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Research Article

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CRISPR-Cas9 Gene Editing in Pediatric Epidermolysis Bullosa

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Abstract

CRISPR-Cas9 gene editing holds promise for treating pediatric epidermolysis bullosa (EB), a group of genetic disorders characterized by severe skin fragility. This literature review comprehensively examines the application of CRISPR-Cas9 in correcting genetic mutations associated with various types of EB. Preclinical studies have demonstrated the potential of CRISPR-Cas9 to target and correct mutations in genes such as COL7A1, LAMB3, and COL17A1, which are implicated in different EB subtypes. Clinical studies are in early stages, with limited but promising data on the safety and efficacy of this gene-editing approach. Various delivery mechanisms, including viral vectors and nanoparticle-based systems, have been evaluated for their efficiency in targeting skin tissues. Viral vectors, particularly lentiviruses and adeno-associated viruses (AAVs), have shown effective gene delivery and sustained expression, while nanoparticles offer a non-viral alternative with a favorable safety profile. Long-term follow-up data, although limited, suggest that CRISPR-Cas9 treatment can achieve durable correction of skin lesions with minimal off-target effects, though comprehensive studies are required to fully ascertain the safety and potential immunogenicity in pediatric patients. Overall, the CRISPR-Cas9 system represents a promising therapeutic strategy for pediatric EB, necessitating further research to optimize delivery methods and ensure long-term safety and efficacy.

Introduction

Epidermolysis bullosa (EB) represents a group of rare genetic disorders characterized by extreme skin fragility and blistering in response to minor mechanical trauma or friction. Inherited in an autosomal dominant or recessive manner, EB varies in severity, ranging from mild forms with localized blistering to severe types with extensive mucocutaneous involvement and systemic complications. The hallmark features of EB include blistering, erosions, and skin fragility, which can occur spontaneously or following minimal trauma. These clinical manifestations stem from defects in proteins essential for maintaining skin integrity, particularly in the epidermis and dermal-epidermal junction (DEJ). Recent epidemiological studies indicate a prevalence of 20-30 per 1,000,000 people and an incidence of 40-60 per 1,000,000, suggesting that EB might be more common than previously thought [1,2].

EB is caused by mutations in genes critical for skin integrity and cohesion. Sixteen genes have been identified as contributing over 30 subtypes of EB, each presenting varying degrees of severity, morbidity, and mortality [3]. These subtypes are categorized into four major groups based on the ultrastructural level within the DEJ where the defect occurs. Understanding the molecular basis and classification of EB is essential for improving diagnosis, patient care, and developing targeted therapeutic interventions. Given the significant challenges of disease management for both patients and caregivers, especially when a permanent cure is unavailable, there is a critical need for innovative therapeutic approaches.

Current management strategies focus primarily on supportive care, including wound care, pain management, infection prevention, and nutritional support. These methods, while essential, are often inadequate for addressing the underlying genetic causes of EB. Consequently, gene therapy stands out as a promising treatment that could offer a curative solution for EB. CRISPR-Cas9, which stands for "Clustered Regularly Interspaced Short Palindromic Repeats" and CRISPR-associated protein 9, is an innovative gene-editing technology that has revolutionized the field of genetic engineering [4]. This system, adapted from a bacterial defense mechanism against viral infections, enables precise modifications to the DNA of living organisms, allowing the correction of genomic errors and the ability to turn genes on or off quickly and easily.

The CRISPR-Cas9 system consists of two key components: the Cas9 nuclease, which introduces double-strand breaks in the DNA, and a guide RNA (gRNA) that directs Cas9 to the specific target sequence [5]. The mechanism by which CRISPR-Cas9 operates involves the Cas9 protein binding to a gRNA, forming a complex that can specifically bind to the target DNA sequence. Upon binding, Cas9 induces a double-strand break at the target site. The cell then repairs this break using one of two primary pathways: non-homologous end joining (NHEJ) or homology-

directed repair (HDR) [5]. NHEJ often leads to insertions or deletions that can disrupt the gene, whereas HDR allows for precise edits by using a DNA template to repair the break.

The specificity of CRISPR-Cas9 is predominantly determined by the 20-nucleotide sequence of the gRNA, although off-target effects remain a significant challenge [6]. Researchers are continually refining gRNA design and developing modified Cas9 proteins to improve specificity and minimize unintended cuts. The potential applications of CRISPR-Cas9 in treating genetic disorders caused by single gene mutations are vast and transformative. In the context of EB, CRISPR-Cas9 can be employed to correct pathogenic mutations directly in patient-derived cells.

Current research is exploring various delivery methods, including viral vectors such as adeno-associated viruses and non-viral approaches like lipid nanoparticles, to enhance the efficiency and safety of gene editing in clinical settings [7]. Additionally, combining CRISPR-Cas9 with other emerging technologies, such as base editing and prime editing, holds potential to further refine the precision and reduce off-target effects of gene editing therapies [8]. These scientific advancements offer promising potential in advancing treatment and improving clinical outcomes for patients with EB. This paper aims to provide a comprehensive review of the current state of CRISPR-Cas9 technology in the treatment of pediatric EB, evaluating preclinical and clinical studies, delivery mechanisms, and future directions for research and clinical application.

