Review

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Organoid technologies meet genome engineering

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Abstract

Three-dimensional (3D) stem cell differentiation cultures recently emerged as a novel model system for investigating human embryonic development and disease progression *in vitro*, complementing existing animal and two-dimensional (2D) cell culture models. Organoids, the 3D self-organizing structures derived from pluripotent or somatic stem cells, can recapitulate many aspects of structural organization and functionality of their *in vivo* organ counterparts, thus holding great promise for biomedical research and translational applications. Importantly, faithful recapitulation of disease and development processes relies on the ability to modify the genomic contents in organoid cells. The revolutionary genome engineering technologies, CRISPR/Cas9 in particular, enable investigators to generate various reporter cell lines for prompt validation of specific cell lineages as well as to introduce disease-associated mutations for disease modeling. In this review, we provide historical overviews, and discuss technical considerations, and potential future applications of genome engineering in 3D organoid models.

Keywords CRISPR; disease modeling; homology-directed repair; organoids; stem cells

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See the Glossary for abbreviations used in this article.

Introduction

Built upon knowledge accumulated through decades of research in developmental biology, miniorgans can now be grown in petri dishes from aggregates of stem cells by stepwise manipulation of molecular signals, mimicking the innate signaling cues during *in vivo* organ development. These stem cell-derived three-dimensional (3D) structures, designated "organoids", recapitulate many aspects of *bona fide* organs in the body in terms of cell fate, cellular organization, and function [1,2]. Over the past few years, a number of organoid models have been established, resembling tissues from the eye [3,4], brain [5–7], intestine [8–11], kidney [12–15], liver [16–18], lung [19–21], and inner ear [22,23], among others. These organoids can be derived from human pluripotent stem cells (PSCs) and thus may serve as human model systems for disease modeling, drug screening, and drug safety testing, as well as providing replaceable tissues in regenerative therapeutics. However, success in these applications would be limited without the ability to modify the genomic contents. For example, although induced pluripotent stem cells (iPSCs) established from patients' fibroblasts can be used for disease modeling, comparison of organoids derived from patient and control iPS cell lines may not reveal disease-relevant phenotypes, but rather may reflect the variation in the genetic backgrounds and the reprogramming history of individual cell lines [24]. Genome editing, on the other hand, can be used to induce specific changes in an otherwise identical genetic background to overcome this limitation. Such an isogenic pair of disease-specific and control cell lines can be generated through genome editing by either introducing mutations in wild-type (WT) cells or correcting mutations in patient-derived cell lines.

Genomic editing with programmable nucleases, especially CRISPR/Cas9 [25], has been one of the major technological breakthroughs of recent years in biomedical research. Although CRISPR-mediated genome editing has generated much excitement, genome editing without a nuclease was accomplished long before the development of the CRISPR technology (Fig 1). In the 1980s, a series of studies demonstrated that genomic sequences can be modified by homologous recombination (HR) between genomic DNA and an exogenous DNA template harboring homologous regions [26–30]. Through HR, modifications made on the template DNA via standard molecular cloning techniques can be precisely introduced to the genomes of mammalian cells including mouse embryonic stem cells (ESCs). In fact, before the programmable nuclease era, HR-mediated genome editing was a standard procedure in generating genomically modified mouse ESC lines as well as generating transgenic mouse strains via blastocyst injections of the genomically modified ESCs [31]. A key disadvantage of HR-mediated genome editing, however, is its discouragingly low efficiency, which is typically at 10^{-6} frequency. Because of this, co-integration of antibiotic selection marker genes is usually required to enrich modified cells [32].

It was later discovered that the HR efficiency can be greatly improved by introducing a DNA double-stranded break (DSB) at the targeted locus [33,34]. While the elevated HR rate was limited to only a few special cut sites due to the non-programmable nature of the nucleases used in these studies, the discovery of programmable nucleases led to the expansion of DSB-stimulated HR to the genome level. Early programmable nucleases, including zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases

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Glossary

2D	two-dimensional
3D	three-dimensional
APC	adenomatosis polyposis coli
Atoh1	atonal bHLH transcription factor 1
Bf1	brain factor 1
C2c1	Class 2 candidate 1
Cas9	CRISPR-associated protein 9
Cas9n	Cas9 nickase
CFTR	cystic fibrosis transmembrane conductance regulator
Cpf1	CRISPR from *Prevotella* and *Francisella* 1
CRISPR	clustered regularly interspaced short palindromic repeats
CRX	cone-rod homeobox
dCas9	nuclease-deactivated Cas9
DKC1	dyskerin pseudouridine synthase 1
DMD	Duchenne muscular dystrophy
DSB	double-stranded break
ESC	embryonic stem cell
FACS	fluorescence-activated cell sorting
FOXG1	forkhead box G1
GFP	green fluorescent protein
GRHL2	grainyhead-like transcription factor 2
gRNA	guide RNA
h (e.g., in hESC)	human
HA	homology arm
HDR	homology-directed repair
HR	homologous recombination
indel	insertion and/or deletion
iPSC	induced pluripotent stem cell
Lgr5	leucine-rich repeat containing G protein coupled receptor 5
m (e.g., in mESC)	mouse
MARCKS	myristoylated alanine-rich protein kinase C substrate
MIXL1	Mix1 homeobox-like 1
NES	nuclear export signal
NHEJ	non-homologous end-joining
NLS	nuclear localization signal
PAX6	paired box 6
PSC	pluripotent stem cell
RFP	red fluorescent protein
RNF43	ring finger protein 43
ROCK	Rho-associated coiled-coil containing protein kinase
Rx	retinal homeobox
SMAD4	SMAD family member 4
SSC	somatic stem cell
ssDNA	single-stranded DNA
TALEN	transcription activator-like effector nuclease
Tbx19	T-box 19
TP53	tumor protein p53
WT	wild type
ZFN	zinc finger nuclease

(TALENs), require labor- and cost-intensive construction of new nucleases for each targeting site. The emergence of the CRISPR-associated RNA-guided nuclease Cas9 has truly revolutionized genome engineering. Rather than assembling various domain arrays into new ZFN or TALEN nucleases to target a particular site, CRISPR/Cas9 only needs the expression of a specific 20-bp guide RNA (gRNA), which can easily be made with standard cloning techniques. In addition to the advantages of simplicity and low cost, CRISPR is highly efficient [35–39] and enables multiplex targeting [36]. Recently engineered high-fidelity Cas9 variants have even reduced off-target effects, once a big concern for CRISPR, down to below detection levels of the most sensitive genome-wide methods available [40,41]. After Cas9, the "new kid on the block", even newer programmable nucleases are being discovered, such as two other CRISPR-associated nucleases Cpf1 [42] and C2c1 [43].

Programmable nucleases can be targeted to virtually anywhere on the genome to create a site-specific DSB. Cells utilize one of two types of pathways to repair the DSB, either homology-directed repair (HDR) or non-homologous end-joining (NHEJ). In the presence of a donor template, the HDR pathway repairs the DNA break via the above-mentioned HR mechanism. Precise modifications, including specific base pair substitutions and insertions ranging from a single base pair to large gene cassettes, can be introduced into the genome through this pathway. The NHEJ pathway, on the other hand, does not use a donor template and is error prone, leading to random-length insertion or deletion mutations (indels) that can often disrupt a gene, especially with frameshift indels [44] (Fig 1). When two DSBs are created on a chromosome, repairs through the NHEJ pathway can result in large genomic deletions (as large as 1,600 kb), inversions, and duplications [45].

Since the earliest HR studies [27,30], genomic editing has been routinely performed on both embryonic and somatic stem cells. As organoids are derived from aggregates of these stem cells and they require genomic alteration for many applications, the marriage between organoid and genome editing technologies is unsurprising. However, the fact that the establishment of organoid models has been built upon the basis of genomically engineered stem cells is often overlooked. In this review, we will highlight how genomic engineering techniques was used for the development and optimization of the earlier organoid models. We will also discuss how genome editing has been catered to model embryonic organ development and disease progression in 3D culture. In addition, we will discuss several technical considerations and provide insights into future applications.

Development of 3D organoid models using genomically engineered reporter cell lines

Traditionally, stem cell differentiation experiments are performed in two-dimensional (2D) monolayer culture conditions. While relatively homogenous populations of differentiated cells can be generated in 2D cultures, the 2D systems face the challenge of a lower degree of physiological relevance. Through several landmark papers published during the late 2000s and early 2010s, Yoshiki Sasai and his colleagues were among the first to not only add one dimension to the differentiation culture system, but also to take one step closer to accurately modeling *in vivo* physiology of an organ [1]. By aggregating dissociated ESCs into spheres and allowing them to differentiate and self-organize in 3D floating culture, Sasai and colleagues invented a new model system that recapitulated development of various organs, representing the cortical tissues, the retina, and the pituitary gland [3,4,46,47]. Though these organoid models were created before genome editing technologies became vastly popular, they relied extensively on genomically edited ESC lines while establishing these organoid models.

