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Precision Gene Editing and Applications in Nephrology

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Abstract

The expanding field of precision gene editing is empowering researchers to directly modify DNA. Gene editing is made possible using synonymous technologies: 1) a DNA targeting platform to molecularly locate user-selected genomic sequences, and 2) an associated biochemical activity that serves as a functional editor. The advent of accessible DNA-targeting molecular systems, like transcription activator like effectors (TALEs) and Clustered Regularly Interspersed Short Palindromic Repeats (CRISPR)-Cas9 gene editing systems, has unlocked the ability to target nearly any DNA sequence with nucleotide-level precision. Progress has also been made in how we can harness endogenous DNA repair machineries to functionally manipulate genetic sequences. The more that is understood about how DNA damage results in deletions, insertions, and modifications the more predictably mutable the genome becomes. These genomic targeting platforms are also useful for locus-specific epigenetic changes and transcriptional enhancement and suppression. This new genome engineering technology builds on a long history of renal science, enabling new animal models of disease as well as novel therapeutic options.

Keywords

Genome Engineering; Gene Editing; DNA Repair; ZFN; TALEN; CRISPR; Cas9; Cpf1; Cas12a; Base Editor; NHEJ; HDR; HR; MMEJ; Renal Disease

Introduction

Engineering DNA is undergoing a far-reaching change with the advent of a new array of genome engineering tools such as CRISPR/Cas9 and TALENs. With the advent of accessible programmable DNA nucleases, genome engineering now includes the growing capability to edit the genome. The initial molecular biologist's toolbox was largely confined to simple rearrangements of DNA through the use of restriction enzymes and ligases *in vitro* or in bacteria. These techniques are inherently limited by the intrinsic sequence-specific nature of restriction enzymes and, consequently, by the functional inability to edit higher order organisms. Indeed, the primary options for manipulating eukaryotic genomes were

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random mutations inducible by chemicals or radiation, directed breeding/evolution through matching of parents with desired alleles, or the use of species-compatible transposon elements or viruses. Despite their limitations, these approaches enabled many advances such as transgenic expression, homologous recombination in embryonic stem (ES) cells to generate knock outs/ins, and led to initial attempts at gene therapy by exogenous gene expression.

The last seven years have, however, been a time of rapid change for the biologists' toolbox, while also empowering a new way of thinking about DNA editing. This review focuses on the development, mechanism, and accessibility of the different DNA editing platforms as well as their targeted interaction with the genome. We will also cover the current understanding of DNA biochemical functions used to make precise modifications for modern precision gene editing.

Custom programmable gene editors (Fig. 1) started with the advent of the zinc finger nuclease (ZFN)[1,2], advanced in precision and usability by the development of the transcription activator-like effector nuclease (TALEN)[3,4] and made highly accessible through the development of CRISPR-based systems[52]. Their shared ability to target unique DNA locations in a targeted genome is an essential core function and continues to be paramount for other editing systems and for others currently in development.

The versatility of precision gene editing has grown through the addition of various enzymatic activities to these programmable DNA binding platforms. Upon interacting with their defined loci, these functionalized programmable DNA binding platforms activate endogenous DNA repair pathways that serve as the molecular pathways to generate a wide range of DNA sequence edits. Current genomic editing uses at least five different DNA repair pathways. A targeted double-strand break (DSB) from an associated nuclease can induce the nonhomologous end-joining (NHEJ) pathway; classical homologous recombination (HR), the more recently deployed method of single strand template repair (SSTR), or microhomology mediated end joining (MMEJ) to induce DNA sequence changes (Fig. 2). A fifth leverages the endogenous base mismatch repair to edit DNA without rupturing the double strand of DNA (Fig. 2). In turn, each of these mechanisms suggests not only the preferred cellular process used, but also the resultant mutation signatures and their potential uses in genomic sciences.

Successful genome engineering depends on the cellular context as well, with editing efficacy being modulated by cell cycle stage, cell type, and other conditions within the cellular environment. In basic science research, DNA editing can be used to study gene function and to create engineered lines of experimental animals. Additionally, this tool represents enormous potential for clinical applications and the generation of accurate disease models in a number of cellular systems. With this new technology, complex genetic disorders can be explored where multiple genetic events and their interactions can be mimicked to understand common renal diseases. Genome engineering allows these diseases to be modeled in diverse cellular and animal models and used to explore both pathology as well as the science underlying health.

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Programmable DNA Targeting Platforms (Fig. 1)

The First Precision Edits: ZFNs

The first custom programmable nuclease was developed from the ~30 amino acid Cys2-His2 Zinc-Finger (ZF) domain, the most abundant DNA binding protein motif utilized in eukaryotic transcription factors[1,5-7] (Fig 1A). In each ZF domain three amino acid clusters or “fingers” each recognize a single specific DNA base and mediate DNA binding[7-9]. Therefore, in principle, through manipulation of the amino acids in each finger, one can create a ZF that binds to any of the 64 possible 3 base permutations[10]. Multiple ZF domains can then be fused together to create a single poly-protein capable of binding long stretches of DNA[11-13].

Although ZFs are still in use today, this programmable DNA binding platform has restricted accessibility due to limitations on synthesis and targeting flexibility. For example, the generation of custom ZFs can be a technically arduous task, as synthesized tandem ZF repeats often exhibit unpredictable context-specific interference that mitigate binding and reduce or alter specificity[7]. Critically, only a subset of the genome has been historically targetable due to these technical limitations for this system[14]. However, recent progress has improved the ZFN platform[15]

ZF scientists were also the first to selectively activate DNA repair pathways. Discussed more in detail below, creating an editor from the ZF DNA binding domain required the attachment of a DNA endonuclease since ZFs have no intrinsic editing activity. This was achieved by the fusion of the FokI endonuclease domain to the C-terminal end of a series of tandem ZF domains to create the Zinc-Finger Nuclease (ZFN)[1,16]. This catalyzes a double-strand break (DSB) between the programmatically targeted ZF binding domains when two ZFNs are bound enabling FokI to dimerize and become catalytically active[6]. The resulting induced double-stranded break repair (Fig. 2) is then harnessed for precision gene editing[17-19]. FokI is thus deployed as a nuclease partner for a custom DNA binding protein, as the enzymatic domain can be functionally uncoupled from its inherent, non-programmable binding domain while maintaining activity[20,21]. Additionally, the FokI nuclease requires the formation of a dimer to be catalytically active. The specificity of the ZF binding platform was thereby enhanced by requiring two ZFNs to bind in close proximity in order to bring together the FokI subunits and enable DNA cutting activity[16]. The specificity of the ZFN system was further increased by modification of the FokI dimerization interface to create an obligate heterodimer system[22-24].

While ZFNs were a critical advancement in the technology of gene editing, the broader deployment of ZFNs has been modest due to the engineering challenges associated with context-dependent assembly constraints[25-27]. The long-term impact by ZFNs on the field of gene editing cannot be overstated, however. Nearly all of the commonly used approaches today were originally tested using this first generation programmable DNA editing platform, and ZFNs are the first custom nuclease system to be deployed in clinical trials. Oftentimes working in relative obscurity (Fig 4), these innovators developed the core technology many use today for modern gene editing.

