

# Gene Editing: Powerful New Tools for Nephrology Research and Therapy

Ayano Miyagi, Aiwu Lu, and Benjamin D. Humphreys

Division of Nephrology, Washington University School of Medicine, St. Louis, Missouri

## ABSTRACT

Biologic research is experiencing a transformation brought about by the ability of programmable nucleases to manipulate the genome. In the recently developed CRISPR/Cas system, short RNA sequences guide the endonuclease Cas9 to any location in the genome, causing a DNA double-strand break (DSB). Repair of DSBs allows the introduction of targeted genetic manipulations with high precision. Cas9-mediated gene editing is simple, scalable, and rapid, and it can be applied to virtually any organism. Here, we summarize the development of modern gene editing techniques and the biology of DSB repair on which these techniques are based. We discuss technical points in applying this technology and review its use in model organisms. Finally, we describe prospects for the use of gene editing to treat human genetic diseases. This technology offers tremendous promise for equipping the nephrology research community to better model and ultimately, treat kidney diseases.

*J Am Soc Nephrol* 27: ●●–●●●, 2016. doi: 10.1681/ASN.2016020146

“I’m all for the scissors. I believe more in the scissors than I do in the pencil.” – Truman Capote

Gene editing can be defined as the process of permanently altering cellular DNA sequences to introduce a desired mutation. Although manipulating DNA has formed a foundation for modern biomedical research efforts for the last 40 years, remarkable new approaches are revolutionizing this process. Mutations can now be introduced into the genomic DNA of cells from virtually any species. These advances have been made possible using custom nucleases, proteins that can now be targeted to specific sites in the genome, where they can correct mutations, introduce new DNA, activate or suppress transcription, and perform other functions. Increasingly, these exciting and powerful approaches are affecting clinical applications in addition to basic research.

Early approaches to site-specific genome modification relied on homologous recombination, a powerful method to introduce a sequence of interest to a specific DNA locus. This discovery was the subject of a Nobel Prize to Mario R. Capecchi, Sir Martin J. Evans, and Oliver Smithies in 2007, but it is also a relatively inefficient and time-consuming technique requiring considerable operator experience.<sup>1,2</sup>

Research into cellular repair mechanisms for DNA double-strand breaks (DSBs) underpins newer approaches to gene editing. Cells have two primary repair pathways: homology-directed repair (HDR) and nonhomologous end joining (NHEJ) (Figure 1). We will discuss a third pathway, microhomology-mediated end joining (MMEJ), below. HDR uses homology regions between the exposed ends of a DSB and a donor DNA template to repair the cut region. By contrast, in the NHEJ pathway, the

broken ends of the DSB are ligated back together. Because this process involves processing of the broken ends to generate compatible ends, the repair often results in small deletions or insertions.<sup>3</sup>

The important concept is that, if one can introduce a DSB at a desired locus, then either mode of repair might come into play, leading to a permanent genetic alteration. As a consequence, investigators have searched intensively for methods of inducing DSBs at a precise location.

The early forms of customized nucleases, such as Zinc Finger Nucleases (ZFNs) and transcription activator–like effector nucleases (TALENs), were engineered to bind specific DNA sequences using knowledge of specific DNA recognition mechanisms shared by transcription factors. For example, zinc finger (ZF) domains in eukaryotic transcription factors<sup>4,5</sup> and transcription activator–like effectors (TALENs) from *Xanthomonas* bacteria<sup>6–8</sup> recognize specific DNA sequences. By designing a cDNA to encode tandem repeats of these ZF or TALE modules, synthetic proteins result that target a specific DNA sequence up to 18 bp.<sup>9</sup> By fusing the synthetic ZF or TALE with a nuclease, such as FokI (a

Published online ahead of print. Publication date available at [www.jasn.org](http://www.jasn.org).

**Correspondence:** Dr. Benjamin D. Humphreys, Campus Box 8126, 600 South Euclid Avenue, St. Louis, MO 63110. Email: [bhumphre@dom.wustl.edu](mailto:bhumphre@dom.wustl.edu)

