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Gene Editing For Rare Genetic Disorders: A Review Of Current Research

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ABSTRACT

Rare genetic disorders collectively affect millions of individuals worldwide, presenting a significant clinical and research challenge due to the diversity and complexity of the underlying mutations. Current treatment options are often limited, focusing on symptom management rather than addressing the root genetic causes. This review article aims to provide a perspective on the evolving field of gene therapy for rare genetic disorders, emphasizing recent advancements, current challenges, and future directions. A comprehensive review of recent advancements in gene therapy for rare genetic disorders was conducted, focusing on therapeutic strategies, delivery systems, and clinical outcomes. Key examples, such as the use of viral vectors and gene-editing technologies (e.g., CRISPR), were highlighted. The challenges, including immune responses and ethical concerns, were also examined. Gene therapy has achieved significant milestones, with the successful development of therapies like Zolgensma for spinal muscular atrophy and Luxturna for retinal dystrophy. However, several hurdles, including efficient gene delivery, immune reactions, and long-term safety, remain unresolved. Gene therapy holds transformative potential for the treatment of rare genetic disorders. While recent successes mark a new era in genetic medicine, ongoing research is required to refine delivery mechanisms, overcome immunerelated barriers, and ensure ethical and safe therapeutic interventions.

Keywords: Gene therapy, rare genetic disorders, Zolgensma, in vivo gene therapy, ex vivo gene therapy.

INTRODUCTION

Rare genetic disorders, often defined as conditions affecting fewer than 1 in 2,000 individuals, represent a vast array of over 7,000 distinct diseases. These disorders affect approximately 300 million individuals globally, with the majority manifesting early in life and often leading to significant health complications, developmental challenges, and, in some cases, early mortality [1]. Rare genetic disorders are often caused by mutations in specific genes, which result in abnormal protein production, loss of function, or other cellular dysfunctions. These disorders encompass a vast range of conditions, including spinal muscular atrophy (SMA), cystic fibrosis, and hemophilia, each typically resulting from a mutation in a single gene. Because of their rarity and genetic diversity, diagnosing and treating these disorders poses significant challenges [2]. Traditional therapeutic approaches, such as symptomatic treatments and enzyme replacement therapies (ERT), have been beneficial for certain conditions. ERT, for example, has shown success in treating lysosomal storage disorders like Gaucher disease and Fabry disease by replacing the deficient enzyme [3]. Similarly, oral medicines are prescribed in case of sickle cell anemia hydroxyurea which reduces the pain and prevents dactylitis in children. However, these treatments are not curative and

often require lifelong administration, which can limit their effectiveness. Furthermore, they do not address the underlying genetic cause of the disorder [4].

2. PREVALENCE AND TREATMENT GAPS

Because each rare genetic disorder affects a limited number of people, developing effective treatments is challenging. Traditional drug development focuses on common diseases with large patient populations, leaving rare diseases with minimal therapeutic options. As a result, most rare genetic disorders lack curative treatments, and the available therapies often only address symptoms rather than underlying causes. This lack of curative options contributes to high morbidity and mortality rates, creating a substantial unmet need for targeted therapies [5].

3. THE PROMISE OF GENE THERAP

Gene therapy offers a transformative approach to treating rare genetic disorders by addressing the root cause: the genetic mutation itself. By introducing, modifying, or repairing defective genes within a patient's cells, gene therapy has the potential to restore normal function at a molecular level. This precision can be particularly effective for rare genetic disorders, which often stem from single-gene mutations, making them ideal candidates for gene correction. Gene therapy has already led to breakthroughs in conditions like SMA and certain inherited retinal diseases, offering hope for future advances across a broader range of rare disorders [6]. Gene therapy operates on the principle of targeting and modifying genes to correct underlying genetic mutations, aiming to treat or even cure diseases at their molecular source. By directly altering DNA within cells, gene therapy can restore or replace faulty genes responsible for causing specific conditions. This approach is particularly promising for rare genetic disorders, as many of these conditions are monogenic, meaning mutations in a single gene cause them. This simplicity enables precise targeting, which increases the likelihood of effective and sustained outcomes. Unlike traditional therapies that manage symptoms, gene therapy has the potential to address the root cause of rare disorders, offering long-term relief and potentially eliminating the need for lifelong treatment [7,8].

4. Gene Therapy: A Revolutionary Approach

Gene therapy represents a transformative strategy for rare genetic disorders by directly correcting or compensating for defective genes. Approaches are broadly divided into in vivo and ex vivo methods.

In vivo gene therapy delivers editing tools or vectors directly into the patient, targeting specific tissues. Examples include Zolgensma for spinal muscular atrophy, which restores SMN1 function via AAV9 delivery, and Luxturna for Leber congenital amaurosis, which replaces the defective RPE65 gene. These therapies have shown remarkable success, halting disease progression and in some cases reversing damage [9,10].

Ex vivo gene therapy involves extracting patient cells, editing them in the laboratory, and reinfusing them after quality control. This approach is widely applied in hematological disorders such as sickle cell disease and β-thalassemia, where corrected hematopoietic stem cells repopulate the blood.

Gene therapy can also be classified as somatic (non-heritable, confined to the individual) or germline (heritable, involving gametes), though germline therapy remains ethically restricted in many countries [11,12].

5. CRISPR-BASED THERAPIES

CRISPR-Cas9 and related systems allow for targeted DNA editing with high precision and efficiency. The Cas9 protein, guided by a short RNA sequence, cuts the DNA at a specific site, enabling gene insertion, correction, or disruption. The discovery of CRISPR-Cas9 has brought a major breakthrough in the field of medicine by enabling the modification of DNA for gene therapy. Variations like prime and base editing have allowed scientists to correct point mutations that cause genetic disorders without increasing the off-target effects. Successful clinical trials have been launched using CRISPR for the treatment of sickle cell disease and beta-thalassemia. The aim is to activate the fetal hemoglobin for all cases of hemoglobinopathies [15,16]. The first trial of in vivo gene editing using CRISPR was carried out in a patient with Leber congenital amaurosis (LCA), which is a rare genetic disorder affecting the eye (Fig. **1**). CRISPR is more efficient and less costly compared to earlier methods. It is also adaptable to multiplex editing, where multiple genes can be edited simultaneously, which is valuable for polygenic disorders [17].

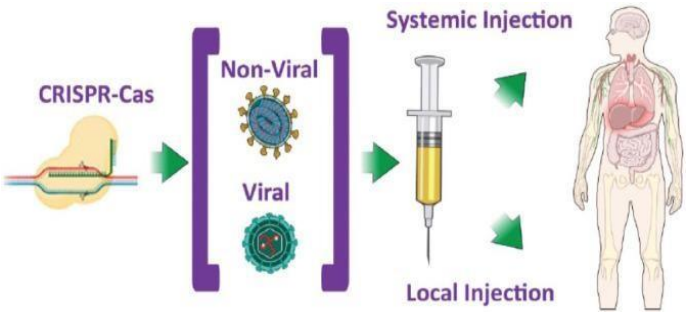

Figure 1 CRISPR/Cas9-based gene therapy.

