



CRISPR engineering in organoids for gene repair and disease modelling

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Abstract

Organoids bridge the gap between 2D cell lines and *in vivo* studies. With their 3D organization and cellular heterogeneity, adult stem cell-derived organoids closely resemble their tissue of origin. The development of CRISPR-mediated genome engineering and the recent additions of base and prime editing to the CRISPR toolbox have greatly simplified the generation of exact, isogenic models for Mendelian diseases. Here, we review recent developments in CRISPR-mediated genome engineering and its application in human adult-stem-cell-derived organoids in the construction of isogenic disease models. These models allow accurate qualification of the impact of allelic disease variants observed in patients. Furthermore, we discuss the use of organoids as models for safety and efficacy of CRISPR for gene repair. Although transplantation of repaired tissue remains challenging, benchmarking CRISPR tools in organoids can bring genome engineering one step closer to patients.

Sections

Introduction

CRISPR–Cas-mediated genome engineering

DSB-free genome editing


Isogenic organoid disease models

Assessing CRISPR-mediated gene repair

Technical considerations

Outlook

Citation diversity statement

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Key points

- CRISPR–Cas9-mediated genome engineering acts by introducing double-stranded DNA breaks into the genome. The damage repair process can be used for gene knockout or precise targeted introduction of exogenous DNA.
- Next-generation CRISPR tools, including base and prime editing, allow for induction of precise base changes and small insertions and deletions, bypassing potentially deleterious double-stranded DNA breaks.
- Owing to their 3D organization, adult-stem-cell-derived organoids closely resemble the tissue of origin and are therefore a good model system to study human health and disease.
- CRISPR–Cas9-mediated genome engineering can be used to create isogenic models to investigate the onset, cause and treatment of human diseases.
- CRISPR tools can be benchmarked for efficiency and safety by studying gene repair *ex vivo* in adult-stem-cell-derived organoids, facilitating CRISPR–Cas9 clinical translation.
- *Ex vivo* repaired adult-stem-cell-derived organoids can potentially be transplanted into patients to relieve disease phenotypes.

Introduction

Variation in the genome has a fundamental role in the onset, course and outcome of hundreds of human diseases. A combination of the increasing number of genome-wide association studies and the decreasing price of whole-exome and whole-genome sequencing has been driving the identification of genetic disease variants^{1,2}. Although the number of detected genetic variants increases, it remains difficult to accurately qualify their impact on disease progression. To obtain a deeper understanding of the molecular mechanisms that underlie genetic variations and to develop novel therapeutic strategies, isogenic human disease models hold great promise. These models consist of human cells that are engineered to accurately model the genetic variant and are matched with wild-type controls with the same genetic background. The development of clustered regularly interspaced short palindromic repeats (CRISPR) as an effective and versatile tool for genome engineering has greatly advanced the generation of isogenic *in vitro* models of human disease^{3–5}.

Classical 2D tissue culture techniques have been used extensively to better understand the homeostasis and pathophysiology of the human body⁶. 2D cell lines are easy to maintain and are amenable to CRISPR–Cas9-mediated genome engineering. However, such cell lines – typically derived from malignancies – do not reflect the cellular complexity of the organ they are derived from. To overcome these issues, the development of more complex *in vitro* tissue culture techniques has gained traction. These efforts have ultimately led to the development of organoids. These ‘mini-organs’ exhibit faithful micro-anatomy and are grown in a matrix that allows for 3D expansion of stem cells, which give rise to cell types present in the native tissue⁷. Current organoid culturing technology exploits either induced pluripotent stem cells or adult stem cells (ASCs). Organoids derived from

induced pluripotent stem cells are taken through an extensive fate-specialization procedure mimicking the embryonic developmental trajectory of the organ of interest⁸. ASC-derived organoids, the subject of this Review, model the adult homeostatic state of organs. They can be derived from most wild-type human and murine epithelial tissues, including large and small intestine⁹ (Fig. 1a), stomach¹⁰, kidney¹¹, pancreas¹², breast¹³, endometrium¹⁴ and cervix¹⁵, liver^{16,17} (Fig. 1b), upper airway and lung^{18,19}, taste bud²⁰, lacrimal gland²¹, prostate and bladder^{22,23} and thyroid^{24,25} (Fig. 1c,d). ASC-derived organoids do not require an extensive maturation process, are genetically stable and can be passaged indefinitely. Moreover, organoids can be clonally expanded from a single adult stem cell, aiding the generation of CRISPR-mediated isogenic 3D cultures that closely resemble the tissue of origin²⁶. Besides variant impact qualification, these isogenic organoid models can be used for drug efficacy screening and *de novo* drug discovery (Fig. 1d).

In this Review we provide the rationale behind the generation of isogenic disease models in human ASC-derived organoids. First, we review the recent advances in CRISPR-mediated genome engineering that enable efficient induction of mutations and genetic variants in the genome. Then, we discuss the efforts made to use these technologies in organoids for modelling and repair of genetic variants that cause disease. Next, we provide technical considerations to generate genetically altered organoids and create complex isogenic disease models. Finally, we provide an outlook on the combination of CRISPR–Cas9-mediated genome engineering and ASC-derived organoids.

CRISPR–Cas-mediated genome engineering

CRISPR is superior to previously developed strategies aimed at altering the genome (Box 1) and has quickly been adopted by laboratories all over the world. To date, six classes of *Cas* genes have been described, of which the class II CRISPR system (which includes *Cas9*) is the most studied²⁷. In conventional CRISPR–Cas9-mediated genome engineering, the effector protein Cas9 is guided towards a genomic target site by an RNA sequence called the guide RNA (Fig. 2). This guide RNA consists of a CRISPR RNA (crRNA) sequence, complementary to the target site, and a trans-activating CRISPR RNA (tracrRNA) that is needed for crRNA maturation and binding to Cas9. The guide RNA was simplified by creating a chimeric crRNA–tracrRNA fusion, yielding a single-guide RNA (sgRNA) for target recognition³. The target site in the genome consists of two elements, the protospacer and a short essential sequence directly downstream of the target site, called the protospacer-adjacent motif (PAM). The PAM motif of the most frequently used Cas9 (derived from the bacterium *Streptococcus pyogenes*, SpCas9) is NGG³. Upon binding of the sgRNA, the Cas9–sgRNA complex screens the genome for PAMs²⁸. After a suitable PAM is found, sgRNA complementarity to the protospacer is tested by opening the DNA around the target site in an R-loop into two single DNA strands (ssDNA). These ssDNA strands are individually cleaved by the RuvC and HNH domain of Cas9, resulting in a double-stranded DNA break (DSB). The cell recognizes this DSB and has two main pathways to repair the damage. In most cases, non-homologous end joining (NHEJ) is initiated. In this error-prone process, the two DNA ends are quickly ligated together, which often results in a small deletion or insertion (indel) at the cut site²⁹. The CRISPR–Cas9-induced indel, if out-of-frame, results in early termination of the targeted protein. Alternatively, the homology-directed repair (HDR) pathway is initiated by supplementing the reaction with an exogenous DNA repair template that contains homology to the DNA adjacent to the DSB and the edit of interest. Therefore, by hijacking the endogenous

