Genome Editing With Precision and Accuracy

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Editing the genetic code of living organisms with word-processing-like capabilities has been a goal of life scientists and engineers for decades. For biomedical applications, changing as little as a single base in the three billion bases of the human genome could potentially cure many disorders, ranging from muscular dystrophy to cystic fibrosis. New genome editing tools have recently been able to develop the "one in a billion" level of specificity, leading to the prospect of new classes of gene and cell therapies. Here I will describe the opportunities and challenges in developing genome editors for biomedical applications.

A cautionary tale

In November 2018, Dr. He Jiankui caught the world by surprise by announcing the birth of two genome edited babies, the so-called "CRISPR twins" (Cyranoski, 2019). It was a momentous step in human evolution: the intentional alteration of the human germline to produce changes that are transferred to the next generation. However, the editing outcomes were not as Dr. He Jiankui expected (Ryder, 2018). The Chinese team injected CRISPR-Cas9 genome editing RNA and proteins into human embryos to modify the *CCR5* gene, which encodes a receptor on the surface of immune cells for human immunodeficiency virus (HIV). Cas9 is a nuclease that can cut DNA, and the location of the cut can be programmed by the sequence of a single-guide RNA (sgRNA). The sgRNAs used by Dr. He targeted two locations in the *CCR5* gene and was designed to generate a deletion of 32 amino acids of the translated CCR5 protein. This delta 32 deletion of the *CCR5* gene abrogates the ability of HIV to infect T cells, and thereby, in theory, would make the treated embryos resistant to HIV. However, genomic analysis of the CRISPR twins indicated that the delta 32 mutation was not achieved, but rather different insertions and deletions of DNA bases were generated in the *CCR5* gene. These insertions and deletions are anticipated to make these twins more susceptible to influenza, with unknown effects regarding their susceptibility to HIV.

While there are many lessons to be learned from this experiment, it is a cautionary tale of using genome editing tools with poor precision. Critiques of the approach indicate suboptimal use of Cas9 nucleases (there are protein-engineered, higher fidelity variants), sgRNAs (other target sequences could have been used) and questionable timing of the intervention (the proteins and RNA could have been introduced at a different stage of embryonic development). The challenges of getting all of these parameters right is a daunting task, and many argue should not even proceed for human embryo editing. However, there is intense activity to attempt to tackle many of these challenges for editing the human body after birth, so-called "somatic genome editing." Below I briefly described current activity to overcome these challenges in obtaining precision and accuracy with genome editing for somatic editing. Four general categories of challenges are shown in **Figure 1** (Mueller et al., 2018).

Challenge 1: On-target nuclease activity

The most common interpretation of "precision" regarding genome editing is the ability to edit the genome at the intended target site, while limiting edits elsewhere within the genome, commonly called "off-target effects." For the CRISPR-Cas9 genome editing systems, Cas9 has been observed to create excessive undesirable mutations (Cradick et al.; Duan et al., 2014; Pattanayak et al., 2013). Now, new methodologies have been developed to controllably introduce genome editing components, such as ribonucleoproteins (RNPs), and strategies to regulate when and where Cas9 is expressed (Chen, Yanhao, Liu, Xiaojian, Zhang, Yongxian, Wang, 2016; Davis et al., 2015; Hemphill et al.). For example, modified Cas9 nucleases can be selectively activated with small molecules to decrease the gene editing time window (Davis et al., 2015).

Further, several groups have modified the nuclease, including engineered "nickase" Cas9 proteins featuring only one active nuclease domain. When used alone, nickases cannot create a full DNA double strand break. However, when two nickases are paired, the resultant break can be repaired via non-

homologous end-joining (NHEJ) within mammalian cells (Ran et al., 2013). While this method lowers off-target effects, the efficiency of genome editing is greatly decreased, as two nickases and two sgRNAs need to be delivered to the nucleus to perform simultaneous cuts. In addition, rational protein engineering approaches to modify the nuclease have also generated high-fidelity variants of Cas9: eSpCas9 (Slaymaker et al., 2016), Cas9-HF1(Kleinstiver et al., 2016), and xCas9 (Hu et al., 2018). Cas9 variants function by decreasing the binding time of the sgRNA to the target sites within the genome, resulting in a decrease in off-target binding and cutting. These high-fidelity Cas9 variants may represent a quick path to clinical relevance as they can greatly reduce off-target events.