6. ZINC-FINGER NUCLEASES (ZFNS) AND TALENS

These are custom-engineered proteins that bind and cut specific DNA sequences. ZFNs were the first tools used in clinical trials for gene editing, particularly in HIV resistance therapies, by disrupting the CCR5 gene in T cells. TALENs (Transcription Activator-Like Effector Nucleases) function similarly to ZFNs but allow for easier targeting and customization. TALENs have been used in experimental therapies, such as treating leukemia by targeting specific genes in T cells. While both ZFNs and TALENs offer high specificity, they are more complex and less versatile than CRISPR systems, which are more widely adopted due to CRISPR's simpler design and ease of programming [18,19].

7. BASE AND PRIME EDITING

This method enables precise point mutations without introducing double-strand breaks (DSBs), making it safer for single-nucleotide polymorphisms (SNPs) associated with genetic disorders. Cytidine deaminase or adenosine deaminase enzymes are fused to Cas proteins to alter specific DNA bases (A to G or C to T). A newer approach can perform targeted insertions, deletions, and all 12 types of base substitutions. Prime editing combines Cas9 nickase with a reverse transcriptase, allowing for complex edits with reduced off-target effects. Both base and prime editing are promising for disorders caused by single-point mutations, like cystic fibrosis, sickle cell disease, and Tay-Sachs disease. They offer increased precision, particularly useful for treating diseases with known, specific mutations [20,21].

8. GENE DELIVERY VEHICLES

8.1. Viral Vectors

Viruses are obligate parasites infecting the human and meanwhile delivering the genome into host cells. This mechanism has made viruses a good vector for gene therapy and development of vaccine. Four major categories of viruses that have been engineered and currently in use are retroviruses (RVs), lentiviruses (LVs), adeno (Advs) and adeno associated viruses (AAVs) [22].

8.2. Retroviruses

These are RNA viruses that are known to cause tumors in rodents. Once inside the host cell, RNA is reverse transcribed to cDNA, and invertase inserts the cDNA into the host genome. Due to this property, they provide a sustainable delivery mode. Retroviruses have been exploited for the treatment of SCID, but the major concer insertional mutagenesis which can lead to the development of leukemia-like symptoms (Fig.2) [23].

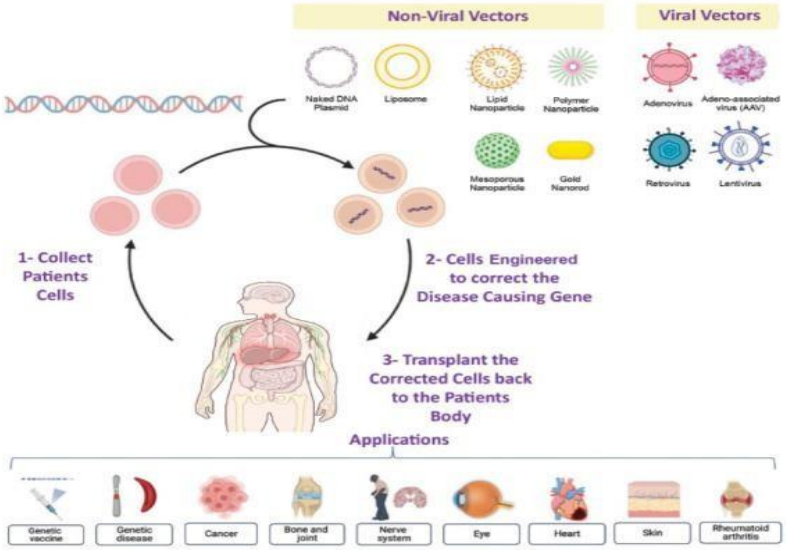

Figure 2 Different methods of viral delivery systems.

8.3. Lentiviruses

They are from the sub-group of retroviruses and possess 2 copies of the RNA genome. LVs can transfer the gene into both dividing and nondividing cells, so this is an efficient vector to be used for stable and sustainable targeting of broad cell types like neurons, liver, and immune cells [24]. After integration, the therapeutic gene shows expression for a long period of time. Lentiviruses have shown promising results in cases of Parkinson's disease and spinal muscular atrophy.

8.4. Adenoviruses

These are double-stranded DNA viruses having a genome size of 36kb and are known for causing respiratory, intestinal and eye infections in humans. They are not integrating means the genome is only delivered into the host nucleus. They are considered ideal for short-term expression. AdV can elicit strong immune responses that may lead to vector clearance. To overcome these limitations, helper-dependent Adenoviral vectors are also known. As gutless vectors are developed that have most of the genomes removed and only retain the inverted terminal repeats and packaging system that are essential for DNA replication and encapsulation. Hence, a large amount of transgene can be incorporated and transported. AdV can deliver CRISPR/ Cas9 system and has been rated as a novel therapeutic delivery strategy [25].

8.5. Adeno-associated Viruses (AAV) in Gene Therapy

Adeno-associated viruses (AAV) are small, single-stranded DNA viruses that naturally integrate genetic material into a specific site on human chromosome 19, making them highly suitable for targeted gene therapy. These vectors offer lasting gene expression, although occasionally uncontrolled, which has proven beneficial for conditions like hemophilia. In hemophilia, AAV can be injected into muscle tissue to produce Factor IX, effectively reducing bleeding episodes. Notably, AAV-based therapies, such as Luxturna (using AAV2 for RPE65 mutations causing Leber congenital amaurosis) and Zolgensma (using AAV9 for spinal muscular atrophy by replacing the SMN1 gene), have shown remarkable therapeutic outcomes [26].

8.6. Non-viral Vectors in Gene Delivery

Non-viral vectors provide a safer alternative to viral vectors, especially for short-term expression or in cases where immune response must be minimized. These methods deliver DNA into cells using physical techniques rather than viruses.

8.7. Liposomes

Liposomes are lipid-based vesicles that can encapsulate therapeutic DNA, allowing it to merge directly with cell membranes to release genetic material into target cells. They are highly versatile, biocompatible, and often have lower immunogenicity than viral vectors, making them safer for repeated administrations. Liposomes can be chemically modified to improve specificity for certain tissues and protect DNA from degradation, which has been particularly useful for targeted cancer therapies and vaccines [27].

8.8. Beta-cyclodextrin Based Nanoparticles

Advancements in non-viral gene delivery, particularly through cell-penetrating nanoparticles like β-cyclodextrin (β-CD), show promise as safer alternatives to viral vectors. These nanoparticles are made up of cyclic oligosaccharides that form a hydrophobic cavity capable of encapsulating therapeutic molecules, making them ideal for both drug and gene delivery. Their non-toxic nature enhances specificity, drug solubility, and stability. β-CD nanoparticles were first used in a clinical trial in 2010 to treat metastatic melanoma patients by delivering siRNA intravenously to downregulate RRM2 Mrna [28]. These nanoparticles have also shown promise in delivering an anti-cancer drug, Paclitaxel, and a siRNA to downregulate the toxic Huntington protein (HTT) in Huntington's disease [29,30]. Due to their stable release properties, β-CD nanoparticles are now being investigated as a potential treatment for Wolfram Syndrome. Their ability to deliver therapeutic genes safely and effectively makes them a promising avenue for curing this rare, monogenic, neurodegenerative disease, which affects nearly 30,000 people worldwide [31,32].