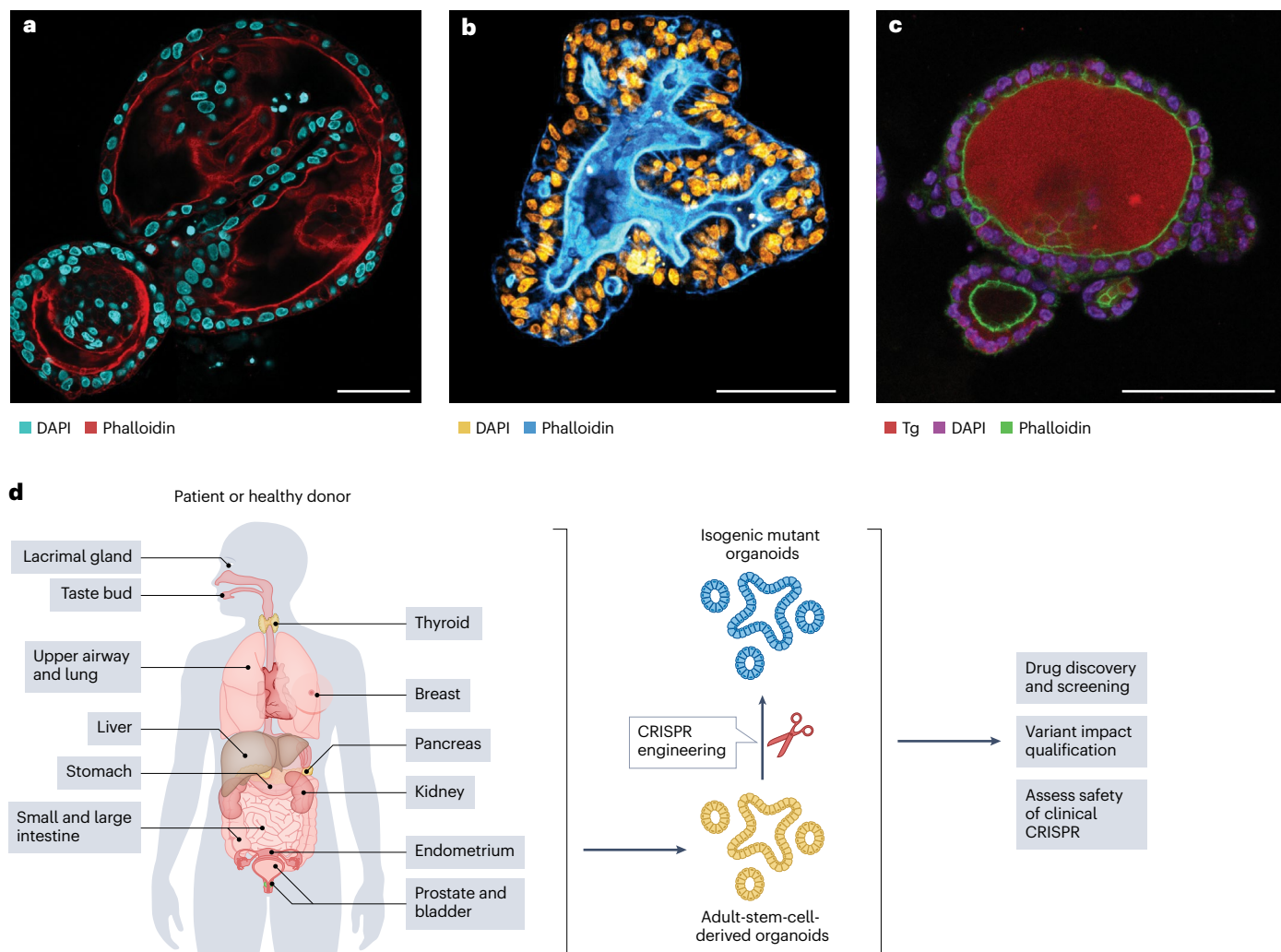


Fig. 1 | CRISPR engineering in adult-stem-cell-derived organoids. Confocal images of adult-stem-cell-derived organoids. **a**, Small intestine organoids. Nuclei are stained by DAPI (turquoise) and actin by phalloidin (red). **b**, Fetal hepatocyte organoids. Nuclei are stained by DAPI (orange) and actin by phalloidin (blue). **c**, Murine thyroid organoids. Nuclei are stained by phalloidin (green) and the hormone carrier protein thyroglobulin (Tg) is stained in red. Scale bars in panels **a** to **c** are 50 μm . **d**, Organoids can be derived

from most epithelial tissues of murine and human origin. By using CRISPR engineering, putative disease variants can be introduced into the genome. By pairing up with wild-type controls, an isogenic system is created that can be used for drug screening, variant impact qualification and drug discovery. Part **a**, image courtesy of Joep Beumer. Part **b**, image courtesy of Shashank Gandhi. Part **c**, image courtesy of Jelte van der Vaart.

DNA repair pathways, CRISPR can be used for genome engineering in a sgRNA-mediated manner³⁻⁵ (Fig. 2).

There are key downsides to using conventional CRISPR–Cas9 for the construction of isogenic disease models. First, because the human genome consists of three billion base pairs, the chances are great that the sgRNA will initiate DSBs at off-target sites³⁰. To overcome this issue, high-fidelity variants of Cas9 such as SpCas9-HF³¹ and hifi-Cas9³² have been developed. Alternatively, off-target free sgRNAs can be selected using profiling strategies such as Guide-seq or Circle-seq prior to use in experiments^{33,34}. Next, even if an off-target free sgRNA is used, on-target DSB repair can result in undesired editing outcomes, such as the induction of large deletions, insertions and translocations³⁵. In extreme cases, CRISPR–Cas9 could lead to chromothripsis, a process of

chromosome shattering and massive structural variation downstream of the sgRNA target site³⁶. Finally, HDR upon CRISPR-mediated DSB induction is required for modelling specific genetic variants. However, HDR can only occur during the G2 and S phase of the cell cycle when sister chromatids are present³⁷, which thereby risks NHEJ repair pathway domination. Cell cycle synchronization and addition of NHEJ inhibitors are two strategies to push the cell towards HDR-mediated DSB repair instead^{38,39}. Nevertheless, because both alleles are often cleaved, the end result of an HDR experiments is often correct variant introduction on one allele whereas the second allele is knocked out owing to the cell's bias towards using the NHEJ for DNA repair²⁹. Because only about 2.4% of disease-causing variants are indels, alternative strategies of genome engineering that do not require DSB induction have been pursued⁴⁰.

Box 1

A brief history of genome engineering

Strategies to alter the genome have been around since the early 1900s. Researchers have used ultraviolet and X-ray irradiation to induce random, unspecific mutations in the DNA¹³². This random mutagenesis was, and still is, used in forward genetic screens to elucidate the function of genes. Most notably, Sydney Brenner performed forward genetic screens to ascribe function to hundreds of genes in the nematode *Caenorhabditis elegans*¹³³, whereas Christiane Nusslein-Volhard and Eric Wieschaus used saturation mutagenesis screens in the fruitfly *Drosophila melanogaster* and discovered — among many other things — the role and function of homeobox genes¹³⁴. Although efficient, these strategies lack the possibility for targeted alterations in the genome.

Genome engineering, defined by the insertion, deletion or replacement of DNA at a specific site in the genome of an organism or cell, really took off in 1979 with the description of targeted insertion of DNA in yeast by Scherer and colleagues^{135,136}. They harnessed the cell's intrinsic process of homologous recombination to seamlessly insert exogenous DNA into the genome of *Saccharomyces cerevisiae*, which was later also demonstrated in mammalian cells^{135,137}. However, homologous recombination is inherently inefficient, therefore, a similar process called homology-directed repair (HDR) was explored for genome engineering. Upon targeted DSB induction in yeast, the cells initiate HDR, resulting in seamless introduction of exogenous DNA¹³⁸. This process exemplified the requirement of a system that introduces targeted DSBs into the genome of living cells for efficient genome engineering. Soon afterwards, different strategies that enabled this feat were developed. Meganucleases are a class of homing endonucleases that recognize large DNA sequences in a similar fashion to restriction enzymes. In 1994, meganucleases

created targeted DSBs in mouse chromosomes¹³⁹ and further engineering of chimeric versions allowed targeting of specific sites in the genome¹⁴⁰. Meganucleases, however, are difficult to engineer, which is why their use has now become mostly obsolete. Two easier-to-reprogram genome-engineering tools, the zinc-finger nucleases (ZFNs) and the transcription activator-like effector nucleases (TALENs), were developed soon afterwards^{141,142}. Both ZFNs and TALENs are fusion proteins of a repeat of sequence-specific DNA binding domains to a non-specific DNA-cleaving nuclease such as FokI. Because of the predictable binding of the individual DNA-binding domains, ZFNs and TALENs are easier to reprogram than meganucleases, but the requirement for protein design and engineering for each subsequent target site still hampered scalable application¹⁴¹. In 2007, Philippe Horvath and colleagues uncovered the biological function of a series of tandem repeats called clustered regularly interspaced short palindromic repeats (CRISPR), found within bacterial genomes¹⁴³. These repeats, together with CRISPR-associated (Cas) genes, constitute a bacterial defence mechanism against foreign DNA¹⁴⁴. Doudna, Charpentier and colleagues then harnessed the system and developed CRISPR–Cas as a genome-editing tool³. The first descriptions of CRISPR to alter the human genome were published soon thereafter^{4,5}. Contrary to meganucleases, ZFNs and TALENs, which require protein engineering for each subsequent target, CRISPR–Cas-mediated genome engineering simply requires a new RNA molecule. Because of its easy retargeting and comparatively low cost, CRISPR–Cas9-mediated genome engineering was quickly adopted by the research community and it came as no surprise when Doudna and Charpentier were awarded with the Nobel prize in chemistry in 2020 for their groundbreaking discovery.

