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GENOME EDITING

Enhanced precision and efficiency

The expression of two DNA repair factors improves the recombination of single-stranded oligodeoxynucleotides with Cas9-induced double-strand breaks, facilitating precise and efficient gene editing.

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CRISPR–Cas9 has ushered in an era in which gene editing is likely to become feasible in any cell and organism (1). The mechanisms that broadly govern the system are known: the Cas9 nuclease induces double-strand breaks (DSBs) at targeted sequences, which undergo editing when DSB repair enzymes re-join the ends (Fig. 1). The repair can occur through the non-homologous end joining (NHEJ) pathway, which immediately re-ligates DNA ends in an imprecise way that risks deleting nucleotides, or by homology directed repair (HDR) mechanisms, which enable precise sequence modifications provided that a double-stranded (ds) or single-stranded (ss) DNA repair template is available (2). In cycling cells, sister chromatids are available as natural dsDNA templates for DSB repair. This situation can be emulated by introducing plasmids with homology sequences into cells to serve as artificial repair templates. These substrates are then recombined with DSBs through the classical homologous recombination (HR) pathway (3), which depends on Rad51 and on numerous other proteins. However, HR is normally drawn on exclusively during the S/G2 phases of the cell cycle, whereas the error-prone NHEJ pathway is dominant and active throughout the cell's different stages. The different activity pattern of the two pathways poses an efficiency problem for biomedical gene-editing applications, for which precision when changing DANN sequences is crucial. Attempts to solve this problem involve trying to find ways to shift the bias from the NHEJ pathway towards HDR-mediated mechanisms3. Interestingly, HDR can also occur with synthetic single-stranded oligodeoxynucleotides (ODNs) as templates (4), probably because they mimic natural recombination intermediates. In these cases, ODN sequences are read into the genome in a process called singlestranded template repair (SSTR), which differs from HR and does not depend on Rad51 (Fig. 1). Synthetic ODNs — convenient, comparably inexpensive reagents — can be synthesized with two homology regions of 50–70 nucleotides, up to a limit of ~200 nucleotides. The extent of the practical uses of ODNs in precision gene editing, however, remains unclear because of questions regarding the nature of the recombination mechanism and whether it can be enhanced. Previous efforts for improving this type of editing have focused on optimizing the design of ODNs with regards to the length and symmetry of the homology regions (5,6), as well as on modifications to its structure (7). Yet, the enzymatic machinery involved remains largely unexplored. Reporting in Nature Biomedical Engineering, Derrick Rossi and colleagues now describe how the ectopic expression of Rad52 and the suppression of 53BP1 activity can be an effective means of enhancing the recombination of ODNs with Cas9- induced DSBs (8). In human HEK293 cells and induced pluripotent stem (iPS) cells, these interventions lead to a 2–3-fold increase in ODN recombination with various target genes. Levels reach up to 34% at single genes and enable the editing of up to four additional loci simultaneously. Importantly, the interventions increase on-target recombination rates, yet do not increase or change the specificity of Cas9 towards potential off-target sites. The approach derives from the fact that 53BP1, when present, plays a determining role in driving the DSB repair pathway towards NHEJ rather than HR. 53BP1 binds ubiquitin and methyl modifications on the histone H2A subunit (9), which serves as a recognition signal for damaged chromatin. Based on this information, the authors counteract the activity of 53BP1 at DSBs by ectopically expressing a truncated fragment that occupies the signal sites on H2A and acts as a dominant negative (dn) 53BP1 mutant. Experiments on 53BP1-knockout cells show that dn53BP1 exerts its effect only as a competitor of wild-type 53BP1. The expression of dn53BP1 alone has a beneficial effect on ODN recombination, but its highest benefit is obtained when combined with the expression of Rad52 (but not of Rad51),

Exo1 or BLM. Rossi and co-authors further show that inactivating the endogenous Rad52 gene does not affect ODN-mediated SSTR, which argues that an as-yet unidentified factor exists that is redundant to Rad52. So far, the functions assigned to Rad52 include its ability to promote the DNA-strand exchange between ssDNA and dsDNA and the stimulation of Rad51 activity (10). In yeast, Rad52 is an essential component of HR, whereas mammalian Rad52 affects survival only when other key mammalian HR proteins such as BRCA1 or BRCA2 are missing.

This observation points to a redundant function of Rad52 as an alternative or

backup in the system. By overcoming a main obstacle in the introduction or correction of small DANN segments — for example, to replace diseaseassociated codons or single nucleotide polymorphisms (SNPs) — the work of Rossi and colleagues presents a significant advance in enhancing the precision of gene editing. Rad52 and dn53BP1 can readily be co-expressed under any conditions suitable for the expression of Cas9 and guide RNAs without further manipulation of the cells. Therefore, this approach makes precise gene editing more efficient by eliminating the need for dsDNA templates and by reducing the efforts in the isolation of mutant cells. By combining this approach with multiplex gene editing in iPS cells, patient-derived mutations or SNPs could be introduced into multiple disease-related loci for the study of their epistatic interactions on the progression of disease-related phenotypes in differentiated cells. Another application will be to realise the potential of ODN substrates and precise gene editing for the correction of mutations in somatic gene therapies (11). This approach has already been applied to correct the sicklecell mutation in the β -globin gene in human CD34+ haematopoietic stem cells (12). Since the rate of gene correction is crucial to ensure therapeutic effect, a 2–3-fold enhancement in somatic cells would be a significant advance towards real clinical applications of Cas9- mediated gene therapies. Further investigations of ODN-mediated HDR aimed at achieving a better understanding of the SSTR recombination process will surely guide the development of even more efficient methods. A recent paper (13) clarifies that the Fanconi anaemia (FA) repair pathway mediates ODN recombination in SSTR (this pathway is known for a role in the repair of inter-strand crosslinks and stalled replication forks (14). The researchers performed a CRISPR knockout screen based on an ODN-mediated DSB repair assay to identify genes involved in SSTR. They found mostly core proteins in the FA pathway, alongside factors such as Rad52 that overlap with HR functions. This suggests a focus for future work: what pushes the FA pathway to repair Cas9-induced DSBs, and how do Rad52 and related proteins act as recombination enhancers? Moreover, methods that support how engineered components assemble at the DSB of interest should help optimize SSTR. This has recently been demonstrated in mouse zygotes using biotin-conjugated ODNs together with a Cas9-avidin fusion protein (15). Thus, the results of Rossi and colleagues open a path towards the use of CRISPR–Cas9 as the basis for the next generation of ‘software’ to edit genomic sequences. By making the method more precise and efficient, the technology may become even easier to use.