Wolfram Syndrome (WFS) is a rare, neurodegenerative disorder caused by mutations in the WFS1 or CISD2 genes, leading to severe multi-organ dysfunction, including diabetes, optic atrophy, and hearing loss. This condition has no cure, and existing treatments are largely ineffective in improving life expectancy or quality of life. Current research is focused on understanding the genetic and molecular mechanisms underlying WFS1, particularly the consequences of WFS1 gene deletion, which causes profound endoplasmic reticulum (ER) stress and proteostasis disturbances. Given the lack of effective treatment, innovative strategies, such as gene therapy, are being explored to address the fundamental cause of the disease [28,29].

8.9. Magnetofection

Magnetofection combines magnetic particles with DNA, allowing the DNA-magnetic complex to be directed into target cells using an external magnetic field. This method enhances transfection efficiency, especially for hard-to-transfect cells such as neurons and stem cells. Due to its precision and high local concentration, magnetofection has potential applications in targeted therapies, particularly for diseases requiring localized gene expression, like in neurodegenerative disorders [33].

8.10. Electroporation

Electroporation uses high-voltage electrical pulses to create temporary pores in cell membranes, enabling DNA to enter. Although highly effective, electroporation can lead to cell damage due to the electric field intensity. To minimize cell loss, optimized protocols and low-voltage variants are being developed, broadening their use in gene therapy for genetic diseases, immunotherapy, and tissue engineering [34].

8.11. Gene Gun/Biolistics

The gene gun, or biolistic delivery, propels DNA-coated gold or tungsten particles directly into cells by high-velocity helium bursts. This approach bypasses cellular uptake limitations and is suitable for tissue-targeted therapies, particularly in areas like skin and ocular applications where localized transfection is necessary. Despite its benefits, gene gun techniques are primarily limited to external or accessible tissues due to the need for direct physical contact with the tissu [35].

9. TRANSCRIPTOMICS: A GUIDE TO GENE THERAPY

Recent advancements in genomic technologies, particularly in transcriptomics, have significantly enhanced our ability to understand dynamic changes in DNA and RNA over time and in response to various challenges. Transcriptomics, which involves the study of all RNA present in cells, plays a critical role in understanding cell phenotypes and diseases [36]. Despite much of the transcriptome not translating into proteins, it still impacts cellular function and provides insight into complex pathologies. This growing field has expanded the understanding of gene functions, particularly by challenging traditional views such as the definition of “pseudogenes,” which have been found to be transcribed and even translated. RNA sequencing, a powerful tool in transcriptomics, has proven essential for analyzing genetic, neurodegenerative, and cancer-related diseases by providing comprehensive sequence data, revealing alternative splicing mechanisms, and identifying key transcriptional regulators [37]. Integrating transcriptomic data with genomic information can refine gene therapy approaches, ensuring that treatments are tailored to the genetic complexities of diseases at the cellular level, ultimately improving the precision and efficacy of therapies. Moreover, transcriptomics aids in evaluating the effectiveness of gene delivery vectors, a critical component for successful gene transfer. As such, transcriptomics is not only instrumental in disease diagnosis and clinical trial design but also serves as a valuable tool in the development of personalized gene therapies, advancing the field of precision medicine
[38].

10. SUCCESS STORIES IN GENE THERAPY FOR RARE DISEASES

10.1. Zolgensma (Onasemnogene Abeparvovec) for Spinal Muscular Atrophy (SMA)

SMA is a severe neurodegenerative disorder caused by mutations in the *SMN1* gene, leading to motor neuron loss and muscle atrophy. Zolgensma, developed by Novartis, is a gene therapy that delivers a functional copy of the *SMN1* gene *via* an adeno-associated virus (AAV9) vector directly to motor neurons [39]. Zolgensma was approved by the U.S. FDA in 2019 for the treatment of pediatric patients under 2 years of age with SMA. Clinical trials have demonstrated that patients treated with Zolgensma showed significant improvement in motor function and survival compared to untreated patients, offering the potential for long-term benefits. Zolgensma has dramatically changed the treatment landscape for SMA, providing a one-time infusion that can halt disease progression, potentially saving lives and preventing severe disability [40].

10.2. Luxturna (Voretigene Neparvovec) for Inherited Retinal Disease

Inherited Retinal Diseases, such as Leber Congenital Amaurosis (LCA), is caused by mutations in the RPE65 gene, leading to progressive vision loss. Luxturna, developed by Spark Therapeutics, is a gene therapy that introduces a functional copy of the RPE65 gene into retinal cells using an AAV2 vector [41]. Luxturna was approved by the U.S. FDA in 2017 for the treatment of adult and pediatric patients with inherited retinal disease caused by biallelic RPE65 mutations. In clinical trials, patients who received Luxturna demonstrated significant improvements in visual function, such as an increased ability to navigate in low-light conditions, which was sustained over time. Luxturna marked a major milestone as

the first FDA-approved gene therapy for a genetic retinal disease, offering the potential to restore vision and improve the quality of life for patients with previously untreatable forms of inherited blindness [42].

10.3. On-going Clinical Trails

Clinical trials in gene therapy for rare disorders have made significant strides, especially in conditions like cystic fibrosis, muscular dystrophy, and hemophilia.

10.4. Cystic Fibrosis (CF)

CF, caused by mutations in the CFTR gene, has seen advances through clinical trials using viral and non-viral vectors to deliver functional copies of the CFTR gene to lung cells. Although challenges remain in achieving efficient gene delivery to lung tissue, ongoing trials have shown improved respiratory function in some patients and continue to refine methods for safer and more effective delivery systems [43].

10.5. Muscular Dystrophy

In particular, Duchenne muscular dystrophy (DMD) has been a major focus for gene therapy. Trials using adeno-associated virus (AAV) vectors to deliver a shortened but functional version of the dystrophin gene have shown promising results, with treated patients experiencing improved muscle strength and reduced muscle damage. This approach, while still under evaluation, offers hope for a therapy that could slow or halt disease progression [44].

10.6. Hemophilia

Hemophilia A and B, caused by deficiencies in clotting factors VIII and IX, respectively, have shown remarkable progress in gene therapy trials. AAV vectors delivering functional copies of the missing clotting factor genes have been able to sustain normal or near-normal clotting factor levels in patients for extended periods. These promising outcomes have led to breakthroughs, with some therapies receiving regulatory approval, offering patients the potential for long-lasting, if not lifelong, relief from frequent clotting issues [45,46].

Overall, these clinical trials underscore the potential of gene therapy to not only treat but potentially cure certain rare genetic disorders by addressing the root cause of each condition. They also highlight ongoing efforts to improve vector safety, dosing protocols, and delivery efficiency to achieve more robust, durable results across various patient populations [47].

