

Expansion of methods of gene editing therapy and analysis of safety and efficacy

9.1 Introduction

Gene therapy involves a number of different approaches. These can include use of factors that block elements within the genes, for example, nucleotides in genes or within immediate product of genes, mRNA. Approaches can also be directed at increasing expression of specific genes.

This chapter will also include discussion of methods to add a normal version of a gene in cases where disease arises due to absent or inadequate function of a specific gene, and more recent efforts directed at editing somatic genes through newly developed methods. In addition, gene therapy approaches to treatment of specific diseases will be presented.

9.2 Therapies designed to block nucleotides or RNA derived from a specific gene

Antisense oligonucleotides were reviewed by [Bennett \(2019\)](#) and [Roberts et al. \(2020\)](#). They are defined as small 18–30 nucleotide single stranded polymers and they were subdivided into steric block polymers and RNase H1 competent polymers. RNase H1 enzyme was reported to recognize RNA – DNA heteroduplex substrates that result from the binding of DNA oligonucleotides and matching RNA. RNase H1 enzyme then leads to destruction of RNA. Specific antisense oligonucleotides referred to as gapmers bind to RNAs and can serve to activate RNase H enzyme leading to mRNA degradation.

9.3 Oligonucleotide therapies

[Roberts et al. \(2020\)](#) summarized aspects of oligonucleotide therapy and specific goals of this therapy; they included the following.

1. Modulation of gene expression, RNA inhibition, target RNA degradation through RNase H mediated cleavage, splicing modulation, noncoding RNA inhibition, gene activation, gene silencing.
2. Oligonucleotides were noted to interact with their targets via Watson – Crick pairing and based on the sequence to be targeted highly specific lead compounds can be designed; thus patient-specific sequences can be targeted.

However, one important difficulty involves delivering therapies to specific organs with the exception of liver. Another important factor to consider is off-target effects of therapy.

9.3.1 Steric block oligonucleotides

These were reported to block RNA transcripts with high affinity but not to lead to degradation of the bound transcript, as these oligonucleotides are not RNaseH1 competent. Steric block oligonucleotides can also be designed to modulate splicing, and lead to exclusion of a specific exon and to inclusion of a specific exon. These oligonucleotides can therefore influence which isoforms are generated from a specific transcript. [Roberts et al. \(2020\)](#) noted that by three splice switching oligonucleotides were FDA (Food and Drug Administration) approved. These include:

1. Eteplirsen that impacts dystrophin exon 51; the target tissue skeletal muscle, administration intravenous.
2. Golodirsen impacts dystrophin exon 53; the target tissue is skeletal muscle, administration intravenous.
3. Nusinersen used for treatment of spinal muscular atrophy, promotes expression of SMN2 exon 7 by blocking inhibitory sequences, target tissue includes spinal cord and nerves, administration is intrathecal.

[Roberts et al. \(2020\)](#) noted that by three RNaseH1 competent allele-specific oligonucleotides had been FDA approved. These included:

1. Fomivirsen for treatment of cytomegalovirus retinitis, the target organ is the eye and administration is intravitreal.
2. Mipomersen for treatment of ApoB-related hypercholesterolemia, the target organ is the liver and administration is subcutaneous.
3. Inotersen for treatment of hereditary transthyretin amyloidosis, the target organ is liver and administration is subcutaneous.

9.3.2 RNA inhibition in therapies

[Crooke et al. \(2018\)](#) and [Roberts et al. \(2020\)](#) reviewed RNA target therapeutics. Short inhibitory RNAs (siRNAs) can guide a specific enzyme complex to degrade a specific mRNA. The siRNA has a guide strand that matches specific mRNA sequence and a passenger strand. Within the cell the siRNA binds to RNA

inducing silencing complex (RISC); the passenger strand is then discarded. The guide strand then binds to the complementary mRNA sequence and the enzyme Argonaute within RISC then cleaves the mRNA.

Two siRNAs have received FDA approval for therapy. These include Patisiran that targets TTR transthyretin that is defective in transthyretin amyloidosis. Patisiran, an interfering RNA that was developed for the treatment of hereditary transthyretin-mediated amyloidosis, was delivered encased in a lipid particle (Yang, 2019). Givosiran, a small interfering RNA directed toward ALAS1 5'-aminolevulinic synthase 1, that is mutated in acute hepatic porphyria (Sardh et al., 2019).

9.3.3 MicroRNAs as mRNA inhibitors

Roberts et al. noted that microRNAs are endogenous RNA inhibitors with physiological roles. MicroRNAs function in posttranscriptional regulation of gene expression. Defects in microRNA function can lead to pathologies and microRNAs are considered to be important drug targets. Roberts et al. noted that steric block antisense oligonucleotides have been developed to impact specific microRNAs.

A specific microRNA mir21 was reported to play a role in the pathology of Alport syndrome (Gomez et al., 2015). Roberts et al. reported that clinical trials were ongoing to target this disorder with an antisense oligonucleotide.

MicroRNAs were shown to play roles in polycystic kidney disease PKD1 and PKD2 (Li and Sun, 2020). A specific antisense microRNA is in clinical trials to target microRNA miR17 that influences pathology in polycystic kidney disease types PKD1 and PKD2.

It is thus important to note that specific therapeutic oligonucleotides can be directed not only against the primary defective gene transcripts but also against ancillary gene encoded transcripts that impact the pathophysiology in a specific disease.

9.3.4 Long noncoding RNAs, small RNAs, endogenous antisense RNAs

Long noncoding RNAs (lncRNAs) have increasingly been found to be involved in the regulation of gene expression. In some cases, endogenous antisense RNAs and small RNAs are being identified and found to influence gene expression. Roberts et al. noted that specific disorders in humans have been attributed to aberrant gene silencing events and there is evidence that oligonucleotides may constitute target for therapies in these disorders. lncRNAs have been reported to be associated with specific disorders including cancer. Gupta et al. (2020) reported the NF- κ B signaling was also reported to be regulated by several long non

lncRNAs. The lncRNA and NF- κ B signaling were reported to crosstalk during cancer and they discussed possibilities for therapies based on these associations.