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The Genome Unlocked: TALENs

After nearly two decades of pioneering ZFN work, a new programmable DNA binding platform was developed from DNA binding factors identified in the plant bacterial pathogen *Xanthomonas*[28,29] (Fig 1B). The Transcription Activator-Like Effector (TALE) domains consist of a series of modular 33-35 amino acid repeats, each repeat binding a single specific DNA base[28,30]. Two hyper-variable residues central to the repeat determine the binding specificity of each repeat[28,31]. These Repeat Variable Di-residues (RVDs) are flanked on either side by constant amino acid sequences. The 33-35 amino acid repeats can be stitched together to form a long polypeptide capable of binding long stretches of DNA in excess of 20 bases[32]. TALEs represent a second-generation programmable DNA binding platform.

Based on the prior ZF work, different functional domains were added to the TALE DNA binding system to access the endogenous cellular repair mechanisms (Fig 2B). Nuclease activity is conferred via the fusion of the TALE domain to monomers of the FokI endonuclease creating the TALE Nuclease (TALEN)[3,29]. A pair of TALEN arms is needed to target the cut site and proximate the FokI monomers[3,29]. Therefore, a targeted TALEN, made up of two arms and a spacer region between them, can be designed to recognize over 40 bases of DNA[29]. As a result of both this high base pair recognition count and the high innate specificity of each TALE repeat module, the commonly deployed TALEN systems exhibit high intrinsic binding accuracy with low off-targeting profiles [33-35].

Another important difference between the TALEN and ZFN systems is the TALENs' lack of any known context-specific assembly constraints[4,36]. No RVD combinations have yet been identified as complicating either binding ability or specificity, and the targeting constraints of the TALEN system are also very few. The only regular constraint is the common inclusion of a 5' T recognition motif in front of each TALEN arm for enhanced binding affinity; although this sequence requirement has been eliminated using next generation TALE scaffolds[37]. In addition, some reports include a differential binding by TALENs in a region of DNA containing a methylated CpG island[38,39]. TALENs have also proven to be far more easily assembled than ZFNs, with several published techniques for high-throughput assembly of TALEN that use as little as a single tube for synthesis[29,39-42]. TALEs have the fewest restrictions imposed on the design of the DNA binding system and thus represent the single-most programmable DNA platform to date. The increase in facility of use can be seen in the increase in gene editing publications in the wake of TALEN development (Fig 4). However, the requirement for the synthesis of two custom proteins for each experimental TALEN setup represents a barrier to the use of TALEs in many labs, especially to those with limited experience in genome engineering.

Gene Editing Democratized: CRISPR

Gene editing went mainstream when a naturally occurring ribonucleoprotein (RNP) complex was discovered to function as a new class of DNA recognition domain. In this system, a common protein binds to a short guiding RNA that is used to target the resulting RNP to the specific region in the genome (Fig 1C). Initially identified as an integral component of many bacterial immune systems, the first characterized CRISPR protein (Cas9) was shown to function endogenously to target and degrade invasive phage or plasmid DNA[43-47]. This

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defense system functions via the storage of previously encountered viral sequences within the bacterial genome in the form of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs)[43,44,48,49]. The viral DNA is coded in the spacers while the repeats themselves serve as regulation and processing domains[48,50,51]. These spacers are then transcribed along with the accompanying repeats and processed into individual CRISPR RNAs (crRNAs) that, together with a constitutive Trans-Activating CRISPR RNA (tracrRNA), bind to a Cas9 endonuclease[50,52,53]. The crRNA sequence then acts as the guide for the endonuclease, directing it to the complementary foreign DNA sequence, or protospacer. Upon matching its sequence via standard Watson Crick base pairing[35,54], the Cas9 induces a DSB[50-52,55,56]. Additionally, binding specificity is also dependent on the presence of a specific three-nucleotide sequence flanking the 3' end of the protospacer known as the protospacer adjacent motif (PAM)[51,57,58]. This sequence is (N)GG (where N is any nucleotide) in the common CRISPR/Cas9 system from *Streptococcus pyogenes* and is determined by protein-DNA interaction[59]. This region is important endogenously as it enables the system to avoid cutting the spacers stored in genomic CRISPR DNA, which do not have the appropriate PAM sequence.

The CRISPR-Cas9 system was studied in relative obscurity for several years until this system was shown to cut double strand DNA after DNA binding[60-66]. One important update that made the Cas9 system highly accessible was the fusion of the crRNAs and tracrRNAs into a single guide RNA (sgRNA). As a result, the only requirement to create a custom DNA interacting complex is to synthesize a single piece of ssRNA co-delivered with Cas9. This simple, two-component system with a single programmable element that can be readily generated by any modern molecular biology laboratory has democratized gene editing.

There are, however, some notable limitations to the targeting specificity of the commonly used CRISPR-Cas9 system[67-70]. This DNA binding and cutting platform can exhibit notable off-target cutting[71]. The mechanism underlying DNA/RNA matching with Cas9 and gRNAs often tolerates mismatches in the interaction especially beyond the first 12 bases immediately adjacent to the PAM (known as the seed region), resulting in a measurable proclivity for non-specific cutting[67,72-74]. Whether this reduced specificity is a significant limitation in a particular gene editing application is highly project-dependent. The search for improvements to CRISPR gene editors is ongoing as exemplified by a 2018 call by the NIH for proposals for improved DNA editing systems and methods to detect off-target effects (https://commonfund.nih.gov/editing/fundingopportunities).

Many potential solutions to the problem of reduced CRISPR-Cas9 specificity have been developed[34,57,70,74,75]. One option mimics the behavior of the TALEN and ZFN systems, fusing FokI monomers to a pair of catalytically inactive (dead) Cas9 proteins[76]. The catalytic inactivation of the Cas9 turns this DNA endonuclease into a simple DNA recognition element[77]. This modification to the system increases specificity by doubling of the binding activity required to create a DSB by the pairing of FokI dimers[78]. Another specificity enhancing option is to use a pair of partially compromised Cas9 proteins designed to create a pair of trans-strand single-stranded DNA nicks in close proximity to one another[79]. While this may reduce the risk of potentially toxic off -target cutting[69], this solution can attenuate gene editing efficiency[79]. A third option for increasing specificity is to

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find or engineer a more specific CRISPR system. Multiple modified Cas9 proteins with enhanced specificity have been reported, with varying levels of success[80,81].

The ease of use and lowered cost of engineering with the CRISPR-Cas9 platform has ushered in the rapid acceleration and expansion of gene editing into many laboratories in academia and industry. The technology is now accessible to the life-science community as a whole and, while not without faults, the CRISPR/Cas9 platform is a major step towards the democratization of gene editing and represents the first easily accessible custom DNA endonuclease for gene editing (Fig 4).

CRISPR systems are common to many bacteria, and this rich family of related proteins is being used to identify new DNA editing platforms such as Cas12a (formerly Cpf1)[82]. The Cas12a family has some features not common to the Cas9 family including the ability to trim its own gRNA and to induce a DSB that has base overhangs (Fig 1D). In contrast to the GG-rich PAM sequence in SpCas9, Cas12a proteins make use of a variety of AT-rich PAM sequences making it easier to target Cas12a in certain areas of the genome. Some Cas12a proteins also appear to have higher innate specificity than many Cas9 proteins. In addition, the generation of the first RNA binding platforms using gRNAs has been reported (C2C2 Cas13)[83,84]. Other potential programmable RNA binding systems have been characterized reminiscent TALEs consisting of a modular series of protein motifs that each binds a single RNA base[85]. The ability to target RNA could establish an entirely new branch of genome engineering.