11. CHALLENGES IN GENE THERAPY FOR RARE GENETIC DISORDERS

Despite the promise, several hurdles remain in advancing gene therapy for rare genetic disorders. One significant challenge is the immune response to viral vectors, Particularly AAVs which can limit the effectiveness and safety of the treatment. Additionally, delivering gene therapy to target tissues, such as crossing the blood-brain barrier for neurological disorders, remains a complex obstacle [48]. Long-term efficacy is another concern. The longevity of the therapeutic effect, as well as potential adverse effects from gene integration into the genome, needs further investigation through long-term clinical follow-ups. To overcome these barriers, research is focused on improving vector design, utilizing non-viral delivery systems, and advancing CRISPR-based gene-editing technologies, which offer greater precision and reduced risk of off-target effects [49] Gene augmentation, which involves introducing functional copies of defective genes, has seen significant success, particularly in monogenic disorders such as spinal muscular atrophy (SMA). CRISPR-Cas9, a precise gene-editing tool, has been instrumental in correcting mutations responsible for conditions like Duchenne Muscular Dystrophy (DMD) [32]. These non-viral methods offer advantages in transient gene expression, ideal for conditions needing temporary gene expression or frequent redosing. However, improving delivery efficiency and minimizing side effects remain key challenges. Current research focuses on enhancing target specificity, optimizing delivery mechanisms, and developing hybrid systems that combine non-viral and viral delivery benefits. By improving these aspects, non-viral vectors are set to play an increasingly significant role in safe and personalized gene therapies provided the vectors address the significant bottleneck of precise tissue targeting [50,51]

12. BIOLOGICAL AND TECHNICAL BARRIERS-IMMUNE REACTIONS

Most gene therapy techniques rely on viral vectors (*e.g.*, adeno-associated viruses (AAV), lentivirus) to deliver therapeutic genes into cells. However, the body's immune system may recognize these vectors as foreign invaders, leading to an immune response that can neutralize the viral vectors before they deliver their genetic payload. This immune reaction may also lead to inflammation or tissue damage, limiting the effectiveness of the therapy [22]. In some cases, individuals may already have pre-existing immunity to

certain viral vectors, particularly AAVs, due to prior natural infections or vaccinations. This can significantly reduce the efficacy of the gene therapy, as the immune system rapidly clears the vectors before they can be effective. To mitigate these risks, researchers are exploring novel, less immunogenic viral vectors and immune suppression strategies to enhance vector persistence [50].

13. DELIVERY TO TARGET TISSUES (CROSSING THE BLOODBRAIN BARRIER)

One of the main challenges in gene therapy is the ability to deliver the therapeutic gene to the specific tissues or cells that require treatment. For example, in diseases like cystic fibrosis, the therapeutic gene must be delivered specifically to the lung cells, and in muscular dystrophy, the muscle cells. The size and type of vector used in gene therapy may limit its ability to reach target tissues [51] efficiently. For instance, AAV vectors can be too large to penetrate certain tissues or organs effectively, particularly in the case of tissues like the brain or lungs. Additionally, ensuring that the gene is delivered to the correct cells within the target tissue (*e.g*., motor neurons, liver cells) remains a significant challenge. While *ex vivo* gene therapy (where cells are modified outside the body and then reintroduced) offers more control over the cell delivery process, *in vivo* therapy (direct delivery to the patient's body) faces more challenges in achieving targeted and precise gene delivery [52].

14. LIMITATIONS IN GENE-EDITING PRECISION- OFF-TARGET EFFECTS

One of the primary concerns in gene editing techniques, such as CRISPR-Cas9, is the potential for off-target mutations. These unintended changes to the genome could lead to harmful consequences, including disruption of other essential genes or activation of oncogenes, which could potentially lead to cancer. While technologies like CRISPR are highly precise, they may not always make the desired edit in every cell, leading to incomplete or inconsistent therapeutic outcomes. Achieving 100% editing efficiency in all target cells remains a significant challenge [53].

15. ETHICAL CONSIDERATIONS

One of the most significant ethical concerns in gene therapy, particularly with CRISPR and other genome-editing technologies, is germline editing—modifying the genetic material in embryos or reproductive cells. Germline modifications could be passed on to future generations, raising concerns about unintended consequences, such as the introduction of harmful mutations and the potential for "designer babies." Another ethical issue is the potential for gene therapy to exacerbate existing health disparities. High treatment costs and limited availability of these therapies could mean that only wealthier populations have access, leaving others without a life-saving treatment. The ethical implications of gene therapy also extend to the unknown long-term effects. While short-term clinical trials may show promise, there is still uncertainty about how these therapies will perform over decades, especially when the treatments involve irreversible genetic modifications [54].

16. REGULATORY LANDSCAPE

The approval of gene therapies is a complex and lengthy process that involves extensive clinical trials to demonstrate safety and efficacy. Regulatory bodies like the U.S. FDA and the European Medicines Agency (EMA) are still developing frameworks to handle gene therapies, which do not always fit within the traditional drug approval process. The lack of clear and efficient pathways for approval can delay access to promising therapies. Gene therapies often involve permanent changes to a patient's genetic material, necessitating longterm post-treatment monitoring to assess potential delayed side effects, such as the development of cancer or immune-related issues [13,14].

17. High Cost, Complex Development, Ethical Concerns, and Limited Patient Availability

Gene therapy remains one of the most expensive treatment modalities due to the long timeline of research, clinical trials, advanced technologies, and specialized expertise required. Manufacturing viral or non-viral vectors demands complex facilities, strict safety protocols, and rigorous quality control, further driving costs. Only a fraction of therapies succeed in trials, adding to the expense of approved treatments. Examples include Hemgenix for hemophilia B ($2.1 million per dose), both designed as one-time therapies to replace lifelong treatment. Beyond cost, ethical concerns, complex developmental procedures, and limited patient availability challenge the broader application of gene therapy**.**

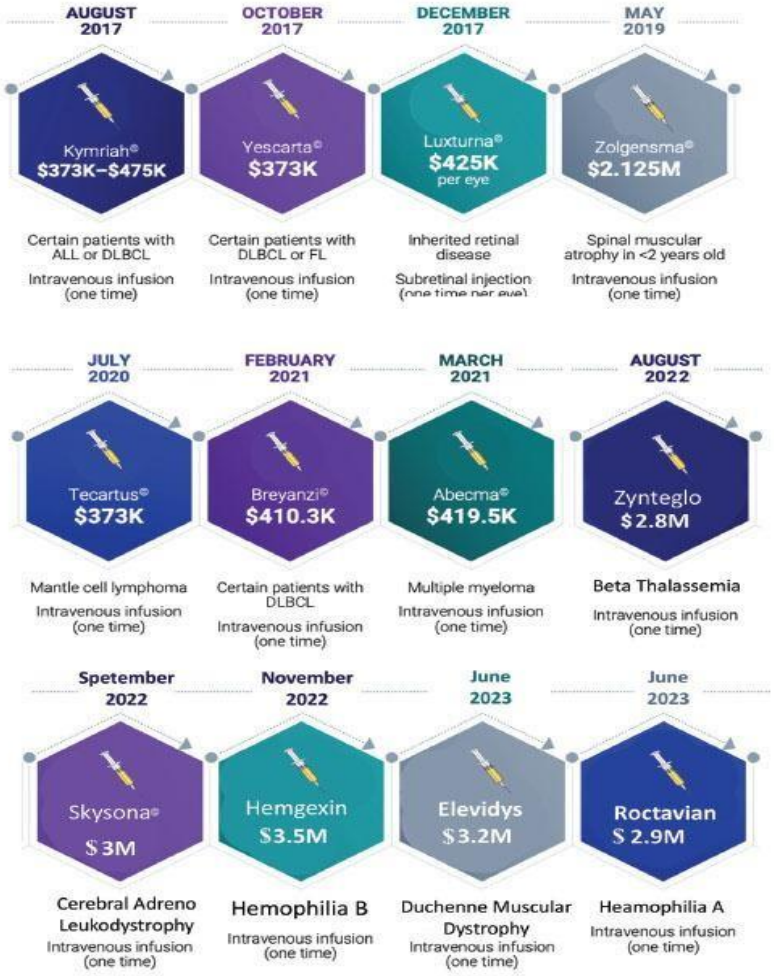

Figure 2 Different methods of viral delivery systems.

