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Evolution of organoid genetics

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ABSTRACT

Organoids have revolutionized *in vitro* research by offering three-dimensional, multicellular systems that recapitulate the structure, function, and genetics of human tissues. Initially developed from both pluripotent stem cells (PSCs) and adult stem cells (AdSCs), organoids have expanded to model nearly every major human organ, significantly advancing developmental biology, disease modeling, and therapeutic screening. This review highlights the progression of organoid technologies, emphasizing the integration of genetic tools, including CRISPR-Cas9, prime editing, and lineage tracing. These advancements have facilitated precise modeling of human-specific pathologies and drug responses, often surpassing traditional 2D cultures and animal models in accuracy. Emerging technologies, such as organoid fusion, xenografting, and optogenetics, are expected to further enhance our understanding of cellular interactions and microenvironmental dynamics. As organoid complexity and genetic engineering methods continue to evolve, they will become increasingly indispensable for personalized medicine and translational research, bridging gaps between *in vitro* and *in vivo* systems.

1. Introduction

Organoids are well-established *in vitro*, 3D, cellular systems that can recapitulate their *in vivo* counterparts (Huch and Koo, 2015; Kim et al., 2020; Sato et al., 2009). Next to spatial organization and cell type variety, organoids also mimic transcriptomic and epigenetic profiles with a stable genome. Since the introduction of organoids, their applications have been ever-increasing. As of 2025, their usage is worldwide and thousands of published papers have utilized this culture system. These statistics are largely attributed to their ease of use and overall accessibility. However, the difficulty and skills required to culture organoids are highly variable depending on the organ of choice and related culture method.

Organoids can be classified as pluripotent stem cell- (PSCs) or Adult Stem Cell- (AdSCs) derived (Kim et al., 2020). Here, PSC entails both induced pluripotent stem cells (iPSCs) and embryonic stem cells (ESCs). Various reviews have already touched upon their differences and the array of organoid types currently available (Corrò et al., 2020; Kim et al., 2020). The latter, in short, reads that organoids of most organs have been established through either method. However, outstanding issues regarding inadequate recapitulation of some organoid types are mostly attributed to a lack of cell-type recapitulation. Which, in turn, is due to the current lack of knowledge regarding appropriate media compositions (Hofer and Lutolf, 2021).

There are several key differences between PSC- and AdSC-derived organoids. One of the first examples of PSC-derived organoids were cortical organoids, which were introduced before the AdSC-derived intestinal organoids from Sato et al. in 2009. They created three-dimensional cortical tissues from embryonic stem cells that also exhibited self-organization (Eiraku et al., 2008). Though widely recognized as the first PSC-derived organoids, they were not coined "organoids" but simply referred to as tissues or a three-dimensional aggregation culture. Soon after the term organoid was coined in 2009 (Sato et al., 2009), many PSC-derived 3D tissues were reported as organoids, such as retinal, brain, gastruloid, and kidney (Eiraku et al., 2011; Lancaster et al., 2013; Spence et al., 2011; Takasato et al., 2016; Van Den Brink et al., 2014). Most PSC-derived organoids are based on first creating aggregates and guiding *in vitro* organ development through stepwise differentiation protocols. AdSC-derived organoids, as the name suggests, come directly from organ-specific adult stem cells. These organoids can be maintained in culture with stem cells and differentiated cells. They can also be kept in a stem cell-enriched cystic state and

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further differentiation can be triggered through modifications of culture conditions. In general, PSC-derived organoids more accurately mimic organ development while keeping various cell types. Therefore, they tend to form more complex structures with epithelial tissue surrounded by niche cell types. It is recommended to use PSC-derived organoids when studying developmental processes such as organogenesis. AdSC-derived organoids are readily available from their tissue of origin and generally have simpler structures e.g., only having an epithelium (Hofer and Lutolf, 2021; Kim et al., 2020). Additional cell types have been introduced to improve overall cellular diversities. AdSC-derived organoids are more useful in banking patient materials and can be relatively easy to culture, depending on their tissue of origin.