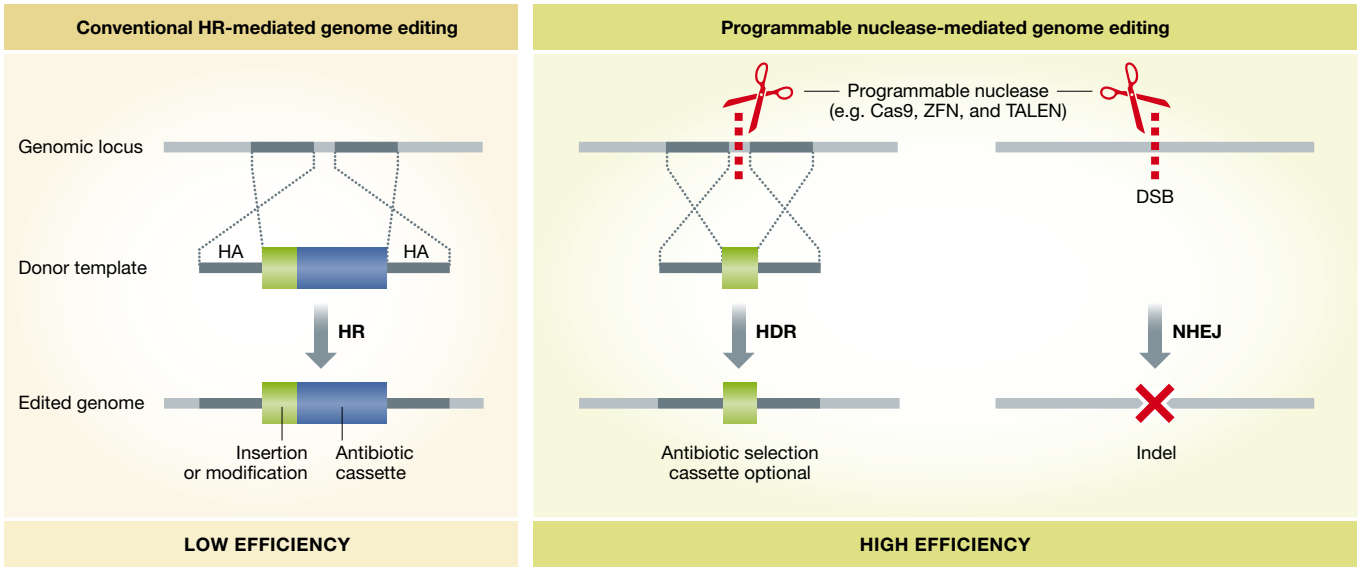

Figure 1. Genome editing mediated by conventional HR or programmable nucleases.
Genome editing has been traditionally achieved to low efficiency without the use of a programmable nuclease. Programmable nuclease-mediated DNA double-stranded breaks dramatically increased the efficiency of genomic editing.

For example, a genomically engineered reporter ESC line was essential for deriving retinal organoids from aggregates of mouse ESCs [3]. Previously, Sasai's laboratory established a protocol to promote retinal differentiation from mouse ESCs, but the differentiated cells lacked spatial patterning and did not morphologically resemble retinal epithelial structures [48]. While they wisely used the transcription factor Rx as a marker for neural retinal precursors in their culture, the lack of a readily identifiable fluorescence reporter compromised their efforts in identifying optimal culture conditions to promote the formation of self-organized retinal tissues. In their subsequent study, a mouse *Rx*-GFP reporter ESC line was generated to overcome this hurdle. Using conventional HR, the investigators inserted GFP after the start codon of one of the two alleles of the *Rx* gene, so that cells would be GFP^+ upon their differentiation into retinal lineages [49]. This fluorescence reporter cell line not only allowed for prompt identification of retinal precursor cells arising in culture, but also enabled easy tracking of the morphological changes of their residing tissues. Indeed, with the help of the *Rx*-GFP cell line, a stepwise differentiation method for 3D retinal organoids was developed. The derived organoids contained GFP^+ cells that bore retinal marker gene signatures. Moreover, these cells self-patterned into neural retina and retinal pigment epithelium domains. Remarkably, the self-patterned retinal cells morphologically self-organized into an optic cup structure that is strikingly similar to an optic cup developed *in vivo* [3]. A number of assays in this study, including fluorescence-activated cell sorting (FACS), multiphoton long-term 3D live imaging, 3D reconstruction of tissue and cell morphology, and cell migration tracking, were made possible due to the *Rx*-GFP fluorescent cell line.

Spurred by the success of optic cup organoid generation from mouse ESCs, human optic cup organoids were generated using genomically engineered fluorescent reporter cell lines as well (*RX*-Venus and *CRX*-Venus human ESC lines) [4]. It should be noted that for these two optic cup organoid studies, conventional HR without a programmable nuclease was used to establish the mouse ESC line [49], while the programmable nuclease ZFNs were utilized for the genomic modifications to the human ESC lines [4]. This is likely because conventional HR approaches, while considered standard in mouse ESCs before the era of the programmable nucleases, have been proven to be very difficult in human ESCs [24].