Discussion

Background on EB

Epidermolysis bullosa simplex

Epidermolysis bullosa simplex (EBS) is the most common and typically milder form of EB, accounting for approximately 70% of all EB cases. EBS is characterized by a defect in the epidermis and is inherited in an autosomal dominant manner [3]. Mutations in the KRT5 or KRT14 genes, which encode keratin proteins 5 and 14, respectively, disrupt the structural stability of basal keratinocytes, leading to intraepidermal blistering [9]. EBS manifests with superficial blistering, often at sites prone to friction, such as the hands, feet, and knees. Although the blisters are non-scarring, they can lead to erosions, infections, and chronic wounds in more severe cases.

Dystrophic EB

Dystrophic epidermolysis bullosa (DEB) can be inherited in either autosomal dominant or recessive patterns, with recessive inheritance typically leading to more severe phenotypes [10]. Four types of dominant DEB and six types of recessive DEB have been identified, including a particularly severe subtype characterized by compound heterozygosity. All forms of DEB involve skin fragility below the lamina densa due to mutations in the COL7A1 gene, which encodes type VII collagen, essential for anchoring fibrils that secure the epidermis to the dermis [11]. This deficiency in collagen VII results in skin fragility, blistering, scarring, and mucosal involvement. Unlike other types of EB, DEB affects deeper layers of the skin, leading to severe mucosal scarring and fibrosis.

Over time, DEB can severely disable patients through contractures and pseudosyndactyly. Additionally, DEB presents with severe scarring, milia formation, nail dystrophy, and esophageal strictures, which further complicate the condition [12]. The propensity for developing metastatic squamous cell carcinoma (SCC) adds to the critical nature of DEB [10]. Due to these significant complications, careful management and monitoring are crucial. The severity of DEB, coupled with the high risk of fatality from metastatic SCC, underscores the urgent need for effective therapeutic interventions and comprehensive patient care.

Junctional EB

Junctional epidermolysis bullosa (JEB), accounting for about 5% of all EB cases, is a severe subtype inherited in an autosomal recessive manner. JEB is characterized by a fragility defect in the lamina lucida of the basement membrane, leading to extensive erosions and ulcerations that significantly affect the skin and mucous membranes, resulting in high morbidity [3,10]. There are eight identified subtypes of JEB, associated with seven distinct mutated genes, including LAMA3, LAMB3, LAMC2, COL17A, ITGA6, ITGB4, and ITGA3. Approximately 70% of JEB cases result from mutations in the LAMA3, LAMB3, and LAMC2 genes, which encode laminin-332, a critical component of the DEJ [13]. Intermediate JEB is often associated with mutations in COL17A1, which encodes type XVII collagen [14].

Mutations in the LAMB3 gene result in a malfunctioning laminin-332 protein. This defect disrupts anchoring filament function and keratinocyte migration during wound healing. Laminin-332 is also crucial for maintaining epithelial function in various organs, including the brain, lungs, eyes, kidneys, thymus, and gastrointestinal tract [1]. Consequently, defective LAMB3 leads to a range of severe complications. In the severe subtype of JEB, the complete absence of laminin-332 causes airway compromise, often resulting in death before the age of two [3]. Furthermore, repeated injury to the lamina lucida increases the risk of SCC, contributing to the early mortality observed in this disease [15].

Mutations in COL17A1 create defective collagen XVII proteins, which are crucial for maintaining hair follicle stem cells, melanocyte stem cells, and cell migration [3]. These proteins are also essential for the stability and integrity of hemidesmosomes, which anchor the epidermis to the dermis. Defects in collagen XVII impair hemidesmosome function, leading to weakened adhesion between the epidermis and dermis. This results in skin fragility and blistering at the level of the lamina lucida within the basement membrane zone, increasing susceptibility to mechanical trauma. Consequently, individuals with these mutations experience skin atrophy, hair loss, dyspigmentation, mucosal involvement, and a heightened risk of carcinogenesis [16].

Kindler EB

Kindler epidermolysis bullosa (KEB) is the rarest of the four major types, with an estimated 400 cases reported globally [3]. KEB involves mutations in KIND1, which encodes kindlin-1, resulting in fragility across any plane of the DEJ. Kindler Syndrome, caused by mutations in the FERMT1 gene, affects keratinocyte adhesion and migration, leading to mixed levels of skin separation [17]. This subtype is characterized by skin fragility, photosensitivity, and progressive poikiloderma, with varying degrees of blistering and mucosal involvement [18].