18. INSERTIONAL MUTAGENESIS

Post-approval, administering these therapies demands skilled professionals and often specialized healthcare settings. Many gene therapies target rare genetic disorders, meaning fewer patients contribute to covering the costs, unlike drugs for widespread conditions.

Many gene therapies use viral vectors to deliver therapeutic genes into cells. However, these vectors can integrate their genetic material into the host genome, potentially disrupting normal cellular processes. Insertional mutagenesis occurs when this integration happens near an oncogene (a gene that can cause cancer), potentially leading to tumorigenesis. This was a significant concern in earlier gene therapy trials, particularly in hematopoietic stem cell gene therapies, where unintended integration caused leukemia in some patients [27]. Newer vector designs and gene-editing technologies aim to minimize the risk of insertional mutagenesis by targeting safer, less gene-dense regions of the genome or using non-integrating vectors, but this remains a critical area of research. While gene therapies may show promising results in the short term, the long-term effects of permanent genetic alterations are still largely unknown. For example, changes to the immune system, off-target mutations, or the persistence of therapeutic genes could have long-term consequences. There is a need for continuous monitoring and long-term follow-up in clinical trials to fully understand the durability of the treatment and the potential risks associated with these permanent changes [55].

19. FUTURE DIRECTIONS

19.1. Analyzing Population-based Genetic Diversity

The future of gene therapy relies heavily on expanding our understanding of genetic diseases through studies that incorporate a diverse range of populations. Historically, genetic studies have focused primarily on single ancestral populations, limiting the scope of our knowledge. However, recent genome-wide multi-ancestral studies have significantly broadened this understanding [56,57]. For example, a multi-ancestry genome-wide study has uncovered multiple target genes for early prediction of systemic lupus erythematosus. The inclusivity of diverse ancestries in genetic research allows for the identification of a broader spectrum of genetic variants, which can inform more effective, personalized treatment strategies [56]. Similarly, another large-scale multi-ancestry meta-analysis of Parkinson's disease (PD), involving over 49,000 PD cases and more than 2.4 million controls from various populations (European,

East Asian, Latin American, and African), identified 78 genome-wide significant loci, including 12 potentially novel ones. These findings provide valuable insights into PD's genetic basis across different ethnic groups and pave the way for future research in nonEuropean populations [57] . Furthermore, the data generated from these studies should be shared globally to enhance gene therapy development, ultimately benefiting global health. In cancer genomics, the creation of standardized DNA reference datasets has also been crucial. Reference call sets from paired tumor-normal genomic DNA samples from a breast cancer cell line have helped identify somatic mutations and germline variants with high confidence despite not being directly representative of primary cancer cells. These reference samples play a key role in minimizing biases in sequencing technologies and assays, and they serve as a valuable resource for improving tumor genomics analyses. Together, these populationbased reference samples and multi-ancestry studies contribute significantly to advancing gene therapy and precision medicine, ensuring treatments are tailored to the genetic diversity of global populations [56,57].

19.2. Advances in CRISPR/Cas9 and Genome Editing

The CRISPR/Cas9 system has revolutionized the field of genome editing, allowing for precise and efficient modification of the genome. Advances in CRISPR technology, such as base editing and prime editing, are improving precision and reducing the risks of off-target effects. [58]

1. Genome Editing for Cystic Fibrosis

CRISPR (clustered regularly interspaced short palindromic repeats) was first identified in *E. coli* in 1987 as part of a microbial adaptive immune system. Among CRISPR-associated proteins, SpCas9 from *Streptococcus pyogenes* is the most widely studied. In 2012, Doudna and Charpentier engineered CRISPR/Cas9 for programmable gene editing, followed by Zhang and Church's demonstration of eukaryotic genome engineering in 2013. These breakthroughs revolutionized medicine by enabling direct correction of disease-causing genes.

A decade later, 76 CRISPR/Cas clinical trials have been registered with the FDA, including landmark successes in sickle cell disease and β-thalassemia, where sustained cures were achieved. These advances have inspired efforts to apply CRISPR-based therapies to other genetic disorders, notably cystic fibrosis (CF).

2. CF Gene Mutations and Clinical Diseases

2.1 CF Genomic Mutations

Cystic fibrosis (CF) is a common life-threatening genetic disorder, most prevalent in Caucasians, with >100,000 patients worldwide. In 1989, CFTR mutations were mapped to chromosome 7 (7q31.2). CFTR encodes a cAMP-activated chloride/bicarbonate channel. Over 2000 mutations have been identified, classified into five groups:

- Class I: Nonsense mutations (e.g., G542X) → truncated protein
- Class II: Misfolded protein degraded in ER (e.g., F508del, ~85.5% of cases)
- Class III: Full-length protein with defective gating (e.g., G551D)
- Class IV: Reduced channel conductance
- Class V: Reduced protein synthesis

2.2 CF Clinical Diseases

CF is a multisystem disease, but lung pathology dominates (>90% morbidity/mortality). Defective CFTR impairs chloride/bicarbonate secretion, increases sodium absorption, dehydrates airway surface liquid, and disrupts mucociliary clearance → chronic infection, neutrophilic inflammation, bronchiectasis, and respiratory failure.

- Pancreas: Early involvement; ductal obstruction → fibrosis, fatty replacement, destruction. ~85% are pancreatic insufficient; 40–50% of adults develop CF-related diabetes.

- Intestine: Meconium ileus in ~20% neonates; lifelong malabsorption, DIOS, constipation, SIBO, dysbiosis, and fibrosing colonopathy.
- Liver: ~30% affected; features include steatosis, biliary cirrhosis, cholestasis, gallbladder abnormalities.
- Reproductive system: Most males have congenital absence of vas deferens; females show reduced fertility.
- Immune system: CFTR loss impairs neutrophil phagosomal chloride transport → defective antimicrobial activity and dysregulated inflammation. Neutrophils and macrophages show hyper-reactivity, abnormal ROS, delayed apoptosis, and impaired bacterial killing.
- Musculoskeletal: 2–8.5% develop CF-related arthropathy; 2–7% hypertrophic pulmonary osteoarthropathy with periostitis and clubbing.