Copyright © 2016 by the American Society of Nephrology



**Table 1.** CRISPR/Cas9 resources

Resource Name	Description	Web Address
Benchling-crispr	Highly integrated sgRNA design tool that supports $\geq 40$ reference genomes; automatically annotates genome coordinates with the exon and coding DNA sequence	<a href="https://benchling.com/crispr">https://benchling.com/crispr</a>
Optimized CRISPR design, Zhang laboratory, Massachusetts Institute of Technology	Input sequence region of interest sequence and software lists candidate sgRNA sequences with scores on the basis of the number of off targets and positions of mismatches; 16 different species	<a href="http://crispr.mit.edu/">http://crispr.mit.edu/</a>
E-CRISP, Boutros laboratory, German Cancer Research Center	Highly customizable sgRNA calculator including efficiency and possible off-target sites	<a href="http://www.e-crisp.org/E-CRISP/">http://www.e-crisp.org/E-CRISP/</a>
CRISPRdirect, Database Center for Life Sciences, Japan	Search for sgRNAs by gene accession number, genomic location, or sequence; >200 species and sgRNA priority on the basis of the number of off-target sites	<a href="http://crispr.dbcls.jp/">http://crispr.dbcls.jp/</a>
The CRISPR page at CNB	A compendium of landmark articles, background information, and links to other CRISPR resources	<a href="http://wwwuser.cnb.csic.es/~montoliu/CRISPR/">http://wwwuser.cnb.csic.es/~montoliu/CRISPR/</a>
Google Group	A forum for real-time discussion of all topics related to CRISPR/Cas; currently, 2290 topics and 3959 members	<a href="https://groups.google.com/forum/#!forum/crispr">https://groups.google.com/forum/#!forum/crispr</a>
Addgene's CRISPR/Cas9 Plasmids and Resources	Excellent overview of CRISPR/Cas9 and the best place to acquire all plasmids needed for gene editing	<a href="https://www.addgene.org/crispr/">https://www.addgene.org/crispr/</a>

As of May of 2016.

The sgRNA target sequence is typically synthesized as a partially complementary oligonucleotide and cloned into a plasmid that encodes a cassette for expression of the sgRNA and Cas9. The plasmid may also encode either a fluorescent marker or resistance gene, enabling enrichment of transfected cells. The delivery method varies depending on the cell line: standard transfection methods may be sufficient for easy to transfect cell lines, whereas electroporation or viral delivery can be used for difficult to transfect cell lines. The activity of sgRNAs can be tested by several straightforward methods (Table 2).

### BASIC KNOCKOUT AND KNOCK-IN CRISPR/CAS APPLICATIONS

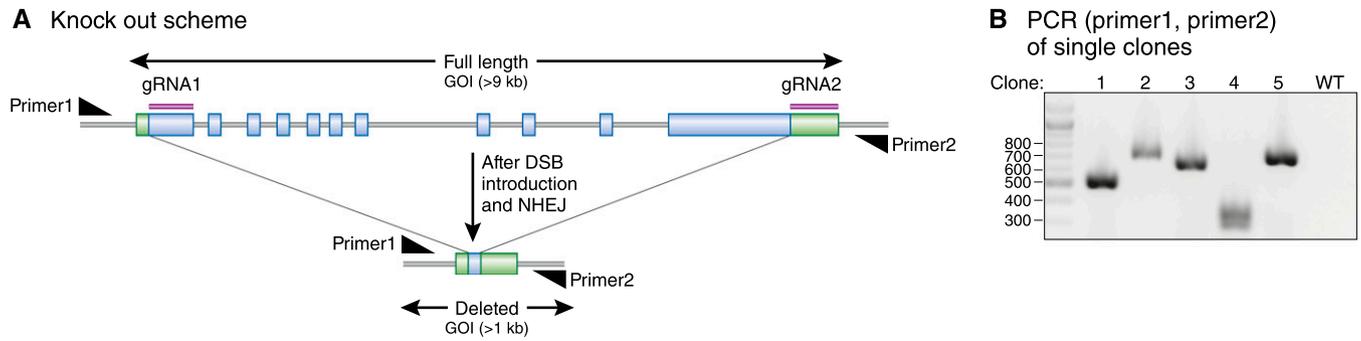
To knock out a gene, an sgRNA can be designed to bind to the 5' end of the coding region. After a DSB is introduced and repaired through NHEJ, small deletions or insertions will be created, many causing frameshift mutations that result in nonsense-mediated decay. Alternatively, two sgRNAs can be designed at the 5' and 3' ends of the gene to delete the whole genic region (Figure 2A). In the former approach, it may be time consuming to screen clones, because DNA from each clone has to be individually

sequenced to identify frameshift mutations. In the latter approach, screening can be carried out efficiently by PCR. For example, Figure 2 depicts one such strategy, in which two sgRNAs flank a long genic region, and the screening primers are located upstream and downstream of the sgRNAs. With a 1-minute extension PCR, deleted alleles yield PCR products, whereas the wild-type allele is too large to amplify (Figure 2).

To introduce specific genetic alterations, such as reporters and disease-specific mutations, knock-in strategies may be used. In this case, an additional DNA template must be provided along with the plasmid-encoding Cas9 and the sgRNA. This additional DNA must have two homology arms and the desired new DNA sequence in between them. Insertion of small epitope tags can be accomplished with single-stranded oligonucleotides using homology arms of about 60 nucleotides. Insertion of a larger sequence, such as green fluorescent protein, requires a plasmid donor with longer homology arms of around 0.8 kb. When this donor DNA with homology arms adjacent to the cut site is present, the HDR pathway may be

**Table 2.** Screening of sgRNA activity

Method	Description
Surveyor or T7E1 mismatch cleavage assay	Uses enzyme to cleave mismatched heteroduplex DNA after denaturation and reannealing; cleaved fragments represent mutated DNA on agarose gel, allowing estimation of efficiency
Tracking of indels by decomposition ( <a href="https://tide.nki.nl/">https://tide.nki.nl/</a> )	Online program that determines the ratio of mutated alleles in a background of wild-type alleles; chromatogram from sequencing of the region around the sgRNA site is uploaded, and the algorithm calculates the ratio of mutated alleles
Deep sequencing	The most sensitive method: it provides not only efficiency of sgRNA edits but also, detailed sequence-based information on the kinds of mutations that each gRNA can generate