A lncRNA is associated with the neurodevelopmental disorder Angelman syndrome (Meng et al., 2015). Use of antisense oligonucleotides to downregulate this long RNA is in clinical trials.

9.4 Delivery challenges in oligonucleotide therapies

Roberts et al. noted that oligonucleotides are large hydrophobic polyanions that do not traverse plasma membranes of cells easily. Within the extracellular space they can be degraded by nucleases. In addition, they may be lost through renal clearance. If they do traverse cell membranes, they may be taken up by endosomes and degraded. For these reasons, a number of therapeutic oligonucleotides have been administered locally, for example, in the intravitreal space in the eye or in the intrathecal space.

The liver was noted to have high concentrations of receptors on cells and that oligonucleotides can be modified to bind to these receptors to expedite uptake.

Additional modifications have been designed to enhance uptake of therapeutic oligonucleotides. These include alterations of ribose or phosphate components of oligonucleotides. Roberts et al. noted that lipid covalent conjugation was being explored to promote delivery of siRNAs.

Conjugation of antisense oligonucleotides with N-acetyl galactosamine (GalNac) was being used to promote oligonucleotide uptake into liver cells through specific receptors.

Antibody coupling of oligonucleotides has been proposed to promote binding to specific cellular proteins. Nanoparticles and particularly cationic polymers are also being explored as carriers for oligonucleotides.

Hammond et al. (2021) reported that a main challenge in nucleic acid-based therapeutic is delivery to the target tissue. They noted that use of conjugates and nanoparticles has proven useful in some cases. Conjugates used included fatty acids, N-acetyl-galactosamine (GalNac), and cell penetrating peptides. Antibodies to specific ligands including cellular receptors are also used.

9.5 Splice mutations and diseases

Aberrant splicing occurs in a number of different diseases. Montes et al. (2019) reviewed splicing defects and development of animal models to help develop therapies. They noted that mistakes in splicing can lead to alterations in transcript code and also to frame shift mutations.

Accurate splicing was noted to be dependent on factors within transcript splice sites and on associated factors and in cofactors involved in splicing. Montes et al.

emphasized that transcripts of a specific gene may undergo splicing at different sites and that there is evidence that 95% of genes are alternatively spliced. Alternative splicing of a specific gene may occur at different stages of development, or in different tissues.

Important elements in splicing include splice regulatory elements (SREs) that occur on primary transcripts; other important elements include trans factors, RNA binding proteins such as serine-arginine rich proteins, and heterogeneous ribonucleoproteins.

Montes et al. noted that 10% of all mutations documented in human disease mutation databases impacted splice sites. In the Human Mutation database (Stenson et al., 2020), mutations that impact trans splicing factors are also documented.

Montes et al. noted the importance that animal models can play in studies of splicing and on the impact of its alterations and that studies of such models are important for development of therapies directed at aberrant splicing.

Montes et al. noted that specific splice switching oligonucleotides have been developed to bind to transcripts of SREs to prevent binding of important proteins. Splice switching oligonucleotides have also been developed to alter activity of splice enhancer or splice inhibitory factors.

For analysis of splicing mutations and development of possible therapies, mouse models of specific mutations have often been developed. Examples include the mdx mouse for studies of DMD mutations leading to Duchenne Muscular Dystrophy. A Duchenne-like phenotype in Golden Retriever dogs arises due to mutation and these dogs also serve as a model for therapeutic applications.

In addition to splice switching oligonucleotides, small molecules have been identified that target splicing factors. Examples include small molecules that impact spliceosomes, such as Spliceostatin. Questions arise regarding the targeting of splice modulators to specific cell types.

Montes et al. reviewed splicing alterations in specific diseases including myelodysplasia syndromes characterized by proliferation of specific subtypes of hematopoietic cells. In myelodysplasia and in acute myeloid leukemia, mutations were reported in splice factors SRF2, U2AF, SFBP1. Splice modulators were developed to target these mutations.

9.6 Antisense therapies under investigation

Bennett et al. (2019) reviewed use of antisense nucleotides under investigation for therapies in specific neurodegenerative diseases, including Huntington's diseases, Alzheimer disease, and amyotrophic lateral sclerosis. They emphasized that beneficial medical therapies were not available for many neurodegenerative disorders including those cited above.

They noted that mechanisms through which antisense RNAs function must initially be tested in cell culture system. Modifications of antisense RNAs were noted to impact their pharmacological properties, tissue distribution, and clearance. Specific modifications include morpholino modifications and phosphorothioate modifications.

It is important to note that after intrathecal antisense oligonucleotides administration, a high percentage of the compound (up to 80%) appeared in the circulation. Studies on the half-life of administered antisense oligonucleotides indicate that this may be 6 weeks – 6 months following injection.

Several conditions where specific strategies to downregulate mutant RNAs are under investigation. These include autosomal dominant diseases due to nucleotide triplet repeat expansions. One example is myotonic dystrophy type 1 due to CUG repeat expansion in the DM1 gene that encodes a protein kinase. A chimeric antisense oligomer was designed to induce degradation of the mutant RNA.

C9ORF 72 mutations that comprise expanded hexanucleotide repeat lead to amyotrophic lateral sclerosis. Investigations to silence this repeat expansion with a gapmer are underway. These investigations are being carried out in neuronal cells. In animal models of the disorder, studies are being carried out using intraventricular administration of the antisense gapmer.

Other therapeutic approaches in specific neurological diseases use silencing to regulate expression of a specific gene. Examples include downregulation of DNM2 dynamin 2, a microtubule associated protein in centronuclear myopathy and downregulation of SOD1 superoxide dismutase 1 in ALS (Amyotrophic lateral sclerosis).

9.7 Genomic data leading to therapeutics

Kaczmarek et al. (2017) noted that the two major classes of compounds included in FDA-approved therapeutics were noted to be small molecules and proteins. Small molecules were noted to be hydrophobic organic compounds that bind to specific sites on proteins. However, such binding sites were noted to be relatively sparse. Antibodies may bind with high specificity to specific proteins; however, antibodies are of large size and are of limited solubility.

