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CKJ Review

CKJ REVIEW

Genome editing and kidney health

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ABSTRACT

Genome editing technologies, clustered regularly interspaced short palindromic repeats (CRISPR)-Cas in particular, have revolutionized the field of genetic engineering, providing promising avenues for treating various genetic diseases. Chronic kidney disease (CKD), a significant health concern affecting millions of individuals worldwide, can arise from either monogenic or polygenic mutations. With recent advancements in genomic sequencing, valuable insights into disease-causing mutations can be obtained, allowing for the development of new treatments for these genetic disorders. CRISPR-based treatments have emerged as potential therapies, especially for monogenic diseases, offering the ability to correct mutations and eliminate disease phenotypes. Innovations in genome editing have led to enhanced efficiency, specificity and ease of use, surpassing earlier editing tools such as zinc-finger nucleases and transcription activator-like effector nucleases (TALENs). Two prominent advancements in CRISPR-based gene editing are prime editing and base editing. Prime editing allows precise and efficient genome modifications without inducing double-stranded DNA breaks (DSBs), while base editing enables targeted changes to individual nucleotides in both RNA and DNA, promising disease correction in the absence of DSBs. These technologies have the potential to treat genetic kidney diseases through specific correction of disease-causing mutations, such as somatic mutations in *PKD1* and *PKD2* for polycystic kidney disease; *NPHS1*, *NPHS2* and *TRPC6* for focal segmental glomerulosclerosis; *COL4A3*, *COL4A4* and *COL4A5* for Alport syndrome; *SLC3A1* and *SLC7A9* for cystinuria and even *VHL* for renal cell carcinoma. Apart from editing the DNA sequence, CRISPR-mediated epigenome editing offers a cost-effective method for targeted treatment providing new avenues for therapeutic development, given that epigenetic modifications are associated with the development of various kidney disorders. However, there are challenges to overcome, including developing efficient delivery methods, improving safety and reducing off-target effects. Efforts to improve CRISPR-Cas technologies involve optimizing delivery vectors, employing viral and non-viral approaches and minimizing immunogenicity. With research in animal models providing promising results in rescuing the expression of wild-type podocin in mouse models of nephrotic syndrome and successful clinical trials in the early stages of various disorders, including cancer immunotherapy, there is hope for successful translation of genome editing to kidney diseases.

Keywords: CRISPR-Cas technology, gene therapy, genetic kidney disease, genome editing, prime editing

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INTRODUCTION

Chronic kidney disease (CKD) is a growing health issue. It is estimated that ≈3.9 million people in the UK will be living with this condition by 2033, with a cost to the National Health Service of £6.4 billion [1]. Given the linear correlation between advancing age and the prevalence of CKD [2] coupled with the world's population aging [3], CKD represents one of the major threats to global health. By the year 2040, projections suggest that CKD will have risen to the position of the fifth most prevalent cause of mortality [4].

CKD is progressive and irreversible, slowly leading to loss of kidney function, and new treatments for this disease are urgently needed [5]. It can be either monogenic, with a single gene causing the disease phenotype, or polygenic, where a combination of mutations in different genes leads to disease manifestation [6–8]. The heritability of CKD is estimated to be substantial, ranging from 30 to 75% [4], with 10% of adults patients and most children suffering from inherited forms of kidney disease [9].

New techniques such as total human exome capture and large-scale genome sequencing provide a growing body of information on the disease-causing mutations that can be targeted for the development of new drugs or therapies [10]. For instance, given the frequency of *COL4A5* gene splice site mutations with an in-frame deletion at the transcript level in male X-linked Alport syndrome cases, Yamamura *et al.* [11] have developed an exon-skipping therapy using antisense oligonucleotides that successfully prevents the progression of kidney failure in mouse models by replacing the truncating variant with an in-frame deletion variant in the *COL4A5* gene. Additionally, Lin *et al.* [12] have shown that micro-injection of the Col4a3 transgene into the single-celled embryos of Col4a3$^{-/-}$ mice restored the normal collagen $\alpha3\alpha4\alpha5$(IV) network and led to structural repair in the glomerular basement membrane (GBM). Gene therapy using nanosized viral and non-viral delivery systems have already been successful in ameliorating renal fibrosis *in vivo* [13]. From a clinical perspective, one exciting development is the potential ability to specifically correct these mutations using genome editing techniques such as clustered regularly interspaced short palindromic repeats (CRISPR)-Cas technology. This is especially relevant for monogenic diseases, where mutations in only one gene lead to manifestation of the disease. CRISPR-based treatments, both *ex vivo* and *in vivo*, have already entered clinical trials and proved to be promising in treating many diseases, ranging from cancer to human immunodeficiency virus infection [14]. Genome editing has been mainly used for immunologically privileged tissues or organs (organs with tolerance against antigens without eliciting inflammatory responses in case of antigen exposure) such as the eyes, brain and liver. For example, considering that proprotein convertase subtilisin/kexin type 9 (PCSK9) functions mainly as an antagonist to the low-density lipoprotein cholesterol receptor (LDLR), Ding *et al.* [15] designed an adenovirus expressing Cas9 and a PCSK9-targeted CRISPR guide RNA that successfully disrupted the mouse *Pcsk9* gene *in vivo*, and this was associated with an increase in hepatic LDLR levels, decreased circulating PCSK9 levels and a 35–40% reduction in the cholesterol levels in plasma. To date, many preclinical studies have shown that, by means of different delivery vectors, gene editing technologies can be used to treat a wide variety of diseases [14].

In this review we will focus on current gene editing approaches for treating CKD, with a particular focus on CRISPR-based approaches. We will discuss the implications of the most recently developed CRISPR editing tools, their safety, delivery methods and possible applications for treating CKDs.

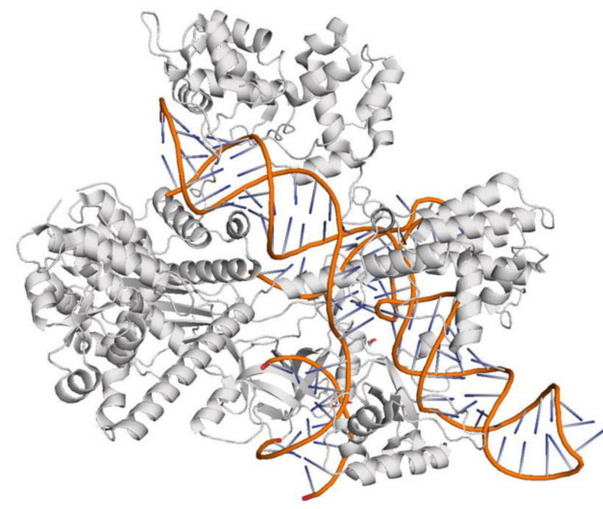

Figure 1: The three-dimensional structure of *Staphylococcus aureus* Cas9 in complex with sgRNA and target DNA (PDB: 5AXW).

THE CRISPR REVOLUTION

Innovations in genome editing enable precise, intentional changes to the genetic information of an organism, allowing scientists to add, delete or modify specific genes for different purposes, such as research or developing novel therapeutic treatments. Available genome editing tools are evolving rapidly, ranging from meganucleases and zinc-finger nucleases (ZFNs) to transcription activator-like effector nucleases (TALENs) and finally to the CRISPR technology [16, 17]. The first three tools are DNA-binding proteins that must be engineered specifically for each DNA sequence of interest. These proteins introduce a double-stranded DNA (dsDNA) break (DSB) that is then repaired by the inherent repair mechanisms of the cells and accompanied by incorporating site-specific modifications into the genomic DNA. However, even though justifiable for the clinical setting, the need to re-engineer the protein for each target sequence is both time-consuming and expensive, severely limiting the versatility of these technologies [18].

