 vectors which had a similar transduction efficiency to the heart and muscle as wild-type AAV9 but 10- to 25-fold lower infection of the liver [168]. The modification of the AAV capsid could be a solution to improve AAV-mediated gene therapy in CVDs.

12 Successful Viral-Based Gene Therapy in Clinical Trials

While every new gene therapy trial helps advance this technique of repairing the genome, crucial safety concerns have arisen with the development of this treatment option. One of the major benefits of AAVs is their low potential to produce

immunological responses due to the absence of viral protein expression and the extremely limited viral elements present in the vector. The cellular immune response decreases without presentation markers on the surface of cells transduced by the AAV [169]. This is not to say, however, that AAVs cannot produce any immune response. A limitation to using AAVs involves an adaptive humoral response which occurs in an organism when they have been previously infected by an AAV of the same serotype. Neutralizing antibodies (NAbs) have the capacity to neutralize this additional infection from the same AAV serotype in 30–60% of humans [170]. NAbs are capable of limiting this possibly lifesaving gene delivery by blocking AAV transduction into cells of a person who was previously infected. The delivery of alternate serotypes is one possible solution, although some NAbs against one specific serotype have also been seen to neutralize additional serotypes as well [170]. To overcome this obstacle, studies have been performed such as one that simultaneously administered anti-CD20 antibodies in order to reduce the internal titer of NAbs to reduce the neutralization of the added gene therapeutic vectors and to also engineer AAV capsids as was previously discussed above [171].

With many decades of research, gene therapy was eventually successful in clinical trials. In 2017, a study was published where the survival motor neuron 1 (SMN1) gene was delivered to patients born with a mutation or deletion that led to spinal muscular atrophy type 1 (SMA1). SMN proteins are produced primarily through the SMN1 gene, as the SMN2 gene is missing an exon, leading to a reduced protein production from this gene alone. Therefore, having an SMN1 deletion and only copies of SMN2 almost guarantees that a patient will have SMA1 as the SMN2 gene alone produces an insufficient level of protein for neuronal cells. Without the SMN1 gene, motor neurons lose the ability to function, resulting in severe motor disabilities, leading to lifelong ventilation and/or death in 75% of patients before 2 years of age [171–173]. Zolgensma (biologically known as AVXS-101), first approved for use in the USA in 2019, is a gene therapy developed using an AAV9 vector to deliver a healthy copy of the SMN1 gene to motor neurons to hinder disease progression and improve the quality of life of these infants. The study showed improvements in motor function in 11 out of 12 patients in the initial trial with more than half not requiring further ventilation and even two gaining the ability to walk [172]. Interestingly, Zolgensma was not the first FDA-approved drug to treat SMA. In late 2016, an antisense oligonucleotide drug known as Spinraza (nusinersen) was first approved to treat SMA through a multi-dose system approach in patients from the early stages of birth [174]. This treatment option was administered through direct injection into the cerebrospinal fluid four times in the first 64 days of the trial [175]. It was determined at the completion of the study that Spinraza would need to be consistently administered for the duration of the patient's life [173]. It is important to note that the mechanism of action by which this antisense oligonucleotide works is quite different to that of Zolgensma. The aim of Spinraza is to have more full-length SMN proteins expressed in motor neurons by targeting the pre-messenger RNA of the existing copy of SMN2 [175]. Since the therapy only interacts at an RNA level, a continuous treatment plan is required to manage disease progression. Zolgensma, on the other hand, is a direct form of gene replacement therapy that only involves a single administration of a healthy SMN gene via an AAV vector, which can then directly produce full-length SMA proteins without consistent manipulation at the RNA level [173].