Kaczmarek et al. emphasized the therapeutic potential of RNA and DNA because of their high specificity. They noted, however, that therapeutic single stranded RNA is prone to nuclease degradation. It cannot easily cross cell membranes and must be protected from degradation in endosomes.

Engineered adenoviral vectors have been particularly used for RNA delivery. However, nonviral vehicles involving nanoparticle encapsulation are being increasingly explored. Polymers were noted to be used for nanoparticle formation.

Cationic polymers were used for condensation. Amine containing polymers were reported to be used for RNA delivery; these included synthetic poly-L-lysine, a polyaminoamine, and polyethylene-amine.

Howard et al. (2006) reported use of a naturally occurring polymer chitosan for RNA delivery. In delivery of DNA polyaminoesters were reported to be useful.

Kaczmarek et al. reported that lipid-like nanoparticles were also being used as nucleic acid delivery vehicles. Ionizable lipids were thought to escape endosomal uptake and degradation more easily. Semiautomated synthesis of lipid-like molecule synthesis for RNA delivery was being explored.

Kaczmarek et al. noted that an additional approach involved conjugation of N-acylgalactosamine to RNA as this could target RNA to specific receptors on liver cells.

9.7.1 Additional RNA modifications to further improve use in therapy

Specific chemical modifications are also being investigated to identify strategies to improve RNA therapy. Strategies explored include modification of the ribose sugars and modifications of phosphate linkages.

9.8 Pluripotent stem cells for investigation of disease manifestations and effects of therapies

Neural stem cells derived from pluripotent stem cells of patients with lysosomal storage diseases have been developed to obtain further insight into stages of pathology in these disorders. Studies are also undertaken on these cells to investigate the impact of certain therapeutic agents.

Luciani and Freedman (2020) noted that pluripotent stem cells can also be developed into brain organoids; neural stem cells can also be developed to radial glial cells. They specifically considered utilization of pluripotent cells differentiated to neural stem cells in the investigation of certain therapeutic agents proposed to treat lysosomal storage disorders. In Niemann – Pick disease Type 1, patient's neural cells were developed from patient pluripotent stem cells to study effects of certain pharmacologic agents including cyclodextrins, tocopherols, reported promoter autophagy, and reduced accumulation of sphingomyelins.

Effects of gene therapy have been studied in neural stem cells differentiated from patients' cells in cases of mucopolysaccharide storage disease, Sandhoff disease, and Tay – Sachs disease.

Hematopoietic stem cells (HSCs) have also been investigated to determine the effects of gene therapy.

9.9 Gene therapy by adding genes

Specific forms of gene therapy in current use were noted to include viral-based delivery of a specific protein coding gene and evidence that the introduced gene may integrate within the host genome or remain extrachromosomal as an episome. Problems encountered with this strategy involved lack of information on where the introduced gene would integrate in the host genome and potential insertional mutagenesis. Nonintegrating genes that remain as episomes in the host were noted to potentially be safer.

There is evidence that episomes may be lost during cell division; therefore episomes for gene therapy are used primarily for editing nondividing cells.

9.10 Gene therapy

9.10.1 Early gene therapy applications

In a review of gene therapy [Zittersteijn et al. \(2021\)](#) noted that early therapies included treatment of adenosine deaminase (ADA)-related immunodeficiency. ADA encoding sequences were cloned into retroviral sequences for therapy of patient T-cells. Rescue of the ADA deficiency in patients was reported. Another form of immunodeficiency, treated around the same time, involved use of sequences encoding the IL2RG receptor cloned into retroviral vector. It subsequently became clear that the retroviral vector bearing the IL2RG receptor had integrated into the patient genome close to an oncogene and integration was also associated with deletion of a tumor-suppressor gene CDKN2A leading to leukemia in 5 of 19 treated patients. Fortunately, in these cases, leukemia was reported to respond to chemotherapy. None of the ADA-deficient patient treated developed leukemia.

Studies revealed that gamma retroviruses tended to integrate into transcription start sites of active genes.

[Shirley et al. \(2020\)](#) reported that gene therapy with viral introduction is used to treat a number of inherited diseases and also a number of acquired diseases. They noted that innate and adaptive immune responses to viral vectors constitute problems to therapy.

They noted that the viral vector must be capable of carrying and delivering the therapeutic gene and the viral vector must be stripped of pathogenic and replicative elements. Viral vectors in use on gene therapy were noted to include adenovirus, adenoassociated virus, lentivirus, and herpes simplex virus.

Specific viral vectors and gene therapies that have been approved for use in clinical trials include adenoassociated vectors carrying genes for therapy, which are present in LUXturna to treat some forms of congenital blindness and in Zolgensma to treat spinal muscular atrophy.

Shirley et al. outlined additional factors that influence selection of a viral vector for gene therapy in given conditions. They include specific cells or tissue to be targeted, the packaging capacity of the vector, and potential for genome integration of the virus. The adenoassociated viral (AAV) vector has frequently been the most favored vector used in gene therapy. Specific host factors to take into account include pattern receptors that recognize foreign proteins on vectors.

Adenoassociated viruses were reported to elicit a weak inflammatory response and to therefore have more favorable safety. However, CD8 T-cell responses to the vector have been reported in some studies (Nidetz et al., 2020).

Shirley et al. also noted that immunotoxicities can result if high doses of vector are infused in gene therapy. These can impact CNS, liver, skeletal, lung, cardiac muscles, and eye. These responses were considered to involve responses to capsid antigens. An additional concern that has emerged is the risk of antigen response to the transgene product. This was noted in some cases where large doses of the therapeutic product were administered.

Transient therapy with immune-suppressive agents was noted to be potentially valuable in these cases.

Lentiviral vectors were reported to be useful in therapy of both dividing and nondividing cells. Lentiviral vectors derived from human immunodeficiency virus have been used in cancer therapy. The incidence of preexisting immunity against lentiviral vectors was reported to be low.

Shirley et al. concluded that immune response to vectors or transgenes remains as hurdles to gene therapy.