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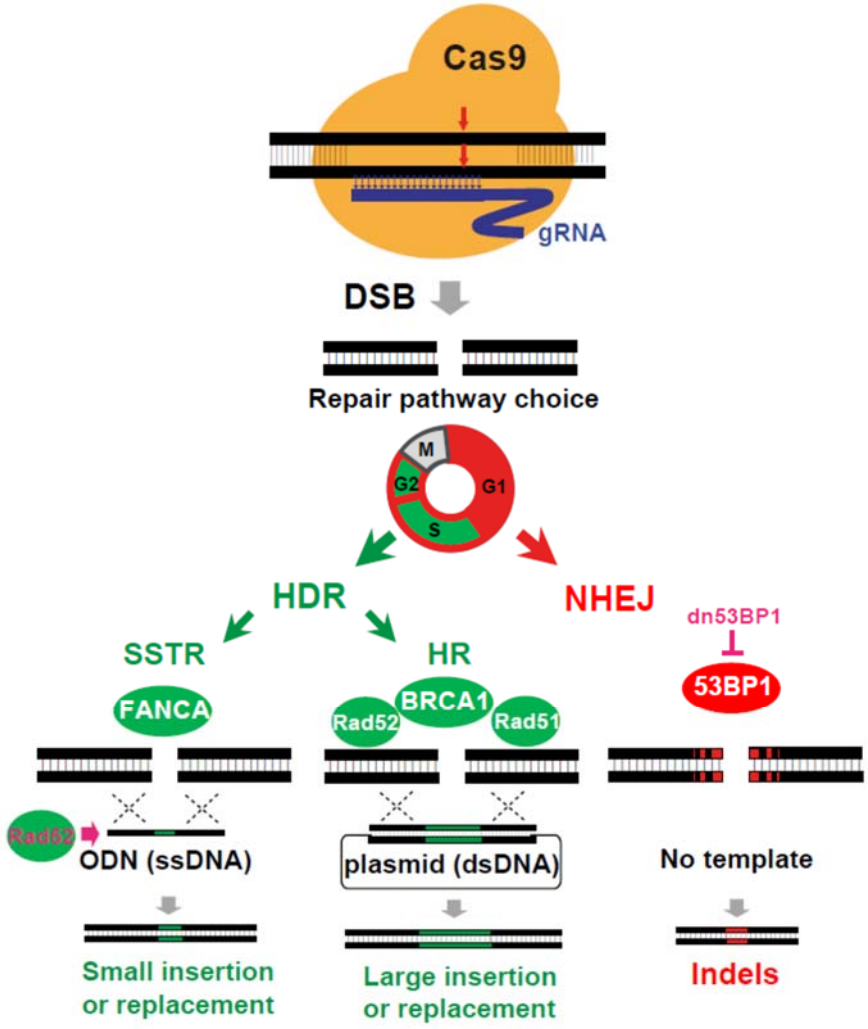

Fig. 1 | Schematic of the DSB-repair pathways used in Cas9-induced genome editing. A double-strand break (DSB) can be induced by Cas9 and a specific guide RNA (gRNA) at a selected target site. The choice of pathways used for DSB repair depends on the cell-cycle phase and on the presence of homologous-repair template molecules. 53BP1 initiates repair via the non-homologous end joining (NHEJ) pathway (bottom right), which is active throughout the cell cycle and re-joins DSB ends without repair templates, frequently leading to the loss or insertion of multiple nucleotides (indels). Precise sequence modifications can be achieved by homology-directed repair (HDR) mechanisms within the S/G2 cycle phases (bottom left), if single-stranded DNA (ssDNA) oligodeoxynucleotides (ODN) or double-stranded DNA (dsDNA) plasmids harbouring homology sequences are available as repair templates. The homologous recombination (HR) pathway, which depends on BRCA1, Rad51 and other proteins, repairs DSBs via recombination with dsDNA templates and enables the insertion or replacement of large sequence segments. SSTR is an alternative, Rad51-independent HDR mechanism mediated by core proteins of the Fanconi anaemia repair pathway (such as FANCA), and accepts synthetic ODNs as templates, thus enabling the insertion or replacement of smallsequence segments flanked by two homology regions of 50–70 nucleotides. The work of Rossi and colleagues

(8) shows that precise gene editing via SSTR and ODNs is enhanced by ectopic expression of Rad52 (purple), together with a dominant negative subfragment of 53BP1 (dn53BP1) counteracting the endogenous 53BP1.