DSB-free genome editing

A single histidine residue at position 840 of the HNH domain of SpCas9 cleaves the PAM strand, whereas the aspartate at position 10 in the RuvC domain cleaves the opposite strand³. Mutating both amino acids to alanines (D10A and H840A) results in nuclease-inactive or 'dead' Cas9 (dCas9). dCas9 still recognizes its target site and opens up the DNA in an R-loop but does not induce DSBs. The binding of dCas9 to its target site alone can function as a repressor of transcription and is dubbed CRISPR interference (CRISPRi)⁴¹. Alternatively, dCas9 can be used as a vehicle to localize DNA effector proteins to the genome. Examples of this strategy are CRISPR activators (CRISPRa)⁴² and CRISPR–DNMT3 fusion proteins for targeted methylation⁴³. To induce genetic variants, DNA-alteration enzymes are fused to dCas9 to overcome the limitations associated with DSB induction in genome engineering. These ongoing strategies could facilitate CRISPR-based genome-engineering clinical translation (Box 2).

Base editing

The first base editor fuses dCas9 to the rat cytidine deaminase apolipoprotein B mRNA editing catalytic polypeptide-like (rAPOBEC1), which catalyses the conversion from cytidine to uracil. The cell repairs

this uracil into thymidine, resulting in a construct (BE1) that replaces a C•G by a T•A base pair, called a cytosine base editor (CBE)⁴⁴. First-generation CBEs were inhibited by uracil glycosylation. Therefore, second-generation base editors (BE2) were developed by fusing an uracil glycosylase inhibitor (UGI) to the dCas9–rAPOBEC1 fusion⁴⁵. To increase editing efficiency, dCas9 can be converted into a nickase SpCas9–D10A. In this optimized base editor architecture (BE3), the strand that is not modified by rAPOBEC1 is cleaved. The cell detects the nick and initiates DNA repair to resolve the damage. The strand containing the base change is then used as a template for repairing the nick, yielding stable integration of the edit with an efficiency between 15% and 75% depending on the sgRNA⁴⁴. The BE3 architecture was further improved by fusing an additional UGI in combination with linker optimization, resulting in the fourth-generation cytosine base editor (BE4). BE4s have improved editing efficiency (by around 50%), with two-fold reduction of unintended byproducts such as indels and point mutations⁴⁶. Subsequent codon optimization⁴⁷ and ancestral reconstitution⁴⁸ led to a CBE architecture that currently enables the most robust base editing in 2D cell lines, organoids and in vivo by improving expression and nuclear localization of the proteins⁴⁹ (Fig. 3a). A similar base editor, that enables C-to-T base changes, was developed by fusing

cytidine deaminase 1 (CDA1) to SpCas9^{D10A} in a system called Target-AID⁵⁰. This base editor has a shifted activity window (from positions 4–8 in the sgRNA for CBE to positions 1–5 in target-AID)⁵⁰. Moreover, C-to-G and C-to-A changes are frequently observed in Target-Aid. These unwanted byproducts also occur in first-generation BE3, but have been resolved in newer iterations of CBE such as BE4 (ref. 46).

The opposite base change can be performed with the use of adenine base editors. For example, tRNA-specific adenosine deaminase, TadA, is a protein that enables editing of adenine residues in the DNA⁵¹. Because wild-type TadA does not act on DNA, the protein was evolved using a process called phage-assisted continuous evolution (PACE)⁵². Fusion of the seventh generation of evolved TadA in a heterodimer with a wild-type TadA to SpCas9-D10A results in an adenine base editor (ABE) with an A-to-G editing efficiency of up to 50% depending on the target site, which is comparable or higher than that of third-generation CBEs^{44,51}. As opposed to CBEs, the TadA heterodimer in ABEs deaminates adenine residues, which are then converted to inosine. Upon cleavage of the non-edited strand and resolving of the DNA mismatch, the inosine residue is converted into guanine, effectively resulting in an A-T to G-C edit (Fig. 3b). The applicability of ABEs was further improved by codon optimization and additional PACE-mediated directed evolution, resulting in optimized eighth-generation base editors with a 1.5–3.2-fold improvement in editing efficiency for ABE8 (ref. 53), and a 9.4–24-fold increase for ABE8e⁵⁴, depending on the sgRNA and nucleotide location in the editing window⁴⁹.

The deaminases fused to Cas9 in base editors function only on ssDNA. Therefore, base editors act only on a few bases of the single-stranded R-loop that is generated upon Cas9 target recognition^{3–5}. This so-called ‘editing window’ roughly spans four nucleotides, between positions 4 and 8 from the 5’ end of the protospacer (Fig. 3c). This ssDNA dependence greatly reduces the sgRNA-mediated off-target effects of base editors but requires very specific localization of Cas9 to induce the desired edits. Relaxing the PAM requirements and increasing the target space of Cas9 has resulted in a series of evolved SpCas9 variants. For example, PACE and structural guided evolution resulted in xCas9 and SpCas9-NG, which recognize an NGN PAM^{55,56}. Further structural modification led to nearly PAMless SpCas9 variants that target NRN (where R = A or G) and NYN (where Y = C or T)⁵⁷. An alternative strategy to increase the target space of base editors relies on Cas9 homologues such as *Streptococcus aureus* (PAM = NNGRRT). Other approaches resulting in evolved SpCas9 variants and SpCas9 homologues with alternative PAM requirements and editing windows have also been developed^{40,58}.

However, base editors have limitations. Although ABEs essentially yield zero off-target effects, genome-wide profiling of CBEs has shown genome-wide C>T mutations owing to the overexpression of APOBEC in the cell^{59,60}. Evolved APOBEC domains can be used in CBE to decrease these side effects⁶¹. Moreover, CBE-induced uracil residues sometimes yield adenine and guanine residues instead of the desired thymidine owing to unwanted cellular uracil DNA glycosylation during the base excision DNA repair pathway^{44,47}. Despite these editing outcomes being undesired, this observation has led to the development of new classes of base editor. For example, removal of UGI from the CBE architecture pushes DNA repair towards guanine instead of thymidine and has allowed the development of C>G base editors^{62,63}. Furthermore, not all desired point mutations can currently be generated by base editors. Finally, base editors cannot introduce indels or larger genetic variants. Prime editors have been developed to overcome these limitations and allow for more versatile DSB-free genome engineering.