Clinical implications for pediatric patients

The clinical implications of EB in pediatric patients are severe and multifaceted, with the disease typically manifesting shortly after birth. Affected infants suffer from widespread blistering and erosions caused by minimal friction or trauma, leading to significant pain and discomfort. The chronic nature of these wounds requires intensive and meticulous wound care, including daily dressing changes to manage exudate and prevent infections. This routine is not only painful but also time-consuming and emotionally taxing for both the patients and their caregivers.

Persistent skin damage results in scarring and the formation of contractures, particularly around joints, which severely limits mobility and function. Pseudosyndactyly, or the fusion of digits, further impairs hand and foot function. Nutritional challenges are common due to the high metabolic demands of chronic wound healing and complications such as esophageal strictures from mucosal involvement, making eating painful and difficult. Consequently, growth retardation and delayed developmental milestones are often observed in these children, stemming from the combined effects of chronic illness and nutritional deficiencies.

Moreover, there is a heightened risk of developing SCC in areas of chronic wounds, especially in patients with severe forms of EB like recessive DEB [3]. This adds a significant long-term health threat to the already extensive clinical challenges faced by these patients. These severe clinical manifestations necessitate a multidisciplinary approach to care, involving dermatologists, nutritionists, pain specialists, physical therapists, and psychological support services to manage the complex needs of pediatric patients and enhance their quality of life. Given the severe morbidity and mortality associated with EB, innovative treatments are urgently needed.

Management and treatment challenges

The significant disease burden on EB patients, particularly those with severe forms, highlights the need for effective treatment options. Currently, the management of EB is predominantly supportive, emphasizing wound care, pain management, infection prevention, and nutritional support. Hubbard et al. highlighted the complex challenges in meeting the nutritional needs of children and adults with EB [19]. Severe forms of EB, especially JEB and severe DEB, are associated with growth impairment and nutritional deficiencies due to poor oral intake and malabsorption. The study advocated for the use of gastrostomy tube feeding in severe EB to provide essential medication and nutrition, potentially enhancing the quality of life for patients and their families. Nutritional assessment and supplementation, often with enteral feeding support, are crucial for optimizing growth and development.

Wound care is a critical aspect of EB management. Techniques such as sterile dressing changes, non-adherent dressings, and topical agents to stimulate wound closure are vital. Effective wound management is paramount to prevent infection, promote healing, and minimize scarring, though it can be challenging. Parents of children with severe EB often find bandage changes difficult [20]. This underscores the importance of specialized care and support in alleviating caregiver burdens.

Moreover, EB patients are at heightened risk for complications such as infections, sepsis, and SCC, especially in severe forms. Vigilant monitoring, prophylactic antibiotics, immunizations, and regular dermatologic surveillance are critical for early detection and management of complications. As research has highlighted, these requirements result in increased expenses in EB management and become a significant barrier to care and burden to families [20]. Therefore, addressing the financial challenges faced by EB families is essential to ensure comprehensive and accessible care, requiring a multidisciplinary approach to address the diverse clinical manifestations and complications associated with the disease.

A study by Bruckner et al. showed that EB imposes a significant burden on patients, caregivers, and their families [21]. The results demonstrated that the condition adversely affected the quality of life for patients and placed a financial strain on their families. This research also highlighted the psychosocial burdens that EB can cause, prompting holistic management and support for patients and their caregivers. Kearney et al. supported these findings by identifying five specific healthcare needs of EB patients, including supporting and managing physical healthcare issues associated with the condition, access to community and home-based services, EB-specific information and psychosocial support, effective interaction with healthcare professionals, and advice regarding benefits and entitlements [20].

CRISPR-Cas9 Gene Editing Technology

Mechanism of CRISPR-Cas9

Understanding the implications of CRISPR-Cas9 gene editing in EB necessitates a brief overview of its mechanism. Initially discovered as a bacterial immunity method to prevent harmful plasmid transfer and pathogenic infection, the CRISPR-Cas9 system has been repurposed by biotechnologists into a revolutionary genome editing tool. This versatile technology extends beyond genome editing to include epigenetic modulation, genome imaging, and transcriptional perturbation [22]. CRISPR-Cas9's ability to analyze and manipulate multiple genes simultaneously offers profound insights into complex pathological processes and the potential for novel treatments and cures for genetic diseases [23]. Its impact on clinical genetics has been transformative.

The backbone of CRISPR-Cas9 genomic editing lies in its precise targeting of genes of interest. The system operates by introducing specific double-stranded breaks in DNA via RNA-guided DNA endonuclease activity at predetermined sites [22]. Single guide RNA (sgRNA) sequences, short nucleic acid molecules, are designed to signal the break site interest. These synthetic sgRNA molecules recruit the Cas9 protein to direct the appropriate cleavage and subsequent correction. The flexibility to create unique sgRNA sequences targeting any gene is a key advantage of this technique [23]. For pediatric EB, target genes include COL7A1, LAMB3, and COL17A1. Mutations in COL7A1 and COL17A1 disrupt collagen production, affecting the DEJ, while LAMB3 mutations impact laminin, a basement membrane protein [24]. Designing corresponding sgRNA sequences allows for precise management of these genes.