Associated Biochemical Activities Critical For Precision Gene Editing

Binding DNA is only the first step in gene editing. Following the creation of a DNA lesion, various endogenous repair pathways function to create the actual chemical change in the genome (Fig 2). Gene editing thus revolves around the cell's ability to repair its DNA. The most prominent repair pathway deployed is double-strand break repair, the cellular response to double-strand DNA breaks caused by FokI, Cas9, or Cpf1/Cas12a. Four main categories of DSB repair are used in gene editing and will be discussed below. These mechanisms range in both efficiency and accuracy, and thus possess dynamic mutagenic signatures. The use of different DNA endonuclease platforms can also affect the pathway and the result of DSB repairs[86-91]. While we are focused on double strand break repair, different genomic insults such as a single-strand breaks have also been explored to diversify and expand the gene editing toolkit[92,93]

Deletion: NHEJ

Non-Homologous End Joining (NHEJ) is a prolific response to DSBs that functions to maintain genomic integrity[92,92]. In the process of NHEJ, genomic repair enzymes identify the DSB, following which, either or both DNA strands can be resected or filled in as necessary, usually creating blunt ends. It is then also possible for new bases to be polymerized and incorporated into the DNA sequence. These ends are then ligated to restore continuity to the DNA molecule. The lack of template and the somewhat random resection and polymerization of the DNA ends results in the creation of various length insertions or deletions (indels, Fig 2A). The indels created by this repair can produce variable mutational

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signatures, so NHEJ is commonly used to create frameshifting mutations in the coding region in order to knockout a protein. Alternatively, two distant cis-DSBs can generated to delete the sequence between them, allowing NHEJ delete whole genes (Fig 2B).

There are many benefits to using NHEJ to make indels or create large deletions. First, NHEJ seems to be active across multiple species so most model systems can be edited. There are also no known context restrictions to NHEJ so edits can be made anywhere in the genome. Though NHEJ is most likely to introduce short indels or large deletions it is also possible to make use it for large insertions by introducing blunt ended DNA template[94]. These insertions are, however, subject to the same randomness of repair and the ends of the insertion will most often contain indels.

Large Insertions: Homologous Recombination

Operating in tandem, though normally with less frequency than NHEJ, are several distinct yet interconnected mechanisms of repair that rely on the use of a DNA template homologous to the DNA sequence flanking the DSB[92,95]. Collectively these pathways are referred to as Homology Directed Repair (HDR), with the best-characterized being Homologous Recombination (HR). Following a DSB, HR repair proteins use a template molecule of dsDNA to correct damaged DNA[96]. The donor contains long stretches of DNA sequences (>500 bps) homologous to either side of the break that is used as a template for repair. The result is a newly synthesized stretch of genomic DNA that is identical to the donor molecule (Fig 2C).

While being useful to the cell in repairing DSBs, HR has been utilized to introduce exogenous DNA to the chromosome[97]. This is accomplished by designing a donor molecule with long homology arms that flank the integration cargo. By co-delivering this exogenous template with a custom nuclease it is possible to incorporate the desired cargo into the genomic DNA. Unlike NHEJ, however, HR is usually relatively inefficient and often requires antibiotic selection or an equivalent enrichment to identify chromosomes that contain the newly synthesized sequence, limiting its application.

Small Insertion: Oligo-mediated Homology Directed Repair

To address the shortcomings of HR, a variety homologous donors to act as templates for repair have been explored. The simplest of these templates has been single strand oligodeoxynucleotides (ssODNs) with very short homology arms (< 100 bps, Fig 2D) by a repair pathway often called single strand template repair (SSTR). While the precise mechanism of repair used to integrate the ssODN sequence is still unclear, this process harbors both an HDR signature (precision integration of the template) and NHEJ (indel formation) repair outcomes depending on the model system used. Recent in vitro work has implicated the requirement for Rad51 activity[98-101]. While this method can often lead to higher rates of integration than traditional HR, it is typically deployed for integrating sequences of 50 base pairs or less[102].

The wide-ranging mechanisms of HDR combined with the faithful accuracy of template dependent repair empower the creation of almost any desired DNA change. Unfortunately, the lower relative frequency of this event compared to NHEJ often requires exhaustive trials

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and screens to find the line or cell with the intended change. However, more work is underway to understand what makes a good candidate site for HDR, suggesting that the use of oligo-mediated HDR for gene processing will continue to increase.

Microhomology Mediated End Joining (MMEJ)

Emerging as yet another mechanism of DNA repair to be used for gene editing is a method that shares characteristics with both NHEJ and HDR. Microhomology Mediated End Joining (MMEJ, sometimes called alt-NHEJ or alt-EJ) repairs a DSB by annealing small homologous regions on each side of a DSB, generating a predictable small deletion in between. The precise contextual and cellular conditions that bias a DSB to be repaired via MMEJ rather than classic NHEJ are not fully understood[103,104].

During MMEJ and upon the recognition of the DSB, the 5' strands of both ends of the break are resected leaving overhanging 3' tails on each molecule[105]. These overhangs are then aligned through short regions of homology, leaving unmatched "flaps" of DNA at each side of the paired homologous region. The remaining ssDNA regions are used as template for new DNA synthesis while the flaps are cleaved off allowing the nick between the newly synthesized DNA and the homologous region to be ligated resulting in the restoration of continuity within the DNA molecule[106]. The resection of the DNA flaps created by the homologous matching results in the removal of a short stretch of nucleotides central to the original DSB along with one of the original homology arms. This intrinsic deletion pattern unique to regions predisposed to engage in MMEJ activity makes the creation of specific indels a more predictable and reproducible event (Fig 2E).

The enhanced predictability of this repair pathway also makes it uniquely useful for the precise integration of exogenous DNA[88,91,107]. The introduction of a double strand template DNA flanked with short microhomology arms (<100 bp) has been shown to result in precise integrations[108] (Fig 2F). This integration has the potential to be as accurate as repair with classical homologous recombination and, in some cases, as efficient as NHEJ[109]. However, the factors that play a role in efficient integration are not currently understood. For example, it is currently unclear how the balance of NHEJ to MMEJ plays a role in the proclivity to integrate a donor molecule using this method. It should here be noted that while the mechanism by which MMEJ proceeds is not yet fully understood, some attempts at characterization have demonstrated that some repair enzymes show preferential activity in different repair pathways.

As MMEJ becomes better characterized and more predictable it may prove incredibly useful for its propensity to produce a small subset of predictable mutations as efficiently as NHEJ, but may also be utilized for precise integration of DNA ranging from point mutations to several kilobases long (Fig 2F). This combination of efficiency and accuracy makes MMEJ an especially potent mechanism that may unlock entirely new options for gene editing.

Direct Editing of Single Nucleotides: Base Editors

Another emerging method for precision gene editing avoids double stranded breaks and instead modifies a single DNA base by activating a series of mismatch repair mechanisms capable of creating single nucleotide substitutions. These base editor systems function via

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the fusion of a nucleotide-modifying enzyme such as a deaminase to an existent DNA recognition system such as a catalytically inactivated Cas9 protein[110]. Upon binding their target, the functional enzyme modifies a single DNA base, for example, by deaminating a cytosine to a uracil. Nicking of the complementary strand forces strand excision during repair, resulting in the use of uracil as template, and the net insertion of an adenine. During DNA replication, uracil is read as Thymine resulting it a C to T transition (Fig 2G). To prevent the removal of Uracil before and during replication a UDG (Uracil-DNA Glycosylase) inhibitor is often used. This technique is promising for gene editing as many genetic disorders are caused by missense polymorphisms, and base editing offers a path to repair that does not rely on causing further damage to the DNA via double strand breaks[110-118]. Recently, a new base editor has been generated through enhanced molecular evolution capable of deaminating adenine[119]. While improvements to efficiency and specificity are still being developed for base editing, this strategy has the potential to deliver targeted single substitutions much more efficiently than homology-dependent repair integrations.

