DNA repair and gene editing: the director's cut

Kelly Doughty (Formerly: Solent University Southampton, UK)
Robert A. Baldock (University of Gloucestershire, UK)

Novelists and screenwriters have bombarded our imaginations with the idea of genetic engineering. From superhero origin stories to theme parks inhabited by dinosaurs, the prospect of re-writing the genetic code has inspired many and raised many ethical questions. The potential for these tools in medicine and biological sciences to prevent genetic diseases is readily being explored. Recent successes include destruction of simian immunodeficiency virus DNA from infected rhesus macaque monkeys (synonymous to the human immunodeficiency virus). The diverse power of these tools is also helping to control mosquito populations and supress the spread of malaria. New gene-editing tools have made genome editing faster, more accurate and cheaper than ever before, but how do they work? And how do we know whether the desired edits will be made?

Breaking the DNA

There are several tools that enable scientists a way of altering the genome, including zinc finger nucleases (ZFNs), transcriptional activator-like effector nucleases (TALENs) and the more recent CRISPR (or clustered regularly interspaced short palindromic repeats as it is more reluctantly written). You may be surprised to hear that none of these tools are responsible for editing the genome. Instead, these tools damage DNA at a targeted location. The systems described are essentially enzymes which can be programmed to create double-strand breaks (DSBs) at a defined location within the genome (summarized in Figure 1). It is how the cell tries to repair these breaks that result in edits to the genome (i.e., in many cases incorporating mistakes or using templates to guide repair). Depending on the DNA repair pathways used to fix the breaks, either a knock-out (loss of gene function) or knock-in (an edit in the sequence of a specific gene) will result. Crucially, to avoid unwanted or 'off-target' edits, it is important to understand how this process is controlled.

Gene-editing tools that rely on DSB generation

ZFNs were the first editing tools to be developed; used in pairs, they consist of a DNA binding domain and a DNA cleavage domain. The DNA binding domain determines the location of the cut site using zinc fingers which will then guide the cleavage domain (engineered from the FOKI endonuclease) to cut the target. FOKI only cleaves one strand and must dimerize to generate a DSB.

TALENs use the same principles as ZFNs with the binding domain built using proteins secreted by the bacterium *Xanthomonas*. The proteins, known as transcription activator-like effectors or TALEs, recognize DNA. However, unlike ZFNs, they recognize single nucleotides making TALENs highly specific. Again, the cleavage domain of TALENs also uses the restriction enzyme, FOKI.

CRISPR was developed from a system that occurs naturally in certain bacteria and offers a defence against phages (viruses that target bacteria). The Cas endonuclease is targeted to viral DNA using an RNA guide. DNA is cut adjacent to a trinucleotide motif called a protospacer adjacent motif (PAM). In cutting the DNA, the bacterium blocks the virus's takeover bid. In 2012, Nobel prize-winning scientists Jennifer Doudna and Emmanuelle Charpentier realized that the guide RNA could be used to program the nuclease to create DSBs at specific locations. This technology has enabled scientists to modify the genomes of many organisms. By altering the guide RNA sequence, they can target DSBs to locations within the genome with minimal expense.

This readily adaptable system can be seen in action; DSBs introduced by gene editing can be visualized by microscopy using DNA repair proteins fused to a fluorescent reporter. Figure 2 shows DNA repair protein, RAD51-associated protein 1 (RAD51AP1) fused to a red fluorescent protein, being recruited to a CRISPR-induced break at the LMNA1 gene locus.

How does the cell process DSBs?

When a cell encounters a DSB, either it must be repaired or it will potentially result in the death of the cell. There are several ways in which the cell can respond, often at the expense of genetic change or mutation! In the case of gene editing a genetic change may be the desired

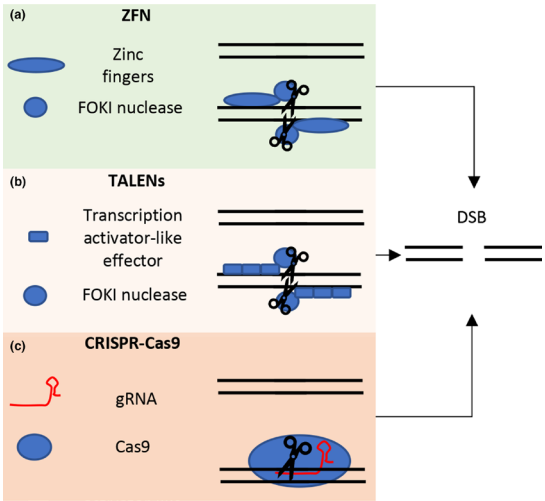

Figure 1. Editing tools that rely on generation of targeted DSBs. ZFNs and TALENs rely on the FOKI nuclease to introduce the break whereas CRISPR relies on Cas9. Black lines represent double-stranded DNA and scissors represent the cutting activity of these enzymes.

response. However, there are several potential changes that can occur after a DSB and because of this, gene editing is far from infallible.