Besides the optic cup organoids, Sasai and his colleagues also took advantage of early genome editing technologies to establish a number of other reporter ESC lines to aid in the identification of other stem cell-derived organs and tissues. Examples include *Bf1*-Venus mouse ESC lines generated via conventional HR for the cerebral cortex organoids [47], *Lim3*-Venus mouse ESC lines generated via ZFN for the pituitary organoids [46], and *FOXG1*-Venus and *PAX6*-Venus human ESC lines generated via ZFN for the human neocortex organoids [7].

In addition to these ES cell-derived organoids, pioneering work on generating organoids from tissue-resident somatic stem cells also used fluorescently labeled reporter cells. Hans Clevers and his colleagues built intestinal organoids with FACS-sorted single *Lgr5*-GFP somatic stem cells [8]. The *Lgr5*-GFP cells were isolated from transgenic mice created via HR-mediated genomic editing in mouse ESCs followed by a blastocyst injection and transplantation into foster mice [50]. Besides the mouse intestinal organoids, genomically engineered human ESC and iPSC lines, with *LGR5*-GFP reporters built with ZFNs, have been used to generate human intestinal organoids [51].

Genome editing technologies used in organoid studies

As discussed above, organoid models were established with reporter PSC lines created by genomic engineering. Genome engineering was

also applied to these newly established organoid systems for inducing or correcting mutations in order to elucidate pathological conditions (Table 1). Many of the above-mentioned reporter cell lines were created by inserting GFP or Venus after the start codon of one of the two alleles of a gene of interest [4,7,46,47,49]. This labeling strategy resulted in disruption of the inserted allele, making the cells heterozygous mutants for the targeted gene. The same labeling strategy can be used to create homozygous mutant cells, simply by screening for cell lines in which both alleles have been inserted with a fluorescence protein [52]. In the pituitary organoid study [46], a mouse ESC line knocked out for the transcription factor gene *Tbx19* was created. The knockout was achieved by selecting for ZFN-mediated biallelic insertion of Venus at the start codon of *Tbx19*. During *in vivo* pituitary development, Tbx19 drives the differentiation of the cell lineage that produces the adrenocorticotropic hormone. To test whether the *in vitro* derived pituitary organoids recapitulate *in vivo* development, $Tbx19^{Venus/Venus}$ knockout ESCs were guided toward pituitary development in 3D culture. As expected, the expression level of the adrenocorticotropic hormone was significantly reduced, confirming the same cell lineage specification requirements between 3D culture and *in vivo* pituitary development.

The discovery of CRISPR/Cas9 was a major milestone in genome engineering. Just a few months after the first studies of CRISPR-mediated editing of mammalian genomes were published [36,37], Clevers and colleagues successfully applied this technology to intestinal organoids for mutation correction [53]. A mutation on the anion channel gene *CFTR* (F508del) is known to cause cystic fibrosis, a disease affecting multiple organs including lung and intestine. The function of CFTR can be assessed in a forskolin-induced swelling assay [54], in which healthy intestinal organoids respond by rapid swelling due to fluid secretion through the forskolin-activated CFTR channels, while organoids derived from patients with the *CFTR* mutation do not expand their surface area. The investigators used CRISPR to correct the *CFTR* mutation via co-transfection of a repair template vector. The genetically corrected intestinal organoids demonstrated a functional repair, as they rapidly expanded the organoid surface area upon forskolin treatment [53]. Together with previous successes of intestinal organoid transplantation in mice [55], this study provided a potential CRISPR/organoid-based therapeutic strategy for intestine symptoms of cystic fibrosis. In addition to this study, functional correction of disease genes has also been performed in intestinal organoids on the *CFTR* gene using ZFN [56] and on a telomere maintenance dysfunction-related gene *DKC1* using CRISPR [57].

Before performing the *CFTR* gene correction, Clevers and colleagues first optimized the CRISPR system in intestinal organoid cultures by targeting the *APC* gene [53]. As APC is a negative regulator of the Wnt pathway, *APC* null mutant organoids can grow in the absence of the normally essential ingredients Wnt and Wnt agonist R-spondin. This system provided an excellent opportunity to perform functional selection on the CRISPR edited organoids, as only mutant intestinal organoids devoid of APC function can survive and expand in the culture medium lacking Wnt and R-spondin. This elegantly designed study serves as a prime example of how targeted clones can be selected promptly and unequivocally based on the target gene function in the organoid culture.