**Figure 2.** Schematic illustration of a gene knockout using CRISPR/Cas9 gene editing. (A) Two gRNAs were designed on the coding start region and 3' untranslated region (UTR), respectively, of gene of interest (GOI) to cut out about 9 kb. The light blue rectangles and green rectangles show coding sequences and UTRs, respectively. The screening primers are located outside of the gRNAs. (B) PCR is performed on DNA isolated from individual clones of cells subject to gene editing. The extension time is purposefully brief, so that the 9-kb WT allele cannot be amplified. Different sizes of deleted alleles are detected, because each clone has different deletion lengths near the DSB.

triggered, and the novel sequences located in between the homology arms will be incorporated. Figure 3 shows successful introduction of a 2.6-kb insert using 0.8- to 1-kb homology arms. A donor-specific primer and a primer that anneals outside the region spanned by the homology arms should be used to screen clones with knock-in alleles. Clones with the inserted alleles should give PCR bands, whereas wild-type alleles do not (Figure 3B).

It is best practice to design sgRNAs and donor homology arms in such a manner that the homology arm sequences do not contain the full sgRNA target sequences to ensure that the plasmid donor and repaired locus will not be subject to mutagenic NHEJ outcomes. This can be accomplished by mutating the PAM sequence of the donor plasmid.

### NEW APPLICATIONS OF CRISPR USING MODIFIED CAS9

Although the CRISPR/Cas9 system produces DSBs efficiently at target sites, it can also cause off-target mutations at unintended sites.<sup>20</sup> This is because sgRNAs can tolerate mismatched nucleotides and even noncanonical PAMs. Recent developments, however, have significantly improved targeting specificity. One solution involves a nickase version of Cas9 containing a D10A or N863A mutation to inactivate one of two conserved nuclease domains.<sup>21,22</sup> These

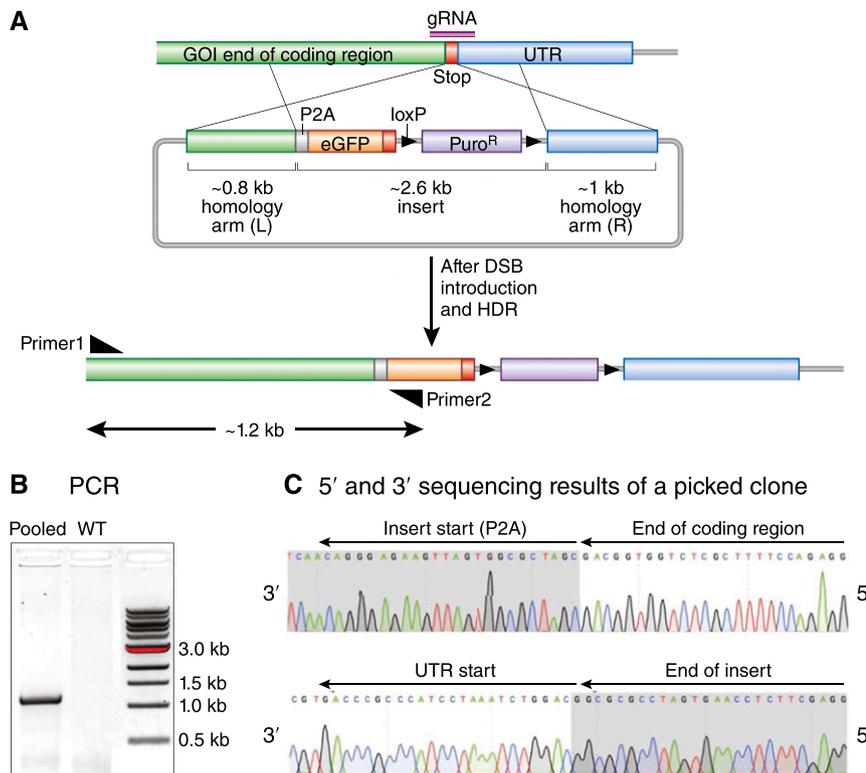
nuclease mutants use two sgRNAs on opposite strands to make paired single-strand breaks. Off-target nicks caused by single nicks are less mutagenic than DSBs, because nicks are repaired with high fidelity.<sup>23</sup> The newest addition is enhanced specificity SpCas9 nucleases, which contains triple mutations to neutralize positive charges in the nontarget strand groove, weakening off-target strand binding. SpCas9 is the simplest solution available and outperforms another recent strategy—the truncated sgRNA strategy, which uses sgRNAs shortened by two or three nucleotides to increase sensitivity to mismatches.<sup>24,25</sup>