Safe harbor gene therapy involves introduction of new DNA into a safe location, into a suitable position or into a location with suitable epigenetic modifications (Pellenz et al., 2019). This study identified 35 new sites for targeted transgene insertion that have the potential to serve as new human genomic “safe harbor” sites (SHS). SHS include potentially 35 sites located on 16 different chromosomes, including both arms of the human X chromosome.

In a report of gene therapies approved for clinical treatment in the United States and Europe, High and Roncarolo (2019) reported that since 2016 six different gene therapies were approved. Two of these were therapies for B-cell cancers. Four were approved for treatment of monogenic disease, these included beta thalassemia, a form of vision loss, spinal muscular atrophy, and a form of immunodeficiency.

They also noted that clinical developments involving cell and gene therapies were underway for more than 800 different diseases for which therapies were not available in 2019. The goals of therapies were to integrate therapeutic genes into precursor or stem cells, also to ensure that the integrated gene was stable, and extrachromosomal location of the introduced gene was favored. The procedures thus involved transduction of patient stem cells and then transplantation of treated stem cells.

It was noted that specific viral vectors used in gene therapy could be associated with significant immune responses in patients. High and Roncarolo noted that lentiviral and adenoassociated viruses were less likely associated to be associated with immune response and were thus used in gene therapy.

High and Roncarolo noted that lentiviral vectors were suitable for transferring genes into hematopoietic cells. However, large-scale production of lentiviral vectors was problematic. Lentiviral vectors were reported to have been used to treat certain blood disorders and metabolic disorders. They were used in treatment of Wiskott – Aldrich syndrome, adrenoleukodystrophy, and metachromatic leukodystrophy.

Gene therapy using lentiviral vectors for gene transfer into HSC was used in treatments of adrenoleukodystrophy and metachromatic leukodystrophy. There were claims that gene treated bone-marrow stem cells gave rise to cells that migrated to the brain. However, high viral copy number was noted to be necessary in these cases.

Some studies have reported immune response problems when AAV vectors were used in therapy. However, AAV vectors have been approved for treatment of autosomal recessive blindness with RPE65 gene therapy. The retina is known to be an immune-privileged site.

9.11 Stem cells and importance in gene therapy

HSCs and stem cell-derived neurons have also been investigated to determine the effects of gene therapy. Effects of gene therapy have been studied in neural stem cells differentiated from patients' cells in cases of mucopolysaccharide storage disease, Sandhoff disease, and Tay – Sachs disease

9.11.1 Hematopoietic stem cells (HSC) and therapies

Ferrari et al. (2020) reviewed aspects of gene therapy in HSCs. They reported that hematopoietic cell transplants have been used to treat specific genetic diseases for more than 50 years. They noted that in (1968) there were reports of treatment of patients with specific types of immunotherapy using HSC transplants.

Ferrari et al. noted that improvements have been made in donor matching, as incomplete matching of donor and recipient resulted in graft versus host reactions in some cases.

Autologous HSC therapy together with gene transfer has been used for treatment of a number of genetic disorders. In successful cases, the treated autologous stem cells were reported to undergo further division so that the benefits of therapy extended over time. Improvements have been made in gene therapy and viral vectors such as gamma retroviral vectors that presented problems are no longer used. In addition to their use in gene therapy, Ferrari et al. noted that HSCs can potentially be used as delivery vehicles for therapeutic proteins.

9.11.2 Collection of hematopoietic stem cells for therapy

This was reported to require multiple collections of bone marrow samples from iliac crest. In some cases this collection is followed by leukapheresis to

specifically collect CD34 + cells that represent cells that include HSCs and their progenitors.

In some situations specific agents are administered to patients to promote hematopoiesis and if stimulation is significant it is sometimes possible to collect adequate number of stem cells by leukapheresis of peripheral blood. One pretreatment agent used is Plexifor that promotes release of stem cells into peripheral blood. However, plexifor cannot be used in all patients.

Ferrari et al. noted that lentiviral vectors were often used for gene transfer. Modifications to vectors were made to inactivate viral promoter regions, to limit transcription of viruses following introduction, and to produce replication defective viruses.

Ferrari et al. explored evidence of utility of HSC as delivery vehicles for certain enzymes or proteins. Questions arise regarding the efficacy of modified HSC in treating conditions that impacted brain, bone, or other organs.

Ferrari et al. reported that there is some evidence that enzyme proteins released from bone marrow stem cells could be advantageous in reducing enzyme deficiency. There is evidence that HSCs give rise to monocytes and microglia that pass through capillaries and paravascular channels in the interior of the brain.

9.12 Gene editing

9.12.1 Early discoveries

In 1994 [Choo and Klug \(1994\)](#) reported on the capacity of certain zinc finger proteins to bind to DNA. Specific zinc finger units bind to single stranded DNA. Each specific zinc finger binds to three nucleotides and zinc fingers can be linked. The linked zinc fingers can bind to a DNA target of 18 nucleotides. The zinc fingers can also be fused to an endonuclease Fok1. Zinc fingers are designed to bind to two opposite strands of DNA and the endonuclease can then cleave DNA between the bound zinc fingers thus creating a double stranded break. If repair by homologous recombination is required, repair sequence can be included. Specific vectors, for example, adenoviral vectors can be utilized to transport the zinc fingers, the nuclease, and the repair sequence.

TALENs are related to plant transcription factors. Each TALEN is composed of 33–35 amino acids; the different TALENs differ from each other by two amino acids. The two amino acids, one at position 13 and one at position 12, distinguish a TALEN that binds to a specific nucleotide. The TALEN HD binds to nucleotide C, TALEN NI binds to nucleotide A, TALEN HG or NG binds to nucleotide T, TALEN NK binds to nucleotide G; TALEN NN can recognize G or A nucleotide. In addition, the TALEN editing system has an endonuclease position between the left and right arm that targets opposite sides of the DNA strand.

TALENs were noted to be easier to generate than zinc fingers but they are very large ([Chandrasegaran and Carroll, 2016](#)).

In 2020 [Ernst et al. \(2020\)](#) reviewed aspects of gene editing and its entry into the clinic. They noted use of zinc finger nuclease (ZFN), TALENs, and aspects of

CRISPR-Cas editing. ZFN and TALENs were noted to involve induction of double stranded DNA breaks mediated by FOK1 nuclease. In CRISPR-CAS technologies the CAS nuclease leads to double stranded breaks.