Using the same functional selection strategies to isolate successfully edited organoids, researchers from two laboratories independently modeled colorectal cancer in intestinal organoids via CRISPR [58,59]. These groups demonstrated that by sequentially mutating tumor suppressor genes and oncogenes including *APC*, *SMAD4*, *TP53*, and *KRAS* with CRISPR, the mutant intestinal organoids grew independently of niche factors (e.g., Wnt) and formed tumors in hostile niche environments. When coupled with chromosome instability, the mutated intestinal organoids showed invasive and metastatic growth upon transplantation into mice. In another colorectal cancer-related study, researchers induced mutations in a distal hot

Table 1. Published studies of genome engineering in organoids.

Main purpose of genome editing	Type of modification	Organoid type	Cell of origin	Genome editing method	Reference number
Generating new type of organoids	Fluorescence gene knockin	Retinal organoids	mESC	Conventional HR	[3,49]
			hESC	ZFN	[4]
		Cerebral cortex organoids	mESC	Conventional HR	[47]
		Kidney organoids	hESC	Conventional HR	[15,95]
		Pituitary organoids	mESC	ZFN	[46]
		Neocortex organoids	hESC	ZFN	[7]
		Intestinal organoids	hESC and hiPSC	ZFN	[51]
Modeling of development	Gene disruption via biallelic knockin of a fluorescence gene	Pituitary organoids	mESC	ZFN	[46]
		Epiblast organoids	mESC	CRISPR (WT Cas9)	[52]
Modeling of disease	NHEJ-mediated gene disruption and/or HDR-mediated gene modification	Intestinal organoids	m/hSSC	CRISPR (WT Cas9)	[53,58–60]
			hSSC	ZFN	[56]
			hiPSC	CRISPR (Cas9n)	[57]
		Kidney organoids	hESC	CRISPR (WT Cas9)	[13]
Small scale screen	NHEJ-mediated gene disruption	Lung organoids	hSSC	CRISPR (WT Cas9)	[61]

spot region of another negative Wnt regulator gene *Rnf43* with CRISPR, and used the mutant intestinal organoids to study the importance of the *Rnf43* distal hot spot region in colorectal cancer progression [60].

In addition to intestinal organoids, CRISPR genome editing technology has been used in kidney organoids for disease modeling [13], and gene function testing in lung organoids [61]. In the latter example, the simplicity and effectiveness of CRISPR allowed investigators to rapidly mutate and screen the function of multiple genes. These genes were a subset of a previously identified pool of putative target genes of the master regulator of lung development, *GRHL2*. Using CRISPR-enabled small scale screening in lung organoid cultures, the investigators discovered two new downstream effector genes of GRHL2 that play important roles in ciliogenesis and barrier function in the airway epithelium [61].

Technical considerations

CRISPR-based genome editing technology, based on its versatility and broad application potential, is rapidly evolving, and many technical improvements are being made to make genome editing more specific and efficient.

WT Cas9 used in the first-generation CRISPR platform, while highly efficient, is also prone to off-target cleavages [62,63]. Most of the CRISPR-organoid studies discussed above used WT Cas9 (Table 1), and off-target indels were indeed found [53]. Several Cas9 variants have been created with greatly reduced off-target effects while retaining on-target activities comparable to WT Cas9. These next-generation Cas9s include the Cas9 nickase variants [64], and the two recently developed high-fidelity Cas9 variants, eSpCas9 (1.1) [41] and SpCas9-HF1 [40]. More recently, another CRISPR-associated endonuclease Cpf1 [42], in its WT form, is reported to be highly specific [65], at levels approaching that of the high-fidelity variants of Cas9 [66].

The CRISPR components can be transfected into cells in the form of DNA vectors encoding Cas9 and gRNA, or as Cas9 mRNA or protein with *in vitro* transcribed gRNA. To deliver these CRISPR components and donor templates into cells of interest, nucleofection is a highly efficient method, especially for hard-to-transfect cells such as human PSCs [67–69]. Other delivery methods, such as lipofectamine, conventional electroporation, and lentiviral infection, have also been successfully used to transfect embryonic or somatic stem cells [53,69–71]. In addition to transfecting dissociated stem cells followed by aggregation and differentiation into organoids, it is also possible to directly deliver constructs into organoids. For instance, adenoviruses have been shown to mediate efficient gene transfer into intestinal organoids [72], and retroviruses have been used to infect fragments of intestinal organoids [73].