More recently, CRISPR technology has come to dominate the gene editing field, due to its efficiency, specificity and ease of use. The CRISPR-Cas system was originally discovered as a bacterial immune defense mechanism against invading bacteriophages, and it has since been adapted for use in a wide range of scientific applications (Fig. 1) [19]. This system consists of two main components: a single guide RNA (sgRNA) and a nuclease enzyme called Cas9. The sgRNA is a fusion of two RNA components, CRISPR RNA (crRNA), which is a short RNA sequence that is complementary to the target DNA sequence, and trans-activating CRISPR RNA (tracrRNA), that binds to the Cas9 protein and helps guide it to the target DNA sequence [20]. The Cas9 protein has two lobes: nuclease and recognition. The four domains of the recognition lobe, REC 1–3 and the bridge helix, play critical roles in targeted recognition and binding. The nuclease lobe is made of RuvC and HNH nuclease domains as well as protospacer adjacent motif (PAM) interacting domains. RuvC cleaves the DNA strand complementary to the sgRNA sequence, HNH cleaves the opposite DNA strand and the PAM interacting

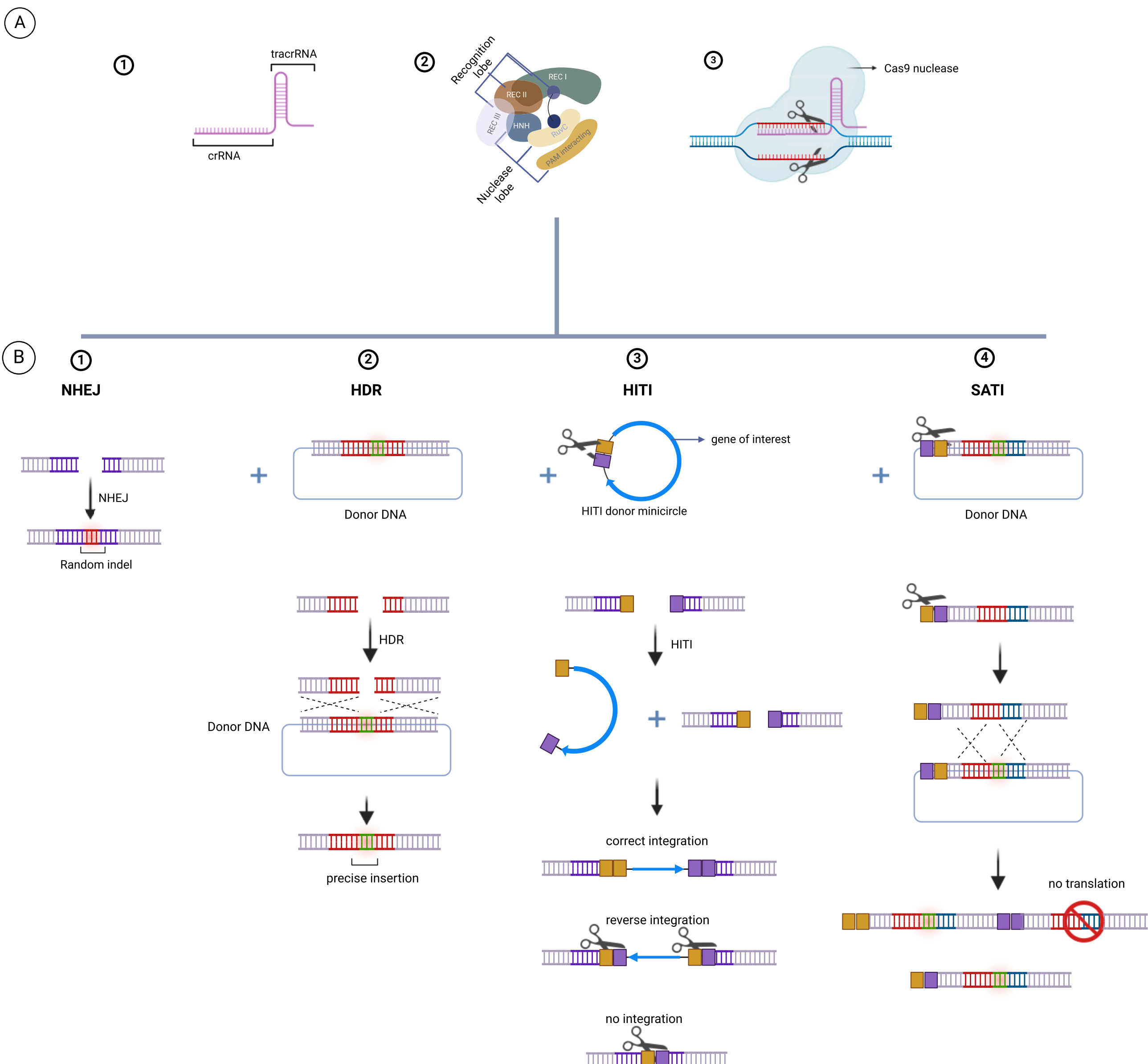

Figure 2: An sgRNA (A1) and a Cas9 programmable nuclease (A2) combine to form an RNP complex that binds and unwinds DNA at a precise locus, complementary to the spacer dictated by the sgRNA. Cas9 produces a DSB 3 base pairs upstream from the PAM (A3). Harnessing the natural repair mechanisms, NHEJ and HDR, the DSB can be repaired, leading to generation of small indels (B1) or, in presence of a DNA donor with suitable homology arms, precise insertion (B2). Other pathways can be exploited for more precise gene editing outcomes, such as homology-independent targeted integration (B3) or single homology arm donor-mediated intron-targeting integration (B4).

domain recognizes a short PAM sequence located adjacent to the target DNA sequence [21, 22]. The PAM sequence plays a crucial role in target recognition and binding by Cas9, ensuring that it binds to the correct DNA site and facilitates the initiation of DNA cleavage [23].

Once the DNA is cut, the DSB is repaired via non-homologous end joining (NHEJ) and homology-directed repair (HDR), which can be harnessed to make precise changes to the genome (Fig. 2). NHEJ is error prone and introduces small insertions/deletions (indels) at the target site and thus can be exploited to generate frameshift mutations for gene disruption. To improve the outcome of the NHEJ-based editing approach, using a dual sgRNA-mediated knock-out strategy can be used, helping to achieve a more precise deletion, irrespective of the ploidy of the cells, by deletion of the intervening fragments and 2–30% inverted reinsertion [24]. HDR, on the other hand, is an error-free repair pathway and can be used to correct or replace the genes when an exogenous donor template is provided [25]. Recently, HDR-based genome modification was further improved to homology-mediated end joining (HMEJ), with the CRISPR-Cas9 not only introducing DSBs in the endogenous genome, but also at the two homology arms in the donor, which linearizes the donor DNA with two long homology arms at the ends. However, studies on whether HMEJ-based knock-in improves the targeting efficiency have been contradictory, with both significant improvements and no changes being observed [26].