Specific clinical applications of gene editing included cancer therapy, HIV infections to disrupt the CCR5 receptor, and particular genetic disorders including hemoglobinopathies, hemophilia, mucopolysaccharidoses, and Leber's congenital amaurosis.

Gene editing methods were reviewed with CRISPR-Cas 9 by [Broeders et al. \(2020\)](#). In the CRISPR-CAS9 systems, nucleotide sequences in the guide RNA can be changed to match other DNA regions and the guide RNA can be coupled to the CAS cleavage system. Guide RNA takes CAS to regions that need to be edited. However, a specific sequence, the PAM sequence, to determine binding of CAS9 must flank the site to be edited in the genome. The cleaved DNA can be repaired using the body's own repair systems or DNA elements can be provided to be inserted following cleavage. Repair pathways following gene editing can include homologous directed gene repair, nonhomologous gene repair, and end joining. Repair by end joining can be used to remove a DNA segment.

Gene editing in patients has thus far involved bone marrow HSCs or T-lymphocytes. Following the editing procedure, cells are cultivated in vitro to determine accuracy or editing; following this they may be introduced into patients.

Ernst et al. noted that off-targeted editing remains a key problem. On-target editing can also be followed by impaired introduction of elements. There are challenges in delivery of gene editing systems to human cells. Different delivery systems are being devised including liposomes and additional vectors and nanotubes.

Ex vivo approaches involve modifying cells outside the patients. Cells used in these approaches include hematopoietic cells, stem cells, or T-cells

9.13 Delivery of reagents for editing

This sometimes involves use of viral vectors, for example, AAV (adenoassociated vectors), problems with developments of abnormal immune reactions following editing to adenoviral vectors have been described.

There are challenges in delivery of gene editing systems to human cells. Different delivery systems are being devised including liposomes, lipid nanoparticles, and additional vectors and nanotubes.

9.14 Preclinical and clinical trials

Ernst et al. reviewed a number of preclinical and clinical trials in place that utilize gene editing with adenoviral vectors. US trials completed include HIV therapy with ZFN.

Ongoing trials include:

1. Gene therapy in hemoglobinopathies, beta thalassemia, and sickle cell disease.
2. Cancer immunotherapy trials and strategies directed toward immune checkpoints. Cancer-related gene therapy investigations were reported to include deletion of HPV (human papilloma virus) in cervical cancer.
3. MPS1 (mucopolysaccharidosis type 1) ZFN therapies.
4. Leber's congenital amaurosis CRISPR-Cas.
5. Deletion of CCR5 (C-C motif chemokine receptor 5) receptor gene in HIV infections.

Ferrari et al. emphasized that targeted gene editing could ensure that the targeted gene remained under control of endogenous regulatory elements. They noted that gene editing proof of concept studies had been carried out in hemoglobinopathies, in severe combined immunodeficiency, and in Wiskott – Aldrich syndrome.

It is known that double stranded DNA breaks induced by gene editing nucleases can be repaired by homologous recombination if donor homologous sequence is available. In some cases, donor sequence elements need to be introduced. There is evidence that introduction of donor template sequence using adenoassociated virus has yielded encouraging results.

DNA double stranded breaks can also be repaired by nonhomologous end joining. This mechanism could be useful to induce knock-out of damaging sequences.

Ferrari et al. noted that chemical modifications in CRISPR-CAS editing reagents were being investigated. These include chemical modifications of the single strand guide RNA and adaptation of the CAS9 ribonucleoproteins. Modifications in agents used to transfer sequences were also being investigated such as use of adenovirus type 6.

Evaluation of the proportion of treated cells correctly modified prior to transfer were necessary. They noted that high doses of modified HSCs were often required.

In some cases, preconditioning of patients was required to reduce the number of cells within the bone marrow so that the transplanted cells could gain a foothold. This preconditioning required immunosuppression and was not recommended in some patients.

Ferrari et al. explored evidence of utility of HSCs as delivery vehicles for certain enzymes or proteins. Questions arise regarding the efficacy of modified HSC in treating conditions that impacted brain, bone, or other organs.

There is some evidence that enzyme proteins released from bone marrow stem cells could be advantageous in reducing enzyme deficiency. There is evidence that HSCs give rise to monocytes and microglia can pass through capillaries and paravascular channels in the interior of the brain.

9.14.1 Delivery of agents for gene editing

[Ates et al. \(2020\)](#) reported that a major barrier to therapeutic gene editing that remained, included scarcity of ideal methods for delivery of reagents. They

also drew attention to liver-directed gene editing for mucopolysaccharidosis and hemophilia B. CRISPR-CAS gene editing tools were noted to have been undertaken for treatments of hemoglobinopathies. They emphasized that in certain diseases, for example, muscular dystrophies, editing needed to occur in widespread cells.

In considering delivery systems they noted use of adenoassociated viruses. They also mentioned hydrodynamic delivery, electroporation, and the use of lipid nanoparticles and cell penetrating peptides.

Ates et al. noted that hydrodynamic delivery was developed primarily for delivery of naked DNA. It has also been used for the delivery of siRNAs and small molecule chemical agents.

9.15 NIH (National Institutes of Health) somatic cell gene editing program

Saha et al. (2021) reviewed the NIH somatic gene editing program designed to investigate and establish safer gene editing methods. The plan includes investigations of new genome editors and effective delivery technologies and also to track editing cells and to assess their effects.

They noted that in some disorders addition of a functioning gene can lead to death, while in other cases there are benefits to editing a defective gene within a patient. Gene editing was noted to include diverse technologies including use of ZFN (zinc finger nucleases, TALENs, (plant transcription factors) or CRISPR-Cas. However, they also noted that challenges remain, including adequate repair of nuclease-induced edits. Other gene editing processes involve nucleotide base editing. Many gene editing studies involve utilization of animal models of disease.

Genome editing processes have been carried out in vivo in some cases while in other cases they are carried out in vitro on patients' cells and treated cells are subsequently administered to patients.

