RNAseq and prime editing - A path toward human genome surgery

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ABSTRACT

The development of next gene sequencing (NSG) technology and prime editing (PE) techniques has made it possible to perform human genome surgery. NGS has revolutionized the development of personalised medicine through rapid DNA and RNA sequencing. To detect genetic disorders and diseases, RNAseq is the basic technique needed for analysing the expressed genes in an individual. Machine learning models analyse RNAseq data to diagnose disease from the reference genome. Sequences provide information about mutations in the expressed genome. Gene editing has been dramatically advanced by the discovery of clustered regularly interspaced short palindromic repeats (CRISPR) and the CRISPR-associated protein 9 (Cas9). It is possible to edit genomes very precisely using the PE tool that has a PE protein and a PE guide RNA (pegRNA). All type of genome surgery/editing is possible with PE and it is therefore a one-size-fits-all solution to many genetic diseases and disorders. Several mutations were corrected using PE, and the tool is evolving rapidly to meet the needs. As molecular therapies advance, PE will ultimately become a cure for most genetic and epigenetic disorders that cannot be treated effectively with chemotherapy.

Keywords - Prime editing, RNAseq, pegRNA, CRISPR Cas 9, Machine learning algorithm, dual AAV, M-MLV RT, sgRNA, genetic disease, cancer therapy, delivery system

I. INTRODUCTION

Gene therapy or personalized medicine is still in its infancy, which requires biotechnology and medicine integration to progress. Personalised medicine (genomic medicine) initially focused on diseases caused by highly penetrant alleles. It has been demonstrated that genomic medicine can assist with diseases such as phenylketonuria, fragile X syndrome, thalassaemia major or homozygous familial hypercholesterolemia. Genomic testing to screen newborns and adults will identify patients with high healthcare costs who are at high risk of acute, critical, potentially curable, life-threatening disorders [1]. Currently, personalised medicine focuses on designing drugs for individuals to avoid adverse drug reactions and boost efficiency [2]. Genomics has recently become a part of medicine with the advancement in whole genome sequencing (WGS) both in efficiency and short duration. Based on the genome of an individual, personalised medicine can be designed using genomic techniques. Genome analysis has become feasible and applicable as a result of advances in genomics technology. RNAseq is the exome/ transcriptome sequencing technique that help in diagnosis of disease. Recent studies have shown that CRISPR and Cas9, components of the natural defense system of bacteria, make PE more efficient than base editing and homology-directed repair in molecular therapy [3,4]. The purpose of this chapter is to discuss RNAseq, PE and their application to human genetic disease therapy.

II. RNA SEQUENCING

A. RNAseq

The RNAseq technique sequences expressed genes and requires isolation of RNA from individuals, creation of cDNA from isolated RNA, and sequencing of the cDNA using various techniques like single molecule sequencing, single molecule fluorescent sequencing, nanopore sequencing and slide sequencing (Fig. 1). Data processing and storage of huge amounts of data must be addressed simultaneously. Integration of information technology, bioinformatics and molecular technologies will solve issues in sequencing, data processing and data storage. WGS identifies genetic variations in DNA samples derived from individuals; however, RNAseq can also identify genetic variations caused by splicing during transcription that are not detected by WGS. Single cell RNAseq (scRNAseq) sequences the transcriptome of cells of same type isolated by flow cytometry to study genes

expressed in cells of same type isolated from a heterogeneous population of cells. ScRNAseq helps to understand the cell-based model to understand the changes in cancer cells so that new therapeutic targets can be discovered [5].

Researchers from Massachusetts General Hospital and the Broad Institute collected RNA from 63 patients exhibiting potential monogenic muscle disorders. They conducted RNA-seq analysis on 184 control samples and identified 13 patients with known disease-causing genetic variants [6]. This study revealed a recurring *de novo* intronic mutation with a high frequency within the COL6A1 gene, which was associated with a dominantly acting splice-gain event and 27 patients with collagen VI-like dystrophy [7].