As mentioned previously, AdSC-derived intestinal organoids and PSC-derived 3D cortical tissues marked the beginning of the organoid field (Eiraku et al., 2008; Sato et al., 2009) (Fig. 1). Regardless of the origin of derivation, this tool quickly became invaluable to developmental studies. In the early stage of organoid technologies, their general structures and recapitulative abilities were first explored. For AdSC-derived intestinal organoids, clear *in vivo*-like, budding crypt structures and villus domains could be observed in the simple single epithelium. These intestinal organoids showed intriguing self-organizing activity with continuous self-renewal powered by so called Lgr5+ stem cells. The stem cell field was revolutionized by the versatile iPSCs (Takahashi and Yamanaka, 2006). Interestingly, it did not take long before these malleable stem-cell cultures were adapted to form PSC-derived organoids. This highlighted the potential of creating 3D, self-organizing, layered structures from 2D cell cultures. Cerebral organoids with complicated brain structures were established not long after and were subsequently utilized for microcephaly studies (Lancaster et al., 2013). Only 4 years later, combined organoid models named assembloids were established (Birey et al., 2017). Organoids from different origins to mimic tissue-tissue interactions were developed to study neural circuit interactions *in vitro*. Organoids showed greater potential in biomedical and developmental studies, compared to 2D cell cultures and simple non-propagating spheroids. Next to stem cell and developmental biology, avenues in drug testing were rapidly realized (Rybin et al., 2021).

Organoids as a whole may not replace *in vivo* animal models but instead will serve as an accessible and scalable intermediate *in vitro* culture that can mimic *in vivo*. This incredible tool has been, and will be, used to identify novel drug candidates and to advance personalized medicine to a new level (Rybin et al., 2021). A primary reason for this is the fact that organoids made from human cells enable human experiments *in vitro*. Animal models are advantageous as an *in vivo* model, but limits remain regarding their ability to precisely model human biology. Organoids are also limited in modeling entire human physiology as an *in vitro* model, but by increasing biological complexity (e.g., cell types and tissue architecture) they can show improved performance in precisely modeling human micro-physiology in an organ specific manner.

In short, organoids of most organs have been established either through the use of PSCs or AdSCs. The same culture technology has been applied to disease-ridden tissues such as tumors, which has led to a cancer biobank with cancer-derived organoids, also referred to as tumoroids or tumor organoids (LeSavage et al., 2021). Furthermore, these organoid biobanks harbor a plethora of organoids across tissues and diseases, allowing for more accurate research through humanized models. In addition, the organoid field has benefited immensely from advanced genetic engineering technologies including various vectors, plasmids, transposons, and viruses. There has also been a lot of synergy with the big bang of CRISPR-Cas9-based gene editing tools. These systems enabled the era of organoid genetics, the main topic of this review.

2. The organoid toolbox

As the popularity for organoids rapidly increased, so did the variety of organ types. Within a decade, the human organoid library expanded to all the major organs, including the brain, heart, liver, pancreas, kidney, lung, gastrointestinal tissues, and sexual organs (Corrò et al., 2020; Kim et al., 2020). There was an increasing demand for novel ways of utilizing these 3D cell models. The genetic toolbox evolved with the organoid field and many ways of genetic manipulation in organoids have been developed for both PSC and AdSC-derived organoids (Fig. 1) (Teriyapirom et al., 2021). Here, these advancements will be shortly described with their principles including respective advantages and disadvantages (Table 1).

The first logical step towards utilizing organoids for biological studies was genetic manipulation. Genetic engineering and genetics have been the fundamental basis of human disease modeling, especially when using animal models. Animal models have been instrumental as a proxy to understanding human biology. However, it had been a long wait for better human disease modeling until the beginning of the human organoid era. Since organoids can be derived directly from human tissues or cells, biological studies with human organoids can be efficiently translated, compared to animal models with potentially different physiology (Huch and Koo, 2015; Kim et al., 2020). Furthermore, organoids mimic the original structures of their respective organs and retain cell type composition, giving clear advantages over 2D cultures regarding in-body resemblance (Sato et al., 2009). Although organoids still have limits in completely simulating human physiology *in vitro*, they provide unprecedented details with 3D structures for mimicking micro-physiology. Moreover, in combination with co-culture, bio-chips, imaging, and advanced analysis tools such as single cell and spatial omics techniques, organoids provide a platform that closely resembles *in vivo* human tissues (Hofer and Lutolf, 2021).