Locus-Specific Epigenetic Targeting

In addition to using the available DNA binding platforms to modify the actual nucleotide sequence of DNA, it is possible to modulate how the cell interprets DNA through direct chemical modification or through the creation of artificial transcription factors to modify gene expression (Fig 3). These methods confer many potential advantages as they do not require the formation of a DNA lesion such as a DSB or a mismatched base and that these epigenetic modifications are usually reversible. Three primary methods are used to modulate DNA and gene expression: methylation, acetylation, and programmable transcription factors. Each of these can be accomplished by the fusion of the appropriate functional domain to any of the DNA binding systems referred to in this review.

DNA Methylation (methyltransferases and tet proteins)

DNA methylation and the inverse process of demethylation can be used to physically mark genes and distinguish DNA for a range of cellular readouts [120]. By fusing DNA binding domains to a sequence non-specific methyltransferase, a methyl group can be added to a cytosine nucleotide that is followed by a guanine nucleotide in the 5’ to 3’ direction, commonly referred to as a CpG site[121] (Fig. 3A). Promoter and enhancer regions with high methylation of these sites modulate transcription of the associated genes[122]. This effect can be reversed by utilizing programmable DNA binding platforms fused to demethylase domains such as the ten eleven translocation (TET) enzymes that oxidize methylated cytosines[123]. Both of these approaches have been applied with ZFs, TALEs and CRISPR systems to activate and deactivate locus-specific regions within the genome[120,124-127].

Histone Acetylation

Acetylation and deacetylation is another common chemical method of modifying expression, although it typically acts using a different mechanism compared to DNA methylation[120]. Histone acetylatransferases (HATs) and histone deacetylases (HDACs) target lysine residues on the tale chains of histone proteins, leading to altered charge of the lysine[128,129]. In the case of acetylation, the positive charge of the lysine is neutralized,

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reducing the histones ability to bind the negatively charged backbone of DNA[120]. Therefore, acetylation acts indirectly on the DNA expression both by modulating protein-protein interactions and by increasing the accessibility of the targeted regions, which has been shown to increase the steric favorability of promoter and enhancer binding[130]. Histone deacetylase fused to dCas9 and other programmable DNA binding platforms has been shown to have the opposite effect depending on the cell line used[131]. The lack of consistent outcomes in targeted deacetylase activity is assumed to be the result of deacetylases having non-specific activity, and altering more histones than expected. Determining the specificity of acetylation and deacetylation targeted epigenetic technology is an active research area.

Artificial Transcription Factors

The concept of using programmable DNA binding platforms as guided and programmable transcription factors returns to the natural origins of both zinc fingers and TALEs. Zinc finger domains can be found in the transcription factors of eukaryotes and even archaea[132-134]. TALEs have a similar natural history in that they originated in pathogenic bacteria to activate genes within their plant hosts[135]. Both of these systems can be easily modified to activate or repress genes by the fusion of different transcription regulating domains (Fig 3C). CRISPR systems that have been catalytically inactivated (dCas) can also be used in the creation of artificial transcription factors[136]. The most commonly used activator domain is from VP16, which is a Herpes simplex 1 viral protein[137]. This domain is can be used individually but is more often repeated 4 times creating what is referred to as the VP64 activation domain[138]. In addition to being fused to various DNA recognition elements, KRAB is a commonly used repression domain in synthetic transcription factors and is found on 30% of endogenous zinc finger related proteins[132]. This domain can be utilized to repress genes by targeting it to the promoter or enhancer region of the gene target[139].

History and Current State of Genetic Engineering in Renal Research

Genetic engineering has attracted the attention of Nephrology research for over 20 years (Table 2). Studies as early as the late 1990s showed the potential of gene delivery *in vivo* in a variety of rodent models of kidney disease including hypertension, cardiovascular disease, glomerulonephritis, renal tubular damage, and renal interstitial fibrosis [140,141] Delivery to the kidney has always been an issue, and in these early studies, with genes mainly provided systemically as naked DNA or in viruses by intramuscular, intravenous, intraportal, or intraperitoneal injection. These non-targeted approaches resulted in widespread expression including the kidney, heart, aorta, lung, liver, muscle, serum, and urine, with relatively short-lasting benefits lasting from 1-6 weeks following injection. An early, more targeted approach was injection of genes packaged in liposomes into the renal pelvis, artery, or parenchyma with expression lasting 3 weeks after injection[142]. While showing promise, these studies also showed the challenge of obtaining targeted, high-level, long-term expression in the kidney. Furthermore, the relatively short-lasting effects may indicate instability and lack of integration of the transgene. It should here be noted that transgene integration is likely to cause another set of problems due to potential disruption of endogenous genes.

Over time, studies examining the effects of exogenous gene expression have evolved to include modifiers of endogenous gene expression such as DNA enzymes [143], decoy

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oligonucleotides [144,145], siRNA [146,147], and antisense oligonucleotides (Table 2)[148,149]. Additionally, multiple delivery methods have been explored including hydrodynamic injection into the tail vein [145,150], renal electroporation [143,151] ultrasound with microbubbles [152-154] and expression of transgenes within mesenchymal stem cells [155]. Delivery by injection directly to the kidney has shown benefits in models of diabetic nephropathy, ischemia/reperfusion injury, tubulointerstitial and glomerular fibrosis [144,155-158]. These genome engineering systems and cellular delivery knowledge are being deployed anew with gene editing tools as cargo.

Employing Gene Editing for Gene Inactivation to Understand Kidney Disease Pathogenesis

The development of new tools for gene editing, such as ZFN, TALEN, and CRISPR/Cas9, has enabled new approaches including editing endogenous genes in a variety of cellular and *in vivo* model systems (Table 2). Many studies have applied these technologies to the kidney using renal cell culture [159] and kidney organoids [160]. Additionally, gene editing can target multiple models and is not limited to HR in mouse ES cells. Especially valuable is the ability to generate vertebrate models ranging in complexity from zebrafish through pig. Accordingly, kidney diseases are now being modeled using precision gene editing tools in a variety of organisms including zebrafish [161], tilapia [162], *Xenopus* [163], mouse [164-167], rat [168-170], and pig [171]. These studies have primarily involved truncating mutations induced by NHEJ to produce what are effectively gene knockouts that have proven central to characterizing the roles of genes and proteins in a variety of conditions, such as glomerulonephritis, hypertension, polycystic kidney disease, renal agenesis, and renal fibrosis [161,165,168,169,172,173]. For example, a TALEN-induced mutation of PDE1A demonstrated its role in polycystic kidney disease in mice [166,167], and three groups used CRISPR/Cas9 to validate identification of a novel renal agenesis gene, GREB1L [165,172,173].