Challenge 2: "Scarless" incorporation of new sequences

While the intended on-target gene can be modified using a targeted DNA break, attempting to insert of specific bases, in other words "writing in" sequences into the genomic target site, adds another layer of complexity. DNA repair pathways within the cell dictate whether new nucleic acids can be inserted, and a major pathway used is the homology-directed repair (HDR) pathway. HDR can generate perfect incorporation of the desired sequence without modifying any other bases in the genome, herein termed "scarless editing". Researchers have thus attempted to increase both the overall efficiency of HDR as well as the ratio of precise edits to imprecise mutations. While researchers have attempted to modify DNA repair pathways (Chu et al., 2015; Yu et al., 2015), these methodologies are most applicable for in vitro cell culture applications where potential toxicity is less limiting. For short insertions, single-stranded oligodeoxynucleotide (ssODN) templates hold significant promise for treating disease variants due to their ease of synthesis. However, sequence changes encoded by the ssODN are infrequently incorporated after editing (<10%), and desired edits are typically outnumbered by other sequence outcomes (presumably from NHEJ). Several groups have tried strategies to link the ssODN to Cas9 (Carlson-Stevermer et al., 2017; Lee et al., 2017) which helps increase HDR. Finally, other methods attempt to avoid the use of HDR altogether, and instead leverage other DNA repair pathways (Suzuki et al., 2016).

Base editors are particularly attractive for clinical translation, as they avoid DNA double strand breaks entirely. They employ a catalytically dead version of Cas9 fused to a DNA deaminase to modify existing base pairs in the sequence proximal to the sgRNA target. Base editors deaminate cytidine bases to form uridine. These modified bases are then recognized by the cell as mismatched and corrected to thymidine (Komor et al., 2016). Current work in this area mostly focuses on C > T (or the analogous G > A) conversions, although future versions will aim to allow modifications of any single base (Gaudelli et al., 2017).

Challenge 3: Precise transcriptional control

Even if challenges 1 and 2 above are met with perfect accuracy and precision, expression of edited gene to generate RNA transcripts can vary over time, as well as across cell differentiation and behavior patterns. Misregulation of the edited transcript can compromise therapeutic efficacy or lead to adverse events. Therefore, it is critical to consider strategies to maximize transcriptional control of any edited transcripts, especially when inserting new bases. A striking discovery regarding the necessity for precise transgene expression recently emerged in the Chimeric Antigen Receptor (CAR) T cell therapy field. In the CAR T paradigm, a synthetic CAR transgene targeting a cancer-enriched antigen is inserted into the patient's T cells *ex vivo*, which are then expanded and reinfused, thereby engineering the immune system to recognize and target cells bearing the antigen (Piscopo et al., 2018). One group recently used CRISPR-Cas9 to generate CAR T cells featuring a transgene at the T cell receptor alpha (TRAC) locus, which ensured that CAR expression was regulated by the endogenous TRAC promoter (Eyquem et al., 2017). These CAR T cells demonstrated striking results in a leukemic mouse model, and also displayed fewer biomarkers of dysfunctional CAR T cells, thus suggesting that precise transgene control may yield a more potent cell product.

Challenge 4: Precise editing within specific cells and tissues

Precise delivery of editing components to the right cells and tissues remains an extant challenge within

the field, as many delivery agents suffer from low efficiency, high toxicity, and immunogenicity. Both viral and nonviral delivery agents have been engineered to achieve cell and tissue specificity. Viral constructs can also be engineered to harbor cell and tissue-specific promoters driving expression of the gene editing system (Ran et al.; Swiech et al., 2014), such that editing machinery is not expressed in non-desired cell types. For nonviral strategies, several designs have demonstrated high gene-editing efficiencies when used with RNPs, ranging from 30 to 40% in cell lines, and up to 90% delivery efficiency (Alsaiari et al.; Mout et al.; Sun et al.; Zuris et al., 2015). In addition to increasing the overall efficiency of delivery, custom biomaterials can be engineered to direct genetic payloads to specific tissue types to allow gene editing *in situ*, thereby bypassing many of the biomanufacturing challenges associated with *ex vivo* therapy. Researchers recently developed DNA nanocarriers with the capacity to deliver CAR transgenes to T cells in a leukemic mouse model by coupling anti-CD3 ligands to polyglutamic acid (Smith et al., 2017). These nanocarriers demonstrated specificity to circulating T cells over other blood cell types shortly after delivery, causing tumor regression.