3. General Considerations in Gene Editing Design for CF

- Systemic disease: CF affects multiple organs; systemic delivery is ideal to reach all tissues.
- Mutation diversity: >2000 mutations exist; targeted insertion of full-length CFTR cDNA offers a pan-mutation solution.
- Durability: Editing stem/progenitor cells ensures long-term correction beyond short-lived epithelial cells.
- Timing: Early intervention is critical, before irreversible organ damage; therapies must be suitable for young patients.
- Safety: Minimizing off-target effects and avoiding germline modification are essential for long-term safety.

4. Gene Editing Tools for Selection

4.1. Cas Nuclease Editors

CRISPR/Cas systems are divided into Class I (Types I, III, IV) with multi-protein complexes, and Class II (Types II, V, VI) with single multidomain proteins. Class II systems, especially Cas9, are the most widely used.

- Cas9: Guided by crRNA and tracrRNA (or fused sgRNA), induces double-strand breaks (DSBs). Variants include:
  - SpCas9 (requires 5′-NGG PAM)
  - xCas9 (expanded PAM recognition)
  - nCas9 (nickase, requires two sgRNAs for DSBs, higher specificity)
  - dCas9 (catalytically inactive, used for transcriptional regulation)
- Cas9 orthologues (e.g., *S. thermophilus*, *N. meningitidis*, *S. aureus*, *C. jejuni*) expand PAM diversity.
- Cas12a: Creates staggered DSBs, ideal for multiplex editing.
- Cas13: Targets single-stranded RNA, enabling transcriptome manipulation.

4.2. Base Editors

Base editing uses a different design to achieve genome modification by the direct generation of precise point mutations. DNA base editors comprise a catalytically impaired Cas nuclease fused with a base modification enzyme that induces base alteration on a single strand of DNA. Then the cellular DNA repair machinery intervenes to repair the mismatch on the complementary strand to complete the intended base conversion. There are two classes of DNA base editors that have been reported: (1) cytosine base editors that convert a C•G base pair into a T•A base pair, and (2) adenine base editors that convert an A•T base pair into a G•C base pair.

4.3. Prime Editors

Prime editors combine nCas9 with reverse transcriptase and use pegRNA templates to introduce substitutions, small insertions, or deletions. Advantages include:

- High precision: Minimal indels.
- Flexibility: Less PAM restriction, edits possible farther from PAM sites.
- Broad applicability: Effective across cell cycle phases, independent of homologous recombination.

5. CRISPR-Based CFTR Gene Editing

5.1 In Vitro Correction

CRISPR/Cas9 has successfully corrected CFTR mutations in patient-derived intestinal stem cells and iPSCs, restoring chloride transport in organoids and airway epithelia. Beyond F508del, premature stop codons have been repaired using CRISPR/Cas9, adenine base editors, and prime editors. Full-length CFTR cDNA has been inserted into the CF locus and safe-harbor sites, demonstrating the feasibility of pan-mutation correction.

5.2 CF Models

CRISPR has enabled the creation of diverse CF cell lines (e.g., F508del, G542X, W1282X, G551D) with isogenic controls, advancing drug testing and mechanistic studies. Animal models—including CF mice, rats, rabbits, ferrets, and lambs—have been generated via pronuclear injection and nuclear transfer, expanding resources for pathogenesis and therapy development.

5.3 In Vivo Correction

Early in vivo studies show promise for lung-targeted CRISPR therapies. Shuttle peptides and base editors achieved airway editing in mice (~12–13%) and rhesus monkeys (~5.3%), with edited epithelia persisting up to 12 months. These findings highlight the potential of CRISPR-based strategies for CF lung disease, with the next step being therapeutic testing in CF animal models.

6. Non-CRISPR-Based CFTR Gene Editing

Beyond CRISPR, several alternative gene-editing platforms have been explored for CFTR correction. Early approaches used zinc finger nucleases (ZFNs) and TALENs, achieving functional CFTR repair in patient-derived airway cells and ~5% targeted integration in adenoviral systems.

A novel peptide nucleic acid (PNA) system has also emerged, enabling site-specific DNA recognition via triplex formation and recombination. When delivered with donor DNA in biodegradable nanoparticles, PNAs corrected F508del mutations in airway epithelial cells. Importantly, systemic delivery in CF mouse models achieved in vivo correction across multiple epithelial tissues (nasal, lung, intestinal), with correction rates of ~0.1–2% and measurable improvements in lung inflammation and chloride transport.

These advances highlight that non-CRISPR editors, though less widely used, remain valuable complementary strategies for CFTR gene correction.

7. Comparison of CRISPR and Non-CRISPR PNA Editors for CF Gene Editing

Both CRISPR and peptide nucleic acid (PNA) systems have demonstrated feasibility in correcting CFTR mutations in vitro and in vivo, though both remain at early stages for therapeutic application. Current challenges include low editing efficiency, unclear target cell spectrum, limited durability data, and untested long-term safety.

Mechanistically, each system offers distinct advantages:

- CRISPR requires PAM sites but carries its own editing enzymes (e.g., base/prime editors), enabling efficient DNA modification independent of endogenous repair.
- PNA does not require PAM sites, offering greater flexibility. Its small size allows packaging into nanoparticles for systemic delivery across epithelial barriers, and low immunogenicity supports repeated administration.

Together, these complementary approaches renew optimism for CF genetic therapy and warrant further optimization before clinical translation [59].

8. Barriers to Overcome to Improve In Vivo Gene Editing Efficiency for CF

From the existing data, there is an apparent gap in gene editing efficiency between in vitro and in vivo applications in both CRISPR- and non-CRIPSR-based gene editors. Thus, delivery is a determining factor in the success of translating this new technology into any clinical benefits. As CF predominantly affects epithelium-lined organs, directing CF gene editors to the epithelial cells is believed to be essential for potential CF therapy. There are only two feasible routes to deliver the gene editors: (1) through epithelial lumens, and (2) through the circulation. Each route has its own barriers to overcome.

8.1. Gene Editor Delivery through Epithelial Lumens

For a luminally released gene editor to reach the nucleus of a target epithelial cell, multiple layers of barriers need to be overcome (Figure 1). First, on the top of an epithelium, there usually exists a mucus and/or surface liquid layer that gene editors have to penetrate. Second, an epithelium-lined organ usually evolves a mucociliary clearance mechanism for the purpose of host defense. This mechanism can also act to clear luminally delivered gene editors. Third, epithelial cells are typically polarized, and their subcellular structures, e.g., cytoskeleton, in the apical and basolateral compartments differ, which limits gene editor entry from the apical side. Fourth, epithelial stem and progenitor cells need to be targeted for any lasting gene editing. These cells sometimes are anatomically located underneath other epithelial cells and are hard to reach without losing the overlaying cells.