Once the target genes are cleaved, DNA repair mechanisms either repair the genetic material without defects or incorporate new genetic material. The two repair pathways are NHEJ and HDR. NHEJ directly ligates DNA disruptions without a template, often leading to genetic unpredictability due to new insertions and deletions [25]. In contrast, HDR uses complementary template strands of DNA, allowing for more seamless and accurate repair [26]. However, the effectiveness of

these repair mechanisms depends on their timing within the natural cell cycle.

Despite the transformative potential of CRISPR-Cas9, concerns about precision and off-target effects remain. High frequencies of off-target mutations and instability in functional genes have been reported, with unintended cleavage occurring in sequences slightly mismatched to sgRNA templates. Mitigating these effects require preliminary screening for potential off-target sites [6]. Although some genetic screening methods are complex and costly, they are essential to minimize undesirable outcomes. Validation techniques to ensure gene editing accuracy include Western blotting of cell lysate products, immunohistochemical methods, and flow cytometry. Considering the influence of the cell cycle, methylation, and chromatin structure will enhance future validation methods [27]. While opportunities for refinement exist, CRISPR-Cas9 has undeniably revolutionized genome editing.

Applications in EB

The potential applications of CRISPR-Cas9 in treating genetic disorders caused by single gene mutations are vast and transformative. In the context of EB, CRISPR-Cas9 can correct pathogenic mutations directly in patient-derived cells. For instance, in DEB, CRISPR-Cas9 can target and correct mutations in the COL7A1 gene, potentially restoring the production of functional type VII collagen and alleviating disease symptoms [28]. Preclinical studies have shown promising results in correcting these mutations in induced pluripotent stem cells (iPSCs) derived from patients with EB, demonstrating restored expression of the corrected genes and improved cellular function [28]. These corrected iPSCs can differentiate into keratinocytes to regenerate healthy skin tissue.

Gene replacement therapies use viral vectors to deliver corrective genes, while gene editing therapies use nucleases like CRISPR-Cas9 to target specific genetic mutations. These therapies can be administered either ex vivo or in vivo. Ex vivo gene therapy involves isolating the patient's skin cells via biopsies, treating these cells in vitro to express the corrected protein, and then creating autologous skin grafts from these corrected cells, which are transplanted back onto the patient. In contrast, in vivo gene therapy directly treats the skin locally. Most gene therapies for EB have been designed as ex vivo due to the shortage of efficient and safe carriers for gene delivery. However, recent breakthroughs have shown promise for in vivo gene therapy, including beremagene geperpavec (B-VEC), a gene replacement therapy recently approved by the FDA, and gene editing approaches [1].

As gene replacement therapies for EB heavily rely on viral vector-based gene delivery, there are inherent risks associated such as random integration and potential insertional mutagenesis. These risks were underscored by previous studies where leukemia development was reported following retrovirus-based therapies for severe combined immunodeficiency and chronic granulomatous disease, despite no carcinogenesis being observed in EB gene replacement strategies to date [29]. This historical context emphasizes the need for caution and rigorous monitoring. Consequently, the FDA recommends a 15-year follow-up period for patients undergoing viral vector gene replacement therapies to ensure long-term safety and monitor any delayed adverse effects [30].

Ex Vivo Gene Replacement

LAMB3

In a phase I/II clinical trial involving a 36-year-old JEB patient, patient keratinocytes were transduced with a retroviral vector expressing full-length LAMB3 cDNA, and these corrected keratinocytes were transplanted onto the patient. This resulted in improved skin integrity and normal laminin-332 expression four months post-transplantation [31]. A follow-up 6.5 years later showed stable skin with normal laminin-332 expression levels and no adverse events [1,32]. More recently, a 7-year-old boy with a LAMB3 mutation, who suffered from an infection leading to 80% epidermal loss, was successfully treated with the same approach. Five years later, normal laminin-332 expression and restoration of Langerhan cells, Merkle cells, sebaceous glands, and sweat glands were observed [33,34]. A phase II/III clinical trial is ongoing to explore the safety and efficacy of this treatment [35].

COL7A1

A phase I clinical trial used a similar retroviral vector strategy for DEB patients [36]. A majority of wounds treated in the trial were present for more than five years, and three months post-transplant, 21 of the 24 graft sites showed wound healing. Moreover, 90% of the biopsied graft sites showed collagen VII expression, though within the first year, collagen VII expression declined from 90% to 42%. A later phase clinical trial with seven DEB patients treated with the same strategy reported improved wound healing over two years, though collagen VII expression declined similarly over time [37]. A five-year follow-up confirmed the treatment's safety and improved wound healing, though effectiveness waned over time, with 93% of treated areas showing 50% or more wound healing after six months, decreasing to 70% after five years [38]. A phase III clinical trial evaluating the safety and efficacy of COL7A1 autologous grafts has been completed, with results pending [39]. Another phase III trial is actively recruiting and expected to be completed in 2025 [40]. Overall, the ex vivo gene therapy approach in DEB has been less satisfactory compared to JEB, highlighting the complexity of EB subtypes and its impact on therapeutic success.