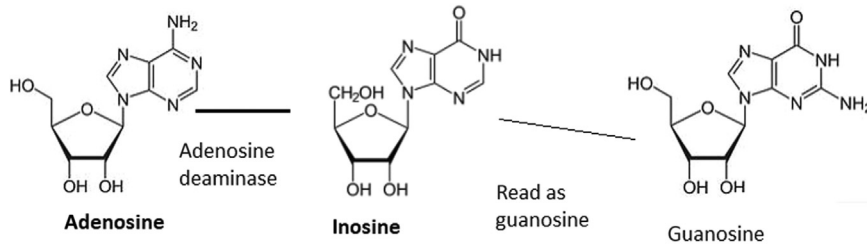
One consideration is that the patient may develop immunity to the delivery cells or to the delivery product.

There has also been progress in use of gene editing of patient T-cells for subsequent immunotherapy in cancer.

Development of safe and effective delivery systems is a key goal. Many of the currently used delivery vectors were not considered to be optimal.

Risks of off-target effects including delivery to inappropriate tissues remained a concern. In addition, currently there are limits in the number and types of tissues to which agents for editing can be delivered.

Editing platforms currently available were noted to include CRISPR system with Cas9 and Cas 12 homologs. Other systems being investigated included a CasX system. Liu et al. (2019) reported a new form of Cas protein referred to

**FIGURE 9.1**

Example of Base editing.

as CasX and sometimes referred to as Cas12e. It was determined to be an RNA-guided DNA endonuclease that generated staggered double stranded DNA breaks with 20 nucleotides complementary to the guide RNA present in CasX. A particular advantage was the small size of CasX protein, 1000 nucleotides approximately, its cleavage characteristics, and the fact that it was derived from ground water organisms rather than from an infectious agent. The guide RNA could constitute 26% of the mass. CasX was shown to be capable of cleaving human DNA.

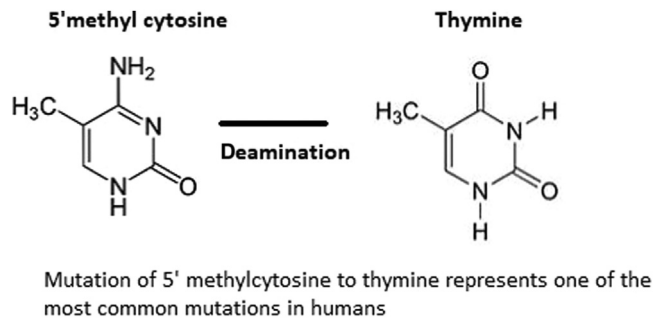
Other editing systems being considered included enzymes DNA helicases, transposase, and epigenetic editors being considered (Fig. 9.1).

9.16 Base editing

(Porto et al., 2020) emphasized that many genetic disorders are known to be caused by single base mutations. They explored possibilities for gene editing of these and emphasized the need for editing tools with high on-target efficiency and minimal off-target effects. Tools initially used included ZFN or TALEN nucleases that required protein synthesis. The CRISPR-CAS9 system was noted to represent a more usable tool. Newer versions of CAS, including CAS12 and CAS13, were noted to be more efficient.

9.17 Programmable base editing

Lewis et al. (2016) reported that deamination of cytosine and of methyl-cytosine occurred frequently in microorganisms. Also this change was frequently observed in mutations in human disease. They examined the mechanism of cytosine deamination. Hydrolytic deamination of cytosine was reported to yield uracil. Uracil may be removed by uracil DNA glycolase.

**FIGURE 9.2**

Example of Base editing.

5-methyl cytosine can undergo deamination to yield thymine and ammonia. The 5-methyl cytosine to thymine conversion was reported to be the most common nucleotide conversion and cytosine to thymine conversion (C to T mutations) was reported to represent common mutations.

Adenine undergoes conversion to inosine, which is read as guanine by polymerase.

Gaudelli et al. (2017) reported that AT to CG mutations represented half of the pathogenic mutations in the human genome.

The development of adenine editors and cytosine editors therefore became a priority.

Base editors were sometimes referred to as Cas9 nickases; Cas 9 was linked to enzymes that included cytidine deaminase or adenine deaminase.

In a 2020 review Kantor et al. noted that base editing could correct point mutations without first inducing double stranded DNA breaks (Fig. 9.2).

9.18 Prime editing

Anzalone et al. (2019) noted that as of July 2019, 75,122 known pathogenic human gene variants were documented in ClinVar.

They described a process that they described as prime editing. This involved use of a modified catalytically impaired Cas9 fused to a reverse transcriptase, and to a prime editing RNA (peg RNA).

The peg RNA specified the target site correction and also the edit. This method enabled editing of insertions and deletions and point mutation. Using prime editing systems they targeted insertions, deletions, and 12 point mutations.

In 2021 Zittersteijn noted that prime editing included a Cas form that nicks DNA and has a guide RNA (pegRNA) that is attached to a reverse transcriptase to synthesize a new DNA from the guide RNA.

9.19 CRISPR-Cas theta

In 2020 Pausch et al. (2020) reported use of a CRISPR-Cas specific system derived from large bacteriophage. The system includes a CRISPR array and Cas theta. It was noted to be a compact system with Cas theta being a 70 kilodalton protein. The molecular size of Cas theta was noted to be half that of Cas9 or Cas12a. The new system was noted to have significant advantages in cellular delivery.

9.20 RNA editing

RNA editing involves modification at the transcript levels and has a lower risk of off-target genomic modifications. Fry et al. (2020) noted that specific RNA editing technologies utilize enzymes that induce adenosine to inosine conversion A to I or cytosine to uracil conversion. Site-directed reagents are used to deliver RNA editing enzymes to targets in RNA that correspond to coding sequence mutations G to A or T to C.

Specific local sequence context can influence the ability of ADAR1 ADA RNA-specific, to deaminate adenosine. There is also some evidence that specifically induced mutations in ADAR1 can influence the editing capability.

ADAR2 has a more limited tissue distribution. However, specific mutations have been introduced to ADAR2 to improve the utility.