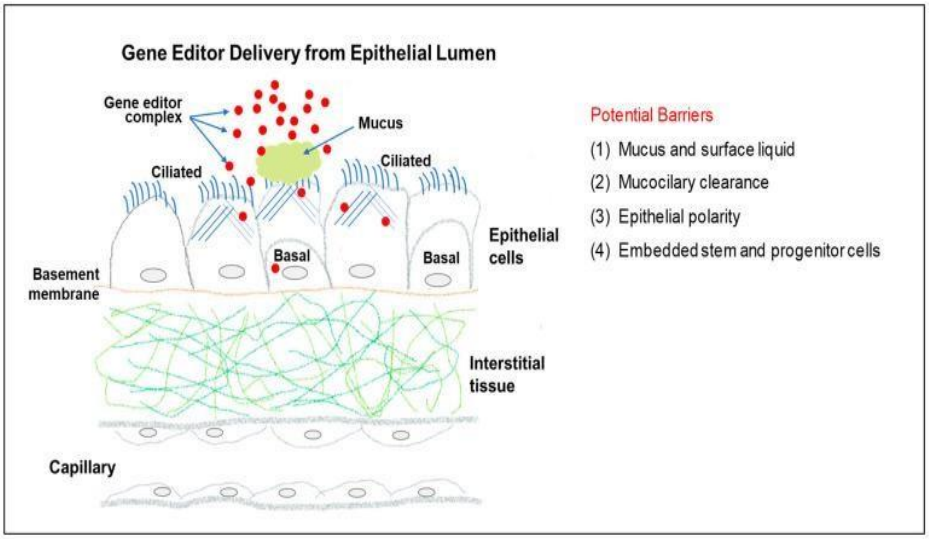

Figure 4. Gene Editor Delivery from Epithelial Lumen

8.2 Gene Editor Delivery through the Circulation

Systemic (circulatory) delivery offers the potential to reach all cell types, but several barriers limit efficiency. Gene editors must cross the endothelial layer, diffuse through the interstitial space, and penetrate the epithelial basement membrane to access target cells.

Key design considerations include:

- Size: Smaller editor complexes favor penetration and diffusion.
- Barrier modulation: Temporary manipulation of epithelial/endothelial junctions can facilitate escape.
- Vehicles: Engineered carriers with shuttle peptides or receptor/ligand targeting enhance penetration and specificity.

Altogether, circulation-based delivery requires optimized editor and vehicle designs to achieve effective systemic correction.

19.3. Development of Non-Viral Delivery Systems

Non-viral delivery systems, such as nanoparticles, are gaining attention as safer alternatives to viral vectors. These systems could potentially reduce immune reactions and enhance the targeted delivery of therapeutic genes.[59]

19.4. Personalized Gene Therapies

The future of gene therapy lies in personalized approaches, where treatments are tailored to the individual's genetic makeup. Advances in next-generation sequencing and personalized medicine are paving the way for customized gene therapies [58].

CONCLUSION

Gene therapy represents a transformative frontier in medicine, offering curative potential for rare genetic disorders once considered untreatable. Breakthroughs such as Zolgensma and Luxturna highlight its ability to reverse disease progression and improve quality of life, while ongoing innovations in CRISPR, non-viral delivery, and personalized approaches promise to expand its reach. Yet challenges remain, from immune responses and delivery barriers to ethical and regulatory concerns. Continued research, coupled with robust frameworks for safety and accessibility, will be essential to ensure these therapies benefit the widest patient populations. With sustained progress, gene therapy is poised to redefine the future of rare disease treatment, bringing lasting hope to patients and families worldwide.

REFERENCES

1.Nguengang Wakap S., Lambert D.M., Olry A. Estimating cumulative point prevalence of rare diseases: Analysis of the Orphanet database. European Journal of Human Genetics. Springer Nature, 2020; 28(2):165–173.
2.Paddison P.J., Silva J.M., Conklin D.S. A resource for large-scale RNA-interference–based screens in mammals. Nature. Springer Nature, 2004; 428(6981):427–431.
3.Rogers K., Chen X. Biogenesis, turnover, and mode of action of plant microRNAs. Plant Cell. American Society of Plant Biologists, 2013; 25(7):2383–2399.
4.Gheysen G., Vanholme B. RNAi for plant disease resistance. Plant Physiology. American Society of Plant Biologists, 2007; 145(4):1460–1464.
5.Dykxhoorn D.M., Novina C.D., Sharp P.A. Killing the messenger: RNA interference meets cancer therapeutics. Cancer Cell. Cell Press, 2003; 3(3):171–175.
6.Lee R.C., Ambros V. An extensive class of small RNAs in Caenorhabditis elegans. Science. American Association for the Advancement of Science, 2001; 294(5543):858–862.
7.Liu Q., Chen Y. Insights into the regulation of plant microRNA biogenesis. Plant Cell Reports. Springer Nature, 2016; 35(9):2215–2228.
8.Bartel D.P. MicroRNAs: Target recognition and regulatory functions. Cell. Cell Press, 2009; 136(2):215–233.
9.Axtell M.J. Classification and comparison of small RNAs from plants. Annual Review of Plant Biology. Annual Reviews, 2013; 64(1):137–159.
10.Voinnet O. Origin, biogenesis, and activity of plant microRNAs. Cell. Cell Press, 2009; 136(4):669–687.
11.Hall Z.W., Talbot K., Wattjes M.P. Recombinant expression technologies in modern therapeutic protein production. Nature Biotechnology. Springer Nature, 2018; 36(12):1142– 1150.
12.Berkowitz S.A., Engen J.R., Mazzeo J.R. Characterization of recombinant therapeutic proteins. Analytical Chemistry. American Chemical Society (ACS), 2012; 84(2):97–118.
13.Liu H.F., Ma J., Winter C. Improving protein folding in microbial expression systems. Biotechnology Advances. Elsevier, 2014; 32(2):435–440.
14.Heidary Z., Abnous K., Taghdisi S.M. Production of recombinant insulin using plantbased systems. Plant Biotechnology Journal. Wiley–Blackwell, 2020; 18(8):1683–1695.
15.Penno M., Müller C., Antošová Z. Recombinant DNA–derived insulin analogs: advances and future prospects. Diabetes Therapy. Springer Healthcare, 2017; 8(4):763–780.
16.Krogh A., Larsson B., von Heijne G. Protein targeting and secretion in recombinant systems. Journal of Molecular Biology. Elsevier, 2001; 312(4):709–723.
17.Sørensen H.P., Mortensen K.K. Soluble expression of recombinant proteins in E. coli: Strategies and applications. Microbial Cell Factories. BioMed Central, 2005; 4(1):1–8.
18.Tokatlidis K., Dhurua S., Singh S. Advances in yeast expression platforms for biopharmaceutical production. Current Opinion in Biotechnology. Elsevier, 2019; 59(1):72– 80
196.Fischer R., Schillberg S. Molecular farming of recombinant proteins in plants. Biotechnology Advances. Elsevier, 2004; 22(6):653–670.
20.Doran P.M. Foreign protein production in plant tissue culture. Journal of Biotechnology. Elsevier, 2000; 76(3):241–253.
21.Lico C., Chen Q., Santi L. Viral vectors for production of recombinant proteins in plants. Journal of Virology. American Society for Microbiology, 2008; 82(13):6766–6777
22.Ma J.K., Drake P.M., Chargelegue D. Plant-made antibody therapeutics. Current Opinion in Plant Biology. Elsevier, 2005; 8(2):223–229.
23.He Y., Ning T., Xie T. Large-scale production of recombinant proteins in plants. Protein Expression and Purification. Elsevier, 2014; 94(1):1–8.
24.Gomord V., Chamberlain P., Jefferis R. Biopharmaceutical production in plants: problems, solutions and opportunities. Trends in Biotechnology. Elsevier, 2005; 23(11):559– 565.
25.Walsh G. Biopharmaceutical benchmarks. Nature Biotechnology. Springer Nature, 2018; 36(12):1136–1145.
26.Gasser B., Prielhofer R., Marx H. Pichia pastoris: protein production workhorse in biotechnology. Microbial Cell Factories. BioMed Central, 2013; 12(1):1–17.
27.Jefferis R. Glycosylation of therapeutic proteins. Biotechnology Progress. Wiley– Blackwell, 2005; 21(1):11–16.