CRISPR/Cas9 can also be used to recruit effector domains to turn gene transcription off or on (CRISPRi or CRISPRa). Catalytically dead (dCas9) variants, which lack nuclease activity but retain DNA binding capacity, can repress transcription sterically, either by blocking transcription initiation or elongation, when the sgRNA directs dCas9 binding to a promoter or exon. Transcriptional repression (CRISPRi) can also be achieved by fusing dCas9 to a transcriptional repressor domain, such as *Krüppel-associated box repressor*, and targeting the sgRNA near a transcriptional start site.<sup>26</sup>

Activation of target gene transcription (CRISPRa) is also readily accomplished using dCas9. Fusion of an artificial repeat of the VP64 transcriptional activation domain to dCas9 is

sufficient to recruit RNA polymerase and initiate transcription at a site determined by dCas9 binding (for example, near a transcriptional start site).<sup>27</sup> This system has recently been improved by fusing several different transcriptional activation domains to dCas9 in tandem. A tripartite construct consisting of the activation domains VP64, p65, and Rta, when fused to dCas9, showed 22- to 320-fold greater activation of endogenous target genes. This dCas9-VP64, p65, and Rta approach also allowed multiplexed activation of up to four genes in the same cell simply through introduction of a collection of guide RNAs targeting those four genes.<sup>28</sup> Finally, dCas9 fused to the catalytic domain of the human acetyltransferase p300 has been reported to accomplish gene-specific epigenome editing. The fusion protein catalyzes histone H3 lysine 27 acetylation around genomic sites targeted by sgRNAs.<sup>29</sup>

CRISPR/Cas technology has also been used successfully for large-scale, high-throughput functional screening. For example, by using a lentiviral knockout sgRNA library, a group has identified genes essential for the intoxication of cells by anthrax and diphtheria toxins.<sup>30</sup> Their method involved lentiviral infection of human cultured cells with the sgRNA library to establish library cell lines followed by toxin screening. Surviving cells were then analyzed for their individual sgRNA sequence by deep sequencing, thereby



**Figure 3.** Illustration of a C-terminal P2A-eGFP-flxed puromycin expression cassette knock-in for gene of interest (GOI) by CRISPR/Cas9 editing. (A) A gRNA was designed to overlap with the stop codon of a GOI, and the donor vector contained homology arms of 800–1000 bp. After HDR, a P2A-eGFP-flxed puromycin expression cassette will be inserted in frame just after the coding region and before the stop codon. Screening primer 1 was designed outside of the left homology arm to prevent detection of the donor plasmid. The insert-specific primer 2 was designed on eGFP. (B) Results of pooled genomic PCR from mouse embryonic stem cells that detects the insertion of the desired sequence into the genome. (C) Sequence results from a single clone indicate precise integration of the desired sequence into the genome. Only the results of the 5' sequence are shown. UTR, untranslated region.

identifying the gene knockout that protected the cell from death by either anthrax or diphtheria.

## GENOME ENGINEERING IN MODEL ORGANISMS

CRISPR/Cas9 technology can be readily applied to genome modification in model organisms. Genetically modified mice, for example, are used routinely for analysis of gene function, but generating mouse lines with one or multiple targeted mutations remains expensive and time consuming. The fastest timeline for creating a mouse knockout by standard homologous recombination is about 1

year, and this does not include time required to construct the targeting plasmid. With a CRISPR approach, injection of an sgRNA and Cas9 mRNA or protein into the one-cell embryo creates germline knockout mice in one generation—4 weeks.<sup>31</sup> Moreover, multiple genes can be targeted at once, which is impossible using standard approaches.<sup>32</sup>

One-step generation of mouse knock-ins is also possible, but it is less efficient than creating knockouts. Insertion of small epitope tags can be accomplished by coinjecting an sgRNA, Cas9, and single-stranded oligonucleotide containing the epitope tag and homology arms of about 60 nucleotides. Larger insertions, which require plasmid donors with

longer homology arms as described in the previous section, are considerably more challenging to accomplish in a one-step procedure, despite early reports to the contrary.<sup>33</sup> Some investigators have resorted to creating the knock-ins in mouse embryonic stem cells using CRISPR/Cas9 followed by traditional blastocyst injection.

Although gene editing with CRISPR/Cas9 has been described in myriad other model organisms, including rat, zebrafish, frog, monkey, mosquito, pig, plants, and more, there still remains room to improve gene knock-in efficiency and applicability to organisms with a low HDR frequency. An intriguing recent development, called CRISPR/Cas9 precise integration into target chromosome, takes advantage of a separate DNA repair mechanism called MMEJ to facilitate knock-in. In contrast to the conventional HDR, the MMEJ process requires only short homologous sequences of 5–25 bp. This method features a donor plasmid with short homology arms, and the whole template region is flanked by sgRNA target sites. Therefore, Cas9 generates DSBs in both the genomic DNA and the donor vector, exposing the microhomology sequences that are then used for MMEJ-mediated knock-in of the insert.<sup>34</sup> The precise integration into target chromosome system has been reported to have about 2.5-fold higher efficiency than HDR-mediated knock-ins in cultured cells and has been successfully used in silkworm, zebrafish, and frog. It certainly seems to be a promising technique that needs to be tested in mouse embryos.<sup>35</sup> Additional studies into MMEJ repair mechanisms might improve the methodology further to enable precise and efficient knock-in of large insertions in whole-animal applications.