Outlook

It is likely that strategies to meet these challenges will be complementary, ultimately enabling more precise genomic surgery within patients' cells. For *in vitro* applications, drug discovery is likely to be accelerated by enhanced tools for disease modeling, target validation and toxicological studies. Meanwhile, in *ex vivo* uses, precision-engineered cell and tissue therapies could incorporate more functionality from synthetic circuits (Weinberg et al., 2017). Finally, for *in vivo* somatic gene editing applications, injectable viral and nanoparticle strategies could specifically edit stem cells to regenerate tissues and correct disease-causing mutations. Successful strategies to overcome the challenges described above may pave the way for a new wave of transformative therapeutics.

References

Alsaiari, S.K., Patil, S., Alyami, M., Alamoudi, K.O., Aleisa, F.A., Merzaban, J.S., Li, M., and Khashab, N.M. Endosomal Escape and Delivery of CRISPR/Cas9 Genome Editing Machinery Enabled by Nanoscale Zeolitic Imidazolate Framework.

Carlson-Stevermer, J., Abdeen, A.A., Kohlenberg, L., Goedland, M., Molugu, K., Lou, M., and Saha, K. (2017). Assembly of CRISPR ribonucleoproteins with biotinylated oligonucleotides via an RNA aptamer for precise gene editing. Nat Commun *8*, 1711.

Chen, Yanhao, Liu, Xiaojian, Zhang, Yongxian, Wang, H. (2016). A Self-restricted CRISPR System to Reduce Off-target Effects. *24*.

Chu, V.T., Weber, T., Wefers, B., Wurst, W., Sander, S., Rajewsky, K., and Kühn, R. (2015). Increasing the efficiency of homology-directed repair for CrIsPr-Cas9-induced precise gene editing in mammalian cells. Nature Biotechnology *33*.

Cradick, T.J., Fine, E.J., Antico, C.J., and Bao, G. CRISPR/Cas9 systems targeting b-globin and CCR5 genes have substantial off-target activity.

Cyranoski, D. (2019). The CRISPR-baby scandal: what's next for human gene-editing. Nature *566*, 440.

Davis, K.M., Pattanayak, V., Thompson, D.B., Zuris, J.A., and Liu, D.R. (2015). Small molecule-triggered Cas9 protein with improved genome-editing specificity. Nature Chemical Biology.

Duan, J., Lu, G., Xie, Z., Lou, M., Luo, J., Guo, L., and Zhang, Y. (2014). Genome-wide identification of CRISPR/Cas9 off-targets in human genome. Nature Publishing Group *24*.

Eyquem, J., Mansilla-Soto, J., Giavridis, T., van der Stegen, S.J.C., Hamieh, M., Cunanan, K.M., Odak, A., Gönen, M., and Sadelain, M. (2017). Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection. Nature *advance online publication*.

Gaudelli, N.M., Komor, A.C., Rees, H.A., Packer, M.S., Badran, A.H., Bryson, D.I., and Liu, D.R. (2017). Programmable base editing of A•T to G•C in genomic DNA without DNA cleavage. Nature *551*, 464–471.

Hemphill, J., Borchardt, E.K., Brown, K., Asokan, A., and Deiters, A. Optical Control of CRISPR/Cas9 Gene Editing.

Hu, J.H., Miller, S.M., Geurts, M.H., Tang, W., Chen, L., Sun, N., Zeina, C.M., Gao, X., Rees, H.A., Lin, Z., et al. (2018). Evolved Cas9 variants with broad PAM compatibility and high DNA specificity. Nature Publishing Group *556*.

Kleinstiver, B.P., Pattanayak, V., Prew, M.S., Tsai, S.Q., Nguyen, N.T., Zheng, Z., and Joung, J.K. (2016). High-fidelity CRISPR–Cas9 nucleases with no detectable genome-wide off-target effects CRISPR–Cas9 nucleases enable highly efficient genome editing in a wide variety of organisms Alteration of SpCas9 DNA contacts. Nature *529*.

Komor, A.C., Kim, Y.B., Packer, M.S., Zuris, J.A., and Liu, D.R. (2016). Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. Nature *533*, 420–424.