28.Trivedi M.V., Laurence J.S., Siahaan T.J. Hydrophobicity of therapeutic proteins. Current Protein & Peptide Science. Bentham Science, 2009; 10(6):614–625.
29.DeLisa M.P., Li J., Rao G. Folding stress in E. coli: strategies for improving recombinant protein secretion. Applied and Environmental Microbiology. American Society for Microbiology, 2003; 69(7):4319–4325.
28.Chen Q., Lai H. Plant-derived biologics. Biotechnology Advances. Elsevier, 2015; 33(6):1172–1181.
29.Torres M., Palomares L.A., Ramírez O.T. Insulin-based therapeutics. Applied Microbiology and Biotechnology. Springer Nature, 2005; 68(2):151–160.
30.Wang T., Li Y., Shi J. Expression and purification of recombinant insulin analogs. Protein Expression and Purification. Elsevier, 2016; 120(1):106–113.
31.Jepson C.E., Sang Y., Fang R. Plant-derived recombinant insulin: advances, challenges, and therapeutic potential. Frontiers in Plant Science. Frontiers Media, 2021; 12(1):1–12.
32.Sharma A., Kumar D., Singh R. Optimization of E. coli systems for high-yield recombinant insulin production. Bioprocess and Biosystems Engineering. Springer Nature, 2018; 41(5):679–692.
33.Anila M., Thomas S., Mathew A. Transgenic plant bioreactors for low-cost insulin manufacturing. Applied Biochemistry and Biotechnology. Springer Nature, 2020; 190(3):889–905.
34.Ahmad F., Alam P., Rahman S. Engineering Camelina sativa seeds as green bioreactors for human pro-insulin. Plant Biotechnology Journal. Wiley–Blackwell, 2024; 22(1):112–124.
35.Bajpai R., Kondhare K., Handore K. Expression of biologically active insulin analog SCI57 in Nicotiana benthamiana. Frontiers in Pharmacology. Frontiers Media, 2019; 10(1):1–10.
36.Boothe J.G., Zeman S., Sato S. Transgenic expression and recovery of biologically active recombinant human insulin. Protein Expression and Purification. Elsevier, 2008; 59(2):243– 250.
37.Walsh G. Biopharmaceutical benchmarks: recombinant insulin and the evolution of rDNA therapeutics. Nature Biotechnology. Springer Nature, 2014; 32(10):992–1000.
38.Bhattacharya S., Banerjee S., Ghosh R. Advances in microbial fermentation strategies for insulin biosynthesis. Journal of Industrial Microbiology & Biotechnology. Oxford University Press, 2017; 44(9):1331–1344.
39.Middelberg A.P.J. Process-scale disruption of microorganisms. Biotechnology Advances. Elsevier, 1995; 13(3):491–551.
40.Baneyx F. Recombinant protein expression in Escherichia coli: advances and challenges. Current Opinion in Biotechnology. Elsevier, 1999; 10(5):411–421.
41.Demain A.L., Vaishnav P. Production of recombinant proteins by microbes and their role in modern biotechnology. Microbial Biotechnology. Wiley–Blackwell, 2009; 2(1):2–15.
42.Walsh G. Post-translational modifications of protein biopharmaceuticals. BioPharm International. MJH Life Sciences, 2010; 23(6):20–27.
43.Tokatlidis K. Protein folding mechanisms in recombinant expression hosts. Journal of Molecular Recognition. Wiley–Blackwell, 2000; 13(1):1–10.
44.Gomord V., Faye L. Post-translational modifications of recombinant proteins produced in plants. Current Opinion in Plant Biology. Elsevier, 2004; 7(2):171–181.
45.Berkowitz S.A. Characterization of biotherapeutics: analytical profiling and stability challenges. Analytical Biochemistry. Elsevier, 2006; 355(1):1–20.
46.Houde D., Berkowitz S.A., Engen J.R. Protein higher-order structure analysis by mass spectrometry. Analytical Chemistry. American Chemical Society (ACS), 2011; 83(17):6237– 6245.
47.Liu Y., Chen X., Zhang X. Improving fermentation efficiency for recombinant insulin production in yeast. Yeast. Wiley–Blackwell, 2016; 33(5):235–246.
48.Huang T.K., McDonald K.A. Bioreactor engineering for recombinant protein–producing transgenic plants. Biotechnology Journal. Wiley–VCH, 2012; 7(10):1218–1228.
49.Xu J., Dolan M.C. Plant cell culture as a biomanufacturing platform for recombinant proteins. Critical Reviews in Biotechnology. Taylor & Francis, 2011; 31(3):215–232.
50.Fischer R., Buyel J.F. Molecular farming — plant-made pharmaceuticals and industrial proteins. Biotechnology and Bioengineering. Wiley–Blackwell, 2020; 117(4):1031–1051.
51.Terpe K. Overview of bacterial expression systems for recombinant protein production. Applied Microbiology and Biotechnology. Springer Nature, 2006; 72(2):211–222.
52.Jayaraman J., Luo Y. Recombinant insulin production in microbial systems: challenges and solutions. Journal of Biotechnology. Elsevier, 2014; 184(1):1–12.

53.Valverde R., Lozano J., Sánchez-Murcia P.A. Advances in protein engineering for improved insulin analogs. Biochemical Journal. Portland Press, 2017; 474(22):3767–3784.
54.Spök A., Karner S., Schiemann J. Regulatory and safety considerations for plantproduced biopharmaceuticals. Transgenic Research. Springer Nature, 2008; 17(4):581–596.
55.De Muynck B., Navarre C., Boutry M. Production of therapeutic proteins in plants: current status and future outlook. Plant Biotechnology Journal. Wiley–Blackwell, 2010; 8(5):564–580.
56.Sørensen H.P. Towards improved protein production in E. coli: strategies for high solubility. Microbial Cell Factories. BioMed Central, 2010; 9(1):1–10.
57.Murray J.B., Bello A.M. Structure–function relationships of insulin and insulin analogs. Journal of Molecular Biology. Elsevier, 2014; 426(1):94–110.
58.Hansen J., Storm K. Stability and formulation of recombinant insulin products. Pharmaceutical Research. Springer Nature, 2010; 27(5):999–1010.
59.Carvalho S.B., Silva M.M., Tavares T. Advances in purification processes for recombinant insulin. Separation and Purification Technology. Elsevier, 2018; 205(1):97–108.