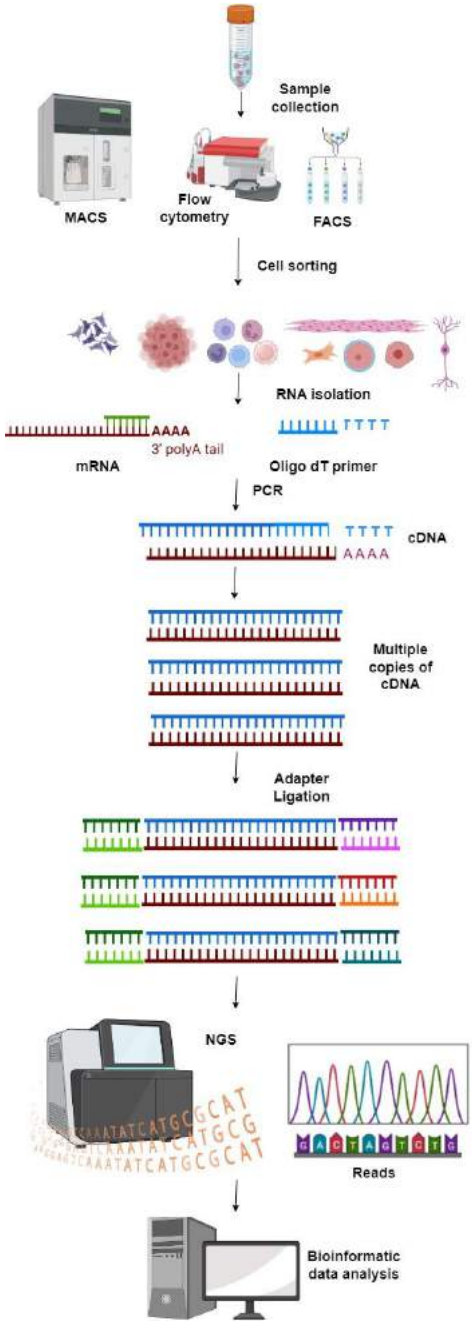

Figure 1: Single cell RNAseq

B. Machine learning analysis of disease diagnosis using RNAseq data

To diagnose and predict diseases, such as cancer, a pivotal process involves the deposition of RNAseq reads from patients into the NCBI database. Subsequently, these reads are meticulously compared against the reference human genome. Here, the prowess of machine learning algorithms (ML) comes into play, showcasing an impressive capability to predict and diagnose diseases with an accuracy surpassing 95%.

Within machine learning, a repertoire of canonical algorithms assumes significance. These encompass familiar names like k-Nearest Neighbour (kNN), Logistic Model Tree (LMT), Random Tree (RT), Random Committee (RC), Classification and Regression Trees (CART), Support Vector Machine (SVM), Bagging SVM (bagSVM), Poisson Linear Discriminant Analysis (PLDA), and the esteemed Random Forest (RF). Additionally, the domain of Deep Learning (DL) introduces classifiers like the 1-D Convolutional Neural Network (1-D CNN), Long Short-Term Memory (LSTM), and Bidirectional LSTM (BiLSTM) as potent tools. Machine learning unfolds in a systematic workflow encompassing preprocessing of data, extraction of features, model learning, and the eventual evaluation of the model, as illustrated in Figure 2.

Figure 2: Pipeline of Machine Learning Algorithm

Drawing insights from a study involving RNAseq data extracted from colon cancer patients and standard human RNAseq data from the NCBI, noteworthy findings emerged. In the context of cancer prediction accuracy, the canonical ML algorithms, namely RC, LMT, and RF, exhibited an impressive performance of 97.33%. Similarly, RF stood out in cancer stage classification, again registering an accuracy of 97.33%. Delving deeper, a distinct standout was observed with applying the 1-D CNN classifier, achieving an exceptional cancer prediction accuracy of 97.67%. Expanding this perspective to cancer stage classification, the BiLSTM classifier showcased unparalleled performance, achieving a remarkable accuracy of 98% [8].

III. PRIME EDITING

A. Prime editing tools

The PE process requires a Prime editing fusion protein (PEFP) called nCas9-RT that combines CRISPR-nCas9 with a reverse transcriptase (RT) and an RNA called pegRNA (Fig.3).

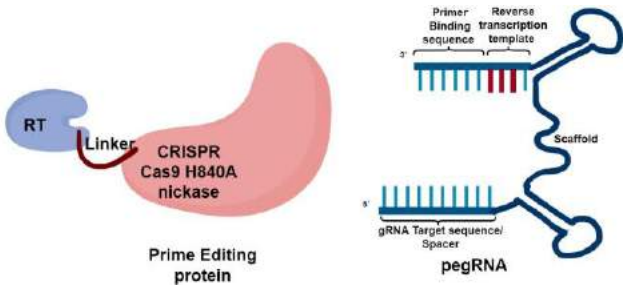

Figure 3: Prime editing tools

CRISPR nCas9 is an enzyme with cascade of protein that are originally present in bacteria as a defense sysytem and help to form single strand nick (SSN) . Reverse transcriptase (M-MLVRT) is an enzyme isolated from virus that cause Moloney murine leukemia which helps to convert the RNA into DNA. The pegRNA consist of a 3’ extension, a scaffold and a 5’spacer or gRNA target sequence [9]. The 3' extension is composed of a primer binding sequence and a reverse transcription template (RTT) containing edited RNA sequences. PegRNAs carry complementary sequences of the target DNA in their 5' spacer or gRNA sequence. PE mechanism is described in Fig.4.