In Vivo Gene Replacement

While ex vivo gene replacement therapies offer benefits, their results indicate that repeated application may be necessary for significant clinical benefits. Additionally, these therapeutics require hospitalization, anesthesia, and invasive surgical procedures. In contrast, in vivo therapies offer reduced intervention burden. A 2022 study described the first clinical trial of topical gene therapy in children with EB [41]. The phase I/II study enrolled nine adult and pediatric patients with generalized recessive DEB and confirmed COL7A1 gene mutations. B-VEC, a replication-defective herpes simplex virus type 1 containing two copies of the COL7A1 coding sequence, was applied topically to wounds. All wounds, except one chronic five-year foot wound, achieved closure within three months, and biopsies showed positive linear deposition of full-length collagen VII [41]. This novel, accessible gene therapy demonstrated a potential for reversing genetic disease through repeated topical applications, eliminating the need for long-distance travel, patient biopsies, anesthesia, or hospitalization. Despite its potential, B-VEC therapy has notable limitations. Its inability to penetrate intact skin restricts its application to recessive DEB wounds only. Moreover, B-VEC does not provide a permanent correction, requiring repeated applications

to maintain efficacy [42]. The financial aspect also poses a significant challenge; Krystal Biotech projects an annual treatment cost of $631,000 per patient, rendering it impractical for many individuals [43].

Gene Editing

While gene editing therapies for EB, such as CRISPR-Cas9, remain in the preclinical stage, their potential is underscored by their successful application in clinical trials for other diseases. As of July 2024, nearly 60 clinical trials utilizing CRISPR-Cas9, primarily for hemoglobinopathies, are listed on the clinicaltrials.gov (accessed on 13 July 2024) database [44]. In a 2020 study, patients with sickle cell disease and beta-thalassemia were treated with CRISPR-Cas9 edited CD34+ hematopoietic stem and progenitor cells, genetically modified to reactivate fetal hemoglobin production [45]. More than a year after therapy, these patients exhibited sustained increases in fetal hemoglobin, achieved transfusion independence, and, in the case of the sickle cell patient, elimination of vaso-occlusive episodes.

Gene editing technologies offer a promising safety profile by specifically targeting the affected gene and enabling permanent repair without relying on viral vectors. This targeted approach significantly reduces the risk of random genetic integration and associated complications. The aforementioned 2020 study demonstrated no off-target editing using CRISPR-Cas9, further validating the precision and reliability of this technology [44]. Such precision is crucial for the safe and effective treatment of genetic disorders, making CRISPR-Cas9 an attractive option for future clinical applications in EB and beyond.

In preclinical studies, CRISPR-Cas9 has shown considerable promise for treating EB. For example, in a 2019 study, researchers employed CRISPR-Cas9 to delete faulty COL7A1 exons in patient-derived skin cells, successfully correcting the mutation in approximately 85% of DEB keratinocytes [46]. The restored type VII collagen exhibited resistance to blister formation, and no significant off-target effects were observed. This level of precision is vital for ensuring the safety and efficacy of gene editing therapies, particularly in conditions as delicate as EB. The ability to correct mutations at a high rate without introducing unintended genetic changes represents a significant milestone in the development of CRISPR-Cas9 based treatments for EB and other genetic disorders. The advancement of CRISPR-Cas9 in both preclinical and clinical settings underscores its transformative potential in treating genetic disorders. While gene replacement therapies have made significant strides, the precision and reduced risk profile of gene editing technologies like CRISPR-Cas9 offer a promising future for more effective and safer treatments.

Delivery Mechanisms for CRISPR-Cas9

The success of CRISPR-Cas9 therapy hinges on developing efficient delivery systems capable of accurately targeting affected tissues. Current research explores various delivery methods, including viral vectors such as adeno-associated viruses (AAVs) and non-viral approaches like lipid nanoparticles, to enhance the efficiency and safety of gene editing in clinical settings [7]. Additionally, combining CRISPR-Cas9 with other emerging technologies, such as base editing and prime editing, holds potential to further refine precision and reduce off-target effects of gene editing therapies [8]. These advancements offer significant potential for improving treatment outcomes and clinical efficacy for patients with EB.

Viral Vectors

Viral vectors, including lentiviruses and AAVs, are commonly used to deliver CRISPR-Cas9 components into target cells due to their high in vivo transfection efficiency and sustained gene expression [47]. Lentiviruses integrate into the host genome, ensuring long-term expression of the delivered gene, which is particularly useful for chronic conditions like EB where continuous expression of the corrected gene is necessary for maintaining skin integrity [48]. A study by Woodley et al. demonstrated that a single lentiviral vector injection could provide stable type VII collagen at the basement membrane zone for at least three months, highlighting the potential of engineered lentiviral vectors in treating DEB [49].