The DSB can be repaired by non-homologous end joining (NHEJ). NHEJ ligates the broken DNA ends back together to repair the DSB. However, processing of the broken ends by nucleases involved in this process can lead to small deletions. This process involves protein Ku and the DNA-dependent protein kinase, DNA-PK, which binds the broken ends and recruits the nuclease Artemis.

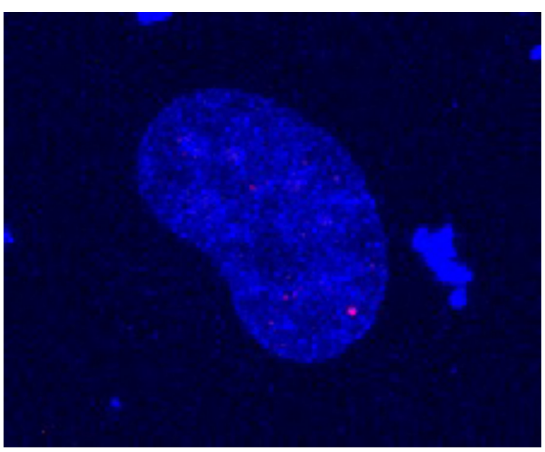

Figure 2. Fluorescently tagged DNA repair protein recruited to a DSB induced using CRISPR-Cas9. Image shows a HT1080 cell nucleus 24 hours after co-transfection with RFP-RAD51AP1 and Cas9 targeted to the LMNA1 gene with an appropriate guide RNA. Credit: Kara A. Bernstein, University of Pittsburgh.

Artemis can trim the ends of the DNA before Ligase IV, XRCC4 and XLF/Cernunnos re-join the broken DNA ends. Critically, the loss of bases would likely lead to a frameshift which results in a non-functional gene (i.e., a gene knock-out).

Alternatively, if the cell is at S or G2 phase (i.e., after having replicated the DNA), homologous recombination (HR) can be used. HR requires resection of the broken DNA ends to reveal a stretch of single-stranded DNA (ssDNA) capable of base pairing with a homologous target. Base pairing with a homologous target creates a displacement loop (D-loop) and genetic information is then copied from the target using the template. If the reverse strand is also captured, a double Holliday junction is formed that can require further endonucleases for resolution. The result is that the repair is largely error-free with no loss of genetic material. This mechanism can be exploited to insert a specific genetic sequence into the genome from a donor template sequence (which would result in a genetic knock-in).

Both DSB repair mechanisms are summarized in Figure 3 and explain how desired genomic edits (either knock-outs or knock-ins) can be introduced. A point of note is that other DSB repair mechanisms can operate, which are variations on the above such as microhomology-mediated end joining (MMEJ) which uses resection of the DNA ends but ligates following pairing of regions of similarity from the resected regions rather than exact homologues – the result of which is a more extensive deletion. Reciprocally, homology-directed repair using synthesis-dependent strand annealing (SDSA) follows the mechanism of HR using a homologous template but does not result in formation of a Holliday junction. Table 1 highlights the mechanisms of DNA repair and the associated types of edits that will be made.

Importantly, the use of one pathway versus another is not always clear – homology-directed repair is largely limited to post-replication S/G2-phase cells, but NHEJ remains the predominant repair mechanism used by cells throughout the cell cycle, at least in mammalian cells. This means that knock-out changes are a more likely outcome of an engineered DSB. This presents a significant challenge to the efficacy of gene editing.

The limitations of DSB-associated gene editing

Despite the significant advances in gene-editing tools such as ZFNs, TALENs and CRISPR, they have limitations, e.g., it is quite costly to design ZFNs and TALENs to cut specific regions of the genome. A major advantage of CRISPR is that the enzyme is readily reprogrammable by the redesign of the guide RNA.