Lee, K., Mackley, V.A., Rao, A., Chong, A.T., Dewitt, M.A., Corn, J.E., and Murthy, N. (2017). Synthetically modified guide RNA and donor DNA are a versatile platform for CRISPR-Cas9 engineering. ELife *6*, e25312.

Mout, R., Ray, M., Tonga, G.Y., Lee, Y.-W., Tay, T., Sasaki, K., and Rotello, V.M. Direct Cytosolic Delivery of CRISPR/Cas9- Ribonucleoprotein for Efficient Gene Editing.

Mueller, K., Carlson-Stevermer, J., and Saha, K. (2018). Increasing the precision of gene editing in vitro, ex vivo, and in vivo. Current Opinion in Biomedical Engineering *7*, 83–90.

Pattanayak, V., Lin, S., Guilinger, J.P., Ma, E., Doudna, J.A., and Liu, D.R. (2013). High-throughput profiling of off-target DNA cleavage reveals RNA-programmed Cas9 nuclease specificity. Nature Biotechnology *31*.

Piscopo, N.J., Mueller, K.P., Das, A., Hematti, P., Murphy, W.L., Palecek, S.P., Capitini, C.M., and Saha, K. (2018). Bioengineering Solutions for Manufacturing Challenges in CAR T Cells. Biotechnology Journal *13*.

Ran, F.A., Cong, L., Yan, W.X., Scott, D.A., Gootenberg, J.S., Kriz, A.J., Zetsche, B., Shalem, O., Wu, X., Makarova, K.S., et al. In vivo genome editing using Staphylococcus aureus Cas9.

Ran, F.A., Hsu, P.D., Lin, C.-Y., Gootenberg, J.S., Konermann, S., Trevino, A.E., Scott, D.A., Inoue, A., Matoba, S., Zhang, Y., et al. (2013). Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. Cell *154*, 1380–1389.

Ryder, S.P. (2018). #CRISPRbabies: Notes on a Scandal. The CRISPR Journal *1*, 355–357.

Slaymaker, I.M., Gao, L., Zetsche, B., Scott, D.A., Yan, W.X., and Zhang, F. (2016). Rationally engineered Cas9 nucleases with improved specificity. Science (New York, N.Y.) *351*, 84–88.

Smith, T.T., Stephan, S.B., Moffett, H.F., Mcknight, L.E., Ji, W., Reiman, D., Bonagofski, E., Wohlfahrt, M.E., Pillai, S.P.S., and Stephan, M.T. (2017). In situ programming of leukaemia-specific T cells using synthetic DNA nanocarriers Designing nanocarriers to achieve CAR expression in T cells.

Sun, W., Ji, W., Hall, J.M., Hu, Q., Wang, C., Beisel, C.L., and Gu, Z. Drug Delivery Hot Paper Self-Assembled DNANanoclews for the Efficient Delivery of CRISPR–Cas9 for Genome Editing.

Suzuki, K., Tsunekawa, Y., Hernandez-Benitez, R., Wu, J., Zhu, J., Kim, E.J., Hatanaka, F., Yamamoto, M., Araoka, T., Li, Z., et al. (2016). In vivo genome editing via CRISPR/Cas9 mediated homology-independent targeted integration. Nature *540*, 144–149.

Swiech, L., Heidenreich, M., Banerjee, A., Habib, N., Li, Y., Trombetta, J., Sur, M., and Zhang, F. (2014). In vivo interrogation of gene function in the mammalian brain using CRISPR-Cas9.

Weinberg, B.H., Hang Pham, N.T., Caraballo, L.D., Lozanoski, T., Engel, A., Bhatia, S., and Wong, W.W. (2017). Large-scale design of robust genetic circuits with multiple inputs and outputs for mammalian cells. Nature Biotechnology *35*.

Yu, C., Liu, Y., Ma, T., Liu, K., Xu, S., Zhang, Y., Liu, H., La Russa, M., Xie, M., Ding, S., et al. (2015). Small molecules enhance crispr genome editing in pluripotent stem cells. Cell Stem Cell *16*, 142–147.