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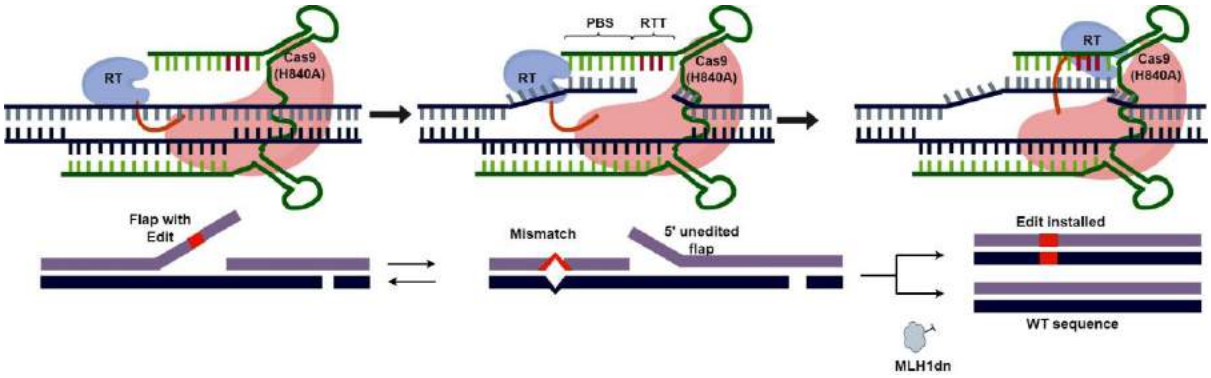

Figure 4: PE mechanism

B. Prime Editing systems

There are five types of PE systems: PE1: wild type M-MLV RT, PE-2: M-MLV pentamutant, PE3: PE2 coupled nicking sgRNA, PE4: PE2 coupled MLH1dn, and PE5: triplex complex of PE2 coupled nicking sgRNA and MLH1dn. The first PE system, PE1, was created by coupling Cas9 H840A nickase and wild-type M-MLV RT enzyme. The PE2 system comprises the PE2 enzyme, a fusion of *Streptococcus pyogenes* Cas9 [SpCas9] H840A nickase, and an engineered RT alongside a pegRNA. Within this system, the RT component of PEFP undergoes five specific mutations to enhance thermostability, processivity, and DNA-RNA substrate affinity, ultimately improving its overall functionality.

In the PE3 system framework, an additional layer is introduced. Here, a single guide RNA (sgRNA) is combined with a pegRNA to facilitate the nicking of the non-edited strand, thereby amplifying editing efficiency. This augmentation involves a standard gRNA, which guides the Cas9 H840A nickase element of PEFP to create a nick in the genomic DNA at a neighboring site, albeit on the opposing strand from the original incision.

The significance of MLH1 governs the DNA mismatch repair pathway. As a strategic measure, a genetically encoded MLH1 inhibitor, represented by the dominant negative mutant MLH1dn (truncated MLH1 D754–756, devoid of the endonuclease domain), is incorporated into the prime editor configuration, leading to the formation of PE4 (PE2 + MLH1dn) or PE5 (PE3 + MLH1dn+nicking sgRNA). This integration yields heightened editing efficiency and concurrently curtails indel formations across various cell types [10]. The engineered MLH1dn component elevates the precision and efficacy of PE by mitigating the occurrence of byproducts arising from mismatched nucleotides. A compelling capability arises from this system, enabling the execution of all 12 types of conversions, encompassing both transversions and transitions, alongside the addition and deletion of base pairs and indels, across the human genome via PE [11].

IV. MOLECULAR THERAPY OF HUMAN DISEASE

Human genome surgery is carried out by two strategies viz., *ex vivo* PE and *in vivo* PE. Ex vivo PE involves reprogramming isolated cells from patients in the laboratory using PE tools and transplanting the edited/reprogrammed cells into patients. In contrast, *in vivo* PE involves injecting the PE tool directly into the patient. PE system efficiency can be evaluated by Western blotting of expressed protein, and off target editing can be assessed by NGS analysis of genome (Fig. 5).