2.1. RNA interference

To genetically manipulate organoids, various methods can be employed. The easiest of which is RNA interference (RNAi). Gene expression can be silenced at a post-transcriptional level by simply transfecting short-hairpin or short-interfering RNAs. The neurodevelopmental disorder microcephaly was modeled through the knockdown of CDK5RAP2 in PSC-derived cortical organoids (Lancaster et al., 2013). Additionally, RNAi was also used to elucidate the roles of m^6A RNA methylation during cortical neurogenesis and of OLIG2 in interneuron production (Xu et al., 2019; Yoon et al., 2017). These examples highlight the value of utilizing RNAi as a form of genetic manipulation in PSC-derived organoid systems, especially for neurodevelopmental fields. Genetic knockdown is potentially an effective and easy-to-use system in many organoids without the need for specialized cell lines. However, the efficiency of knockdown varies significantly from target to target, even when targeting the same mRNA. RNAi does not cause a full knockout, meaning that the expression level of the target gene is lowered but not reduced to zero. Moreso, off-target effects have been observed, making it even more complicated when assessing gene function adequately through the knockdown strategy. For this, genetically engineered knockouts or knock-ins can give better insights due to clearer phenotypes.

To stably modify the genome of organoids, several strategies can be employed. For AdSC-derived organoids, genetically modified mice can be utilized to establish organoids from. This is quite advantageous for quick up-scaling and testing. However, making new organoid lines this way is time-consuming. It is also not possible to introduce novel mutations to humans for genetically modified human models, although patient materials with germline or somatic mutations can still be used for similar purposes. To circumvent this problem, both CRISPR- and non-CRISPR-mediated techniques have been developed for genetic tagging or gene editing in organoid systems.