Due to the low efficiency of HDR, even in cultured cells, and the repair being restricted mostly to the cells in S/G2 phases [27], most therapeutic clinical trials to date have used CRISPR-Cas9 to disrupt the genes and not to edit the sequence [28]. In 2016, however, Suzuki *et al.* [29] developed an NHEJ-based homology-independent strategy for introducing precise DNA knock-ins in both dividing and non-dividing cells. This strategy, called homology-independent targeted integration (HITI), overcomes the limitation of NHEJ but is limited to targeted knock-in and cannot remove pre-existing mutations. Further improvement in genome editing systems has led to the development of intercellular linearized single homology arm donor mediated intron-targeting integration (SATI), harnessing both NHEJ and HDR repair strategies for not only targeted gene knock in, but also correction of point mutations [30]. The wild-type Cas9 enzyme forms an editing complex that tolerates some mismatches resulting in unintended cuttings and off-target effects [31]. The other forms of Cas9 are a catalytically inactive Cas9 [endonuclease-deficient Cas9 (dCas9) or null mutant Cas9] that lacks the nuclease activity and cannot create DSBs and Cas9 nickases (nCas9), with mutations in either the RuvC or HNH domain, that can create single-strand breaks instead of DSBs [32, 33].

In addition to Cas9, a variety of CRISPR-associated programmable nucleases have been described. Among these, Cas12a is one of the most widely used, with features such as the ability to trim its own guide RNA and the ability to induce a DSB with staggered ends. It also uses a variety of AT-rich PAM sequences, making it easier to target certain areas of the genome [31]. To improve HDR efficiency, different strategies such as supressing NHEJ as the main repair competitor, using single-stranded oligodeoxynucleotide template that contains homology arms or arresting the cell cycle at the late S and G2 phase where sister chromatids are available to be used as templates have been suggested [25].

Improvements in CRISPR-Cas technology continue to be made to enhance its efficiency, accuracy and specificity. For instance, using the CRISPR-Cas tool, the introduction of biallelic indels happens more often than monoallelic editing and this can be adjusted by controlling the Cas9 activity and dosage. To understand the relationships between Cas9 activity, editing outcomes, adverse effects and allelic configurations, Kawamata *et al.* [34] developed an allele-specific indel monitoring system that quantifies the editing patterns in each allele using a fluorescence-based approach. Using this system, they showed that the addition of cytosine stretches to the 5′-end of the gRNAs reduces the editing activity of Cas9 by changing the intracellular fitness of the gRNA-Cas9 complex and that this reduction is commensurate with the length of the stretches. Additionally, recent advances in CRISPR-Cas technology have resulted in the development of new editing systems that will be described below.

PRECISE EDITING USING DSBS PRODUCING CAS9 VARIANTS

Base editing

This technology enables changes to individual nucleotides both in RNA and DNA. Base editing can correct disease-causing mutations without cutting the DNA. Unlike traditional genome editing methods, such as traditional CRISPR-Cas9, which cut the DNA and rely on the cell's own repair machinery to make changes, base editing directly converts one DNA base to another without cutting the DNA. However, the editing specificity of the base edits is limited by the width of the activity window; in other words, base edits tend to operate and edit bases within an area that can be from 4 to 15 nucleotides in length. This increases the chance of having bystander mutations or unwanted base conversion within the editing window, which can lead to off-target editing [35]. Base editors typically consist of two components: a catalytically inactive Cas9 protein (dCas9) and a base-modifying enzyme. The dCas9 protein is used to target a specific location in the genome and the base-modifying enzyme then converts a specific nucleotide base to a different base. It is worth mentioning that instead of the dCas9, the nickase version of Cas9 has also been used in the structure of base edits, and this was accompanied by an increase in both the editing frequency and number of indels [36].

There are two main types of base editing technologies for DNA [37]: cytidine base editors (CBEs) and adenine base editors (ABEs). CBEs use a modified version of the CRISPR-Cas9 system in which the Cas9 protein is fused with a cytidine deaminase enzyme. The CBE can convert a C·G base pair to a T·A base pair by converting the cytidine to uridine, which is then recognized as thymidine during DNA replication. ABEs use a similar approach as CBEs, but with a different deaminase enzyme that can convert an A·T base pair to a G·C base pair by converting adenine to inosine, which is then recognized as guanine during DNA replication.

Many major improvements have been introduced to the base editing system for DNA, one of which is the addition of DNA glycosylase inhibitor (UGI) to the CBE complex [38]. Deamination of the base cytosine happens quite frequently in the cells, but uracil N-glycosylase (UNG) corrects this mismatch by cleaving the N-glycosidic bond and starting the base excision repair (BER) pathway [39]. Moreover, as is evident in Fig. 3, the ceiling of the editing efficiency for the first DNA base editing system is 50%, as editing is only happening in one of the strands. To solve this limitation, Komor *et al.* [40] used nickase Cas9 instead of dCas9 to introduce a nick in the non-edited DNA strand. This nick triggers the G-containing strand being resynthesized using the U-containing strand as the template. This, coupled with the inhibition of BER, can increase the base editing efficiencies in mammalian cells from 0.8–7.7% to up to 75% [28]. Meanwhile, the addition of the Gam protein of bacteriophage Mu, which prevents the degradation of dsDNA by binding to and protecting the ends and linkers between the components of the abovemotioned base editing constructs, can further improve the efficiency while reducing the number of undesired by-products [41].

There are also two different types of base editing techniques that have been developed for RNA editing, including adenosine to inosine (A-to-I) and cytidine to uridine (C-to-U) editing. A-to-I editing is the most common type of RNA base editing and involves the conversion of adenosine to inosine in RNA molecules by the enzyme adenosine deaminase acting on RNA. C-to-U editing is the other type of RNA base editing and involves the conversion of cytidine to uridine in RNA molecules. It is typically achieved using the CRISPR-Cas system coupled with cytidine deaminase enzyme or the apolipoprotein B mRNA editing enzyme complex-1 [42]. However, the challenge with RNA-based systems is the increased chance of off-target effects, which can be improved by switching Cas9 to Cas13 endonuclease [43]. Cas13 has four subtypes (a–d) and is smaller than Cas9. Cas13 enzymes form a complex with crRNA, and after targeting the desired RNA sequence, their catalytic domain introduces a cleavage in the target sequence [44].