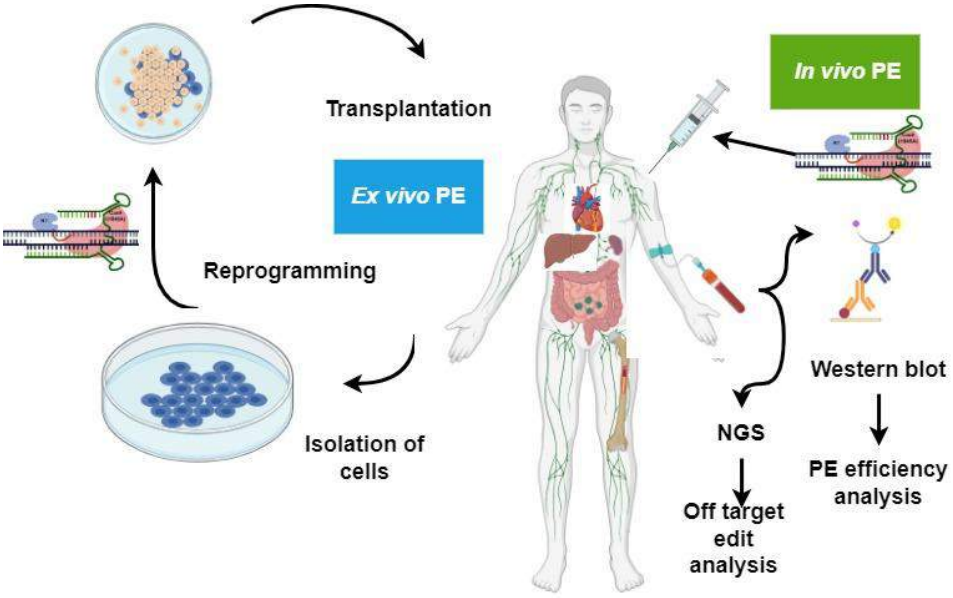

Fig 5. Strategies of human genome surgery

A. Hepato-genetic disease

Diacylglycerol-acyltransferase (DGAT1) deficiency leads to lipotoxicity due to the incapability to form lipid droplets. DGAT1 mutations cause congenital diarrhoea and protein loss enteropathy upon lipid ingestion. By using intestinal tissue from a DGAT1 deficient patient, a 3D organoid was grown and PE3 was used to correct the mutation with high editing rates and low level of byproducts in 3D- organoids. The ABCB11D482G mutation, which is a frequent cause of bile salt export pump (BSEP) deficiency, and in-frame deletions causing heterozygous mutation in CTNNB1, which are a cause of Wilson disease, were also prime edited in liver [12].

A new protein, PE2, (NLS-optimized SpCas9-based prime editor), enabled editing of somatic genome in the liver of adult mice in order to correct alleles causing pathogenic disease and to enable the creation of cancer models by creating mutations that will drive the growth of tumours. SERPINA1 gene, a serpin family A member, contains a PiZZ homozygous mutation (1024 G > A, E342K), resulting in deficiency of 1-antitrypsin. Patient-derived induced pluripotent stem cells (iPSCs) were prime edited with PE3 and found to be less efficient due to poor delivery [13].

In patients with phenylketonuria (PKU), phenylalanine amino acid accumulates in the body due to PAH gene mutation, which produces the enzyme to break down phenylalanine. The correction of phenylalanine levels in neonates with the mutation Pahenu2 was achieved with a correction efficiency of 11.1% (up to 17.4%) without inducing off-target mutations or prolonging liver inflammation using PE2 [14].

PE was performed *in vitro* on a HT1 mouse model with hereditary tyrosinemia type 1 mutation implanted with chemical-derived hepatic progenitors in order to correct the mutation. The reprogrammed hepatocytes were then transplanted into the same mouse to increase its survival. Cells that have been prime edited *in vitro* can be transplanted to cure liver disease [15]. PE2 and PE3 were delivered by hydrodynamic injection in mice with the genetic liver disease hereditary tyrosinemia. PE precisely corrected the disease-causing mutations and led to the amelioration of the disease symptoms without detectable off-target edits [16].

B. Ophthalmic genetic disease

Leber congenital amaurosis (LCA), an inherited retinal disease, has found a promising avenue for correction through the optimized dual-adeno-associated virus (AAV) split-intein system (AAV-Split PE3). Remarkably, this system has demonstrated an efficiency of up to 16% in precisely addressing the condition. Notably, this correction has not been achieved without any detectable off-target edits. This therapeutic intervention has yielded the restoration of RPE65 gene expression, leading to retinal function reestablishing and preserving photoreceptors within rd12 mice [17]. Furthermore, in a separate study, introducing PE2 via an AAV vector ameliorated disease symptoms in mice afflicted with LCA. The notable achievement in this endeavor was the absence of detectable off-target edits, further enhancing the potential for precise and effective gene correction [16].