2.2. CRISPR-Cas9

The best-defined organoids are the AdSC-derived mouse small

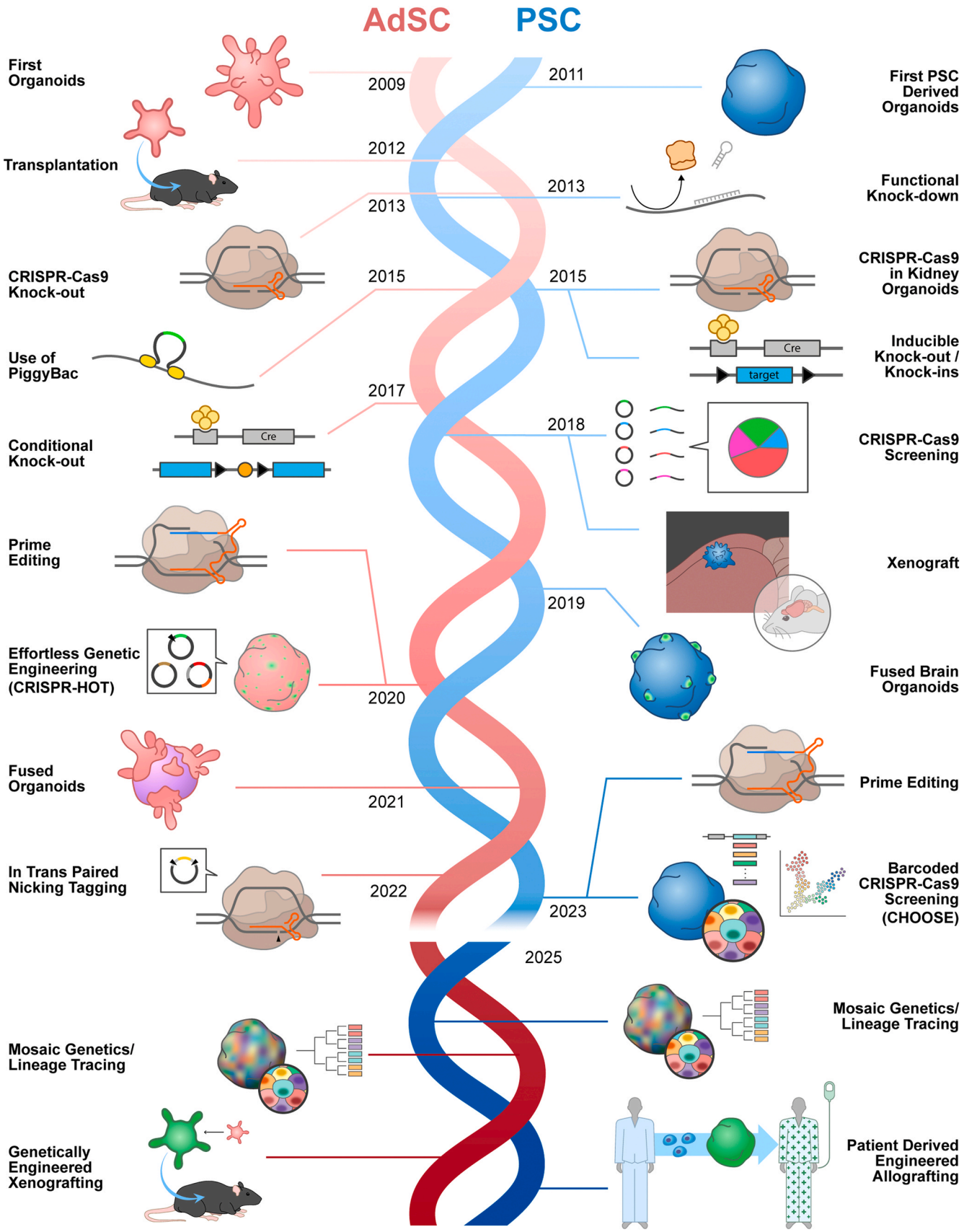

Fig. 1. Schematic overview of the developed techniques and milestones for both PSC-derived organoids and AdSC-derived organoids over time with expected upcoming developments. Reference papers for AdSC from top to bottom are Sato et al. (2009); Yui et al. (2012); Schwank et al. (2013b); Andersson-Rolf et al. (2017); Schene et al. (2020); Artegiani et al. (2020); Bollen et al. (2022), respectively. Reference papers for PSC from top to bottom are Eiraku et al. (2011); Lancaster et al. (2013); Freedman et al. (2015); Bian et al. (2018); Bian et al. (2018); Linkous et al. (2019); Chao Li et al. (2023); Chong Li et al. (2023), respectively.

Organoid limitations

AdSC-derived organoids are constrained by their inability to fully recapitulate all cell types of the target tissue and their limitation in modeling early developmental processes. Whilst PSC-derived organoids can mimic early development, they often display batch-to-batch variability and lack full maturation capacity. Recognizing their limitations and strengths is crucial for using organoids as a model system. A major value lies in serving as substitutes when in vivo experiments are not feasible like human cases.

intestinal (SI) organoids. Since these SI organoids were the first organoids to be established, they have been readily combined with various genetic engineering tools. Firstly, retro- and lentiviral vector-based genetic overexpression and knockdown have been tried as the first attempt of *in vitro* genetic modification in SI organoids (Koo et al., 2011). However, it has been less popular due to being time-consuming and disadvantageous in ease of use. Direct delivery of plasmids or bacterial artificial chromosomes (BACs) has also been tried through relatively inefficient electroporation or lipofectamine-mediated transfection methods (Schwank et al., 2013a). These methods did not gain much attention until the time of the CRISPR revolution.