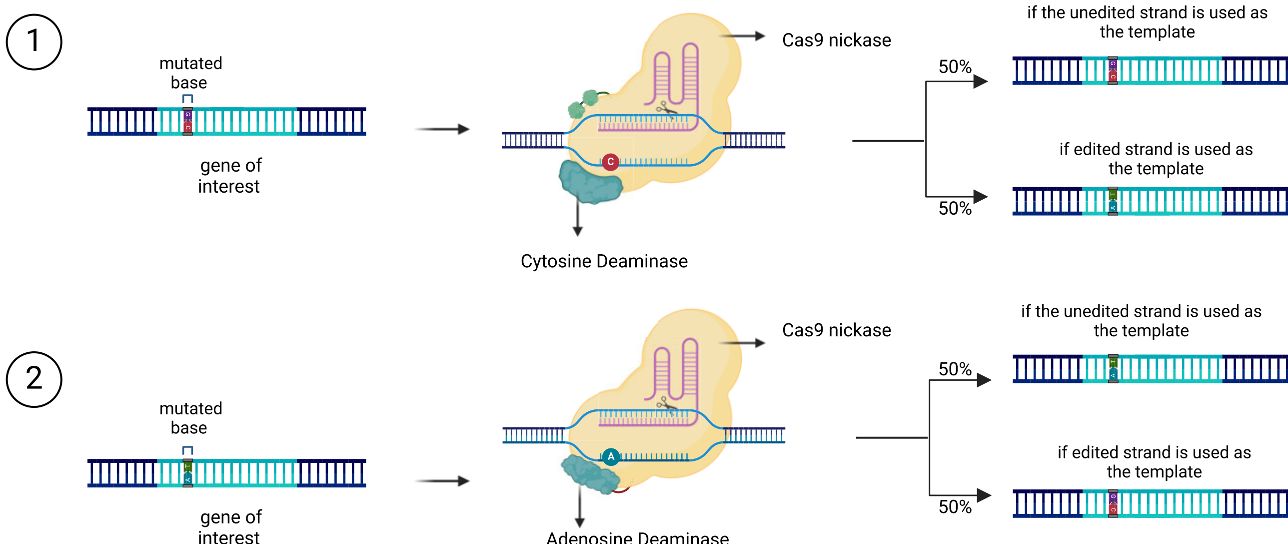

Figure 3: Base editing of cytosine deaminase enzyme: 1) CBEs incorporate a cytosine deaminase enzyme catalyzing the conversion of cytosine (C) to uracil (U) within the DNA sequence. The modified uracil is then recognized as a thymine (T) during DNA replication and repair processes, resulting in a permanent C-to-T base substitution. 2) ABEs use an adenine deaminase enzyme, which is an evolved form of TadA (tRNA adenosine deaminase). This enzyme converts adenine (A) to inosine (I), which is recognized as guanine (G) during DNA replication and repair processes, leading to the permanent substitution of A to G.

Prime editing

In 2019, Anzalone *et al.* [45] developed a new form of CRISPR-Cas technology, prime editing, that enables precise and efficient editing of the genome (nucleotide changes, insertion or deletion) without inducing DSBs. Prime editing allows for more complex edits to be made to the genome than with traditional CRISPR-Cas systems. The prime editing complex is composed of a nickase Cas9 fused to a reverse transcriptase along with prime editing guide RNA (pegRNA) (Fig. 4). The pegRNA is an extended version of sgRNA coupled with a primer binding site (PBS) and an editing guide sequence (Fig. 4). The PBS is a short sequence (8–17 nt) that is complementary to the genomic DNA immediately upstream of the target site. This region serves as a binding site for the primer extension activity of the prime editor protein and enables the pegRNA to anneal to the target DNA. The editing guide sequence (10–20 nt) is the reverse transcriptase template that is specific to the target site and guides the prime editor protein to the correct location for editing. The guide RNA directs the Cas9 protein to the target site, while the pegRNA contains the desired edit and directs the Cas9 protein to the precise location in the genome where the edit should be made.

However, prime editing system has several disadvantages. First is the need for a PAM sequence near the target sight. For instance, the original system, the most commonly used is Cas9 from *Streptococcus pyogenes* (spCas9), needs a 5′-NGG-3′ PAM sequence, and this confines the sites editable with this system. To address this issue, over the past few years more attention has been paid to engineering Cas9 variants with altered PAM specificities or, ideally, with reduced or even eliminated PAM requirements. For instance, a so-called near-PAM-less spCas9 version has been developed that needs either NRN or NYN PAMs (N: A, G, C or G; Y: C or T; R: A or G) [46]. Moreover, simultaneous delivery of all the necessary components can be challenging, especially because PE2 editors are much bigger in size than Cas9. This delivery requires the use of multiple vectors, which decreases the efficiency. To address this issue, Aulicino *et al.* [27] developed an all-in-one baculovirus delivery system that allows efficient single and multiplexed prime editing in different human cell lines. The other challenge is that the mismatch DNA repair pathway (MMR) can substantially inhibit prime editing efficiency. While transient MMR inhibition via overexpression of dMLH1 can rescue prime editing efficiency [47], this strategy inevitably increases the number of required components.

When it comes to editing technologies, prime editing stands out as an exceptionally safe method, far surpassing any other alternatives. Compared with ongoing Cas9 clinical trials, where off-target rates can be as high as 10 or even 20%, prime editing shines as a game-changer, with its average off-target rates well below 0.5%. Since 2019, attempts have been made to improve this system, and many changes have been introduced to overcome the inconsistent editing efficiency of prime editing [48]. While it is true that off-target effects still pose some concerns, this low off-target rate is practically undetectable without next-generation sequencing. More importantly, prime editing has the unique characteristic of not generating DSBs, setting it apart as the sole system that exclusively produces the intended outcomes or leaves the genome in its original wild-type state. In contrast to base editing methods, prime editing offers complete control over the editing process, facilitating the ability to achieve all types of base pair substitutions as well as short deletions or insertions. This level of control opens up a wide range of possibilities for precise genetic modifications.

CRISPR-mediated epigenome editing

CRISPR-Cas9 technology is a cost-effective and easy-to-use method for epigenome editing. CRISPR-mediated chromatin modification happens through targeted transcriptional regulation, histone modification, DNA methylation or demethylation or the relocation of non-coding RNAs (Table 1). The basics of all these methods are the same, and the only difference is the component that is coupled to the dCas9 protein [49].