In comparison, AAVs are known for their lower immunogenicity and ability to transduce both dividing and non-dividing cells, making them suitable for targeting various cell types involved in skin regeneration [50]. Petek et al. found that an AAV gene-targeting vector corrected a gene in EBS, resulting in a fully functional epidermis for 20 weeks post-grafting onto severe combined immunodeficiency disease mice [51]. Both lentiviruses and AAVs demonstrate high efficiency in delivering CRISPR-Cas9 components to skin cells, with numerous preclinical studies showing successful mutation correction in keratinocytes and fibroblasts [52]. The sustained expression achieved by these vectors ensures that therapeutic effects are maintained over time, reducing the frequency of treatments.

However, the use of viral vectors is not without risks. Lentiviruses, while offering high cloning capacity and less immunogenicity, can lead to insertional mutagenesis and random integration into the host genome. Advances in engineering non-integrating lentivirus vectors (NILVs) aim to mitigate these drawbacks [47]. Similarly, while AAVs offer advantages such as minimal integration into the host genome and versatility in cell targeting, they face limitations in viral packing capacity and potential immunogenicity of the viral capsid. Recent advancements include delivering sgRNA and Cas9 separately in a dual-delivery method and utilizing smaller Cas9 and alternative Cas effectors [47].

Non-Viral Approaches

Non-viral delivery methods, such as nanoparticle-based systems, offer a promising alternative to viral vectors for CRISPR-Cas9 delivery. These systems are engineered to encapsulate CRISPR-Cas9 components and deliver them directly to target cells. Nanoparticles can be designed to release their payload in a controlled manner, enhancing the precision of gene editing. Modifications to the surface of nanoparticles can improve targeting and uptake by specific cell types, making them a versatile tool for gene therapy [53].

Unlike viral vectors, nanoparticles do not integrate into the host genome, eliminating the risk of insertional mutagenesis. Additionally, nanoparticles are less likely to elicit strong immune responses, making them suitable for repeated administrations, which may be necessary for chronic conditions like EB [54]. Despite these advantages, nanoparticle-based systems face challenges related to delivery efficiency and stability. Efficient uptake and expression of CRISPR-Cas9 components in target cells can be difficult due to physiological

barriers in the skin. Ensuring the stability of nanoparticles in the biological environment is also a concern, as premature degradation can reduce treatment effectiveness. A review by Xu et al. identifies other obstacles, including the encapsulation of large CRISPR systems, refining targeted delivery, and enhancing endocytosis [55]. Ongoing research focuses on optimizing nanoparticle design and formulation to overcome these challenges and improve therapeutic potential.

Both viral and non-viral delivery mechanisms hold promise for applying CRISPR-Cas9 in treating pediatric EB. The choice of delivery method depends on balancing the efficiency of gene delivery and expression with the safety profile. While viral vectors offer high efficiency and sustained expression, non-viral alternatives provide a safer option with reduced immunogenicity, although they require further optimization to enhance delivery efficiency and stability. As research progresses, these advancements will likely lead to more effective and safer gene therapies for patients suffering from EB and similar genetic conditions.

Challenges and Future Directions

Safety and Efficacy

The application of CRISPR-Cas9 technology in pediatric populations necessitates a nuanced exploration of ethical and regulatory considerations, given the technology's transformative potential and associated risks. A primary concern is the potential for off-target effects and unintended consequences, which are especially significant in children due to their developing physiology and immune systems. The safety and efficacy of CRISPR-Cas9 therapies for pediatric patients demand rigorous preclinical and clinical testing, with a strong emphasis on long-term follow-up to monitor for delayed adverse effects [45]. This comprehensive evaluation is crucial to ensure that potential risks are identified and managed effectively before these therapies become widely available.

The durability of CRISPR-Cas9 gene editing in pediatric EB hinges on the stability of gene correction over time. Early preclinical and clinical studies have shown promising results, suggesting that CRISPR-Cas9 can achieve sustained expression of corrected genes, leading to long-term phenotypic correction in treated cells [33,34,41,45]. Animal models have demonstrated that gene correction can restore normal skin architecture and function, with benefits lasting several months post-treatment [56]. Histological analysis in Bonafont et al.'s study on DEB revealed that grafts from both healthy and gene-edited keratinocytes exhibited normal skin architecture 12 weeks post-transplantation. This indicates that the gene-editing process did not disrupt the overall structure and integrity of the skin, maintaining a normal histological appearance similar to healthy controls.