Clearly mouse models have contributed innumerable insights into disease pathophysiology, and the value of obtaining consistent results through these studies cannot be overstated. However, there is also great value to the ability to chose the model that best fits the need, Limited studies to date on non-mouse models make it currently unclear which animal models are closest to human. Indeed this may vary according to the specific kidney disease being studied[174]. It is clear, however, from the number of failed clinical trials based on mouse studies, that results often poorly translate from mouse to human. For example, studies testing mTOR inhibitors for PKD showed effectiveness in mouse models but not in clinical trials [175-178]. Rat and pig models may represent more faithful models than mice, making the advent of gene editing in these animals particularly exciting [179]. Use of pigs in particular has the disadvantage of higher cost to raise and maintain lines to achieve genetic homogeneity, which is significant as founders may be mosaics, but the value of this must be considered in comparison to the cost in dollars and human life to that of a failed clinical trial.

Another situation where gene inactivation by CRISPR/Cas9 is potentially valuable for renal health is in generating organs suitable for xenotransplantation. Pigs are a promising source of organs for xenotranplantation provided several obstacles including transmission of porcine endogenous retroviruses (PERVs) can be overcome. Progress has been made on this front using CRISPR/Cas9 inactivation of PERVs in a porcine cell line preventing

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transmission to human cells *in vitro* [180]. Additionally, PERV-inactivated pigs were recently generated using somatic cell nuclear transfer [181]. While several challenges remain such as immunological issues, these studies illustrate the essential contributions of gene editing to this effort [182].

Employing Gene Editing for Engineering Precise Gene Changes Relevant for Kidney Diseases

TALENs and CRISPRs can be employed to make targeted gene edits in cells and model organisms, even enabling the recapitulation of exact disease-causing sequence variations using targeted integration approaches (Fig. 2). An example relevant to the kidney is provided by a study of uromodulin-associated kidney disease (UAKD) [183], where gene inactivation does not recapitulate the human disease phenotype. This study used CRISPR/Cas9 with HR to re-create in mice the p.C147W mutation found in patients. This new mouse model more faithfully reproduced the human phenotype including progressive kidney disease leading to renal failure, provided insight into pathogenic mechanisms of the mutation i.e., the involvement of ER stress, apoptotic signaling, and decreased autophagy, and showed the therapeutic value in mice of blocking TNF alpha to reduce activated caspase-3 and tubule cell death. A few studies have also successfully combined CRISPR with HR for tagging edited genes in *Xenopus* and renal cell culture [159,163]. These tagged proteins allow cellular and subcellular expression and protein trafficking to be better defined by examining the endogenous, albeit modified, protein. This provides an alternative, and potentially more useful approach than standard techniques, such as *in situ* hybridization and antibody labeling.

The Promise and Challenges of Gene Editing for Targeted Treatments in Nephrology

New technologies for precision genome editing in somatic cells are potentially valuable as therapeutics. Recent studies using CRISPR/Cas9 demonstrate proof of principle as a therapeutic approach in a mouse model of Duchenne muscular dystrophy (DMD). In these studies, CRISPR/Cas9 was used to induce exon skipping to avoid a truncating mutation and partially restore dystrophin expression, building on an approach used in a previous clinical trial using morpholino oligomers [184-187]. Possible approaches in Nephrology could be correction of base pair variants in monogenic disease where these types of mutation are common and where a small amount of functional protein may be valuable. In addition, in diseases where gene dosage appears important and a normal allele is present, such as in autosomal dominant polycystic kidney disease (ADPKD), up regulation of the normal copy of the gene may be advantageous.

At present there are questions about the precision of the gene editing and off target effects, which need to be addressed before widespread human treatment is likely. In addition, the extent to which targeted delivery to the kidney is possible is unclear, although, adenovirus, which was used for the DMD studies, has been used previously to deliver genes to the kidney (as well as the liver, aorta and adrenal gland) following injection into the tail vein in rats [188].

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The Real Estate Problem for *In Vivo* Genome Editing in the Kidney.

Despite the promise, there are multiple issues to address to precisely and efficiently perform genome editing in the kidney. These include, effects of pharmacology, vector tropism, and immune responses against vectors. These problems are made worse for renal disease for the simple fact that the kidney is a very effective filter, and it's effective at preventing vectors delivered in the blood from accessing the kidney.

In addition, the liver and spleen are usually the first organ destinations for most injected particles, leaving little to reach the kidneys. Then, the diameter of the afferent arteriole that feeds the glomerulus is a simple and effective barrier to kidney vector delivery. The arteriole is approximately 10 nm in diameter. In contrast, most viral and non-viral gene delivery vectors are 20 to 200 nm in diameter. If a vector can squeeze through the arteriole, it then confronts the stringent molecular weight cut-off of the glomerulus. Only proteins below 50 kDa are thought to readily pass through this barrier, while most gene therapy vectors are megaDa is size.

For example, adeno-associated virus (AAV) vectors are by far the most popular vectors used for *in vivo* gene therapy and are only 25 nm in diameter (reviewed in [189]). While these small viral vectors can extravasate into other tissues well after intravenous injection to mediate impressive transduction of multiple tissues in mice [146,190], most do a poor job of transducing kidney cells [191]. Rare CD31+ endothelial cells and EpCAM+ proximal tubules can be transduced using this approach, but podocytes are not modified. In no cases does one observe saturation of kidney cells after intravenous injection.

While the favorite intravenous route of vector delivery fails for kidney gene therapy, recent developments suggest additional options are forthcoming. For example, delivering non-viral, AAV, and adenovirus vectors by retro-grade injection in the ureter or directly in the capsule can bypass this delivery problem [192].

The DNA Payload Problem for *In Vivo* Genome Editing in the Kidney.

AAV is popular in part because it is compact. That smallness also has limitations because AAV can also only package 4.5 kilobases (kb) of sequence. This restricted cargo capacity is appropriate for delivering a small gene for renal gene therapy, but it restricts the use of medium to large genes. For example, AAV can easily carry the ~ 3,000 base pair (bp) cDNA for *PKD2*, but it would take three AAV vectors to carry the ~13,000 bp *PKD1* cDNA.

When considering using AAV for renal genome editing *in vivo*, two ZFNs separated by a 2A peptide and furin cleavage sequence with a DNA repair substrate of 750 nucleotides can be squeezed into one AAV vector [193]. A single TALEN expression cassette can easily exceed 3.5 kb, making it impossible to package the two TALENs needed for DNA double strand breaks into one AAV vector [194], let alone both TALENs along with a repair template. CRISPR will require the use of one or two AAVs carrying the sp or saCas9 and gRNA sequences. An additional AAV will likely be needed to carry a repair template restricted to less than 4.7 kb in size. Finally, it is difficult enough to transform many cells with one vector in vivo; delivery approaches needing 2 or 3 vectors *in vivo* may be difficult unless co-delivery is optimized.

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In contrast, adenovirus (Ad) vectors can carry up to 36,000 bp of transgene sequences, reviewed[195]. Most Ad vectors described in the literature are known as first generation Ads (FG-Ads). FG-Ads can carry ~7 kbp of sequence, since these vectors still retain most adenovirus genes and ORFs. As such, FG-Ads still provoke strong immune responses due to leaky expression of Ad proteins in transduced cells [196]. In addition, a similar third generation Ad vector was responsible for the tragic death of Jessie Gelsinger [197]making these tools markedly less appealing when considering them for gene therapy.

However, newer Helper Dependent (HD-Ad) vectors have been designed to address these earlier concerns and are appropriate to use for *in vivo* delivery, particularly if they are confined to the kidney. All adenovirus genes and ORFs are deleted in HD-Ad vectors. This allows sequences as large as 36 kbp to be packaged in the vector. No Ad proteins are produced in HD-Ad vector-transduced cells because all of these viral genes have been removed. Because of this, HD-Ad vectors generate lower Ad T cell responses against transduced cells [198,199]. This reduced immunogenicity enables transgene expression over years [200,201]. For example, in an ongoing study, baboons were treated once with HD-Ads are still expressing the transgene protein more than 7 years later [195].