With respect to RNA editing and CRISPR-CAS, Fry et al. noted that type VI CRISPR-Cas nuclease CAS13 under direction of a guide was noted not to require PAM, protospacer adjacent motif.

Fry et al. noted that nucleotide editing can be used to treat retinal degeneration due to G to A or T to C mutation. RNA editing may be feasible. However, in dominant disease editing of specific alleles may be necessary.

However, they did note that delivery of DNA and RNA base editing reagents is an ongoing challenge.

9.21 Gene therapy in specific diseases

9.21.1 Eye diseases and retinal degeneration

Fry et al. (2021) reported utilization of RNA editing therapy in treatment of genetic disorders associated with retinal degeneration. Examples of large genes with single point mutations leading to specific forms of retinal degeneration include *USH2A* and *ABCA4*.

USH2A encodes usherin, a laminin-like protein found in the basement membrane and may be important in development and homeostasis of the inner ear and retina. Mutations within this gene have been associated with Usher syndrome type IIa and retinitis pigmentosa.

ABCA4 ATP-binding cassette (ABC) transporter. ABC proteins transport various molecules across extra and intracellular membranes. Mutations in this gene are also associated with retinitis pigmentosa-19, cone-rod dystrophy type 3, early-onset severe retinal dystrophy, fundus flavimaculatus.

Defects in these genes were noted to be present in 25% of cases of genetic retinal degeneration in the United States.

Fry et al. noted that genetic therapies are particularly applicable in the retina because of easy access for delivery, and further because of immune privilege of the retina and because functional improvement can be readily assessed. Thus the FDA approval was obtained for gene therapy for gene defects that lead to Leber congenital amaurosis and occur in *REP65*. This gene encodes retinoid isomerohydrolase. *RPE65* is a component of the vitamin A visual cycle of the retina, which supplies the 11-cis retinal chromophore of the photoreceptors, opsin visual pigments (Russell et al., 2017).

Fry et al. noted that AAV vectors were most commonly used in gene therapy for retinal disease, as they were demonstrated to have tropism for retinal pigment epithelium and photo receptors. These structures are most frequently damaged in retinal degeneration. Also overexpression of transgenes can lead to cell damage.

The packaging capacity of AAV was noted to be 4–7 kb. Importantly, given their size the *ABCA4* and *USH2A* could not be accommodated in the AAV vectors. They noted that CRISPR-Cas9 has been used to reprogram photoreceptors in mouse models of retinal degeneration. They emphasized that gene editing approaches that require a donor template for homology-dependent correction of DNA cleavage have a lower success rate. Approaches that involve base editing without double stranded DNA breaks were reported to be more successful.

Fry et al. emphasized that defects in large genes may be beyond the possibilities of gene replacement therapy based on their size. The large genes cannot be accommodated in AAV vectors. They listed the six large genes most commonly implicated in retinal degeneration and the size of these genes.

These genes, their size in kilobases, and their functions are listed below.

1. *ABCA4* 6.81Kb ATP-binding cassette (ABC) transporter.
2. *USH2A* 15.6 Usherin a basement membrane protein.
3. *CEP290* 7,4 centrosomal protein.
4. *MYO7A* 6.65 myosin 7a a mechanochemical protein, serves as an anchor.
5. *EYS* 9.43 eyes shut, protein contain multiple epidermal growth factor and lamin domains.
6. *CDH23* 10.1 cadherin-related 23, encodes calcium dependent cell – cell adhesion glycoproteins.

9.22 Molecular analyses and therapies relevant to hearing loss

Appler and Goodrich (2011) reported new techniques and improved inner ear access have revealed details regarding connections of the peripheral auditory

systems and connection with the central auditory circuit. Further information has been gathered on the arrangement of the inner and outer hair cells, the sound and fluid movements that lead to hair cell movement, and to signaling to the spiral ganglion that is connected via axons to the hair cells and that also sends signals to the 8th cranial nerve. There is evidence that the different spiral ganglion neurons differ in the sounds to which they respond.

Delmaghani and El-Amraoui (2020) reviewed progress in development of inner ear therapies for hearing loss. Disabling hearing loss was reported by the World Health Organization to impact 5% of the world's population. Congenital hearing impairment was reported to occur in 1 in 500 newborns.

The authors of this review noted that increases in knowledge of the molecular mechanism of the auditory and vestibular systems could be applied to analysis of hearing loss. Improved therapy was dependent upon determination of the nature of factors that damage hearing and information on target cells. Cells that were impaired included auditory hair cells, supporting cells, or neurons.

Delmaghani and El-Amraoui noted that development of therapies must take into account auditory hair cells that react to and amplify sound stimuli and inner hair cells that transmit stimulation to the nervous system. Outer hair includes between 9000 and 12,000 cells arranged in three rows. Hair cells were reported to carry actin-rich stereocilia on their apical surfaces. In response to stimuli, inner hair transmit stimuli that ultimately lead to depolarization of cells and release of neurotransmitters.

Early onset of prelingual deafness includes syndromic hearing loss, when hearing loss and other physical or functional abnormalities occur and nonsyndromic hearing loss when hearing loss occurs as apparently the only abnormality. Nonsyndromic hearing loss due to genetic factors includes autosomal dominant, autosomal recessive, X-linked forms, and can arise as a result of mitochondrial defects.

Delmaghani and El-Amraoui noted that defects in more than 140 genes have been reported to lead to hearing loss. The first genetic linkage of hearing loss was reported in 1994 when Guilford et al. (1994) reported mapping of nonsyndromic form of neurosensory, recessive deafness to the pericentromeric region of chromosome 13q. The map region was refined to be 13q12 and the relevant gene was found to encode a gap junction protein connexin 26.

Delmaghani and El-Amraoui documented the different functions impacted by genes with defects that lead to hearing loss. They included genes involved in hair bundle formation and function, genes involved in hair cell adhesion and maintenance. Other genes implicated in hearing loss included gene involved in cochlear ion homeostasis, transmembrane secreted proteins and extracellular matrix components, genes encoding products involved in control of oxidative stress, metabolism, and mitochondrial functions, and genes involved in transcription regulation.