Conversely, grafts from untreated keratinocytes displayed blisters, highlighting the severity of the disease phenotype without gene correction. Immunohistochemical analysis showed no detectable type VII collagen in regenerated tissue from untreated keratinocytes, whereas treated keratinocytes exhibited continuous type VII collagen deposition along the basement membrane zone, similar to healthy donor keratinocytes. This suggests that gene editing can normalize skin function and resilience in patients with EB, which is essential for managing chronic conditions like pediatric EB, where continuous skin integrity repair is necessary.

A significant challenge is the potential reversion to mutant alleles, which can negate therapeutic effects. Strategies such as HDR are being explored to ensure precise and stable integration of the corrected gene. Zhang et al. improved HDR efficiency by employing a double cut donor vector design, significantly increasing HDR by two to five fold compared to a circular donor plasmid [57]. Minimizing the replaced sequence surrounding the double-strand break can further enhance HDR and suppress non-homologous end joining-mediated insertion, increasing the precision of gene editing. To address potential reversion and enhance durability, researchers are investigating combination therapies and supportive treatments. Combining CRISPR-Cas9 gene editing with protein or small molecule therapies may stabilize the corrected gene and its expression. Small molecule inhibitors can significantly increase the precision and efficiency of CRISPR-Cas9 gene editing by optimizing the DNA repair process, suppressing NHEJ, and promoting HDR, leading to more accurate gene edits and reducing off-target effects [58]. Continuous monitoring and follow-up studies are essential to evaluate the long-term efficacy of these approaches, providing critical insights into the longevity and sustainability of CRISPR-Cas9-mediated gene correction in pediatric EB patients.

Monitoring for Off-target Effects

Despite improvements in screening techniques, unintended genomic alterations remain a concern, which could have adverse consequences [6]. Effective delivery of CRISPR-Cas9 components to target tissues, such as skin, is another major hurdle. In vivo delivery systems must overcome barriers related to vector penetration and targeting efficiency, complicating the effective application of the technology [41]. Developing and applying these therapies require advanced validation methods, such as whole-genome sequencing and targeted deep sequencing, to ensure accuracy and safety [59].

Ensuring the safety of CRISPR-Cas9 gene editing involves comprehensive monitoring for off-target effects, which are unintended genomic alterations that could have serious consequences. Advanced techniques such as whole-genome sequencing (WGS) and targeted deep sequencing are employed to detect and quantify these off-target edits, providing a detailed understanding of the genomic changes induced by CRISPR-Cas9. In a mini-review by Guo et al., advanced sequencing techniques like WGS are highlighted as essential tools for detecting both desired and unwanted editing events by comparing genome sequences before and after editing [59]. Alternative techniques such as CIRCLE-seq, GUIDE-seq, and LAM-HTGTS can detect genome-wide off-target sites without the expense of full WGS by integrating DNA fragments into double-strand breaks created by Cas9 to identify off-target cleavage sites [59]. These techniques are crucial for the comprehensive detection and quantification of off-target effects, ensuring the safe application of CRISPR-Cas9 in gene editing technologies for treating pediatric EB.

Bioinformatics tools are also used to predict potential off-target sites based on the specific gRNA sequences used in the gene editing process. Predictive models help design gRNAs with higher specificity, reducing the likelihood of off-target effects. Research emphasizes the importance of optimizing gRNA sequences through iterative design and testing using both in vitro and in vivo models to enhance on-target activity while minimizing off-target interactions [60]. This optimization is critical as these technologies advance toward clinical use,

ensuring both efficacy and safety in therapeutic applications. Continuous optimization of gRNA design and developing modified Cas9 proteins with enhanced specificity are crucial steps in minimizing risks. Combining such technologies with robust monitoring strategies is essential for ensuring the genomic integrity of treated cells, ultimately enhancing the safety profile of CRISPR-Cas9 for pediatric EB.

Immunogenicity Concerns

The immunogenicity of CRISPR-Cas9 components, particularly the bacterial-derived Cas9 protein, poses a significant challenge in gene editing therapies. The human immune system may recognize Cas9 as a foreign antigen, potentially triggering an immune response that could compromise the efficacy and safety of the treatment. This immune reaction could lead to the clearance of edited cells and reduce the therapeutic benefits of CRISPR-Cas9 gene editing. Sun et al. highlighted this concern, citing research indicating that the host immune system can mount adverse responses against the CRISPR-Cas9 components and the gene-edited cells, diminishing therapeutic effectiveness and, in severe cases, potentially leading to fatal outcomes [61]. Their evidence includes findings that a significant presence of T cells reactive to *Streptococcus pyogenes* Cas9 in the adult human population could impede the therapeutic use of CRISPR-Cas9 by triggering immune responses that limit its efficacy [61]. These findings raise significant considerations for pediatric applications. The developing immune systems of children may respond even more vigorously to the bacterial-derived Cas9 protein, potentially exacerbating the risks observed in adult populations [27]. To mitigate this risk, researchers are exploring the use of humanized Cas9 variants designed to be less recognizable by the human immune system. These variants aim to reduce immunogenicity while maintaining the gene-editing efficiency of the Cas9 protein. Further research is needed to fully understand the immunogenic potential of CRISPR-Cas9 and develop effective strategies for its mitigation. Addressing these concerns is critical to ensuring the safety and efficacy of gene-editing therapies for pediatric EB patients, paving the way for broader clinical applications of this groundbreaking technology.