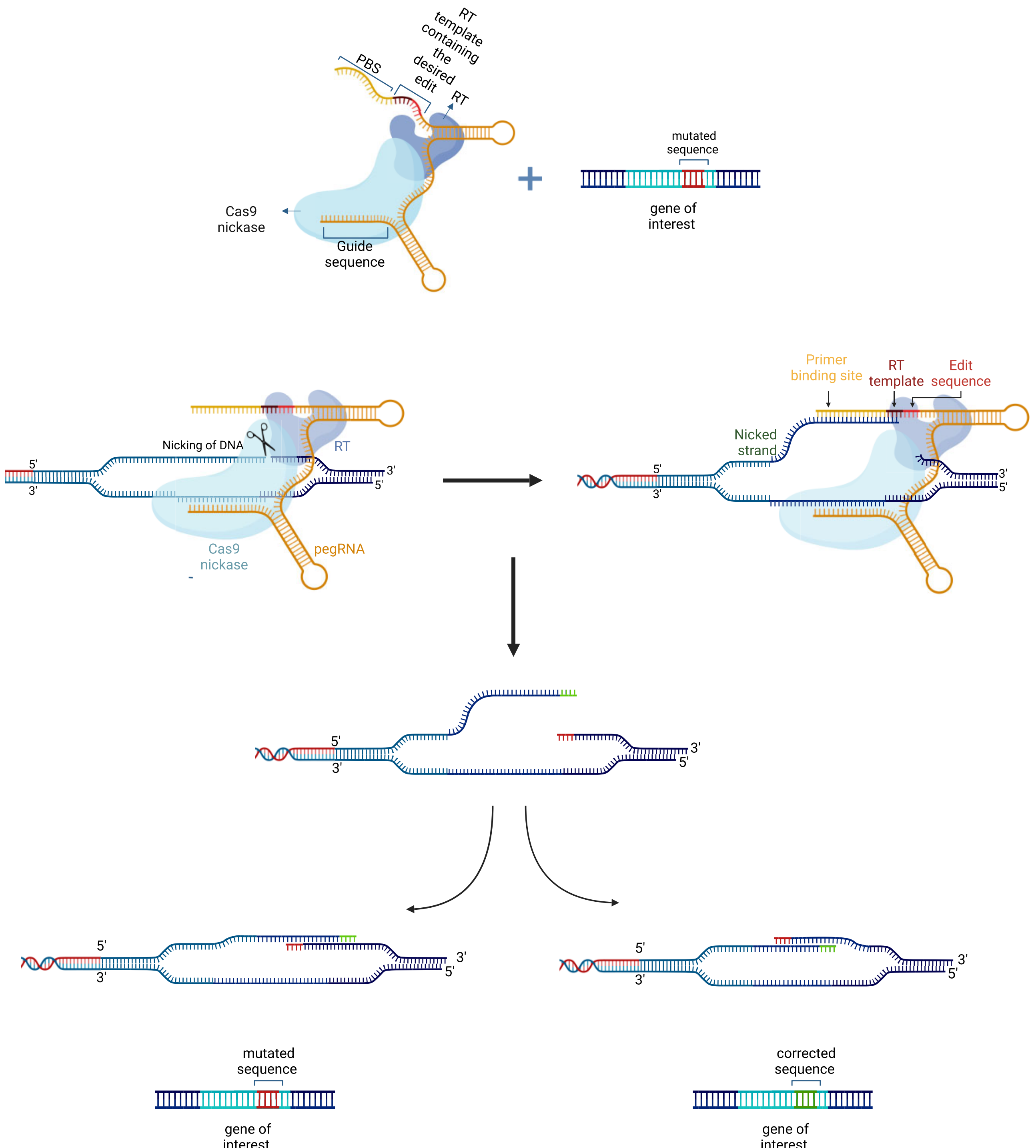

Figure 4: Prime editing steps. The pegRNA and Cas9 protein forms a complex within the cell. The complex scans the DNA and finds the target sequence. This is followed by the dsDNA becoming separated and the guide sequence (the pegRNA spacer) in pegRNA pairing with the complementary sequence. The PAM sequence adjacent to it is recognized by the Cas9, which is necessary for the prime editing protein to nick the target sight at the non-complementary strand to the pegRNA spacer. The PBS binds to the complementary sequence, which acts as primer for RT. The RE uses the 3′ end as the primer and RT template to synthesize the new strand. As the pegRNA contains the edit, the desired base sequence is added to the DNA.

The main strategy is fusing the dCas9 protein, as a DNA-binding domain, with a transcription repressor or activator domain, known as an epigenetic effector, to form the dCas9-epieffector fusion complex. Specific fusions such as the dCas9-KRAB or dCas9-VPR complex have been demonstrated to silence or activate target genes without global gene expression perturbation or off-target effects [50]. Depending on the fusion, different mechanisms can be used for inhibiting gene expression; for example, while dCas9-KRAB primarily functions through transcriptional repression by recruiting co-repressor proteins,

Table 1: The possible epigenic changes that can be achieved by linking dCAS9 to different effectors

Method	Transcriptional regulation	DNA methylation	Histone modification	RNA relocation
Components conjugated to dCAS9	Activator or repressor	Ten-eleven translocation enzyme (TET) or DNA methyltransferase	Histone demethylase, histone methyltransferase, histone acetyltransferase, histone deacetylase, histone ubiquitin ligase	Enhancer RNAs, long non-coding RNAs, promoter-associated RNAs

dCas9-LSD1 acts through histone demethylation. Additionally, other fusions such as dCas9-DNMT3A or dCas9-TET have been shown to be reliable gene activation tools by inducing methylation or rescuing silenced genes via demethylation, respectively [50].

Genome editing applications in kidney health

These new technologies open the exciting possibility of using gene editing techniques to potentially treat genetic forms of kidney disease (Fig. 5). For example, polycystic kidney disease (PKD) is a common inherited kidney disorder and a potential target for therapeutic gene correction. Autosomal dominant PKD (ADPKD) affects 1 in 1000 people and causes renal failure in adulthood. PKD is caused by mutations in the *PKD1* or *PKD2* gene, which could potentially be targeted for gene correction using gene editing technologies [51]. New research has identified potential new targets for treating this disease with gene editing approaches. For example, Onuchic *et al.* [52] recently showed that just the C-terminal tail of polycystin-1 is sufficient to suppress cystic disease, and Lakhia *et al.* [53] identified a binding site for inhibitory microRNAs (miRNAs) that if deleted also ameliorates the development of kidney cysts.

Leveraging CRISPR technologies could offer more sustainable and potentially long-lasting effects in suppressing cystic kidney disease compared with existing approaches. For example, CRISPR-mediated deletion of inhibitory miRNA binding could target and delete the specific binding sites for inhibitory miRNAs within the genome associated with PKD. By precisely editing these sites, it may disrupt the binding of inhibitory miRNAs, potentially offering sustained suppression of the inhibitory miRNA activity and preventing the development of kidney cysts over an extended period. Additionally, CRISPR-Cas9-mediated targeted integration could be employed to insert the C-terminal tail sequence of polycystin-1 into specific genomic loci known to facilitate sustained protein expression. By inserting the C-terminal tail sequence into the genome, it could lead to continuous and stable expression of the therapeutic C-terminal tail of polycystin-1, providing a long-term inhibitory effect on cyst development.

Changes in the epigenome have been known to influence the development and progression of kidney diseases; for example, Ko *et al.* [54] showed that epigenetic dysregulation, in particular cytosine methylation changes, is associated with changes in the transcript level of pro-fibrotic genes and CKD development, and histone methylation and acetylation have been associated with various pathogenic manifestations, including renal fibrosis and inflammation, in diabetic kidney diseases (Fig. 6) [55, 56]. Given the role of these post-translational changes in the pathogenesis of various kidney diseases and the possibility of epigenome editing using new CRISPR technologies (Fig. 7), this article highlights the existing gap and the need to apply new advances in genome editing to study the CRISPR-Cas9 system for modifying DNA methylation in mouse models of PKD. The researchers targeted the promoter regions of genes involved in the development of PKD and were able to decrease the severity of the disease in mice by decreasing the expression of these genes [57]. However, identification of DNA methylation changes in ADPKD remains complex due to conflicting data and internal biases within DNA methylation methodologies. Despite this, recent drug discovery platforms have shown that targeting epigenetic changes may be a way forward for new therapeutic development [57].

As explained earlier, new CRISPR-based gene editing strategies can target and repair these specific disease-causing mutations with greater efficiency than traditional gene therapy methods. These strategies have been used to efficiently disrupt or edit specific genes in various cell lines with minimal off-target mutations. Based on the nature of the disease-causing mutation, the editing tool can be CRISPR-Cas9, prime editing or base editing. Recent studies in animal models have used CRISPR to correct an array of genetic diseases, including muscular dystrophy, Huntington's disease and retinal degenerative diseases [14]. Before using the CRISPR-Cas9 technology in the clinic, there is a need to come up with solutions to resolve the challenges of CRISPR's large genetic cargo size and choose the proper delivery route [58]. Physical approaches like microinjection, electroporation and hydrodynamic injection can be used to deliver CRISPR-Cas components. However, in the scope of delivering them to the kidney of a fully developed organism, these approaches are associated with limitations and challenges. For instance, the application of microinjection is often limited due to challenges related to scale, invasiveness and cell accessibility. In other words, the size of the organism and the complexity of its tissues make it difficult to target kidney cells through these methods without causing damage or to reach the desired location effectively.