The Immune Problem for *In Vivo* Genome Editing in the Kidney.

Foreign genes usually need to be expressed for gene therapy or genome editing. Treating a genetic disease and repairing the expression of a missing piece of a protein risks targeting and cellular ablation by the immune system. Many of the current proteins used in gene editing are entirely foreign and may stimulate immune responses against the modified cells even if they are expressed only transiently. Finally, since some of these editing proteins are bacterial in origin from human pathogens, this may be a pre-existing immune barrier. For example, humans already have memory antibody and T cell against Cas9 from *Staphyloccocus pyogenes* (spCas9) and from *Staphylococcus aureus* (saCas9)[202].

Gene Editing is in the Clinic

Despite reservations about human treatments, several CRISPR, TALEN, and Zinc Finger Nuclease trials are ongoing for treatment of such diseases as various forms of cancer (including renal cell carcinoma), beta thalassemia, hemophilia, HIV, mucopolysaccharidosis, and sickle cell disease (clinicaltrials.gov). Several are located in medical centers throughout the US, including some in phase 2. Clearly many hurdles remain, but the implications of recent advances in gene editing for advancing research and medicine in Nephrology holds tremendous promise for advancing medicine.

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Highlights

- Analysis of common genome engineering tools, ZFN, TALEN, and CRISPR systems.
- DNA repair processes leveraged to make edits in the genome, NHEJ, HDR, MMEJ SSTR.
- The deployment and associated challenges of genome engineering in the study of renal disease.

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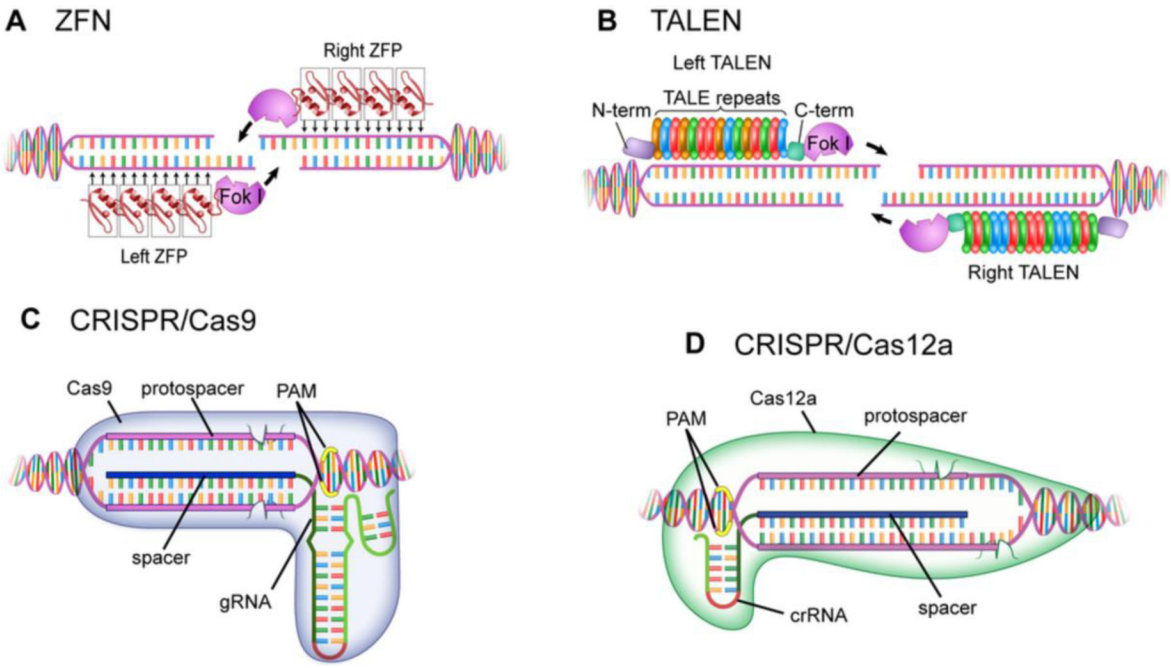

Figure 1. Commonly Used Programmable DNA Platforms:

A diagram showing programmable DNA binding platforms that recognize double-stranded DNA (dsDNA). **A.** A pair of 4-Finger Zinc Finger proteins binding to each side of the desired double-stranded break (DSB) location in the DNA. Each Zinc Finger (ZF) domain binds three bases of DNA; multiple ZF domains can be stringed together to bind longer stretches of DNA. When bound, the attached FokI nuclease (N) dimers become close enough in proximity to activate and catalyze a double stranded DNA break. **B.** A pair of Transcription Activator Like Effector domains (TALEs) bound to each side of the preferred DSB position. TALE domains consist of a series of 35 amino acid repeats attached in sequence. Each of these motifs binds to a single specific DNA base and can be strung together to recognize diverse DNA sequences. **C.** The CRISPR/Cas9 endonuclease system functions through the interaction of a RNA guide with a single protein, Cas9. The RNA guide consists of two domains, a constant poly-hairpin structure that interacts with the Cas9 protein and a programmable guide region that targets DNA through standard Watson-Crick base pairing. Upon binding its target region by interrogating and unwinding (melting) the dsDNA, the Cas9 protein induces a blunt double stranded break. **D.** Another CRISPR system makes use of different class of guide RNA coupled with a different constant protein, Cas12a. Targeting is again determined by Watson-Crick base pairing between the guide RNA and the DNA, following which Cas12a induces a DSB with its signature overhang.

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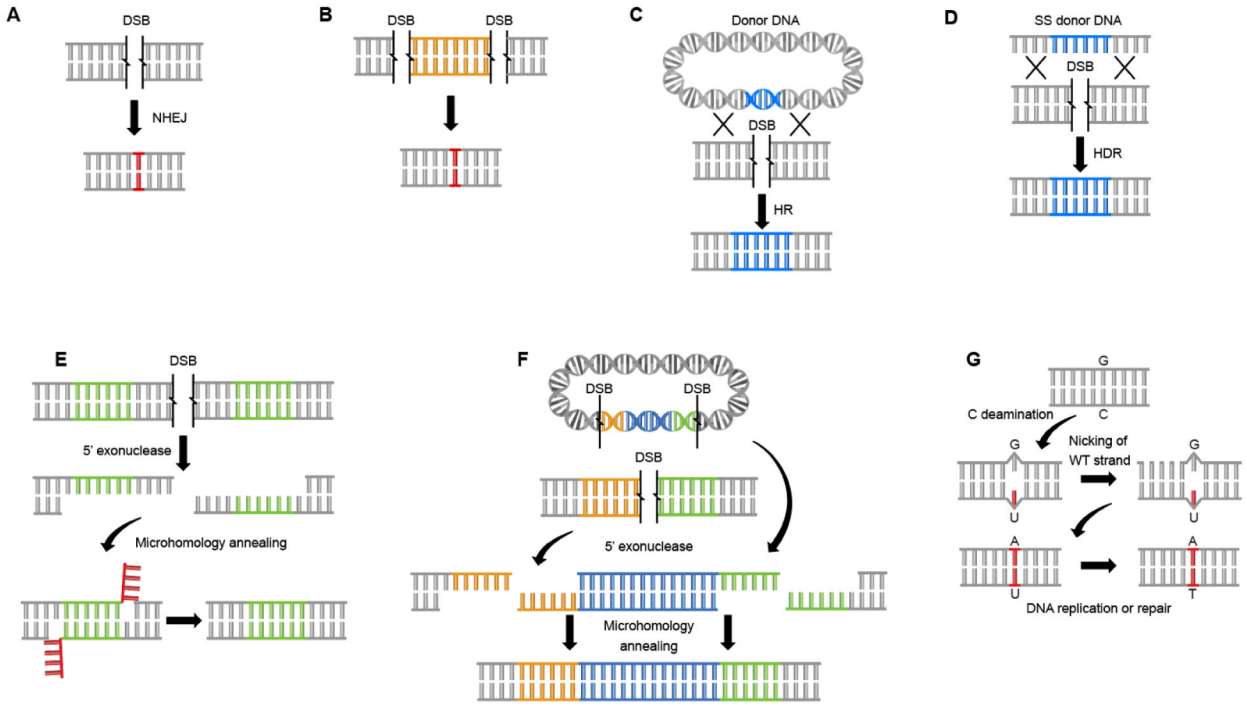