The first CRISPR application in organoids was shown by using homology-directed repair (HDR) mediated by CRISPR-Cas9 to make a single amino acid correction in the CFTR locus of cystic fibrosis patient-derived colonic organoids (Schwank et al., 2013a). This proved the possibility of utilizing Cas9 within organoids. Other studies quickly followed suit and highlighted the use of generating colon cancer-like organoids through CRISPR-Cas9 indel-mediated knockouts (Drost et al., 2015; Matano et al., 2015). An improved version of CRISPR-Cas9 mediated organoid genome engineering that came out in 2020 is CRISPR-HOT (Artegiani et al., 2020). This technique is adapted from CRISPaint for base-specific gene tagging (Schmid-Burgk et al., 2016). CRISPaint is a homology-independent transgenesis technique that enables easily adaptable, non-laborious, site-specific gene editing for either knock-ins or knock-outs. CRISPR-HOT adapted the technique in organoids and showed that Non-Homologous End Joining (NHEJ) could achieve high efficiencies for genomic insertions in intestinal and liver organoids (Fig. 2). Within a few years, this technique has gained considerable popularity. It has been applied in various studies, including revealing the function of IL22 in Paneth cell formation in the intestine and developing fibrolamellar carcinoma organoid models (He et al., 2022; Rüland et al., 2023). CRISPR-Cas9-mediated gene editing has proven to be a revolutionary tool for generating genetically engineered organoids and reporter lines.

In comparison, genetic engineering through CRISPR-Cas9 in PSC-derived organoids is relatively straightforward. As PSC cultures are in 2D, the optimization and generation of CRISPR-Cas9-engineered PSC lines were already readily established and were quickly adapted to generate modified organoid lines (Zhou et al., 2021).

In addition, PSC-derived organoids of all kinds have also been subjected to genetic engineering. An adequate example is the work by Freedman et al., which modeled kidney disease in organoids formed from genetically engineered PSCs (Freedman et al., 2015). They knocked out two polycystic kidney disease genes, resulting in cyst formation. This work highlights CRISPR-Cas9 as an incredibly useful tool for gaining a deep understanding of patient-specific variants through gene editing (Koo et al., 2019; Raja et al., 2016).

Some disease mechanisms that impair stem cell proliferation or survival make it challenging to stably generate knock-outs in PSC populations. In such cases, creating a conditional knock-out targeting a specific subset of cells at a controlled time point becomes necessary. Conditional knock-outs have been well established in hPSCs with either Flp/FRT or Cre/LoxP (Chen et al., 2015). These recombination systems allow for controlled inversions or deletions. Conditional knock-outs can also be achieved using a system that enables sgRNA expression in a cell line constitutively expressing Cas9, providing precise temporal and spatial control over gene editing (Snijders et al., 2019). Originally developed in hPSCs, this technique can be seamlessly applied to organoid systems.

Conditional knock-outs can also be achieved using the insertion of a conditional intron system that targets a specific portion of the gene. In 2017 a conditional knock-out technique was developed called CRISPR-FLIP which was shown to work in ESCs and organoids (Andersson-Rolf et al., 2017). This system requires insertion of a cassette harboring a fluorescent protein or selection marker within an exon which functions as an intron sequence. Without recombination the protein within the cassette is transcribed through a promoter sequence whilst the target protein expression remains the same as the cassette is spliced out without any interference. However, following recombination, the target protein can no longer be transcribed as the splice acceptor is disrupted. The fluorescent protein or selection marker within the inserted cassette is still expressed after recombination. Furthermore, an alternative version of their CRISPR-FLIP harbor additional FLP sites allowing for a reversible knock-out. Recently, another system that utilizes a conditional intron was developed named Short Conditional Intron (SCON). This technique enables inducible knock-out of the gene of interest following the insertion of a small (<200 bp) construct (Wu et al., 2022). In this system, the intron is recombined upon Cre or Flp-mediated recombination so that transcription no longer generates a functional protein, resulting in a knock-out. While this system has been demonstrated to function in organoids derived from genetically engineered mouse lines (Wu et al., 2022), studies utilizing this approach in human PSC-derived organoids have not been published yet. Lastly, though not targeting the gene itself, CRISPR-Cas9 mediated knock-ins of a degron tag could be an alternative approach to conditional genetics. The auxin-inducible degron (AID) system can rapidly deplete protein expression of proteins tagged with degron (Nishimura et al., 2009). Unfortunately, AID does not ablate 100 % of the protein expression as some proteins tagged with degron can escape recognition by E3 ligase, or if the target protein is expressed at high levels. However, their major advantage comes from its reversibility and could therefore be a useful tool within organoids to study knock-downs and reintroduction of proteins.