Prime editing

The rationale behind prime editing is to bring exogenous DNA with the edit of interest close to the Cas9 binding site. In the first generation of prime editors (PE1), a reverse transcription (RT) domain derived from the Moloney murine leukaemia virus was fused to nickase SpCas9-H840A⁶⁴. The RT domain converts RNA into DNA and finds its template in the 3’ extension of the specially designed sgRNA, called the prime-editing guide RNA (pegRNA), that guides the Cas9 in PE1 to the target site. Upon target recognition, the PAM-containing strand is nicked by the active HNH domain of Cas9-H840A. Then, the pegRNA extension binds to the nicked strand at the primer-binding site (PBS), after which the RT domain of PE1 uses the remaining pegRNA (RT template) to synthesize a 3’-DNA flap containing the edit of interest. This DNA-flap

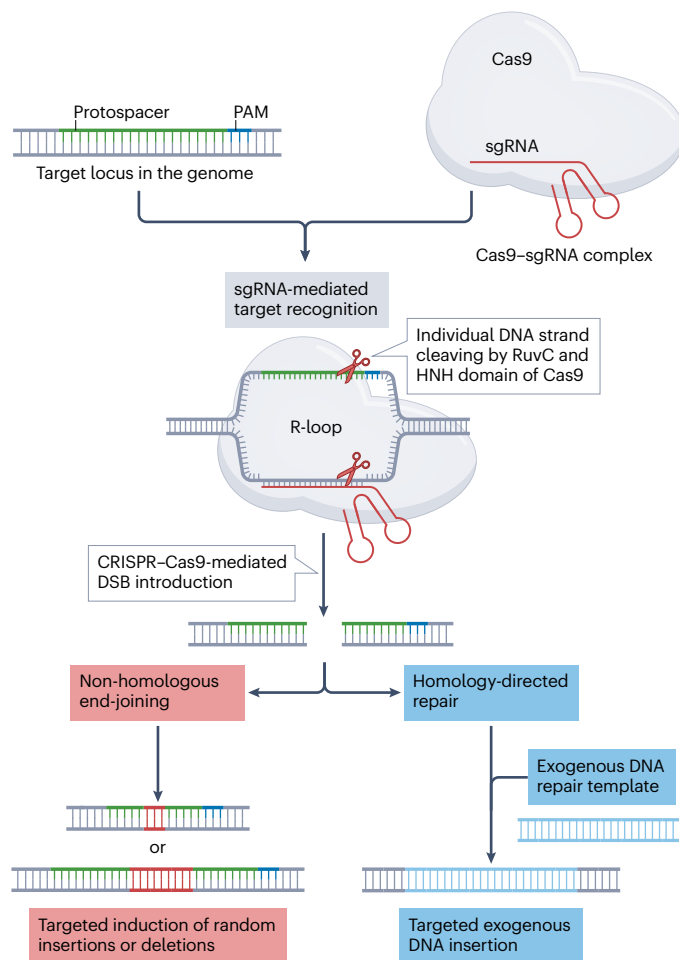


Fig. 2 | Principles of CRISPR-Cas9-mediated genome engineering. Upon binding of the Cas9–single-guide RNA (sgRNA) complex to the genomic target site, which consists of a protospacer and a protospacer-adjacent motif (PAM), the genome is opened in an R-loop, resulting in two single-stranded DNA (ssDNA) strands. These ssDNA strands are individually cleaved by the RuvC and HNH domain of Cas9, resulting in a double-stranded DNA break (DSB). The cell has two endogenous repair mechanisms to resolve DSBs. DSB repair by non-homologous end joining (NHEJ) results in the induction of indels at the target site, which can be used to knock out genes of interest. Homology-directed repair (HDR) can be used to introduce exogenous DNA, containing the genetic alteration of interest at the target site.

Box 2

CRISPR from bench to bedside

The biggest concern for in vivo genome engineering is safety. As the human genome is three billion base pairs in size, there is a good chance that even a single-guide RNA (sgRNA) designed for specificity will create off-target effects. Careful design of sgRNAs can enable genome engineering without off-target effects at other loci in the genome of mice¹⁴⁵, but off-target effects must be mapped for each individual genomic target site to ensure safety in the clinic.

Cas9 mRNA delivery by lipid nanoparticle packaging efficiently target the liver, whereas other organs remain more difficult to target. Therefore, much effort has been put into developing adeno-associated viruses (AAVs). These viruses benefit from not integrating into the host genome and have reduced immunogenicity compared to viral vectors such as lentivirus and adenovirus¹⁴⁶. Nine individual AAV serotypes exist naturally, each with their own tropism towards individual organs¹⁴⁷. Therefore, selecting an AAV serotype that has tropism only towards the target organ can limit Cas9 exposure, thereby reducing unwanted off-target effects. By using AAVs in combination with tissue-specific promoters, expression of CRISPR components in cells that do not need to be repaired can be further decreased¹⁴⁸. Furthermore, by mixing the capsid and genome of AAV serotypes — called pseudotyping — the tropism of AAVs can be further refined¹⁴⁹.

A downside of AAVs is the limited packaging space of roughly 5.2 kb (ref. 150). SpCas9 itself, without the promoter, is already 4.1 kb, leaving little space for anything else in the AAV genome. Delivery of base editors or prime editors in a single AAV is even more challenging, owing to their size. Split inteins can post-translationally

collate proteins and have been developed for base and prime editors for in vivo delivery in mice^{49,151,152}. Alternatively, a completely new delivery method describing a virus-like particle that is engineered out of proteins native to mammals has been proposed¹⁵³. It remains to be seen whether these strategies are translatable to humans.

A much safer strategy is performing CRISPR ex vivo and then transplanting the tissue back into patients. Using CRISPR in blood, similar to strategies used in chimeric antigen receptor (CAR) T cells could be the most straightforward approach¹⁵⁴. CRISPR-engineered bone-marrow stem cells have already been autologously transplanted successfully into patients suffering from β -thalassaemia and sickle cell disease¹⁵⁵. Because ASC-derived organoids can be grown from most epithelial tissues, there could be similar benefits in transplanting CRISPR engineered organoids to patients. Moreover, organoids from most epithelial tissues can be maintained indefinitely and can be split and propagated rapidly to yield the biomass needed for successful transplantation¹⁵⁶. Current efforts to transplant ASC-derived organoids have shown some success in mouse intestine^{124,125,157}. Similarly, cholangiocyte organoids are able to reconstitute bile ducts in the human liver. Despite using human donor livers undergoing ex vivo normothermic perfusion, meaning that there was no patient transplantation, ASC-derived organoids readily integrate into human organs¹²⁶. Nonetheless, there is a need to develop tissue-specific transplantation strategies to unlock the autologous transplantation of CRISPR-repaired organoids throughout the body.

is resolved by cellular DNA repair processes integrating the edit of interest. Efficiencies of prime editing can be further enhanced by using a rationally evolved variant of the RT domain (PE2) and by inducing a proximal second nick in the opposing DNA strand, guided by a second (PE3) guide RNA⁶⁴ (Fig. 4). However, the use of a PE3-guide in prime editing comes with a cost as indel numbers are substantially higher compared to PE2 (6.8% average indels for the sgRNA with the highest editing efficiency)⁶⁴. This issue can be resolved by using a PE3b-guide that matches the edited strand, resulting in a second nick once the edit is made⁶⁵. In addition to all transition and transversion mutations, the first description of prime editing reported the induction of deletions of up to 80 and insertions of up to 44 base pairs⁶⁴. For efficient use of prime editing, extensive optimization of the pegRNA and PE3 guide is required. The length of both the PBS and the RT, as well as the distance between the pegRNA and PE3-guide nick influence the editing efficiencies of prime editing. Optimization can be easily performed in the HEK293T cell line, but is more difficult in organoids or in vivo. However, once fully optimized, prime editing is the most versatile DSB-free genome-engineering technology to date.

Prime editing holds great promise owing to its versatility in potential edits; however, the need for optimizing pegRNA and PE3-guides limits its application in organoids. To overcome this issue, three key modifications have been made to the prime-editing system. First, the use of two pegRNAs in *trans* with overlapping RT domains increases

prime-editing efficiencies in human cells as well as plants^{66–68}. Second, engineered pegRNAs can have evopreq or tmpknot domains fused to the 3' end. These domains increase the stability of the pegRNA, which can increase prime-editing efficiency⁶⁹. Finally, including the R221K and N394K amino acid changes increases the nuclease function of SpCas9, resulting in a more efficient PE2Max⁷⁰.

Isogenic organoid disease models

Because ASC-derived organoids more closely resemble their tissue of origin compared to 2D cell lines, they are more suitable for the study of human physiology. The rapid developments of CRISPR-mediated genome engineering now allow for rapid generation of isogenic organoid models that harbour specific mutations that have a role in the onset and course of human diseases.