Zuris, J.A., Thompson, D.B., Shu, Y., Guilinger, J.P., Bessen, J.L., Hu, J.H., Maeder, M.L., Joung, J.K., Chen, Z.-Y., and Liu, D.R. (2015). Cationic lipid-mediated delivery of proteins enables efficient protein-based genome editing in vitro and in vivo. Nature Biotechnology *33*, 73–80.

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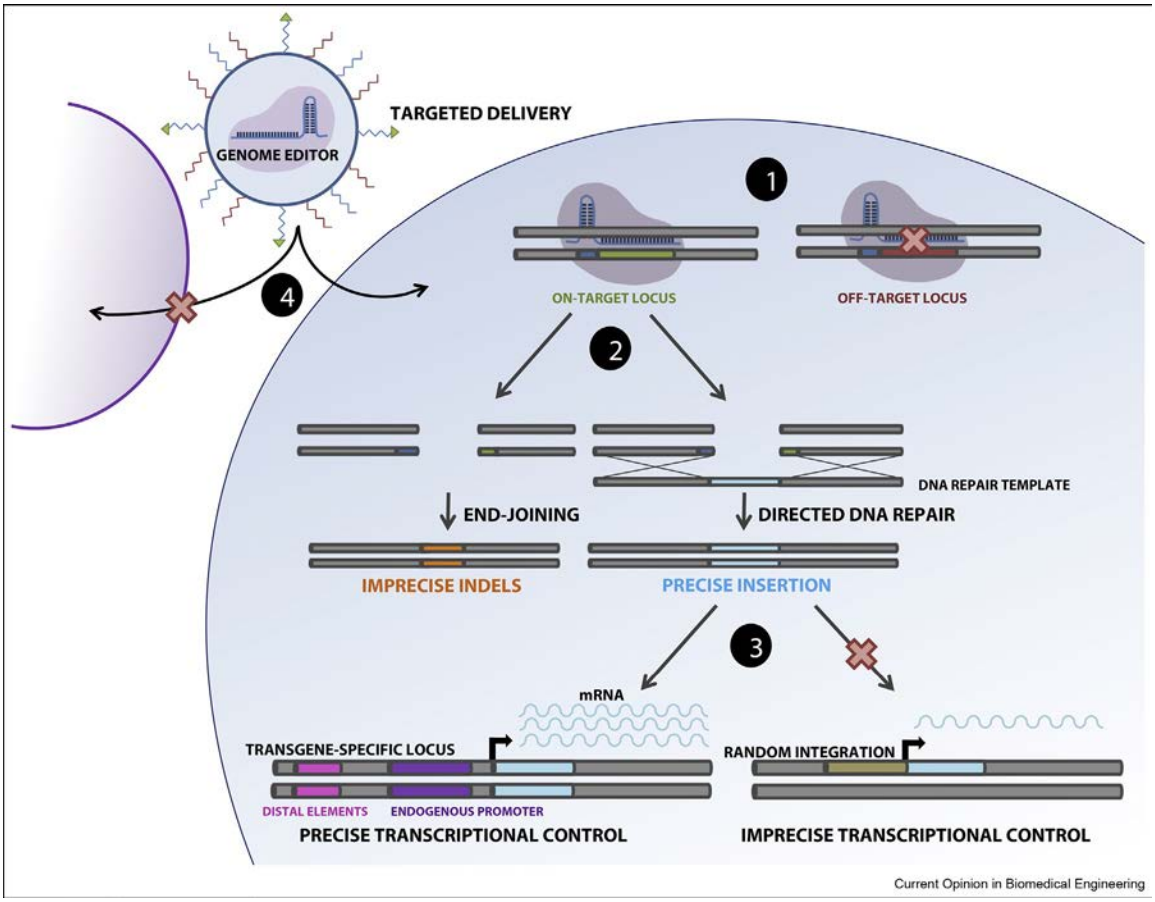

Figure 1. Four different concepts of precision and accuracy in genome editing. Schematic illustrates four ways in which precision and accuracy can be achieved for genome editing therapeutics: (1) the binding of genome editing machinery to the desired target genomic locus, (2) the incorporation of the correct sequence into the edited locus following DNA double strand break formation or after base editing (not shown), (3) precise regulation of integrated transgenes by endogenous promoters and distal elements in comparison to random integration, and (4) delivery to specific cell types by engineered nanomaterials or viral capsids. Reprinted from (Mueller et al., 2018) with permission from Elsevier.