Expanding the spectrum of applications, a cataract disorder in mice was generated through the microinjection of PE3 plasmids. Impressively, this approach yielded a high G-deletion rate of 38%, giving rise to a disease model mouse presenting with nuclear cataracts. This distinctive strategy showcases the versatility of PE in creating accurate disease models for research purposes [18].

The severity of Retinitis Pigmentosa is epitomized by Retinitis Pigmentosa, which is attributed to a 4-bp deletion mutation within the RPGR ORF15. This mutation, identified through Sanger sequencing, happens to be located in the same genomic region with missing X chromosome exome sequencing coverage. To address this, cells harboring the identified mutation were procured from the patient. After establishing a cell line, PE was employed to rectify the mutation. This approach offers a promising strategy for correcting this challenging genetic disorder [19].

C. Dermal genetic disease

Recessive dystrophic epidermolysis bullosa (RDEB) is caused by mutations in the COL7A1 gene that cause loss-of-function due to point mutations and insertion/deletion mutations. A PE procedure was performed on fibroblasts from two patients to correct the mutations in COL7A1. When these PE-corrected RDEB fibroblasts were transplanted to the skin of immunodeficient mice, C7 deposition and anchoring of fibril formation were observed. This gene editing by *ex vivo* strategy can treat RDEB [20]. Oculocutaneous albinism was created in zebrafish by creating the mutation P302L C>T by PE3 system to create a model organism [21].

D. Cardiac genetic disease

The genetic defect causing Duchenne muscular dystrophy (DMD) (c.8713C>T) prevents dystrophin protein from being produced under the sarcolemma. The mutation in the DMD gene was corrected using PE, and the effectiveness of different variations of the RTT sequence was tested. Simultaneous modifications have been introduced into the target sequence, including an alteration in the PAM sequence at position +6 and a silent mutation at position +9. A significant breakthrough was achieved through meticulous exploration of editing efficiency across diverse nucleotides, distance from the target, potential nicking sites, and additional mutations. Specifically, this effort resulted in a remarkable 22% modification rate within myoblasts derived from a DMD patient. This alteration led to the restoration of dystrophin expression; a crucial milestone confirmed through western blot analysis conducted on the formed myotubes [22].

DMD can also stem from frameshift mutations within the DMD gene, which encompass exon 52 deletions or deletions spanning exons 45 to 52. An ingenious PE strategy was adeptly applied to HEK293T cells and human myoblasts to rectify such frameshift mutations. This intervention led to a specific substitution of GT nucleotides at the splice donor site of exon 53, achieving successful correction in up to 32% of HEK293T cells and 28% of patient-derived myoblasts. Splice donor site modifications for exon 51 and 53 resulted in exon skipping events. Consequently, exon 50 was connected to exon 53, and exon 44 was connected to exon 54, ultimately engendering dystrophin protein production and contributing to functional protein restoration [23].

D. Spinal muscular genetic disease

A mutations in the survival motor neuron 1 (SMN 1) gene causes spinal muscular atrophy (SMA) , which leads to infant mortality due to recessive gene of the motor neurons in autosomal chromosomes. Even though SMA patients do not have SMN1, they do have SMN2, which is nearly identical to SMN1. Since SMN2 is alternatively spliced, it cannot prevent development of SMA as most SMN2 transcripts lack exon 7 due to the silencer for intronic splicing -N1. This results in an unstable truncated protein. PE-mediated targeted deletion of silencer for intronic splicing -N1 in SMA patient-specific induced pluripotent stem cells showed a 7/24 efficiency rate. There was a successful restoration of full-length-SMN in the targeted-deletion iPSCs clones and their derived motor neurons [24].

E. Neurogenerative genetic disease

Tay-Sachs disease (TSD) is caused by insertion of four nucleotide base (TATC) into Hexa gene at exon 11 (HEXA ins TATC). In order to create a novel animal model with TSD that accurately mimics HEXA in TATC, various PE systems were used and their efficiency was evaluated [25]. The PE of primary cortical neurons of mice

after mitosis was performed with wide range of efficiency in an attempt to correct a deletion in HEXA for the treatment of Tay-Sachs disease efficiently and to minimize the generation of byproducts [26].

F. Cystic fibrosis

Cystic fibrosis is caused by the phenylalanine deletion due to mutations in ΔF508 and R785 positions in the *CFTR* gene. A PE system was used to correct the cystic fibrosis CFTR-F508del mutation in an organoid. Cystic fibrosis CFTR-F508del mutation was functionally repaired by PE and WGS of prime edited organoids showed no off-target effects [27].