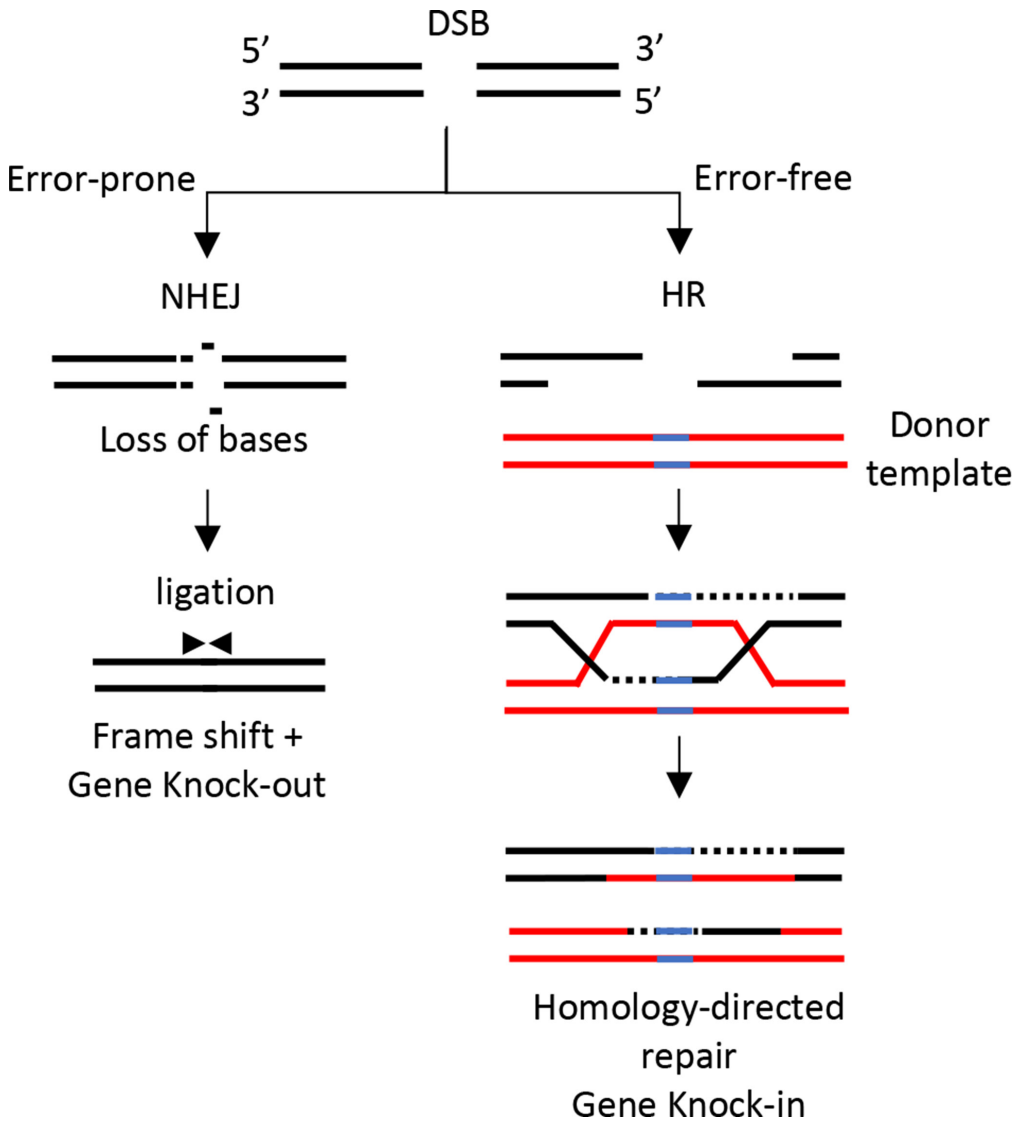

Figure 3. How repair pathway choice dictates the type of gene editing. Black lines represent double-stranded DNA. Broken DNA can be repaired by NHEJ which may lead to a small genetic loss prior to ligation of the DNA ends (likely resulting in a gene knock-out). Alternatively, HR enables the copying of genetic information from a homologous template or donor template during repair (likely resulting in a gene knock-in). Dashed lines show regions of DNA synthesis and blue lines show where changes can be knocked in.

There is a requirement however for a nearby PAM site when using CRISPR, although this varies with different Cas enzymes.

Additional challenges have presented around the prospect of gene-editing-based treatments in humans. In 2019, Charlesworth and colleagues found that human cells can mount an adaptive immune response to Cas9 (the most common DNA cutting enzyme in CRISPR-based editing). The full implications of this have yet to be understood but will likely be an important consideration for any future clinical applications. A second major challenge with this technology is the risk of off-target effects cutting by the enzyme. A recent *Nature* article highlighted three studies demonstrating that CRISPR-Cas9 editing in human embryos can create deletions and rearrangements. In some cases, the deletions were extremely large (several thousand nucleotides) and found close to the intended target. Crucially, the role of DNA repair and the edits or deletions introduced are of key concern as unwanted changes are potentially very dangerous. Defects in DNA repair pathways may lead to unintended gene edits by promoting the use of alternative DNA repair mechanisms. Our research has helped understand which genetic variants in DNA repair coding genes block specific mechanisms of repair such as HR. Alongside influencing cancer susceptibility, these

Table 1. Examples of DSB repair mechanisms and likely gene-editing outcomes

Repair mechanism	Homology requirements	Accuracy of repair	Likely outcome	Processing of DNA ends
NHEJ	None	Error-prone	Knock-out	Minimal processing of DNA ends
MMEJ	Microhomology sequence on both sides of the DSB	Error-prone	Knock-out	Limited resection of DNA ends
HR	HR – limited to S/G2-phase cells (or a donor template)	Error-free	Knock-in	Extensive resection of DNA ends
SDSA	Limited to S/G2-phase cells Template for repair	Error-free	Knock-in	Extensive resection of DNA ends

variants may influence the types of edits that will likely be produced from the repair of DSBs and will likely have implications for future clinical applications.