Figure 2. Associated Biochemical Activities Critical For Precision Gene Editing:

Diagram showing the different cellular repair machineries and corresponding biochemical functions critical for gene editing. **A.** Repair of a DSB by Non-Homologous End Joining (NHEJ) introduces mutation through the creation of small insertions or deletions (indels). **B.** NHEJ repair can delete long segments of DNA (whole genes) by creating two double stranded breaks and removing the intervening region from the chromosome **C.** Homologous Recombination (HR) can be used to insert exogenous DNA though the introduction of an exogenous template flanked by large (often >500 base pairs) dsDNA sequences homologous the sequence adjacent to the DSB. **D.** Oligo-directed HDR can introduce small changes through the introduction of a short single-strand DNA (ssDNA) template flanked with homology matching the regions to either side of the DSB. **E.** Micro-Homology Mediated End Joining can be used to create small reproducible deletions. This repair pathway functions by annealing small homologous regions on each side of the DSB. Unbound DNA flaps are removed and ends are ligated resulting in the removal of one homology arm and the intervening region. **F.** MMEJ can also be used to insert exogenous DNA through the introduction of a template with matching microhomology arms. **G.** Single nucleotide polymorphisms can be introduced without a DSB using a base editor. By targeting a cytosine deaminase to a specific target in the genome it is possible to convert a cytosine to a uracil resulting in a mismatch base. This mismatch is then recognized and repaired, generating a single base change depending on which strand is chosen as repair template, this can be selected by design by repeated nicking of the strand to be modified.

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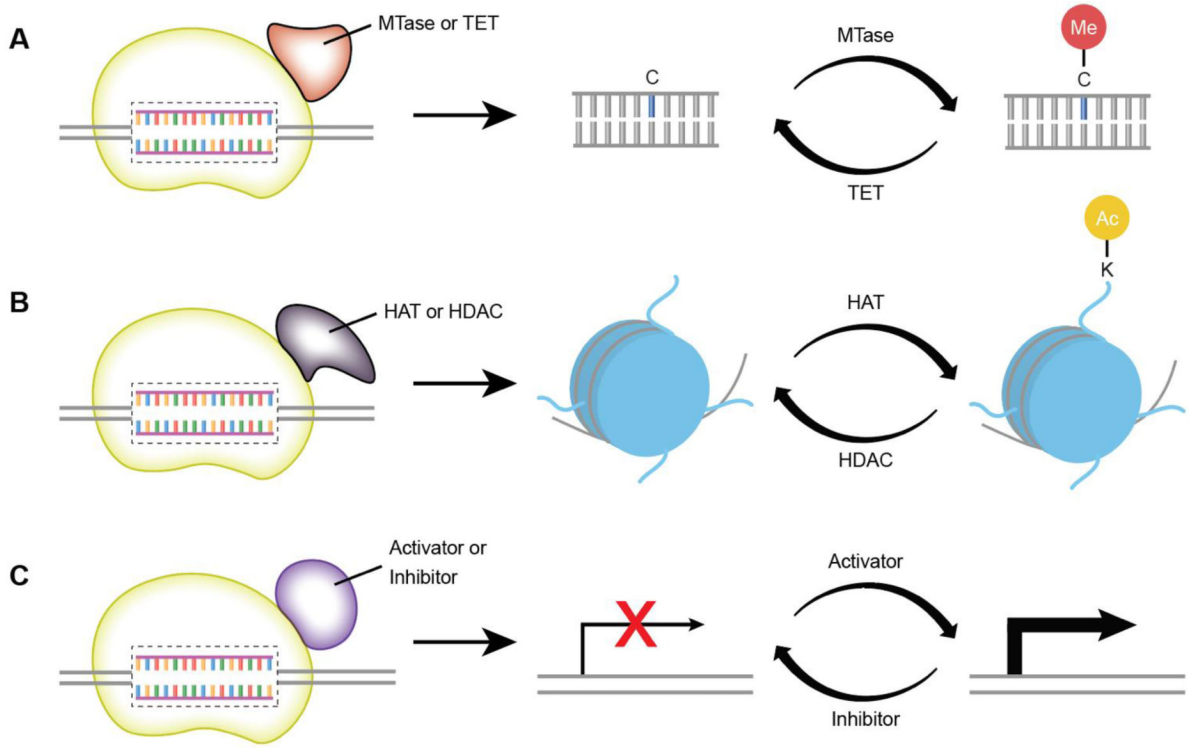

Figure 3. Precision Epigenetic Modulation

A. Programmable methylation of CpG islands in DNA can be achieved by fusion of any of the DNA binding platforms described in this review to a sequence non-specific methyltranferase (MTase) or Trans-eleven Translocation enzyme (TeT) to methylate or demethylate the DNA, respectively **B.** Fusion of a DNA binding system to histone acetyltransferases (HATs) or histone deacetylases (HDACs) enables programmable acetylation or deacetylation of specific lysine residues of the histone proteins associated with the target DNA. **C.** Artificial transcription factors can be created by the fusion of transcription activating or suppressing domains to any DNA binding system. When bound to promoter or enhancer regions, transcription levels of genes can be modulated without any chemical modification to the DNA or associated proteins.