Recent reports also document the first successful application of gene editing approaches to the study of kidney disease. Freedman *et al.*<sup>36</sup> recently reported protocols for the directed differentiation of human pluripotent stem cells (hPSCs) to kidney organoids. Using CRISPR, they knocked out the podocalyxin gene in hPSC.<sup>36</sup> Podocalyxin regulates tight

junctions and is expressed in podocytes in mature kidney. After differentiation of wild-type or podocalyxin knockout hPSC to kidney organoids, Freedman *et al.*<sup>36</sup> reported disappearance of protein tracks of the other podocyte proteins synaptopodin and ZO-1 along with a decrease in the gap width between podocyte-like cells.<sup>36</sup> This proof of principle experiment establishes a novel platform for investigation of kidney genes in hPSCs.

Freedman *et al.*<sup>36</sup> also generated biallelic loss of function mutations in the *polycystic kidney disease 1 (PKD1)* and *PKD2* genes that are responsible for development of human PKD. After differentiation of these lines and wild-type cells into kidney organoids followed by extended culture, Freedman *et al.*<sup>36</sup> observed large cyst-like structures apparently emanating from the tubular compartment in organoids. Although these were uncommon at 6% of organoids, this seems to be a highly promising new approach to model cystogenesis in PKD *in vitro*.

## GENE THERAPY PROTOCOLS USING GENOME EDITING TECHNOLOGIES

One of the fastest growing applications of CRISPR/Cas9 approaches is in gene therapy to cure disease. Sick cell anemia, for example, is caused by a single-nucleotide mutation in the  $\beta$ -globin gene, which encodes a protein subunit of hemoglobin (Hb) found in red blood cells. The mutation does not alter the ability of Hb to carry oxygen, but under hypoxic conditions, it does promote polymerization of intracellular Hb molecules, which in turn, reduces elasticity of the red blood cell and causes shape change called sickling. The now rigid blood cells block small capillaries, causing occlusion and ischemia.<sup>37</sup>

One strategy to cure sick cell anemia is to activate expression of fetal Hb, HbF, which does not require the  $\beta$ -globin subunit and therefore, does not cause sickling. HbF expression is normally downregulated after 6 weeks of age by a

transcriptional repressor encoded by the *BCL11A* gene.<sup>38</sup> A group from the Dana Farber Cancer Institute used a CRISPR/Cas9 to perform saturation mutagenesis on the human enhancer (a noncoding element) that regulates *BCL11A* expression. The group identified sgRNAs that caused complete knockout of *BCL11A*, with subsequent upregulation of HbF expression.<sup>39</sup> This important work provides a framework for therapeutic gene editing in hematopoietic stem cells to restore HbF expression and ameliorate the sickle cell phenotype. The particular enhancer targeted is specific to erythroid elements, thus preserving *BCL11A* expression in nonerythroid tissues and reducing side effects.

Another disease that seems to be amenable to CRISPR-based gene therapy is Duchenne muscular dystrophy (DMD). This muscle disease mainly affects boys who develop progressive skeletal muscle and heart degeneration and die in their mid-20s. DMD is caused by genetic defects in the gene *dystrophin*, a component of the muscle cell cytoskeleton. It has been known for some time that manipulations causing the transcriptional machinery to skip the exon containing the mutation result in truncated dystrophin protein that still functions adequately enough to reverse the muscle pathology.<sup>40</sup>

Three independent groups very recently reported CRISPR strategies to achieve exon skipping in the *dystrophin* gene, resulting in improved muscle function in mouse models of DMD.<sup>41–43</sup> These groups (Olson and coworkers,<sup>41</sup> Gersbach and coworkers,<sup>41</sup> and Wagers and coworkers<sup>43</sup>) all used adeno-associated viruses (AAVs) to deliver the CRISPR/Cas9 components. AAVs are nonintegrating viruses that are safe for human use, and they efficiently transduce skeletal and heart muscle. Investigators designed novel AAVs that encoded the Cas9 endonuclease along with paired sgRNAs that flank the mutated exon. Simple intravenous injection of the custom AAV resulted in efficient excision of the mutant exon, restoring myofiber and cardiomyocyte function. Wagers and coworkers<sup>43</sup> also showed

that excision occurred in muscle stem cells, providing hope that this approach may provide a durable cure if adapted to humans.

## POTENTIAL APPLICATIONS TO KIDNEY DISEASE

Applications of CRISPR-mediated gene editing to the treatment of kidney disease can be envisioned. For example, the shortage of transplantable organs remains a critical roadblock to the treatment of ESRD. Xenotransplantation of porcine kidneys has been considered a viable solution to this problem, but concerns about transmission of porcine endogenous retroviruses (PERVs) to humans has limited progress in this field. PERVs cannot be eliminated by biosecure breeding, because these elements are encoded within the pig genome. Recently, Church and coworkers<sup>44</sup> used a CRISPR approach to eradicate a gene critical to PERV function at 62 independent sites within the genome of a pig cell line during a single round of CRISPR. This result provides a proof of principle that the same approach could be used to create pig embryos with inactivated PERV sequences and subsequently, cloned pigs with kidneys free from retroviruses.