G. Hemato-genetic disease

The IVS-II-654 mutation (C > T) is the underlying cause of beta-thalassemia, inducing an anomalous 5′ donor site within intron 2 of beta-globin. This subsequently leads to irregular mRNA splicing. Employing the PE3 system, microinjection was performed on a mouse model harboring the human beta-thalassemia IVS-II-654 mutation (C > T) to rectify the mutation. The successful correction achieved an editing efficiency of 14.29% [28].

Sickle-cell disease (SCD) emerges due to a mutation within the haemoglobin subunit beta (HBB) gene, leading to an A·T-to-T·A change and consequent Glu 6 to Val substitution [29]. While allogenic hematopoietic stem cell transplantation offers a potential avenue for treating this disease, the scarcity of suitable donors and the specter of graft rejection are significant obstacles preventing the procedure's execution. With the introduction of PE, a ray of hope emerges as it showcases the ability to rectify the SCD allele (HBBS) in hematopoietic stem and progenitor cells (HSPCs) derived from SCD patients, achieving a frequency of 15%–41%. To validate this, prime-edited HSPCs were transplanted into immunodeficient mice, resulting in erythrocytes with diminished sickle haemoglobin content. These red blood cells exhibited adult haemoglobin derived from HBBA at levels ranging from 28% to 43% of the normal range and demonstrated enhanced tolerance to hypoxia-induced sickling [30].

Further advancements are witnessed as human cells were effectively edited to correct a transversion in the HBB gene with minimal byproducts [26]. Pioneering strategies have been devised for preclinical trials involving a CRISPR-Cas9 gene targeting approach utilizing high-fidelity Cas9 paired with chemically modified guide RNAs to facilitate recombinant adeno-associated virus serotype 6 (rAAV6)-mediated HBB gene correction. In the preclinical domain, the feasibility, effectiveness, and safety of HBB gene correction were successfully showcased in plerixafor-mobilized CD34+ cells obtained from healthy individuals and SCD patients with the gcHBB-SCD mutation. The journey towards clinical-scale gcHBB-SCD manufacturing yielded remarkable results, achieving up to 60% allelic correction rates. A 20% gene correction was achieved with multilineage engraftment after transplantation into immunodeficient NSG mice. In conclusion, the gcHBB-SCD drug product engrafted was safe, tumorigenic, and toxicology-free for long-term use. It did not exhibit abnormal hematopoiesis, genotoxicity, or tumorigenicity which led to a phase 1/2 clinical trial initiation in patients with SCD [31].

H. X-linked genetic diseases

The gene encoding interleukin-2 receptor gamma (IL2RG) is mutated in X-linked severe combined immunodeficiency (X-SCID). Employing PE, an *in vitro* model of this condition was meticulously crafted, utilizing K-562 cells and T cells derived from healthy donors harboring the c.458 T>C point mutation within the IL2RG gene. The methods employed to establish these model cells were subsequently harnessed to rectify the IL2RG c.458T>C mutation within patient T cells exhibiting the mutation alongside revertant somatic mosaicism. However, these endeavors yielded less than satisfactory outcomes due to the inherent limitations of proliferation of mutant cells *in vitro* and the intricate nature of somatic reversal processes [32].

I. Personalised oncology

CRISPR-engineered T cells were studied for their feasibility and safety in treating advanced-stage lung cancer, which showed very low off-target editing rates and no severe treatment-related adverse events, thus supporting its general safety for clinical use [33]. PD-1 gene-edited bulk autologous T cells were used to treat 12 patients with non-small-cell lung cancer, and the results supported both the feasibility and safety of gene editing for cell therapy [34]. Three patients with advanced cancer participated in a phase 1 clinical trial to assess the safety and feasibility of gene editing by CRISPR-Cas9. To improve antitumor immunity, CRISPR-Cas9 disrupted TRAC, TRBC, and PDCD1genes in T lymphocytes from patients. Incorporating NY-ESO-1 as a cancer-targeting

transgene served the purpose of tumor recognition. Engineered cells were administered to patients, and their reception was well-tolerated, as evidenced by sustained engraftment throughout the study [35].

A chimeric antigen receptor (CAR) T-cell cancer therapy involves the use of engineered receptors that are capable of recognizing antigens, signaling, and co-stimulating in order to reprogram T-cells in order to specifically target and eradicate cancer cells. Although CAR T-cell therapy has demonstrated remarkable efficacy in treating refractory B-cell cancers, the pursuit of optimal potency for other cancer types and substantial tumors remains ongoing. This endeavor has encountered challenges such as T-cell exhaustion, inadequate persistence of CAR T-cells, cytokine-associated toxicities, and hurdles in the manufacturing process. By integrating the capabilities of PE and synthetic biology, a promising avenue emerges to enhance CAR T-cell therapy for future clinical trials [36].