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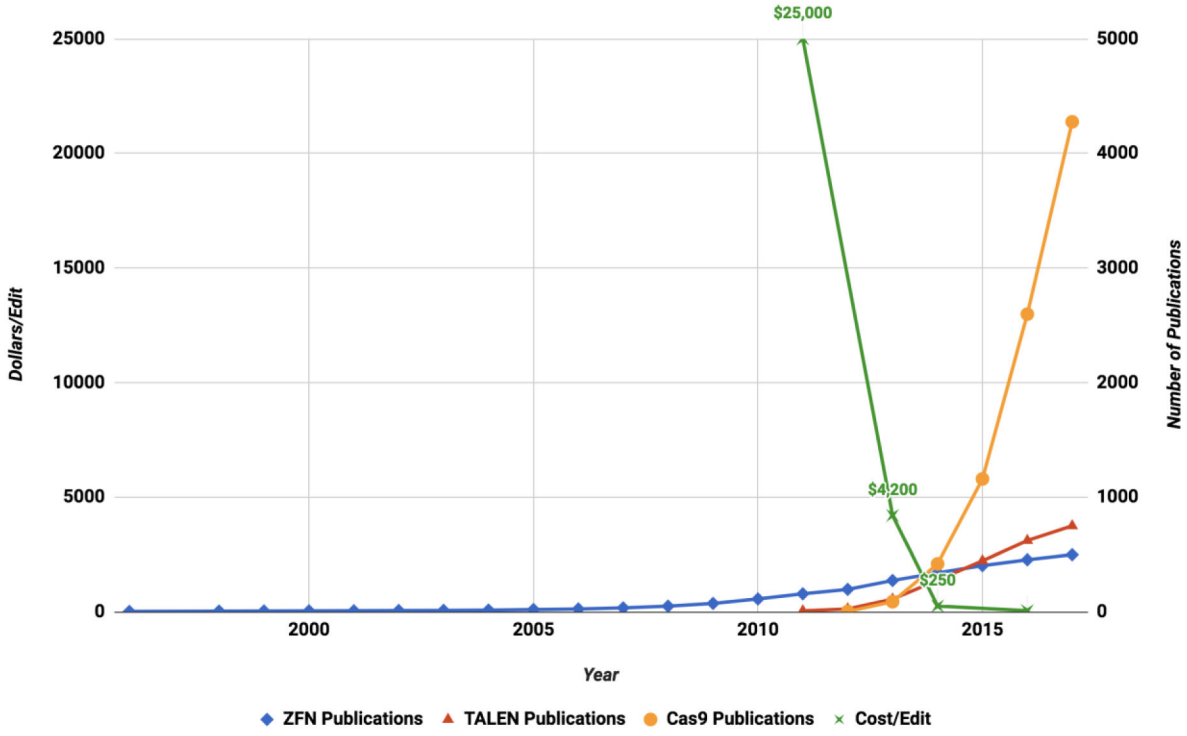

Figure 4. Exponential Reduction in Cost of Gene Editing Tools and Subsequent Rapid Growth of Deployment in Scientific Publications.

Left axis – chart shows representative commercial costs of gene editing tools from ZFNs in 2011 to TALENs in 2013 and CRISPR-Cas9 in 2016.

Right axis -chart shows the number of publications in PubMed using each indicated gene editing platform since 1995. The exponential reduction in cost and the greatly increased access to these new tools is reminiscent of the Moore's Law of continual reduced cost underlying computer technology.

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Table 1.

Major approaches using gene therapy in renal research.

Substance	DNA packaging	Delivery	Disorder	Effect Duration	Source
Decorin gene	cDNA	IM	glomerulonephritis	6 days	Isaka et al 1996
Kallikrein gene	naked or adenovirus	IM, IV, IP, intraportal	hypertension	6 weeks	Chao and Chao 1997
Egr-1 DNA enzyme	NA	ureter+kidney electroporation	UUO tubulointerstitial fibrosis	7 days	Nakamura et al 2002
Smad7 gene	adenovirus	renal pelvis	UUO tubulointerstitial fibrosis	14 days	Terada et al 2002
IL-10 gene	recombinant adenoviral vector	renal parenchyma	glomerulosclerosis and renal failure	20 days	Choi et al 2003
vIL-10 gene	plasmid	tail vein	glomerulonephritis	7 days	Higuchi et al 2003
Smad7	plasmid	renal artery and ultrasound with microbubbles	UUO injury inc renal fibrosis	2 days	Lan et al 2003
AP-1 decoy oligodeoxynucleotides	HVJ-liposome	renal artery	diabetic nephropathy	15 days	Ahn et al 2004
TGFbeta siRNA	plasmid	renal artery	tubulointerstitial fibrosis	14 days	Hwang et al 2006
decoy receptor 3 (DCR3)	NA	tail vein	glomerulonephritis	21 days	Ka et al 2011
ERK2 antisense	adenovirus	perfusion of donor kidneys	renal allograft fibrosis	24 weeks	Ding et al 2011
HGF gene	mesenchymal stem cells	tail vein	UUO-renal fibrosis	14 days	Liu et al 2011
Catalase gene	adenovirus	renal artery	I/R injury	ND	Yang et al 2015
CTGF siRNA	plasmid	medulla+kidney electroporation	renal fibrosis	7 days	Ren et al 2015
AGT antisense oligo	NA	IP	renal fibrosis in PKD2WS25	1 week	Ravichandran et al 2015
Intermedin gene	plasmid	renal artery and ultrasound with microbubbles	UUO injury inc renal fibrosis	7 days	Qiao et al 2015
VEGF gene	mesenchymal stem cells	IV	UUO injury inc renal fibrosis	14 days	Ozbek et al 2015
siRNA cocktail	NA	perfusion of donor kidneys	I/R injury	NA	Zheng et al 2016

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Table 2.

Contributions using new gene editors to renal research.

Target	Approach	Model	Disorder	Source
talpid3	ZFN	zebrafish	cystogenesis	Ben et al 2011
RAG1	ZFN	rat	hypertension	Mattson et al 2013
ROMK	ZFN	rat	hypertension	Zhou et al 2013
TGFb1	ZFN	rat	renal fibrosis	Chen et al 2013
HV1	ZFN	rat	hypertension	Jin et al 2014
PLEKHA7	ZFN	rat	hypertension	Endres et al 2014
apol1	CRISPR/Cas9	zebrafish	nephropathy	Anderson et al 2015
HSD11B2	ZFN	rat	hypertension	Mullins et al 2015
PERVs	CRISPR/Cas9	pig kidney cells	transplant	Yang et al 2015
PKD1	ZFN	Pig	ADPKD	He et al 2015
PKD1	CRISPR/Cas9	kidney organoids	cystogenesis	Freedman et al 2015
PKD2	CRISPR/Cas9	kidney organoids	cystogenesis	Freedman et al 2015
PODXL	CRISPR/Cas9	kidney organoids	glomerular disease	Freedman et al 2015
c21orf29/kurly	CRISPR/Cas9+HR	Xenopus	cilia	Jaffe et al 2016
PDE1A	TALEN	mouse	ADPKD	Ye et al 2016
NOX4	ZFN	rat	hypertension	Cowley et al 2016
sec61al2	CRISPR/Cas9	zebrafish	ADTKD	Bolar et al 2016
Tns2DeltaC	CRISPR/Cas9	mouse	glomerulonephritis	Marusugi et al 2016
GREB1L	CRISPR/Cas9	mouse	renal agenesis	Brophy et al 2017
GREB1L	CRISPR/Cas9	mouse	renal agenesis	De Tomasi et al 2017
greb1l	CRISPR/Cas9	zebrafish	renal agenesis	Sanna-Cherchi et al 2017
HOXA5	CRISPR-Cas9-SunTag-DNMT3A	HEK293	DNA methylation	Huang et al 2017
LAMA5	TALEN+HR	mouse IMCD cells	ADPKD	Hofherr et al 2017
miR-210-3p	CRISPR/Cas9	RCC cells	renal cell carcinoma	Yoshino et al 2017
PKD1	CRISPR/Cas9+HR	mouse IMCD cells	ADPKD	Hofherr et al 2017
PKD1	TALEN	MDCK cells	ADPKD	Hofherr et al 2017
PKD2	TALEN	mouse IMCD+MDCK cells	ADPKD	Hofherr et al 2017
PDE1A	TALEN	mouse	ADPKD	Wang et al 2017
wt1	CRISPR/Cas9	tilapia	glomerular development	Jiang et al 2017
UMOD	CRISPR/Cas9	mouse	UAKD	Johnson et al 2017