Future Research

The future of CRISPR-Cas9 gene editing in the treatment of EB hinges on optimizing delivery methods and conducting large-scale clinical trials to establish its safety and efficacy. Enhancing the efficiency and targeting accuracy of CRISPR-Cas9 delivery systems is critical. Current methods, including viral vectors and nanoparticle-based approaches, require further refinement to improve their specificity for target cells and minimize off-target effects. Advances in the engineering of guide RNAs with higher fidelity and the development of new Cas9 variants with reduced off-target activity are pivotal in this regard [6]. Additionally, innovative delivery techniques such as electroporation and microinjection can significantly enhance the precision and efficiency of delivering CRISPR-Cas9 components to target cells [7].

Novel delivery systems, such as lipid nanoparticles and polymer-based methods, offer safer and more efficient alternatives to viral vectors. These systems can be engineered to enhance cellular uptake, stability, and targeted delivery of CRISPR components. Furthermore, research into biomimetic delivery vehicles, including exosomes and cell-penetrating peptides, holds promise for improving the intracellular delivery of CRISPR-Cas9 complexes. These advancements could reduce immunogenicity and increase the precision of gene editing, thereby improving clinical outcomes for patients with EB [8].

The potential for combining CRISPR-Cas9 with other therapeutic strategies holds great promise for enhancing treatment outcomes, particularly for conditions like EB. Integrating CRISPR-Cas9 with traditional gene replacement therapies could provide a synergistic effect, addressing both the genomic and phenotypic aspects of the disease. For instance, while CRISPR-Cas9 can correct specific genetic mutations, gene replacement therapies can supply functional proteins or other supportive treatments, offering a more comprehensive approach to managing EB [1]. Additionally, combining CRISPR-Cas9 with other modalities, such as topical therapies or regenerative medicine, may improve treatment efficacy by targeting multiple aspects of the disease [1,41]. This holistic approach could address various facets of skin repair and integrity, enhancing overall therapeutic outcomes. Moreover, CRISPR-Cas9 could be used in conjunction with other advanced therapeutic strategies to address multiple mutations simultaneously. This could be particularly beneficial for patients with complex forms of EB, enabling a more integrated treatment approach. By targeting various genetic defects concurrently, combination therapies could potentially reduce the need for multiple interventions and improve patient outcomes.

Large-scale, multi-center, and long-term clinical trials are essential for translating CRISPR-Cas9 gene editing from the laboratory to the clinic. Such trials are necessary to assess the safety, efficacy, and durability of gene-editing therapies in diverse patient populations. Multi-center studies ensure that findings are generalizable and applicable across different demographics and clinical settings [41]. Long-term follow-up is crucial to monitor the persistence of therapeutic benefits and identify any late-onset adverse effects, ensuring that gene-editing therapies can be safely and effectively integrated into clinical practice. Standardizing clinical trial protocols is imperative for the successful development and implementation of CRISPR-Cas9 therapies. Establishing standardized procedures for patient selection, treatment administration, and outcome assessment ensures consistency and comparability across different studies. Protocols should include rigorous criteria for evaluating the safety and efficacy of gene-editing interventions and guidelines for managing potential adverse events [41]. Moreover, standardized protocols facilitate collaboration between research centers and regulatory bodies, accelerating the translation of promising gene-editing technologies into approved therapies. Developing comprehensive, standardized clinical guidelines will be instrumental in advancing the field of gene therapy for EB and other genetic disorders.

Conclusion

The implementation of CRISPR-Cas9 gene editing technology represents a transformative advancement in the treatment of EB, especially for pediatric patients. By targeting and correcting genetic defects at their source, CRISPR-Cas9 holds the promise of shifting from palliative care to potentially curative therapies. Early clinical studies and preclinical models have shown that CRISPR-Cas9 can precisely and efficiently correct pathogenic mutations, restoring skin integrity and function in EB patients. However, challenges such as off-target effects and immunogenicity must be addressed through continuous

advancements in delivery mechanisms, gRNA design, and the development of humanized Cas9 variants.

The future success of CRISPR-Cas9 in treating EB relies on optimizing delivery methods, conducting large-scale clinical trials, and standardizing clinical protocols. Ethical and regulatory considerations are paramount, particularly in pediatric populations, requiring meticulous attention to informed consent, equitable access, and the complexities of gene editing. Despite these challenges, CRISPR-Cas9 technology offers a beacon of hope for EB patients and their families. With further research and development, CRISPR-Cas9 could fundamentally change the treatment landscape for EB, moving from merely managing symptoms to potentially offering a cure for this debilitating condition.

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