When making precise genome modifications, small molecule inhibitors of the NHEJ pathway can be used to promote the efficiency of HDR [74–76], including the NHEJ inhibitor Scr7, which is reported to increase the HDR efficiency by up to 19-fold [77]. Recently, an impressive 60% HDR efficiency has been achieved using an asymmetric single-stranded DNA (ssDNA) donor with optimal homology arm lengths [78]. Chemically modified ssDNA donors with phosphorothioate linkages also enhance HDR efficiency [79]. In addition, restricting Cas9 or Cas9 variants to specific cell cycle stages favors the HDR pathway over the NHEJ pathway, thus increasing the HDR rate nearly twofold [80–82]. Combined use of these approaches may further improve the efficiency of HDR.

When making mutant organoids, it is important to note that not the entire targeted cell population contains indels, even after puromycin selection or FACS sorting when a Cas9-puromycin/GFP co-expressing vector is used. In addition, in-frame indels may not disrupt gene function, as the alteration of a small number of amino acids may not affect the protein function. Even a frame-shift indel, which disrupts the reading frame of a gene, may not be a complete gene knockout if it does not occur at the beginning of the gene. Therefore, to generate monoallelic/biallelic mutant

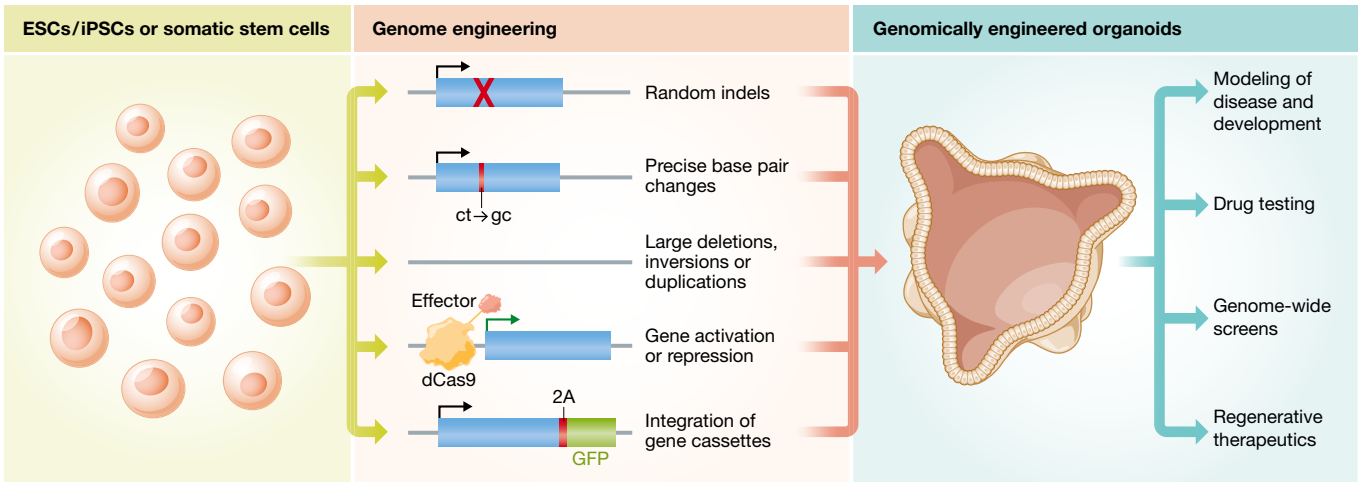

Figure 2. Workflow of genomic engineering in organoids.
Before *in vitro* differentiation into organoids, pluripotent stem cells (ESCs and iPSCs) or multipotent somatic stem cells can be genomically modified in various ways. The resulting genomically edited organoids play critical roles in applications such as disease modeling and drug testing.

organoids, it is essential to design gRNAs to target the beginning of the gene for all its splicing variants, establish cell lines from single cells, perform thorough genotyping and sequencing analysis, and use cell lines with frameshift indels. With regard to establishing clonal cell lines, single-cell survival rate has been a hurdle for clonal cell line generation in human PSCs. This challenge has been largely overcome by the discovery that ROCK kinase inhibitors can dramatically increase the survival rate of single human PSCs [83]. However, care should be taken as small clusters of cells due to incomplete single-cell dissociation may survive better than single cells [84], giving rise to "clonal cell lines" with mixed genotypes.