Tumorigenesis and cancer

The majority of CRISPR-generated isogenic ASC-derived organoid models currently focus on tumorigenesis and carcinogenesis. Two similar studies in human intestinal organoids recreated the Vogelstein model of sequential driver mutation accumulation in colorectal tumorigenesis^{71–73}. By removing selected growth factors or adding small molecule inhibitors, organoids with mutations in *APC* (removal of Wnt), *TP53* (addition of Nutlin), *KRAS* (removal of epidermal growth factor, EGF), *SMAD4* (removal of Noggin) and *PIK3CA* (addition of MEK-inhibitors)

can be generated. Subcutaneous transplantation of these growth-factor-independent organoids in mice results in metastasizing carcinomas^{72,73}. Inspired by these first two studies, multiplexed genome engineering was applied in ASC-derived organoids with subsequent transplantation into mice to elucidate the minimal requirements for tumorigenesis in other tissues. For example, subcutaneous transplantation of CRISPR-mediated knockout of *PTEN*, *TP53*, *RBI* and *NFI* in breast organoids results in tumour formation resembling oestrogen- and progesterone-receptor-positive and human epidermal growth factor receptor 2 (HER2)-negative luminal B breast cancers in mice⁷⁴. Furthermore, CRISPR–Cas9-mediated knockouts of *TP53*, *SMAD4*, *PTEN*, *NFI* and *BAP1* were generated in cholangiocyte organoids to elucidate the

role of the tumour suppressor *BAP1* in cholangiocarcinoma⁷⁵. Loss of *BAP1* results in impaired chromatin accessibility and thus gene expression, crucial for epithelial integrity in the organoids and in mice⁷⁵. Two studies generated isogenic models for pancreatic ductal adenocarcinoma (PDAC) in human ASC-derived ductal pancreas organoids. When combined with oncogenic *KRAS*^{G12V}, CRISPR-based multiplexed mutation of *TP53*, *CDKN2A* and *SMAD4* results in organoids with PDAC phenotypes⁷⁶, whereby overexpression of *KRAS* leads to organoids mimicking PDAC precursor states⁷⁷. Two independent studies created CRISPR–Cas9-mediated knockout models of DNA repair genes. Mutational signature analysis of human colonic organoids with loss-of-function mutations in *MLH1* revealed the predominant occurrence

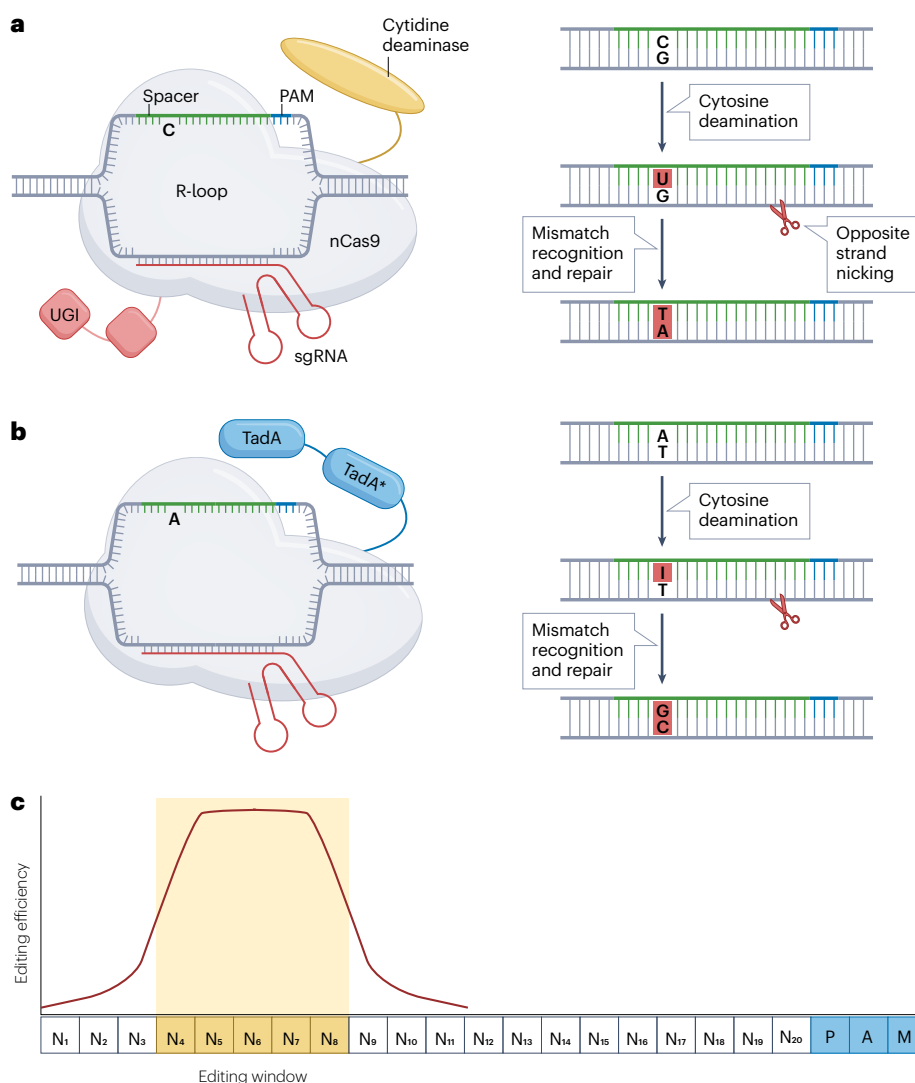


Fig. 3 | Principles of cytosine and adenine base editing. **a**, A cytosine base editor consists of a nickase Cas9–DIOA fused to a cytidine deaminase and a tandem repeat of uracil glycosylase inhibitors (UGI). After binding of the Cas9–single-guide RNA (sgRNA) complex, the DNA opens up in an R-loop, which enables cytidine deamination and conversion into uracil. After nicking of the non-edited opposite strand, the U•G base pair is repaired into a T•A base pair, effectively resulting in C>T base editing. **b**, An adenine base editor consists of a nickase Cas9–DIOA fused to a heterodimer of TadA, an adenine deaminase. The asterisk

indicates the evolved TadA variant and further exemplifies the heterodimer state of the fusion protein. After R-loop generation by binding of the Cas9–sgRNA complex to the target site, adenine is deaminated, effectively turning it into inosine. The inosine residue is converted into guanine by nicking of the non-edited strand, after which DNA repair is guided towards the correct A>G edit. **c**, The cytidine and adenine deaminases function only on single-stranded DNA. Therefore, base editor activity is limited to a small editing window within the R-loop that spans from roughly the 4th to the 8th base from the start of the sgRNA.

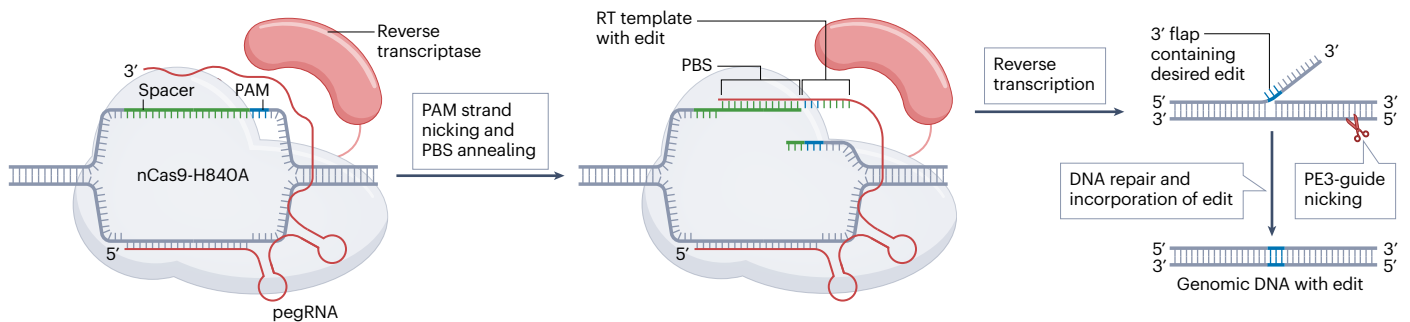


Fig. 4 | Principles of prime editing. The prime-editing guide RNA (pegRNA) complexes with the nickase SpCas9-H840A-RT prime-editing fusion protein and binds to the target DNA. Upon protospacer-adjacent motif strand cleavage by SpCas9-H840A, the primer-binding site of the pegRNA extension binds to the single-stranded DNA, upon which the reverse transcriptase (RT) synthesizes a