PKD represents another potential application for therapeutic gene editing. Most patients develop kidney failure from small mutations in either the *PKD1* or *PKD2* gene. These types of mutations are amenable to repair by the HDR approach and would only require a relatively short oligonucleotide donor template. Two possible therapeutic approaches might be possible. On the one hand, fibroblasts from an affected patient could be isolated and induced to become pluripotent cells (a standard protocol now). The PKD mutation could be corrected by CRISPR at this point, and the pluripotent cells could then be induced to differentiate into a transplantable kidney, which would have the benefit of being immunologically identical to the patient.<sup>36</sup> Unfortunately, it is not clear when or even if a transplantable kidney can be grown in the laboratory.

An alternative approach would be to deliver Cas9, a gRNA, and the donor template DNA directly to kidney tubular epithelia in patients affected with PKD and correct the gene defect in all of the nephrons of that patient. Here, the major limitation is that there is no clear method to deliver this DNA to the kidney. The AAV approach seems to be the most promising and is already in clinical trials in other organs; however, there is no evidence of any AAV serotype capable of infecting kidney epithelia.

## ETHICAL CONSIDERATIONS

The ability to edit the genome with relative ease has led to an intense debate about whether and how this technology might be used to modify the genomes of human embryos. Although *in utero* or preimplantation genetic screening—for Down syndrome, for example—has existed for years, CRISPR/Cas9 could be used to make changes that are inherited, crossing what some consider an ethical boundary. Some scientists have argued that germline genome editing should be banned because of unpredictable effects on future generations, the impossibility of informed consent, and the possible use of gene editing for nontherapeutic purposes (appearance, for example).<sup>45</sup> Others argue that such a ban would reflect a lack of imagination and lack of faith in society to properly regulate a field with enormous potential benefit to patients suffering from incurable disease.<sup>46</sup>

As a result of these concerns, a group of 18 prominent leaders in the field met in 2015 in Napa, California to initiate a discussion on the scientific, medical, legal, and ethical implications of CRISPR/Cas9 gene editing. The group called for a moratorium on the clinical use of germline gene editing until a diverse group of citizens and stakeholders examines the technology and evaluates its risks and potential benefits.<sup>47</sup>

## CONCLUSIONS

Over just a few years, the scientific community has witnessed the development of a

remarkably powerful, flexible, and exciting new tool for studying and manipulating gene function. We have highlighted the development of gene editing and focused on CRISPR/Cas9-based approaches, currently the most flexible gene manipulation available. These technologies offer the rapid, inexpensive, and efficient targeting of one or multiple genes and are easily accessible for any laboratory environment. These approaches offer powerful new tools to those studying kidney disease and are sure to play an increasingly important role in both kidney investigation and therapeutics in the future.

## ACKNOWLEDGMENTS

The authors thank Beno Freedman for helpful discussions.

Work in the laboratory of B.D.H. is supported by National Institutes of Health/National Institute of Diabetes and Digestive and Kidney Diseases grants DK107274, DK103740, and DK103050; Established Investigator Award EIA14650059 of the American Heart Association; and Biogen Idec.

## DISCLOSURES

None.