2.3. Patient derived iPSC organoids

iPSCs can be established from human somatic cells, such as fibroblasts or blood cells, by reprogramming them using the introduction of Yamanaka factors (Takahashi and Yamanaka, 2006). This process reverts the cells to a pluripotent state while preserving their genetic information. These cells can be obtained non-invasively from patients with genetic diseases, enabling the development of patient-specific organoid lines. iPSCs derived from patients with a unique nonsense mutation resulting in LCA5-associated retinal disease, were gene-corrected and exhibited a rescued phenotype (Afanasyeva et al., 2023). Similarly, the SLC12A3 gene was corrected in patient-iPSC-derived kidney organoids to rescue the phenotype of Gitelman's Disease (Lim et al., 2023). These recent examples show the power of utilizing iPSCs from patients to prove gene-correction functions properly through subsequent organoid formation. Following the proof of the reversibility of genetic disease

Table 1
Summary table of discussed systems with their respective function, developed techniques or adaptations, advantages, disadvantages and references. AdSC-derived organoid, standard PSC culture, and PSC-derived organoid applicability are also mentioned, here, "in theory" is used when it has not been published to but can theoretically and realistically be applied.

			Applicable in:					
System	Function	Developed Techniques	PSC cultures	PSC-derived organoids	AdSC-derived organoids	Advantages	Disadvantages	Example Refs
RNA Interference	To introduce either small RNA fragments to block translation of a target gene	shRNA miRNA	Yes Yes	Yes Yes	Yes Yes	Easy to use Low prep time Cheap	Knock-down leaves remnant expression of gene of interest	Lancaster et al., (2013); Yoon et al., (2017)
Conditional Knock-Out	To introduce a cassette into the genome which allows for a complete knock-out a gene at a desired timepoint	OPTiKO CRISPR-FLIP SCON	Yes Yes Yes In theory	Yes Yes In theory Yes In theory	Yes Yes Yes In theory	Temporal control Fast and easy Complete KO Can assess KO effect of embryonic lethal genes at later stages	Laborious For CRISPR-FLIP and SCON not all genes are targetable Requires homozygous tagging making it more difficult	(Andersson-Rolf et al., 2017; Snijders et al., 2019; Wu et al., 2022)
Transposable Element	To introduce gene fragments into the genome randomly through transfection	PiggyBac Sleeping Beauty	Yes Yes	Yes Yes	Yes Yes	Easy to use Cheap Effective	Random integration Sleeping Beauty leaves DNA scar upon excision	Lee et al., (2022); Sandoval-Villegas et al., (2021)
Fusion	To fuse organoids with different genetic backgrounds or origins and assess cellular interactions	GLICO Fused Organoids	No No	Yes No	No Yes	Easy to use Can model interactions between tissues or different genotypes	Need to likely do genetic engineering prior to experiments	Krotenberg Garcia et al., (2021); Linkous et al., (2019)
CRISPR-Cas9	To introduce a double stranded break to create knock-ins or knock-outs	CRISPR-Cas9 CRISPR-HOT	Yes Yes	No No	Yes Yes	Easy to use Well established	Can have low efficiency Can create unwanted indels Making organoid lines is time consuming	Artegiani et al., (2020); Chen et al., (2015); Schwank et al., (2013b)
In Trans Paired Nicking	To introduce a nick into the genome to enable gene tagging	ITPN	Yes	No	Yes	Creates almost no indels	Has limited use	Bollen et al., (2022)
Prime Editing	To introduce small insertions, deletions, or substitutions	-	Yes	Yes	Yes	Highly efficient No indels Easy to use	Has limited functionality Cannot insert big fragments	Anzalone et al., (2019); Chao Li et al., (2023); Schene et al., (2020)
Transplantation	To transplant tissue (in this case organoids) from another animal of the same species	-	Yes	No	Yes	Can be used for assessing interactions with surrounding cell types	Difficult to perform Does not always work	Revah et al., (2022); Tadokoro et al., (2024); Watanabe et al., (2022); Zhang et al., (2023)
Xenografting	To transplant tissue (in this case organoids) from another animal of a different species	-	Yes	Yes	Yes	Can be used for assessing interactions with surrounding cell types by putting human organoids in mice	Difficult to perform Compatibility issues can arise	Bian et al., (2018); Zhang et al., (2021)
CRISPR-Cas9 based KO screening	To assess importance of genes in different genetic backgrounds	-	Yes	Yes Some	Yes	Lots of data High chance for discovering new gene interactions	Labor intensive Requires a good readout Expensive	Michels et al., (2020); Ringel et al., (2020)
Barcoding based screening	To assess the full genome of singular cells upon knock-outs or other perturbations	Tracer CHOOSE CRISPR-LICHT	Yes	Yes	Yes In theory	Lots of data Full transcriptome information Will find new gene interactions	Labor intensive Requires a good readout Expensive	Esk et al., (2020); Chong Li et al., (2023); Lindenhofer et al., (2024)
Concatemer CRISPR-Cas9 based screening	To introduce and assess the effect of paralogue inclusive genetic knock-outs	-	Yes In theory	Yes In theory	Yes	Can detect full knockout without paralogues fulfilling the role in a simple KO screening	Labor intensive Requires a good readout Expensive	Andersson-Rolf et al., (2016)
Visible Lineage Tracing	To introduce a cassette that can assess offspring over time by fluorescent labeling for example	-	Yes In theory	Yes In theory	Yes	Allows for tracing of progeny and spatial information if labeled fluorescently	Largely undiscovered territory in organoids	Huang et al., (2024)
Light inducible systems	To allow for spatial activation of certain genes like CRISPR-Cas9 for site-specific knock-outs	LACE CPTS2.0	Yes	Yes In theory	Yes In theory			Nihongaki et al., (2017); Polstein and Gersbach, (2015)