3'-DNA flap containing the edit of interest. This 3'-flap is resolved by cellular DNA repair processes, which can be further enhanced by introducing a proximal second nick in the opposing DNA strand, guided by a second (PE3) guide RNA. The red scissors indicate the nick site of the SpCas9-H840A. PAM, protospacer-adjacent motif; PBS, primer-binding site.

of COSMIC signature 20 associated with errors made during normal DNA replication^{65,78}. Moreover, knockout of *NTHL1* in colonic organoids results in an increase in C>T transitions, which resembles COSMIC signature 30, whereas *XPC* knockout generates organoids deficient in nucleotide excision repair, yielding COSMIC signature 8 (ref. 79). In a more sophisticated approach, the common fusion genes *DLG1-BRAF*, *PTPRK-RSPO3* and *EIF3E-RSPO2* were modelled into human colon organoids⁸⁰. Co-transfecting two sgRNAs that target both loci of interest results in complex genomic rearrangements only in organoids that lacked TP53 expression⁸⁰. CRISPR-Cas9 strategies can be similarly used to create single- or double-mutant isogenic knockout models of *TP53* in human hepatocyte organoids⁸¹, *ARIDIA* in human gastric organoids⁸², *RBI* in human intestinal organoids to model neuroendocrine neoplasms⁸³ and *RNF43* to model early-onset colorectal cancers⁸⁴.

Multiple genes can also be CRISPR-screened in a single experiment. For example, performing a small targeted CRISPR screen in human intestinal organoids enables mapping of RASGAP dependencies in colorectal cancer progression. Only loss of NF1 results in enhanced RAS-ERK signal amplification⁸⁵. To increase the throughput of CRISPR, genome-wide CRISPR screening platforms have been developed⁸⁶⁻⁸⁸, allowing for positive and negative survivability screens while assessing loss-of-function mutations across all genes in the genome. Furthermore, protocols for genome-wide CRISPR screening have been developed for use in ASC-derived organoids. For example, a positive selection genome-wide CRISPR screen performed in WT, *APC-KO* and *APC-KO*; *KRAS*^{G12D} mutant intestinal organoids, identified genes involved in a previously undescribed link between TGFβ and WNT signalling, revealing *PBRM1*⁸⁹ and *ARIDIA* and *SMARCA4*⁹⁰ as novel hits driving TGFβ resistance. These studies emphasize the possibility of genome-wide CRISPR screening in 3D models.

To model mutations observed in cancer patients accurately, simple CRISPR-Cas9-mediated knockouts by indel formation is not sufficient. Different mutations in the same cancer gene can have drastically different effects, as shown for *TP53*⁹¹ and *KRAS*⁹², highlighting the need for specific mutations instead of 'blunt' CRISPR-Cas9-mediated knockouts. For example, CRISPR-based prime editing for cancer modelling can be performed by introducing common *TP53* mutations in human colon and hepatocyte organoids. Besides prime-editing-mediated induction of *TP53*^{R175H} and *TP53*^{R249S}, it is possible to directly compare ABE to prime editing by introducing the same mutation using both

techniques. Targeting of *TP53*^{Y220C} results in organoids that are able to grow on medium containing the mouse double minute 2 homologue (MDM2) inhibitor nutlin-3, which kills wild-type organoids by stabilizing TP53 (ref. 93). ABE substantially outperforms prime editing with 1.5–2-fold increased editing efficiencies but induces undesired additional base changes⁹³. Similarly, introducing common in-frame deletions in *CTNNB1* exon 3 in human cholangiocyte organoids generates mutant organoids, which can grow without exogenous Wnt⁹⁴. These results highlight the efficacy of DSB-free genome engineering in organoids for cancer modelling.

Isogenic disease models beyond cancer

An example of combining CRISPR with ASC-derived organoids beyond cancer applications is the creation of isogenic models of *DGATI* loss in intestinal organoids as a model for congenital diarrhoeal disorder⁹⁵, resulting in mutant organoids being more susceptible to lipid-induced cell death compared to their controls⁹⁵. Another example comprises a genome-wide positive CRISPR screen to study confounding factors contributing to ulcerative colitis in mice. Despite wild-type organoids dying when treated with interleukin-17A (IL17A), organoids that harbour mutations in *IL17RA* and *NFKB1Z* upon treatment are enriched⁹⁶. Furthermore, organoids are a great model to study the 2019 pandemic causing severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)⁹⁷⁻⁹⁹. To elucidate essential host factors for SARS-CoV-2 entry, a targeted CRISPR screen generated knockouts of 19 previously implicated genes¹⁰⁰. Interestingly, SARS-CoV-2 is not able to infect intestinal organoids harboring mutations in the host genes *ACE2* and *TMPRSS2*, whereas none of the other 17 target genes shows a substantial decrease in infection potential of the virus¹⁰⁰.

Assessing CRISPR-mediated gene repair

Because ASC-derived organoids more closely resemble their tissue of origin than do 2D cell lines, they hold the promise of mapping the efficacy and safety of therapeutic genome engineering in vitro prior to in vivo application. However, genome-engineering tools are benchmarked for efficiency and safety in conventional 2D cell lines such as HEK293T and U2OS^{4,5,44}. Despite the ease of handling of these in vitro cultures, on- and off-target efficiencies can differ vastly from the cell types that are targeted in patients. For example, Cas9 binding can be influenced by methylation of CpG islands and chromatin accessibility, which differs

greatly between cell types^{101,102}. Therefore, testing CRISPR efficiency and safety in the target cell type in ASC-derived organoids could provide more accurate prediction of CRISPR-based genome engineering in patients. Furthermore, transplantation of ASC-derived organoids might complement whole-organ transplantation^{17,103}. In vitro CRISPR-repaired autologous organoids could similarly be transplanted back to patients after rigorous off-target determination by whole-genome sequencing for safety purposes (Box 2).

Cystic fibrosis

The first hereditary disease to be repaired in human stem cells with the use of CRISPR–Cas9-mediated genome engineering was cystic fibrosis¹⁰⁴. Cystic fibrosis is caused by various mutations in the cystic fibrosis transmembrane conductance regulator gene (*CFTR*), with the deletion of phenylalanine-508 being the most common (F508del)¹⁰⁵. ASC-derived intestinal organoids model the function of the *CFTR* channel, in this case, through the forskolin-induced swelling assay, which correlates with clinical disease severity^{106–108}. Sequencing-based screening of co-transfected Cas9 with a targeting sgRNA towards exon 11 and a donor plasmid containing a repaired F508 *CFTR* sequence and an intronic insertion of puromycin reveals correction of the F508del mutation in 17 out of 89 sequenced organoid clones (19.1%). These repaired organoids restore the forskolin-induced swelling response to wild-type levels¹⁰⁴. Another strategy relies on repairing deleterious splice site mutations that disrupt *CFTR* function: 3272–26 kb A>G and 3849+10 kb C>T. Instead of directly repairing the point mutations, an allele-specific disruption of the mutation can be chosen by using Cas12a, a type 5 Cas protein^{109,110}. Lentiviral transduction of the Cas9 and sgRNA into intestinal organoids derived from patients with cystic fibrosis results in 40% allele-specific indel induction depending on the corrected splicing as measured by forskolin-induced swelling¹¹⁰.

An intestinal organoid biobank was subsequently established containing 664 organoid lines that represent 154 distinct *CFTR* mutations¹¹¹. From this biobank, organoids that could be repaired by SpCas9-ABE were selected. Intestinal organoids harbouring the R785* mutation were transfected with base editing reagents, after which forskolin-induced swelling revealed functional repair in about 9% of the transfected organoids. The target space of base-editor-mediated *CFTR* repair was increased by using xCas9-ABEs to repair R553*, R1162* and W1282* mutations in intestinal and upper airway organoids¹¹¹. Similarly, prime editing in intestinal organoids allows DSB-free repair of the F508del mutation⁹³. The lack of genome-wide off targets as measured by whole-genome sequencing of intestinal organoids after *CFTR* repair with base editing and prime editing underlies the safety of DSB-free genome engineering for therapeutic purposes^{93,111}. To increase the in vitro editing efficiency of prime editing, a fluorescent prime editing and enrichment reporter called fluoPEER was developed¹¹². In fluoPEER, mCherry is expressed if active prime editing occurs within the cell. Fluorescence-activated cell sorting (FACS)-based selection of mCherry-positive cells facilitates the generation of isogenic prime-edited organoids. Using fluoPEER, the repair efficiency of *CFTR*^{F508del} is increased to 80%, enabling reparation of the elusive *CFTR*^{G542*} mutation¹¹².