KRAS, a member of the RAS family harboring mutations, presents a complex molecular landscape that poses challenges for targeted cancer treatment. A groundbreaking advancement emerges as a universal pegRNA designed for PE, capable of addressing the diverse G12 and G13 oncogenic KRAS mutations. Demonstrating its potency, this universal pegRNA achieved successful correction across 12 distinct KRAS mutation types, encompassing a staggering 94% of the entire spectrum of known KRAS mutations. The correction rates reached 54.8% in HEK293T/17 cells. The pegRNA methodology was employed to rectify endogenous G13D mutations within human cancer cells in a significant stride. The G13D mutations were successfully reverted to wild-type KRAS sequences, attaining a 40% correction frequency devoid of indel mutations. This universal pegRNA-driven PE approach presents a prospective therapeutic avenue for addressing the spectrum of KRAS oncogene variants in a comprehensive 'one-to-many' manner [37].

In parallel, deploying a biobank housing 31 genetically distinct human pancreatic cancer (PDAC) lines, accurately reflecting the heterogeneity of primary pancreatic cancer tissue both molecularly and phenotypically, allowed for the exploration of gene-drug interactions. Utilizing CRISPR-Cas9 genome editing and drug screening, this biobank facilitated the identification of 26 compounds from a pool of 1,172 FDA-approved compounds that effectively targeted and eliminated PDAC organoids. This encompassed 19 chemotherapy drugs sanctioned for treating other cancer types, revealing a promising strategy for drug repurposing in PDAC treatment [38].

Furthering its utility, the PE tool was harnessed to rectify a TP53 missense C > T mutation (L194F) within a T47D luminal A breast cancer cell line. Additionally, it was employed to introduce the L194F mutation into HEK293T cells. Through rigorous assessment using Sanger sequencing on prime-edited cell pools and single cell-derived clones, no base substitutions were detected within these cell lines. Employing a more sensitive amplicon target sequencing, the anticipated substitution was identified within both T47D and HEK293T cells, albeit with a noted lower editing efficiency [39].

PE ex vivo therapy was evaluated in human clinical trials to determine whether it enhanced the efficacy of T cell therapy. A total of 17 patients have been infused with PD-1-edited T cells obtained by co-transfection of Cas9 and single guide RNA plasmids using electroporation in *ex vivo*. Peripheral blood was detected with edited T cells following infusion. Based on NGS analysis, 0.05% of off-target mutations occurred. Edited T cells had a median progression-free survival rate of 7.7 weeks and a median overall survival rate of 42.6 weeks [40].

V. DELIVERY SYSTEM

The efficiency of the delivery of PE tools *in vivo* is less due to their large size [41]**.** The use of dual AAVs for a PE2 system delivery was shown to be effective in correcting a pathogenic mutation in the mouse liver [42]. A downsized *Sp*Cas9 prime editor that lacked the RNaseH domain was employed, along with an intein-split construct, to enable AAV-mediated delivery of the prime editor to the liver. The intein-split construct involves dividing the PE tool into two separate AAVs, combined within cells to generate the functional PE tool (Fig. 6) [14]. Remarkably, the dual-AAV systems, namely v1em and v3em PE-AAV, showcased a robust delivery efficiency, exceeding 42% in the cortex, 46% in the liver, and 11% in the heart. Encouragingly, the *in vivo* utilization of v3em PE-AAV demonstrated an absence of detectable off-target effects and no notable alterations in liver enzymes or histology [43]. In HT-1 mouse models and LCA mice, PE was administered hydrodynamically and subretinally, respectively [17]. Rather than using viral vectors, lipid nanoparticles (LNPs) conjugated with apolipoprotein E can directly target hepatocytes, improving gene therapy safety [44]. With the use of enhanced LNPs (eLNPs) containing the cholesterol analog β-sitosterol at ideal RNA cargo ratios, a notable 54% prime editing rate was achieved. The distinctive polyhedral structure of the eLNPs, coupled with a fluid membrane state, facilitated superior endosomal escape. This, in turn, enabled the initiation of editing in as little as 9 hours, with peak efficiency reached within 24 hours. It's worth noting that prime editors can be successfully conveyed as

ribonucleoproteins to embryos of zebrafish and human primary cells [21]. Nonetheless, for the assurance of PE's safety profile over time, refining the efficiency of its delivery remains a crucial aspect that demands optimization.