To create a reporter cell line, a fluorescence gene (e.g., GFP) is integrated at a marker gene locus (e.g., *Rx*) whose expression coincides with the emergence of the cell type of interest. The marker genes often play essential roles for the differentiation or the function of the cell type of interest, and therefore, reduced expression levels of the marker genes may decrease the target cell type generation efficiency or may affect the function of these cells. Up to now, most of the reporter cell lines in the organoid field were created at the expense of disrupting one of the two alleles of a marker gene, by inserting a fluorescence-antibiotic gene cassette right after the start codon of the marker gene. We recommend a non-destructive labeling design for optimal differentiation efficiency and normal functioning of the organoids. For example, GFP can be fused in-frame with the marker gene, or alternatively, a "self-cleavage" 2A sequence [85] can be placed between the marker gene and GFP (Figs 2 and 3) so that GFP will be co-expressed during transcription but later separated from the marker

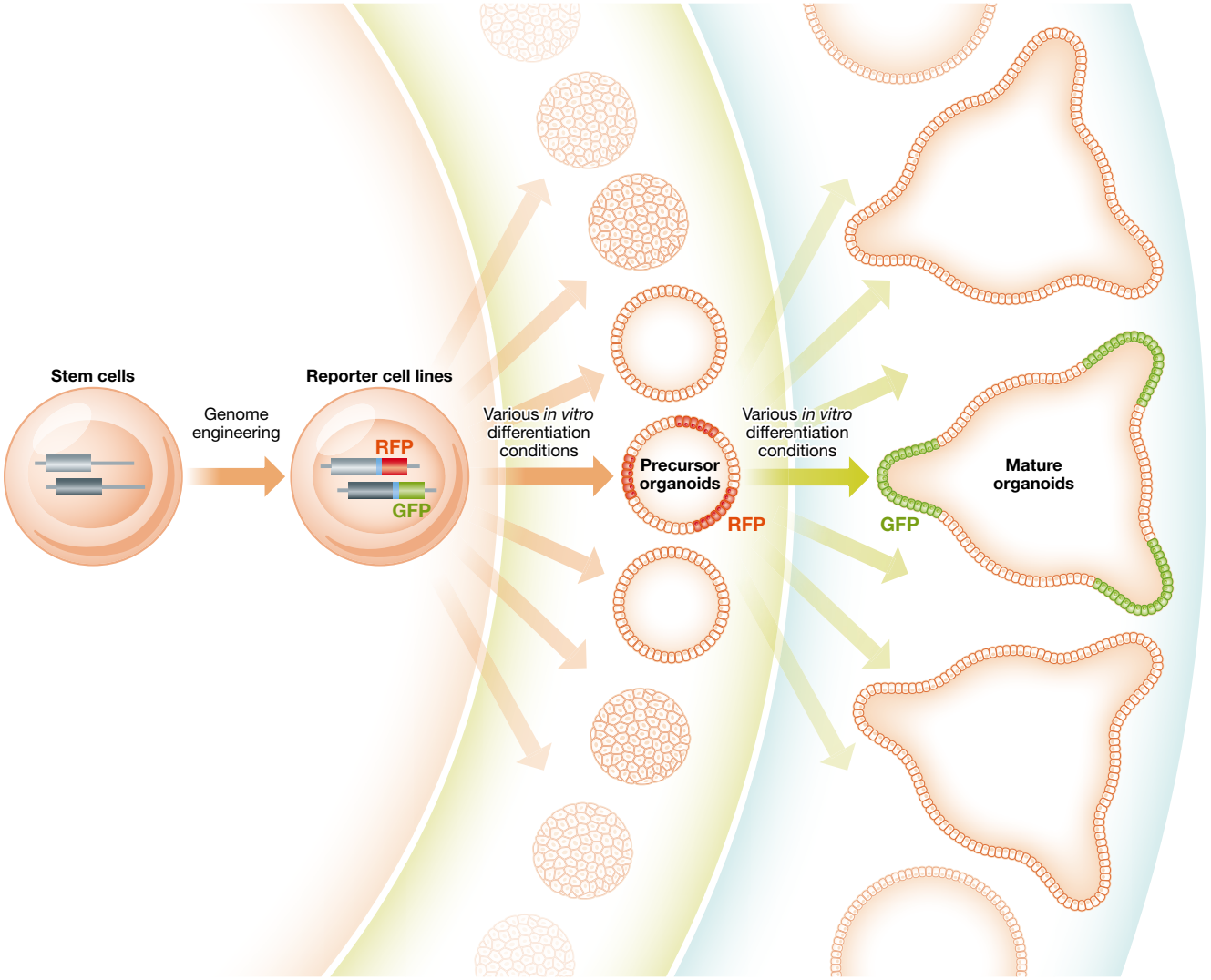

Figure 3. Generating new organoid models with reporter cell lines.
Development and optimization of new organoid generation methods can be greatly accelerated by genomically engineered fluorescence reporter stem cells. In this example, a red fluorescent protein (RFP) gene is integrated at a gene that is expressed in precursor cells. The successfully derived precursor cell-containing organoids will exhibit red fluorescence, allowing quick identification of optimal deriving conditions. Similarly, via multiplex genome editing in the same stem cell line, a green fluorescence protein (GFP) gene integrated at a gene that is expressed in the mature target cell type will assist protocol development for generating mature organoids. A short 2A "self-cleaving" peptide sequence (shown in blue) can be placed between the target gene and the fluorescence protein for non-destructive labeling.