Diseases beyond cystic fibrosis

Applying prime editing in patient-derived isogenic intestinal organoids allows restoration of the most common mutation in *DGATI*, c.629_631delCTT,p.s210del, which causes a defect in fatty acid storage in lipid droplets, as measured by survivability after fatty acid addition to the culture medium⁹⁴. Similar results were observed in an experiment

repairing *ATP7B* mutations in liver organoids of patients with Wilson disease⁹⁴. Subsequently, using the fluoPEER system effectively corrected mutations in *ABCB4* and *ATP8B1* responsible for intrahepatic cholestasis¹¹². CRISPR-engineered isogenic organoids can also be used to study primary cilia dyskinesia disease. Using an organoid differentiation protocol allows visualization of cilia defects in airway organoids from patients with primary cilia dyskinesia¹¹³. From a mini-biobank of patient-derived organoids, organoids harbouring a splicing mutation in the cilia gene *DNAH11* can be repaired using prime editing with efficiencies of up to 85%. Owing to limitations in clonal outgrowth of human airway organoids, no morphological analysis of repaired organoids could be performed¹¹³.

Technical considerations

Because conventional and next-generation CRISPR tools have primarily been developed for 2D cell lines, translation into 3D cell cultures is not straightforward. Therefore, key considerations need to be addressed before using ASC-derived organoids to create isogenic disease models.

The right genome-engineering tool

To circumvent undesired on-target and off-target effects of conventional CRISPR–Cas9, the use of next-generation CRISPR tools, in this case, base editing and prime editing, is advisable. CBE can be effectively used for the introduction of stop codons in the genes¹¹⁴, mediating C>G to T>A base changes turning arginine (CGA to TGA), glutamine (CAA/CAG to TAA/TAG) and tryptophan (TGG to TAG/TGA/TAA) into stop codons. According to the CRISPR-STOP method, because CBE does not require DSBs, lower levels of apoptosis are observed, resulting in cells that are less stressed upon transfection of genome-editing components. If no suitable sgRNAs are available for the CBE-mediated introduction of stop codons, ABE can be used to disrupt either the start codon¹¹⁵ or splice sites¹¹⁶ to effectively create a gene knockout. Importantly, using CRISPR-STOP eliminates the need for Sanger deconvolution or sub-cloning to determine individual indel outcomes of both alleles, as is required for conventional CRISPR–Cas9-NHEJ, resulting in a more efficient genotyping process¹¹⁷.

We recommend first designing the CBE-mediated stop-codon insertion before designing ABE-mediated methods because the cell could still express the protein of interest owing to alternative splicing or use of an alternative start site. Moreover, it is important to realize that the DNA-altering fusion proteins in base editors function in a specific sequence context. For example, a machine-learning protocol called BE-Hive reports that guanine residues in front of the cytosine substantially decrease the levels of base editing when using BE4¹¹⁸. Alternatively, modifications of CBE and ABE, such as evoA-BE4 and ABE-CP, can be used to perform base edits in alternative sequence contexts¹¹⁹. Moreover, base editors function most effectively within the editing window that roughly spans from the 4th to the 8th nucleotide from the start of the sgRNA (Fig. 3c). If a specific edit is required, but editing of additional nucleotides within the editing window would result in unwanted amino acid changes, prime editing is the preferred strategy because the RT-template can be designed to exclusively incorporate the edit of interest.

Delivery and selection

The delivery of genome-editing agents into organoids is considerably more difficult than in popular 2D cell lines. Simple lipofection of plasmid DNA can reach up to 95% transfection efficiency in HEK293T, whereas efficiencies in 3D organoids differ greatly per line and can be

as low as 1.5%¹¹¹. Lentiviral transduction can be a more efficient strategy. However, even if titrated properly, the viral genome is prone to integrate multiple times, which might influence organoid fitness⁹⁰. Electroporation of ribonucleoprotein complexes into organoids substantially increases transfection efficiencies¹²⁰, but it decreases flexibility, because for each subsequent next-generation CRISPR tool, a new protein has to be produced. The low transfection efficiencies in organoids do not limit successful isogenic model generation as long as strategies to select for either the transfected or functionally edited organoids is taken into account during the experimental setup. For functional selection, CRISPR-engineered organoids are selected for based on the introduced genetic variant, which can be based either on survivability or phenotypic changes upon genome engineering. Examples of functional selection based on survivability are the removal of Wnt and Rspodin-1 for selecting WNT pathway mutants such as *APC* and removal of EGF in combination with the addition of the EGFR inhibitor gefitinib or MEK inhibitors for the selection of oncogenic *KRAS* or *PIK3CA* mutations in intestinal organoids, respectively^{72,73}. TP53 mutations can be selected by the addition of MDM2 inhibitor Nutlin-3, which enables straightforward mutagenesis of multiple cancer genes by CRISPR multiplexing with *TP53* (ref. 75). An example of morphology-based functional selection is the swelling response of intestinal organoids that carry either a wild-type (swelling) or mutant (no swelling) *CFTR* gene^{93,104,111}. If no functional selection is available for the desired edit, transfection selection is the preferred strategy. The choice can be made either by FACS sorting based on fluorescence¹⁰¹ or by antibiotic resistance that is acquired upon integration into the coding sequence of a gene to create a knockout⁷⁷ or by co-transfection of hygromycin resistance piggyBac¹¹⁹ (Fig. 5).

Outlook

Conventional CRISPR engineering through active Cas9 nucleases that create DSBs in the genome is highly efficient; however, it can induce DNA damage and be detrimental to the cell. To overcome these issues, next-generation CRISPR tools have been developed that no longer require the induction of a DSB to induce genetic alterations. The first class, base editors, allow for the introduction of either C>T or A>G base changes and have already proved their potential in vitro and in vivo. The need for more versatile genome-editing tools has led to the development of prime editors that can induce all transition and transversion point mutations, as well as introduce DNA insertions and deletions. These developments have allowed modelling or repair of over 90% of all genetic variants described in human disease, simply by selecting the most optimal genome-engineering tool for the desired genetic alteration. The application of CRISPR tools can be extended to organoids that can be derived from ASCs from both healthy and diseased donors. Complex ASC-derived isogenic disease models have been developed to reveal the mechanisms of disease progression.

Although creating knockouts of interest in ASC-derived organoids is not difficult, modelling single-nucleotide variants and larger genomic alterations remains a challenge. CBE and ABE have proved to be efficient for genome engineering and disease modelling of organoids; however, simple and robust application of prime editing could substantially increase the scope of single-nucleotide variants modelling because it can mediate all base changes. The suggested improvements to prime-editing strategies enhance the editing efficiency in HEK293T cells, but it remains to be seen whether they also prove to be effective in ASC-derived organoids, where editing efficiencies appear to be more difficult to predict.