Delmaghani and El-Amraoui noted that the inner ears represent an available site for gene therapy in part because it is relatively isolated, and second because the fluid circulation in ear can disseminate therapeutic agents.

In focusing on specific approaches in gene therapy they noted that using direct administration reagents could be delivered to vestibule structures including the semicircular canal, utricle, and cochlear round window. Injection in the posterior semicircular canal could be used to treat auditory and vestibular dysfunction.

Early in gene therapy, approaches were carried out in mouse models of genetic deafness. Both viral vectors and nonviral vectors were used; the latter included nanoparticles, microsphere of biodegradable polymers, including liposomes.

The most promising viral vectors were reported to be adenoviral vectors and at least 12 different serotypes and variants of these have been identified. Different serotypes of adenovirus were noted to have different tropisms.

Specific serotypes were identified to administer therapy. The adenoassociated virus AAV2 serotypes 2/1, 2/2, 2/9, 26/27 were found to have tropism for hair cells. V9 serotypes were found to be useful in transmitting gene therapies to the cochlea.

However, one disadvantage of the AAV vectors was their limited capacity to accommodate genes larger than 4.8 kb.

Nonviral delivery was shown to be less efficient but more stable and flexible.

9.22.1 RNA-based therapies in deafness

Delmaghani and El-Amraoui noted that antisense oligonucleotides and short interfering RNAs or microRNAs provided possibilities for treating dominant negative deafness. The harmonin encoding gene implicated in USHER syndrome C has a specific mutation c.216 G > A, which leads to generation of a cryptic splice site that in turn leads to a truncated harmonin protein. This defect is associated with early onset profound deafness. Specific antisense oligonucleotides were generated to block the cryptic splice site.

Delmaghani and El-Amraoui noted that RNA interference (RNAi) approaches have also been investigated to counteract the common connexin 26 defects that leads to deafness DFN3. This is an autosomal dominant progressive deafness. RNAi can be used to selectively inhibit expression of the mutant allele. This was achieved using liposomes containing inhibitory RNA that bound to the mutant allele.

A specific microRNA was developed to reduce expression of a function mutation in the gene TMC1 transmembrane channel like 1. It is known to be required for normal function of cochlear hair cells. Mutations in this gene have been associated with progressive postlingual hearing loss and profound prelingual deafness. Gene editing approaches were used in mouse studies to target DFNA36 mutation in TMC1.

9.22.2 Efforts to promote hair cell regeneration

These include overexpression of the MOTH1 transcription factor that is important for differentiation of hair cells. MOTH1 overexpressing genes were introduced into the cochlea.

Local delivery of growth factor such as BDNF and neurotrophin NTF into the cochlea have also been shown to be beneficial in animal models.

Animal models of deafness, particularly mouse models, have been utilized to investigate possibilities for gene replacement, gene editing, and RNA-based therapeutics.

9.23 Therapy of cystic fibrosis including genetic approaches

In an editorial published in 2020, Pedemonte noted that prior to 2012 therapy for cystic fibrosis (CF) focused on cooccurring infections, deficits in mucus clearance in lungs, and intestinal malabsorption (Pedemonte, 2020).

Subsequently, treatment with CFTR (cystic fibrosis transmembrane conductance regulator) gene product modulators or potentiators followed. These included ivacaftor, or combinations including tezacaftor, elexacaftor, and ivacaftor. They noted that in vitro demonstration of modulator efficacy was not always reflected in in vivo efficacy.

Pedemonte concluded that there is still an urgent need for novel drugs. Some of the new approaches included potentiators of expression other gene products, for example, TMEM16a also known as anomactin ANO1, a calcium-activated chloride channel.

Pedemonte noted that airway surface homeostasis is important. Mitash et al. (2020) considered molecular methods including the use of microRNA-based approaches to increase production of components necessary to promote airway surface homeostasis. Specific microRNAs were shown to impact viability of mRNA of certain genes involved in epithelial surface homeostasis including CFTR, ANO1, EnaC (SLNN1A sodium channel) SLC26A9 (Solute carrier 26A9). TGFB1 (transforming growth factor B1) was also shown to impact surface homeostasis through recruitment of specific microRNAs to CFTR. Mitash et al. (2020) noted that elucidation of effects of specific microRNAs on mRNA of gene involved in surface homeostasis could provide insights into new therapeutic approaches.

Maule et al. reviewed gene therapy approaches to cystic fibrosis. A critically important discovery in cystic fibrosis was that it arose due to damaging mutation in a specific gene that encodes a cyclic AMP-regulated chloride channel CFTR. Because of the importance of this channel, particularly in lung epithelial cells, manifestation of CFTR malfunction occurs primarily in the lung and also in other body tissues.

Maule et al. (2020) noted that 352 different CFTR mutations have been found to be disease-causing. Partial progress in treatment of cystic fibrosis resulted from use of small molecules that improved trafficking or modification of mutant CFTR protein. However, 10% of cases were noted to not respond to small molecule therapy.

They noted that there are severe challenges to gene therapy including production of large quantities of mucus and the fact that epithelial cells are constantly being renewed. Maule noted that gene editing is now being considered. In this approach, site-specific DNA cleavage will be carried out followed by target integration of corrected sequence. Correction of the delta F508 deletion is being investigated in vitro. Splicing mutations were reported to occur in 10% of cases and successful in vitro editing of these in CF patient cells was reported. Investigation of base editing approaches in in vitro studies was noted to be under investigation.

However, genetic approaches to therapy have also been proposed (Maule et al., 2020). Other delivery systems being considered are AAV vectors, nonviral delivery systems, engineered guide RNAs, nonviral vesicles including liposomes, peptides, peptide nucleic acid combination.

9.23.1 Ongoing problems needing to be addressed in gene therapies

One translational problem currently being investigated is that the therapeutic payload may not enter the nucleus of the cell.

An ongoing concern is that the patients may develop immunity to the delivery vehicle or the delivery product.

Development of safe and effective delivery systems is a key goal. Many of the currently available gene delivery systems were not considered optimal.

Another concern involves off-target effects. The report noted that there are currently limitations to the number and types of tissues to which agents for editing are delivered.

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