## REFERENCES

- Smithies O, Gregg RG, Boggs SS, Koralewski MA, Kucherlapati RS: Insertion of DNA sequences into the human chromosomal beta-globin locus by homologous recombination. *Nature* 317: 230–234, 1985
- Thomas KR, Folger KR, Capecchi MR: High frequency targeting of genes to specific sites in the mammalian genome. *Cell* 44: 419–428, 1986
- Pardo B, Gómez-González B, Aguilera A: DNA repair in mammalian cells: DNA double-strand break repair: How to fix a broken relationship. *Cell Mol Life Sci* 66: 1039–1056, 2009
- Miller J, McLachlan AD, Klug A: Repetitive zinc-binding domains in the protein transcription factor IIIA from *Xenopus oocytes*. *EMBO J* 4: 1609–1614, 1985
- Pavletich NP, Pabo CO: Zinc finger-DNA recognition: Crystal structure of a Zif268-DNA complex at 2.1 Å. *Science* 252: 809–817, 1991
- Boch J, Scholze H, Schornack S, Landgraf A, Hahn S, Kay S, Lahaye T, Nickstadt A, Bonas U: Breaking the code of DNA binding specificity of TAL-type III effectors. *Science* 326: 1509–1512, 2009
- Moscou MJ, Bogdanove AJ: A simple cipher governs DNA recognition by TAL effectors. *Science* 326: 1501, 2009
- Boch J, Bonas U: *Xanthomonas AvrBs3* family-type III effectors: Discovery and function. *Annu Rev Phytopathol* 48: 419–436, 2010
- Liu Q, Segal DJ, Ghiara JB, Barbas CF 3rd: Design of polydactyl zinc-finger proteins for unique addressing within complex genomes. *Proc Natl Acad Sci U S A* 94: 5525–5530, 1997
- Cermak T, Doyle EL, Christian M, Wang L, Zhang Y, Schmidt C, Baller JA, Somia NV, Bogdanove AJ, Voytas DF: Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting. *Nucleic Acids Res* 39: e82, 2011
- Bhakta MS, Henry IM, Ousterout DG, Das KT, Lockwood SH, Meckler JF, Wallen MC, Zykovich A, Yu Y, Leo H, Xu L, Gersbach CA, Segal DJ: Highly active zinc-finger nucleases by extended modular assembly. *Genome Res* 23: 530–538, 2013
- Mak AN, Bradley P, Cernadas RA, Bogdanove AJ, Stoddard BL: The crystal structure of TAL effector PthXo1 bound to its DNA target. *Science* 335: 716–719, 2012
- Barrangou R, Fremaux C, Deveau H, Richards M, Boyaval P, Moineau S, Romero DA, Horvath P: CRISPR provides acquired resistance against viruses in prokaryotes. *Science* 315: 1709–1712, 2007
- Brouns SJ, Jore MM, Lundgren M, Westra ER, Slijkhuis RJ, Snijders AP, Dickman MJ, Makarova KS, Koonin EV, van der Oost J: Small CRISPR RNAs guide antiviral defense in prokaryotes. *Science* 321: 960–964, 2008
- Marraffini LA, Sontheimer EJ: CRISPR interference limits horizontal gene transfer in staphylococci by targeting DNA. *Science* 322: 1843–1845, 2008
- Travis J: Making the cut. *Science* 350: 1456–1457, 2015
- Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E: A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 337: 816–821, 2012
- Gasiunas G, Barrangou R, Horvath P, Siksnys V: Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. *Proc Natl Acad Sci U S A* 109: E2579–E2586, 2012
- Deltcheva E, Chylinski K, Sharma CM, Gonzales K, Chao Y, Pirzada ZA, Eckert MR, Vogel J, Charpentier E: CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. *Nature* 471: 602–607, 2011
- Fu Y, Foden JA, Khayter C, Maeder ML, Reyon D, Joung JK, Sander JD: High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nat Biotechnol* 31: 822–826, 2013