Apart from physical approaches, genetic material can also be delivered using viral and non-viral vectors. With non-viral delivery systems, different platforms of CRISPR-Cas9 can be used, such as Cas9 protein and sgRNA, Cas9 mRNA and sgRNA or CRISPR-Cas9 plasmid [59]. Non-viral vectors such as cell-penetrating peptides, gold nanoparticles, zeolitic imidazole frameworks, biotinylated oligonucleotides, dendritic polymers and graphene oxide-poly(ethylene glycol)-polyethyleneimine nanocarriers have been reviewed previously [59]. However, only a few nano-delivery systems have been designed for the kidney, and the majority of them target the tubules [60]. Recently, Oniszczuk *et al.* [61] used a gold colloidal template to make nanocarriers with chitosan/DNA multilayer walls designed to deliver miRNA against c-mip specifically to podocyte cells in the kidneys. *In vivo* studies on these nanoparticles showed a significant reduction in proteinuria by targeting c-mip into the podocytes.

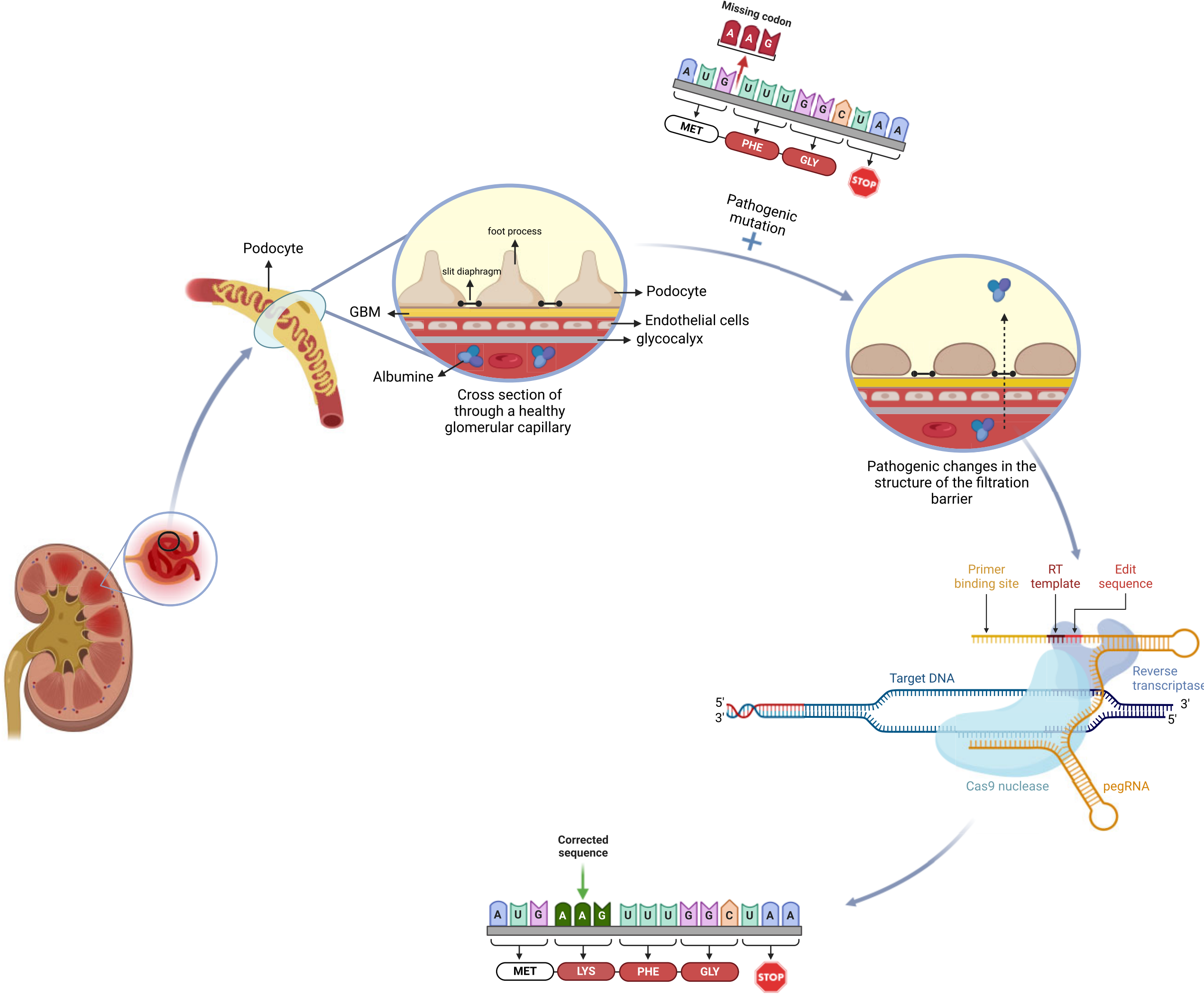

Figure 5: The potential of the CRISPR-Cas9 technology in treating kidney diseases. In the glomerulus, the filtering unit of the kidney, capillaries are wrapped by the podocytes. The cross-section of the glomerular capillaries shows the filtration barrier in the glomerulus, which is made of several layers, including glycocalyx, fenestrated endothelial cells, glomerular basement membrane and podocyte cells. The normal structure of this barrier helps the kidney's role in filtering the blood. Different mutations (here the deletion of the lysin codon) in glomerular cells leads to detrimental changes in the barrier's structure, leading to the leakage of proteins from the blood to the urine. Based on the type of mutation, different CRISPR-Cas technology techniques can be used to correct the DNA sequence, helping the kidneys to retain their normal function.

Viral vectors have also been used to target and rectify the underlying genetic defect in several diseases [62]. Compared with non-viral delivery systems, viral particles have the advantage of higher transduction efficiency, which makes them an interesting tool for researchers. However, currently used viral delivery systems suffer from limited DNA cargo capacity, thus restricting their use, particularly with rapidly developing gene editing technologies that increasingly depend on foreign DNA cargo for cut-and-paste functions that present systems cannot accommodate [63]. Moreover, developing adeno-associated virus (AAV) particles requires at least three plasmids, and this makes them quite expensive. The common viral vectors used for gene delivery include adenoviruses [64], AAVs [65] and lentiviruses [66], which have been reviewed previously. The choice of viral vector depends on the specific application and the target cell type. Adenovirus can infect a wide range of cell types but can elicit a strong immune response. Lentivirus has a larger packaging capacity and integrates into the host genome with a high risk of insertional mutagenesis. AAVs are considered the best option for CRISPR delivery [58] and, compared with lentiviruses, are smaller with lower immunogenicity, but have limited packaging capacity [64], meaning that genes larger than 4 kb cannot be delivered using this delivery system, restricting the number of diseases that can be treated with AAV-based gene therapy.