Box 1: In need of answers

(i) Organoids are typically derived from PSCs through modulation of key signaling pathways by stepwise treatments with small molecules and recombinant proteins. Can such directed differentiation be achieved with CRISPR-based inducible gene activation/repression instead? Will the CRISPR-generated organoid models solve the consistency and efficiency issues that many current organoid models are facing?

(ii) Various novel methods, such as Scr7, asymmetric ssDNA donors, and cell cycle synchronization, have been recently developed to promote HDR. Can any of these methods be combined to further enhance the HDR efficiency?

(iii) Cas9 and its variants are the most popular genome editing tool in academia, but they are not yet available for biopharma and biotech industries due to the ongoing CRISPR patent dispute. Will Cpf1, which has a distinct intellectual property, be the answer for industry users who wish to replace ZFNs and TALENs?

gene during translation. The 2A-GFP labeling approach has been successfully used in human ESCs, demonstrating higher fluorescence intensity (indicating better protein stability) compared to the direct GFP fusion approach [86]. Since the fluorescence protein is separated from the marker gene protein during translation, it will have a pan-cellular localization pattern. If another cellular localization pattern is desired, short localization sequences can be added to the fluorescence gene. For instance, the nuclear localization signal (NLS) or nuclear export signal (NES) can be linked with GFP for localization inside or outside the nucleus, respectively, and a short sequence from the *MARCKS* gene can be added to GFP for plasma membrane localization, which can facilitate visualization of fine cellular morphologies [87].

Future perspectives

The past several years have seen a surge in new organoid models, aided by fluorescent reporter stem cell lines which allow prompt identification and confirmation of lineage-specific cell types arising in 3D culture, thereby greatly facilitating optimization of differentiation protocols. Now with the highly robust means to make genomic modifications at hand, we expect to see a significant increase in different organoid models being created with CRISPR-based reporter cell lines (Fig 3). Aside from developing methods to generate new types of organoids, reporter cell lines can be used to isolate specific cell types for biochemical or genomic assays (e.g., RNA-seq). Moreover, reporter cell lines are a must-have tool to identify rare cell populations in organoids for functional assays. For example, *Atoh1*-GFP (created by random genomic insertion of an *Atoh1*-GFP construct in mouse ESCs [88]) was used to identify sensory hair cells in mouse ESC-derived inner ear organoids, and mechano-electrical transduction currents as well as voltage-gated currents were successful recorded from these GFP-positive cells [89].

For research purposes, *ex vivo* CRISPR-mediated mutant gene correction can be easily achieved by genotyping and selecting for corrected cell lines. For example, a 3-bp deletion (*CFTR* F508 del) and a 1-bp point mutation (*DKC1* A386T) have been corrected with HDR in patient-derived stem cells, and the correct clones were selected for further studies in intestinal organoids [53,57]. However, due to the low efficiency of the HDR pathway, precise gene corrections *in vivo* for therapeutic purposes are technically challenging. Recently, several groups discovered that deleting a mutant exon entirely through NHEJ, in lieu of correcting disease-associated mutations via HDR, greatly improved muscle function in mouse models with Duchenne muscular dystrophy (DMD) [90–92]. This exon deletion approach holds great therapeutic promise as NHEJ is more efficient than HDR. Also, patient iPSC-derived organoids could serve as a beta-test platform prior to clinical trials to validate the therapeutic potential of this approach for various diseases.

In addition to making changes to the DNA sequences, the CRISPR technology can also be repurposed to regulate gene expression. The nuclease-deactivated mutant form of Cas9 (dCas9) can be fused with various effector domains. Bringing these effectors to specific genomic loci results in activation or repression of the genes, depending on the type of effectors [93]. Coupled with commercially available gRNA libraries, genome-wide CRISPR-based gene activation/repression screening should be readily applicable to organoid tissues (Fig 2). For example, gRNA-coding sequences integrated into the host genomes can be detected by deep-sequencing [94]. By comparing the abundance of gRNA-coding sequences that lead to activation or repression of their corresponding genes, it is possible to identify a set of genes essential for the specification of certain cell types.

In conclusion, 3D organoid technology is a new and fast-evolving field in stem cell biology. When combined with powerful programmable nuclease-based genome engineering, this technology provides exciting opportunities for a wide range of biomedical research, from uncovering mechanisms of human organ development to exploiting future clinical applications.

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Conflict of interest

The authors declare that they have no conflict of interest.

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