21. Nishimasu H, Ran FA, Hsu PD, Konermann S, Shehata SI, Dohmae N, Ishitani R, Zhang F, Nureki O: Crystal structure of Cas9 in complex with guide RNA and target DNA. *Cell* 156: 935–949, 2014
22. Ran FA, Hsu PD, Lin CY, Gootenberg JS, Konermann S, Trevino AE, Scott DA, Inoue A, Matoba S, Zhang Y, Zhang F: Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. *Cell* 154: 1380–1389, 2013
23. Dianov GL, Hübscher U: Mammalian base excision repair: The forgotten archangel. *Nucleic Acids Res* 41: 3483–3490, 2013
24. Slaymaker IM, Gao L, Zetsche B, Scott DA, Yan WX, Zhang F: Rationally engineered Cas9 nucleases with improved specificity. *Science* 351: 84–88, 2016
25. Fu Y, Sander JD, Reyon D, Cascio VM, Joung JK: Improving CRISPR-Cas nuclease specificity using truncated guide RNAs. *Nat Biotechnol* 32: 279–284, 2014
26. Qi LS, Larson MH, Gilbert LA, Doudna JA, Weissman JS, Arkin AP, Lim WA: Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell* 152: 1173–1183, 2013
27. Maeder ML, Linder SJ, Cascio VM, Fu Y, Ho QH, Joung JK: CRISPR RNA-guided activation of endogenous human genes. *Nat Methods* 10: 977–979, 2013
28. Chavez A, Scheiman J, Vora S, Pruitt BW, Tuttle M, P R Iyer E, Lin S, Kiani S, Guzman CD, Wiegand DJ, Ter-Ovanesyan D, Braff JL, Davidsohn N, Housden BE, Perrimon N, Weiss R, Aach J, Collins JJ, Church GM: Highly efficient Cas9-mediated transcriptional programming. *Nat Methods* 12: 326–328, 2015
29. Hilton IB, D'Ippolito AM, Vockley CM, Thakore PI, Crawford GE, Reddy TE, Gersbach CA: Epigenome editing by a CRISPR-Cas9-based acetyltransferase activates genes from promoters and enhancers. *Nat Biotechnol* 33: 510–517, 2015
30. Zhou Y, Zhu S, Cai C, Yuan P, Li C, Huang Y, Wei W: High-throughput screening of a CRISPR/Cas9 library for functional genomics in human cells. *Nature* 509: 487–491, 2014
31. Inui M, Miyado M, Igarashi M, Tamano M, Kubo A, Yamashita S, Asahara H, Fukami M, Takada S: Rapid generation of mouse models with defined point mutations by the CRISPR/Cas9 system. *Sci Rep* 4: 5396, 2014
32. Wang H, Yang H, Shivalila CS, Dawlaty MM, Cheng AW, Zhang F, Jaenisch R: One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. *Cell* 153: 910–918, 2013
33. Yang H, Wang H, Shivalila CS, Cheng AW, Shi L, Jaenisch R: One-step generation of mice carrying reporter and conditional alleles by CRISPR/Cas-mediated genome engineering. *Cell* 154: 1370–1379, 2013
34. Sakuma T, Nakade S, Sakane Y, Suzuki KT, Yamamoto T: MMEJ-assisted gene knock-in using TALENs and CRISPR-Cas9 with the PITCh systems. *Nat Protoc* 11: 118–133, 2016
35. McVey M, Lee SE: MMEJ repair of double-strand breaks (director's cut): Deleted sequences and alternative endings. *Trends Genet* 24: 529–538, 2008
36. Freedman BS, Brooks CR, Lam AQ, Fu H, Morizane R, Agrawal V, Saad AF, Li MK, Hughes MR, Werff RV, Peters DT, Lu J, Baccei A, Siedlecki AM, Valerius MT, Musunuru K, McNagny KM, Steinman TI, Zhou J, Lerou PH, Bonventre JV: Modelling kidney disease with CRISPR-mutant kidney organoids derived from human pluripotent epiblast spheroids. *Nat Commun* 6: 8715, 2015
37. Frenette PS, Atweh GF: Sickle cell disease: Old discoveries, new concepts, and future promise. *J Clin Invest* 117: 850–858, 2007
38. Bauer DE, Kamran SC, Lessard S, Xu J, Fujiwara Y, Lin C, Shao Z, Canver MC, Smith EC, Pinello L, Sabo PJ, Vierstra J, Voit RA, Yuan GC, Porteus MH, Stamatoyannopoulos JA, Lettre G, Orkin SH: An erythroid enhancer of BCL11A subject to genetic variation determines fetal hemoglobin level. *Science* 342: 253–257, 2013
39. Canver MC, Smith EC, Sher F, Pinello L, Sanjana NE, Shalem O, Chen DD, Schupp PG, Vinjamur DS, Garcia SP, Luc S, Kurita R, Nakamura Y, Fujiwara Y, Maeda T, Yuan GC, Zhang F, Orkin SH, Bauer DE: BCL11A enhancer dissection by Cas9-mediated in situ saturating mutagenesis. *Nature* 527: 192–197, 2015
40. Cirak S, Arechavala-Gomez V, Guglieri M, Feng L, Torelli S, Anthony K, Abbs S, Garralda ME, Bourke J, Wells DJ, Dickson G, Wood MJ, Wilton SD, Straub V, Kole R, Shrewsbury SB, Sewry C, Morgan JE, Bushby K, Muntoni F: Exon skipping and dystrophin restoration in patients with Duchenne muscular dystrophy after systemic phosphorodiamidate morpholino oligomer treatment: An open-label, phase 2, dose-escalation study. *Lancet* 378: 595–605, 2011
41. Long C, Amoasii L, Mireault AA, McAnally JR, Li H, Sanchez-Ortiz E, Bhattacharyya S, Shelton JM, Bassel-Duby R, Olson EN: Postnatal genome editing partially restores dystrophin expression in a mouse model of muscular dystrophy. *Science* 351: 400–403, 2016
42. Nelson CE, Hakim CH, Ousterout DG, Thakore PI, Moreb EA, Castellanos Rivera RM, Madhavan S, Pan X, Ran FA, Yan WX, Asokan A, Zhang F, Duan D, Gersbach CA: In vivo genome editing improves muscle function in a mouse model of Duchenne muscular dystrophy. *Science* 351: 403–407, 2016
43. Tabebordbar M, Zhu K, Cheng JK, Chew WL, Widrick JJ, Yan WX, Maesner C, Wu EY, Xiao R, Ran FA, Cong L, Zhang F, Vandenberghe LH, Church GM, Wagers AJ: In vivo gene editing in dystrophic mouse muscle and muscle stem cells. *Science* 351: 407–411, 2016
44. Yang L, Güell M, Niu D, George H, Lesha E, Grishin D, Aach J, Shrock E, Xu W, Poci J, Cortazio R, Wilkinson RA, Fishman JA, Church G: Genome-wide inactivation of porcine endogenous retroviruses (PERVs). *Science* 350: 1101–1104, 2015
45. Lanphier E, Urnov F, Haecker SE, Werner M, Smolenski J: Don't edit the human germ line. *Nature* 519: 410–411, 2015
46. Check Hayden E: Should you edit your children's genes? *Nature* 530: 402–405, 2016
47. Baltimore D, Berg P, Botchan M, Carroll D, Charo RA, Church G, Corn JE, Daley GQ, Doudna JA, Fenner M, Greely HT, Jinek M, Martin GS, Penhoet E, Puck J, Sternberg SH, Weissman JS, Yamamoto KR: Biotechnology. A prudent path forward for genomic engineering and germline gene modification. *Science* 348: 36–38, 2015