In the past few years, different strategies have been suggested to overcome this challenge. One is using the dual- or triple-vector approach, where different AAV particles are used to deliver different segments of the desired protein. However, with this technique there are risks of erroneous reassembly, the unexpected expression of one individual split or reduced efficiency compared with the single AAV approach. Moreover, an increase in the number of AAV vectors makes gene therapy even more expensive than it already is. Another approach is the minigene strategy, in which a truncated but functional part of the desired gene is used instead of the whole gene [67]. For instance, Berger *et al.* [68] showed that when there is a deletion (F508-del)

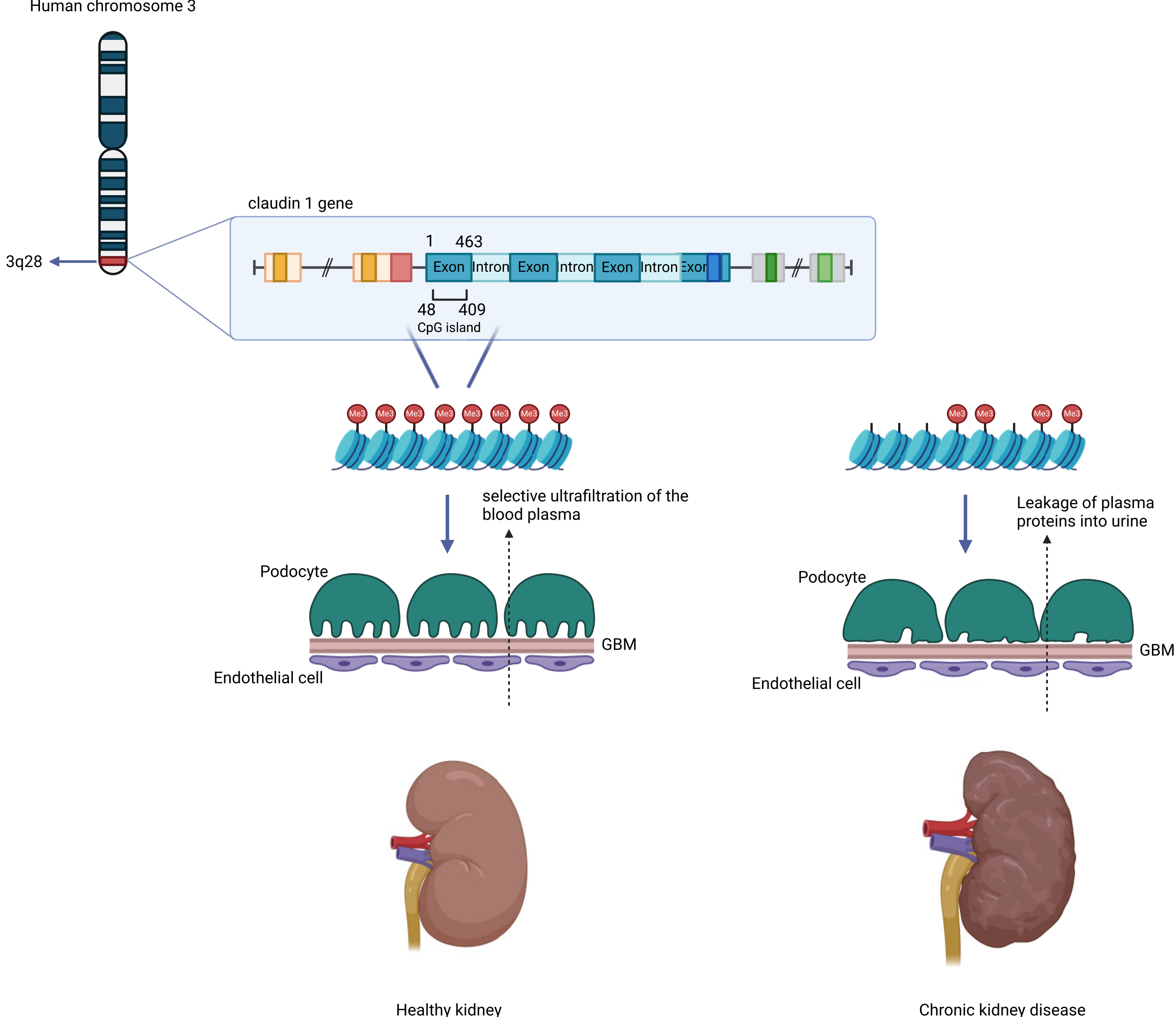

Figure 6: Hypomethylation of the CpG island is associated with the upregulation of the *Claudin-1* gene. Higher levels of claudin-1 in the glomeruli are linked with diabetes-induced albuminuria.

in cystic fibrosis transmembrane conductance regulator (CFTR) protein, the delivery of the truncated version of the protein can lead to trans-complementation, where the function of the CFTR protein is rescued. However, very strong knowledge of the protein and interdisciplinary collaboration are required to spot this truncated structure [67].

AAV has become a popular choice for CRISPR gene delivery due to its safety, ability to infect both dividing and non-dividing cells and sufficiently large cargo capacity for Cas9 and its associated regulatory elements and gRNA. So far, local and systemic administration of AAV vectors encoding CRISPR and gRNA have been studied for gene correction in different diseases, including post-mitotic retina [29, 69], Duchenne muscular dystrophy [70] and Huntington's disease [71]. However, delivering CRISPR-Cas9 systems *in vivo* remains challenging due to numerous physiological barriers. Baculovirus has emerged as a powerful and safe technology to deliver heterologous genetic material to mammalian cell types both *in vitro* and *in vivo* [72]. Crucially, baculovirus has a large capacity for foreign DNA due to its flexible envelope, which adapts to the size of the packaged genome. Baculovirus can transduce both dividing and non-dividing cells and exhibits low cytotoxicity in vertebrate cells [72–74]. Baculoviral genomes are amenable to genetic manipulation and are easily produced and harvested for mammalian applications at high titres in biosafety level 1 laboratories. Importantly, there is no detectable pre-existing immunity to baculovirus in humans, which is a recurrent issue with other viral systems [62, 75]. Baculovirus has a very narrow insect cell host specificity and is a replicative virus only in its insect host, whereas it remains replication- and integration-deficient in mammalian cells, tissues and organisms. Once baculovirus enters mammalian cells, genes encoded in its genome, under control of suitable mammalian promoters, are actively transcribed within 9 h and the cells produce the heterologous gene product.

In 2016, the Berger team developed a specialized baculovirus-derived system, MultiPrime, comprising a modified baculovirus