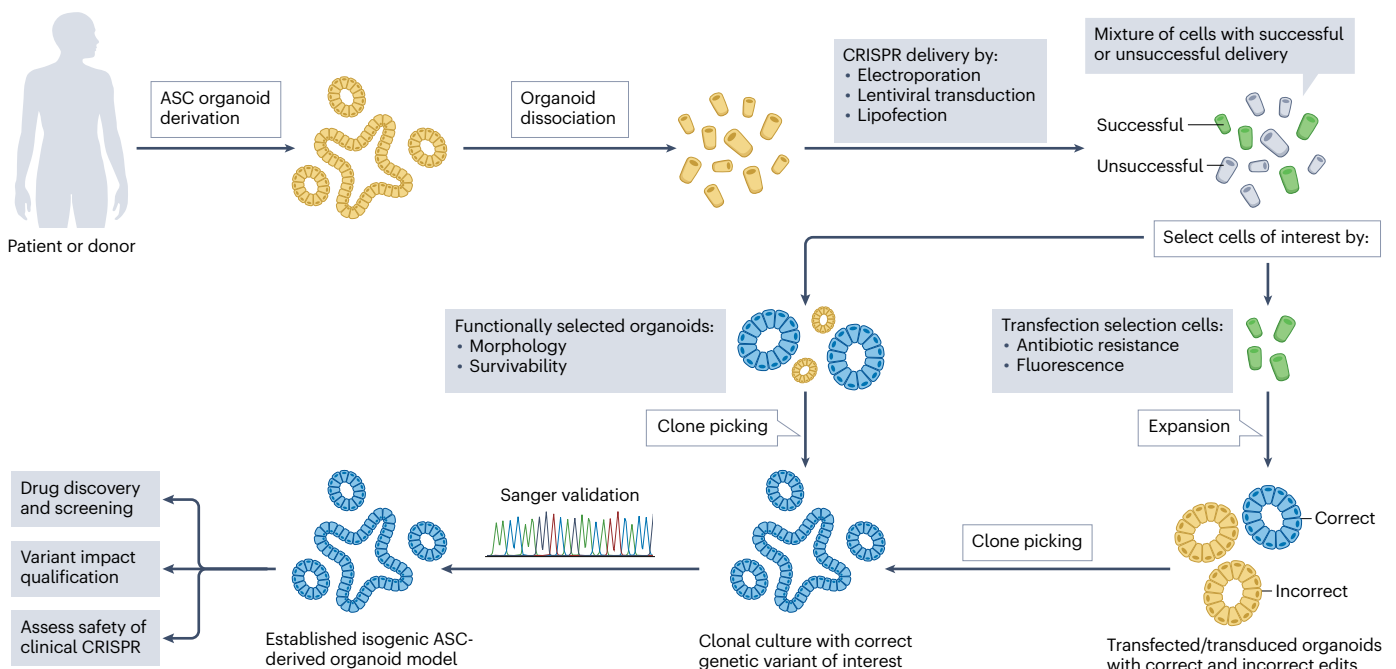


Fig. 5 | The process of creating isogenic disease models in adult-stem-cell-derived organoids. Patient-derived organoids are dissociated into single cells, upon which genome-engineering agents are delivered by electroporation, lipofection or lentiviral transduction. Selection for edited cells can be performed

based on transfection selection or functional selection. After Sanger validation, clonal isogenic organoid pairs can be used for variant impact qualification, drug discovery and screening and assessment of the safety of genome-engineering approaches.

Even if further development of the current genome-engineering toolbox results in more robust genome engineering, we can still engineer 'only' 90% of the genetic variants observed in patients using the current toolset. The remaining 10% of disease-causing mutations involve larger chromosomal alterations such as larger inversion, deletions and insertions, up to the loss or duplication of a complete chromosome. Efforts are ongoing to increase the target scope of genome engineering. Two new iterations of prime editing could be part of the solution. Insertions of up to 5 kb and inversion of DNA pieces of up to 40 kb can be achieved by pairing prime editing with site-specific recombinases⁶⁶. Combining prime editing with integrases increases the potential of genomic integration up to 36 kb without the need for DSBs¹²¹.

Genome-wide CRISPR screens have already been used in organoids to obtain biological insight in tumour development and colitis. However, to perform a high-quality CRISPR screen, a library saturation of up to 500-fold is needed, requiring tens of millions of transfected or transduced adult stem cells⁹⁰. Scalability in ASC-derived organoids is expensive owing to the cost of 3D matrices and growth factors. To resolve this issue, a 3D matrix consisting of only 5% basement membrane extract (compared to the conventional 50–100%) has been benchmarked for CRISPR screens in organoids, resulting in easy and, most importantly, cheap expansion of cancer organoid cells¹²². This protocol adaptation could further simplify genome-wide CRISPR screens in organoids derived from different tissues. Moreover, most genome-wide CRISPR libraries still use NHEJ to create genetic knockouts. Genome-wide base editor screens for DSB-free screening of disease variants have been developed¹²³, which could be expanded to ASC-derived organoids to enable high-throughput and accurate qualification of genetic variants in the future.

The combination of CRISPR and ASC-derived organoids could also benefit the clinical translation of genome engineering. Despite its high efficiency, safety remains the biggest concern for CRISPR–Cas9-mediated in vivo genome engineering. ASC-derived organoids can address safety concerns because gene repair can be performed ex vivo followed by rigorous off-target analysis. Moreover, organoids can be rapidly expanded, and the safely corrected clone can then be expanded and transplanted back into the patient to repopulate the affected organ. However, tissue-specific transplantation protocols do not yet exist for most tissues. Transplantation of ASC-derived organoids have originally focused on the first established organoid system, the mouse intestinal organoids^{4,124,125}, whose success laid the foundations of cholangiocyte, thyroid and salivary gland organoids transplantation^{25,126,127}.

Despite transplantation into humans not being common practice at present, ASC-derived organoids could already improve the safety of genome-engineering technologies. Clinical trials applying CRISPR as a therapeutic strategy have already started, with one standing out in particular. Systemic injection of lipid nanoparticles containing SpCas9 mRNA and a sgRNA targeting *TTR*, whose mutation is associated with transthyretin amyloidosis, substantially decreased the baseline serum TTR in all subjects¹²⁸. Prior to injecting human subjects with CRISPR reagents, the efficacy of the strategy was assessed in cynomolgus monkeys after careful selection of the sgRNA based on on-target efficiency and specificity. However, because this treatment aims to target the entire human liver, billions of cells must undergo CRISPR engineering with nuclease-active Cas9s. It is almost impossible to control off- and on-target adverse effects in such a vast number of cells, because uncontrolled cell growth can be induced by a single chromosomal rearrangement, thereby compromising safety. Moreover, despite validating specificity

in cynomolgus monkeys, no safety experiment has been performed in the cells of patients. ASC-derived organoids could fill this gap.

With the development of DSB-free genome-engineering strategies, the majority of safety concerns can be addressed. Although the first generations of CBEs exhibited extensive off-target effects owing to overexpression of APOBEC, ABEs have not shown any genome-wide off-target effects⁵⁹. Upgraded iterations of CBEs have also reported reduced sgRNA-independent off-target effects¹²⁹. Furthermore, despite the undesirable on-target edits observed in prime editing, edits at off-target sites are mostly absent¹³⁰. One likely reason is the Cas9-nickase architecture, which requires two nicks close to each other to induce a DSB¹³¹. Therefore, we envision that these DSB-free 'next-generation CRISPR tools' will take over the therapeutic space.

CRISPR-based genome engineering in organoids holds great promise for disease modelling and for patient care in the future. The rapid development of new genome-engineering technologies that allow the scalable induction of increasingly complex DNA mutations further highlights the applicability of CRISPR in ASC-derived organoids.

Citation diversity statement

We acknowledge that papers authored by scholars from historically excluded groups are systematically under-cited. Here, we have made every attempt to reference relevant papers in a manner that is equitable in terms of racial, ethnic, gender and geographical representation.

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Competing interests

H.C. is inventor on several patents related to organoid technology; his full disclosure is given at <https://www.uu.nl/staff/JCClevers/>. H.C. is currently head of pharma Research Early Development (pRED) at Roche. H.C. holds several patents on organoid technology. Their application numbers, followed by their publication numbers (if applicable), are as follows: PCT/NL2008/050543, WO2009/022907; PCT/NL2010/000017, WO2010/090513; PCT/IB2011/002167, WO2012/014076; PCT/IB2012/052950, WO2012/168930; PCT/EP2015/060815, WO2015/173425; PCT/EP2015/077990, WO2016/083613; PCT/EP2015/077988, WO2016/083612; PCT/EP2017/054797, WO2017/149025; PCT/EP2017/065101, WO2017/220586; PCT/EP2018/086716, n/a; and GB1819224.5, n/a. M.H.G. is currently a scientist at Xilis BV.

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