Even though the treatment only requires a one-time administration, Zolgensma is currently the most expensive drug on the market, partially due to this single-dose treatment, the cost of developing the drug, and the rarity of the disease itself, highlighting another (in this case, economic) limitation of AAV-based gene therapy [176]. Despite the high costs, this drug is not perfect and can lead to elevated liver enzymes in patients who have taken it, which can cause safety concerns in those with preexisting liver conditions. Other AAV-based therapeutics undergoing clinical trials have also been seen to

cause severe problems, even death, in patients with preexisting liver conditions. The FDA has even halted clinical trials after two patients died while receiving a high dose of AT132 in the Audentes Therapuetics' trial [177]. This AAV8 vector is used to deliver a healthy copy of the myotubularin-1 gene to treat X-linked myotubular myopathy. Since this was the highest dose of AAV gene therapy given to date in a clinical trial and the patients who died as a result also had underlying liver conditions, safety concerns relating to low-dose treatments especially in patients with healthy livers are relatively low. Other trials for AAVs used to treat Duchenne muscular dystrophy have also observed toxicities in patients; however, the knowledge and understanding of gene replacement therapy continues to grow with each trial, especially when complications arise [177]. This was especially true in the case of Jesse Gelsinger who was the first patient to die from an immune reaction to an rAV to treat ornithine transcarbamoylase back in 1999. The severe immune reaction that he experienced that ultimately led to his death was extremely rare as none of the other 4000 patients from other clinical trials experienced the same side effects. The reevaluation that this led to by the FDA to intensely study and determine why and which vectors could be harmful as delivery vehicles has undoubtedly saved lives and ensured safer treatment for all future studies after this tragic loss [178].

13 Novel Therapeutic Target Genes

Current gene therapy candidates in CVDs are mainly focusing on cytokines or calcium-related proteins such as VEGF or SERCA2a. Apart from coding genes which only comprise 1–2% of the human genome [179], noncoding RNA (ncRNA) transcripts (without coding potential) are worth noting as future therapeutic targets. Although the function of most ncRNAs is still unknown, growing evidence has proven that ncRNAs are key modulators in diseases (such as cancer or CVDs) [180]. For exploring a clinical application, numerous independent studies regarding circulating ncRNAs have been reported as biomarkers to predict and monitor the response of CVDs and treatments [181, 182]. Notably, preclinical investigations of ncRNAs are also heading in the direction of potential therapeutic options for CVD patients. For example, a conserved long noncoding RNA (lncRNA) H19 is a powerful ncRNA molecule for the protection of pathological cardiac hypertrophy. Restoration of H19 expression mediated by AAV injection four weeks after induction of chronic left ventricular pressure overload successfully attenuated cardiac hypertrophy in mice. In addition, AAV6-mediated H19 overexpression improves contractility of human engineered heart tissue, highlighting translational potential of H19 [183]. In addition, miRNAs and ncRNAs approximately 20 nt in length have also been evaluated in several preclinical studies for the treatment of CVDs such as miR-181a [184]. Overexpressed miR-181a mediated by AAV9 delivery one week after MI was able to show recovered heart function in mice [185]. Apart from these novel ncRNAs, some traditional protein coding genes were also validated as putative CVD therapeutic targets in preclinical investigations. For example, Tert, a telomerase reverse transcriptase encoding gene, is well known for its role in cellular senescence. Cardiac-specific overexpression of Tert by AAV9 attenuated cardiac dilatation, improved ventricular function, and reduced infarct scarring after an acute MI [186]. Following studies proved that AAV-Tert overexpression protected against cardiac apoptosis and cardiac dysfunction from doxorubicin-induced cardiotoxicity in mice [187]. These encouraging preclinical studies recognized the potential to utilize ncRNAs as well as protein coding genes as novel therapeutic candidates to treat CVDs.

14 Conclusion

After over 30 years from the first gene therapy clinical trial, no successful application has since been reported in CVDs, indicating that this field is still young and needs further development which

is currently pursued with tremendous efforts both in academia and in the pharmaceutical industry. The improvement of gene delivery platforms and preclinical investigation systems, as well as for novel therapeutic candidates, are supporting the development of next-generation gene therapy in rare genetic disorders as well as CVDs.

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