Editing DNA without DSBs

Based on our understanding of different DNA repair mechanisms, additional methods of editing the genome have been developed that do not rely on introducing potentially damaging DSBs. Instead, they rely on other types of molecular changes to DNA bases that when repaired or tolerated would result in the desired genetic change.

One of these techniques, referred to as prime editing, relies instead on the formation of nicks or single-strand breaks (SSBs) that initiate alternative repair mechanisms. By only breaking one strand of DNA, the risk of larger genomic loss is reduced. Prime editing uses a variant of Cas9 only capable of cutting one of the DNA strands rather than both (referred to as a nickase). This is coupled with a reverse transcriptase (RT) enzyme that converts the RNA template into new DNA (thereby creating the edit). Unlike CRISPR-Cas9 editing, the gRNA is also modified, known as the prime editing guide RNA, which incorporates the guide sequence, a primer binding sequence and template RNA to be reverse transcribed.

Another example is base editors; these exploit the cellular machinery used to repair damaged DNA bases that may become altered either enzymatically or spontaneously in response to cellular stress. Scientists have developed two classes of base editors, cytidine base editors (CBEs) which enable changes from C>T and G>A and adenine base editors (ABEs) which enable changes from A>G and T>C. Using CRISPR-Cas9 to target the base editor, specific regions of the genome can be readily targeted without introducing DSBs.

These techniques are currently in their infancy and a long way from clinical use, but as they do not introduce DSBs, they could offer safer gene-editing options in the future. The potential for gene-editing therapies in medicine is extensive and with further research and development could change many patient outcomes. This technology is not just confined to medical science and could be used in other areas such as farming and agriculture; however significant challenges remain regarding the efficacy and accuracy of any edits made. Crucially, understanding DNA repair pathway choice will be central to predicting or even engineering the correct DNA edits and preventing unintended genetic changes. ■

Further reading

- Mancuso, P., Chen, C., Kaminski, R. et al. (2020). CRISPR based editing of SIV proviral DNA in ART treated non-human primates. *Nat. Commun*. **11**, 6065. DOI: 10.1038/s41467-020-19821-7
- Hammond, A.M. and Galizi, R. (2017). Gene drives to fight malaria: current state and future directions. *Pathog. Glob. Health* **111**, 412–423. DOI: 10.1080/20477724.2018.1438880
- Jinek, M., Chylinski, K. , Fonfara, I. et al. (2012). A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **337**, 816–821. DOI: 10.1126/science.1225829
- Ledford, H. (2020). CRISPR gene editing in human embryos wreaks chromosomal mayhem. *Nature* **583**, 17–18. DOI: 10.1038/d41586-020-01906-4
- Anzalone, A.V., Randolph, P.B., Davis, J.R. et al. (2019). Search-and-replace genome editing without double-strand breaks or donor DNA. *Nature* **576,** 149–157. DOI: 10.1038/s41586-019-1711-4
- Rees, H.A. and Liu, D.R. (2018). Base editing: precision chemistry on the genome and transcriptome of living cells. *Na. Rev. Genet*. **19**, 770–788. DOI: 10.1038/s41576-018-0059-1
- Baldock, R.A., Pressimone, C.A., Baird, J.M. et al. (2019). RAD51D splice variants and cancer-associated mutations reveal XRCC2 interaction to be critical for homologous recombination. *DNA Repair (Amst)* **76**, 99–107. DOI: 10.1016/j.dnarep.2019.02.008

Kelly Doughty is a recent graduate from Solent University achieving a first-class honours in biomedical science. During her degree, Kelly investigated the impact of fluoroquinolone antibiotics on mitochondrial function. She is keen to see how scientific advances will change patient outcomes.

Robert A. Baldock is a Senior Lecturer in Biomedical Sciences at the University of Gloucestershire. His research aims to uncover the mechanisms of DNA repair used in maintaining genomic stability. He sits on the early career research publishing taskforce for Portland Press and on the Biochemical Society's Genes Research Area Panel. Email: rbaldock@glos.ac.uk