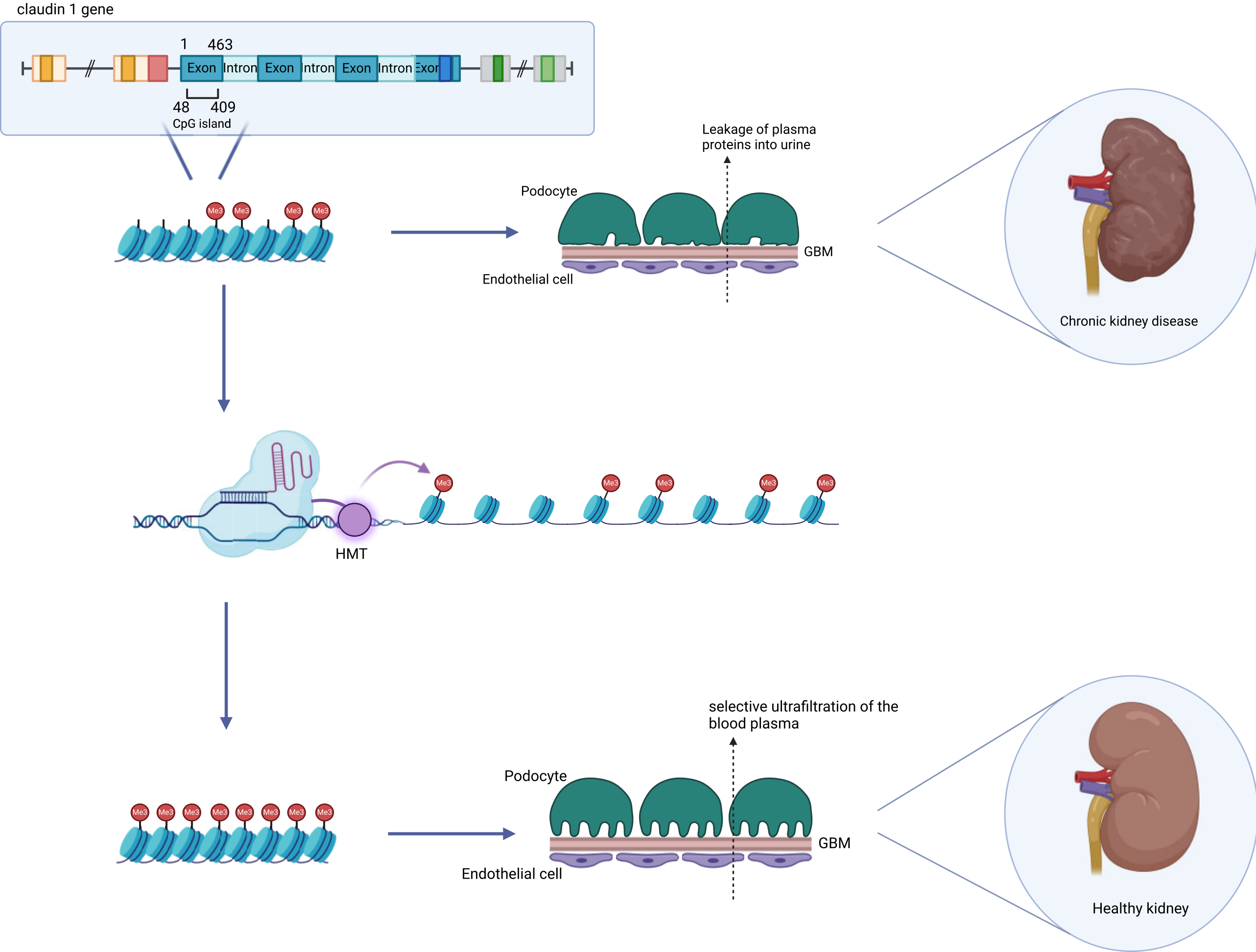

Figure 7: The possibility of correcting the pathogenic hypomethylation of the CpG island in exon 1 of the *Claudin-1* gene using a CRISPR-mediated epigenome editing technique and linking dCAS9 protein with histone methyltransferases (HMT) to transfer methyl groups to the histones.

genome functionalized for mammalian cell transduction by displaying specific protein modalities on the viral envelope, thus pseudo-typing the vector. MultiPrime is a highly efficient multigene delivery system for a wide range of established and primary mammalian cells and tissues [73]. In marked contrast to currently used viral systems, MultiPrime has a very large (>100 kb) DNA cargo capacity and is based on a technology (baculovirus) that has already received US Food and Drug Administration approval as a manufacturing tool and is therefore considered safe for laboratory and therapeutic use. More recently, Aulicino *et al.* [27] further improved this system to deliver a variety of complex CRISPR toolkits to mammalian cells. Using this technology, homology-independent targeted integration of large DNA cargo was used to successfully integrate a wild-type copy of *NPHS2* and a rescue recessive disease-causing gene (*NPHS2 R138Q*) in podocytes derived from steroid-resistant nephrotic syndrome patients. These findings highlight the potential of the newly designed baculovirus in overcoming the limited cargo of other viral delivery systems, opening up new avenues for treating various kidney diseases using CRISPR-based genome editing technologies.

Overall, the advances in CRISPR-Cas technology have expanded the range of applications for genome editing, enabling more precise and efficient editing and opening up new possibilities for treating genetic kidney diseases, improving human health and establishing valuable models of kidney diseases. Recently, studies have focused on using genome editing techniques to develop models of various genetic kidney diseases [76]. These models provide us with invaluable information about the changes happening during disease progression. For instance, Neto *et al.* [77] developed podocyte cell lines with Fabry disease phenotypes by knocking out the *GLA* gene using the CRISPR-Cas9 system. Studying the proteomic profile of the established cell line deficient for α-GAL shed light on the proteins being differently expressed during the disease, and this can be useful for understanding more about the disease pathway and more personalized and efficient treatment. Also, given that the exact mechanism through which autosomal dominant tubulointerstitial kidney disease progresses after the *PKD1* mutation, Kuraoka *et al.* [78] used CRISPR-Cas9-mediated gene editing to compare the cyst formation between ureteric bud organoids derived from homozygous and heterozygous *PKD1*-deleted induced pluripotent stem cells (PSCs) and the ones derived from an ADPKD patient with a heterozygous missense mutation, showing that the established organoids are a good representation of the genesis of ADPKD. Another study used CRISPR-Cas9 gene editing to

generate podocytes from human PSCs and showed that the established cell line is close to the podocytes at the capillary loop stage in terms of ultrastructure, gene expression and mutant phenotypes [79].

Meanwhile, scientists are exploring the use of CRISPR-Cas9 to correct genetic mutations that cause diseases, potentially offering a cure for patients with kidney diseases. Many kidney diseases, such as PKD and glomerulonephritis, have complex genetic underpinnings [80, 81], making them excellent candidates for genomic editing. A recently published article showed that kidney gene therapy is both possible and efficient in treating kidney diseases [82]. In the article, Ding *et al.* aimed to investigate the potential of kidney gene therapy for treating childhood steroid-resistant nephrotic syndrome where mutations in podocin, a podocyte-specific protein encoded by *NPHS2*, causes disease progression. In their study, AAV vectors with a minimal nephrin promoter were administered into the tail vein of inducible podocin knock-out and knock-in mouse models to express mouse podocin, with the results showing improvements in the degree of albuminuria and survival of the mice, demonstrating the first successful AAV-based gene therapy for a monogenic kidney disease.

Currently, clinical trials for CRISPR gene editing are all in the early stages, focusing on the use of CRISPR as an alternative approach for immunotherapies, including the treatment of certain cancers [83]. One clinical trial used traditional CRISPR-Cas for renal cell carcinoma immunotherapy. This study aims to evaluate the safety and effectiveness of allogeneic CRISPR-Cas9-engineered T cells (CTX130) in subjects with advanced, relapsed or refractory renal cell carcinoma with clear cell differentiation, through a phase 1 dose escalation and cohort expansion. However, despite the promising results obtained from the recent advances in CRISPR-Cas technology, none of them has yet entered any kidney clinical trials. Therefore, there is a need for translating the recent advances in CRISPR-Cas technology to translational kidney research in the hope of advancing this area to the clinical trial stage. Additionally, due to its filtration role, the structure of the GBM is specialized in a way that only molecules <10 nm can pass through this filter, making efficient gene delivery to the kidney, particularly via systemic injection, more challenging than for other organs like the liver. Other delivery routes such as direct renal blood vessel, retro-ureteral and subcapsular injections, even though they lead to more efficient delivery of genetic material, are highly invasive, which is not ideal for translating to clinics. Therefore, exploring more efficient and less invasive delivery methods to the kidney should be another area of focus.

As mentioned earlier, there are other challenges that need to be addressed before successful clinical translation of CRISPR-Cas9 gene editing can be achieved. Researchers have improved the packaging capacity and delivery efficiency of AAV vectors by using synthetic surface peptides, splitting the Cas9 protein or using smaller orthologues. However, more optimization is required, including determining the immunogenicity of AAV vectors, dCas9 proteins and guide RNAs, and minimizing off-target effects to ensure clinical safety. Baculoviruses and AAV improvements have shown promising results in overcoming the existing challenges, but the recent improvements in this technique need to be expanded to translational kidney research.

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DATA AVAILABILITY STATEMENT

No new data were generated or analysed in support of this research.

CONFLICT OF INTEREST STATEMENT

None declared.

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