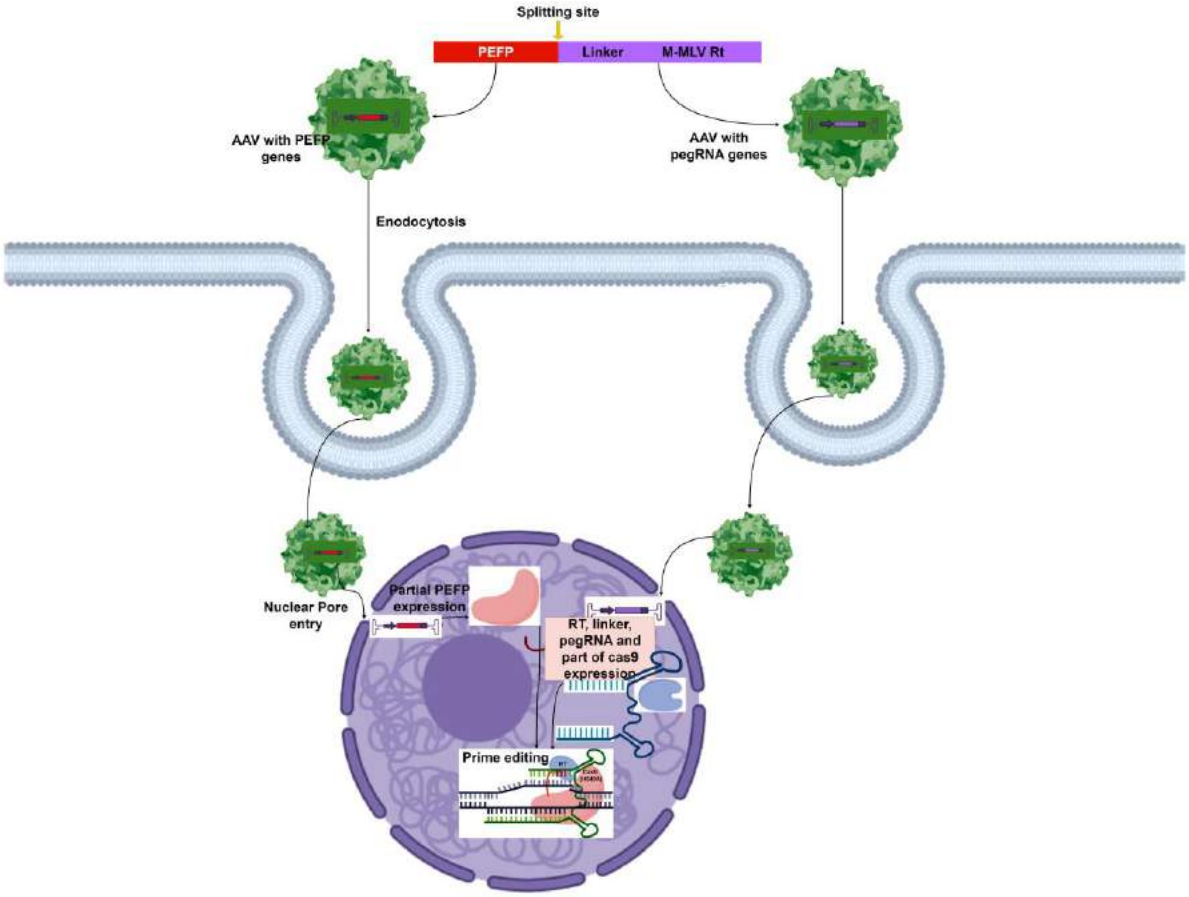

Figure 6: Dual AAV vector delivery system

VI. HURDLES

Specific substantial challenges must be overcome before human molecular therapy can be implemented. These obstacles encompass initiating immune reactions to both the vector and the PE due to elevated vector dosage [14], potential off-target effects prompted by Cas9, undesired ssDNA deamination, arbitrary deamination, and off-target editing [45]. Additionally, hindered editing may arise from regional constitutive heterochromatin and local nucleosome occlusion at target sites. Moreover, the cellular repair mechanisms triggered by PE need to be effectively managed.

VII. CONCLUSION

Currently, PE is used to create disease model animals and cell lines, and correct mutations in organoids, cells, and model animals. Few human clinical trials were carried out, and PE's efficiency was less than 50%, which has to be improved. To overcome hurdles, molecular therapy requires more research in modifying PE tools to reduce its size and design a tissue-specific delivery system. PE systems PE4 and PE5 are more efficient than PE2 and PE3 systems and demonstrate improved efficiency for small insertion and deletion and base substitution [46]. In the near future, it could treat various genetic diseases through genome surgery. However, ethical questions still need to be answered before this technology can be widely adopted. Additionally, more research needs to be done to ensure safety and accuracy when using PE. There are also concerns about the potential for this technology to be utilized for eugenics or designer babies. Governments must craft regulations and laws to ensure this technology is used ethically.

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