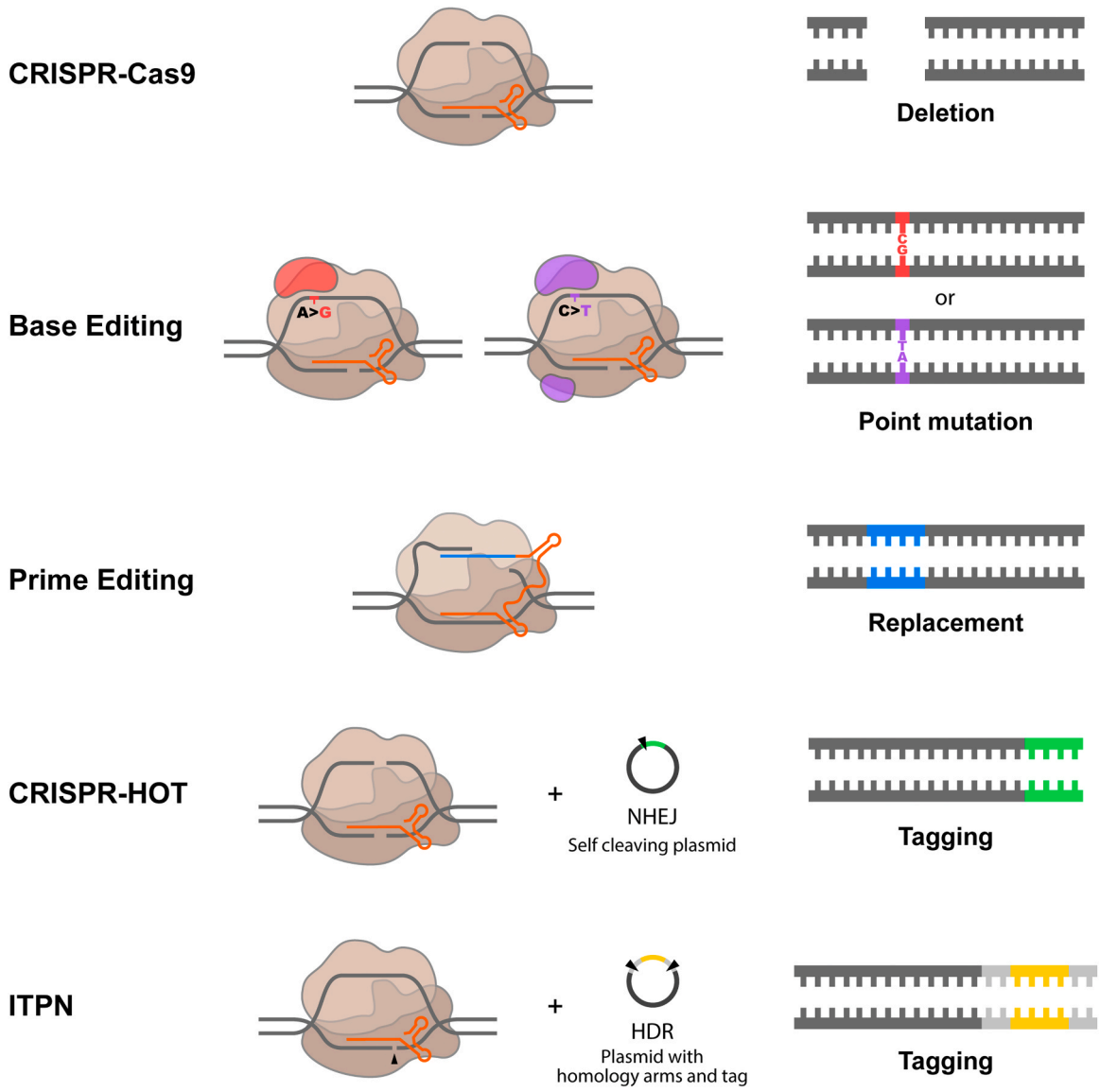

Fig. 2. Schematic overview of developed CRISPR-Cas9 based DNA editing techniques. The examples include classical CRISPR-Cas9, Base Editing, Prime Editing, CRISPR-HOT, and ITPN, with their respective results.

phenotypes by organoids is developing functional treatment. The same organoids would be a great starting point to test realistic strategies that could be translated to *in vivo* to cure genetic mutations.

PSC-derived organoids often show lack of maturation and cannot model epigenetic related conditions. As previously mentioned, PSC-derived organoids also display high variability. The latter issue could potentially be circumvented by increasing the number of patients within a study to increase accuracy. A good example of this is shown by Kimura et al., which utilized a panel of patient derived iPSCs to establish liver organoids (Kimura et al., 2022). Their 24-patient panel study focused on patients with steatohepatitis, a type of fatty liver disease, to address metabolic associated genetic traits. They found disease severity to be associated with a diabetic state and highlighted the potential of utilizing *in vitro* patient panels. Other genetic diseases which are difficult to dissect within patients could also benefit from PSC-derived organoid cohorts.

An alternative take on increasing cohort size is through multiplexed organoids. This strategy can overcome previously mentioned limitations regarding the number of donors used for PSC-derived organoid cohorts. This more inclusive approach utilizes so called chimeroids or mosaic organoids (Antón-Bolaños et al., 2024; Caporale et al., 2024). In both studies, patient-derived PSCs were used to generate cortical organoids, but the mosaic version does not require dissociation and re-aggregation to maintain a balance of individual lines within the organoid. Their models highlighted that individual lines within different combinations prevail which might be linked to genetic backgrounds of said PSCs. These unique strategies can enable high-throughput, easily scalable screening options to utilize for interindividual disease patterning.

2.4. Cas9 nickases and prime editing

Uniquely, In Trans Paired Nicking (ITPN) can be used for double-stranded-break-free gene-tagging (Bollen et al., 2022). This indel-free technique has been shown to allow for single-round triple fluorescent tag knock-ins. The modified Cas9 nicks the DNA in only a single strand, which, when combined with offset nicking sites, significantly reduces the chance for off-target mutations. Due to a reduction in off-targets indels, tagged cells can be pooled, resulting in a population primarily consisting of knock-in cells. This technique reduces labor time for organoid model generation and is an avid competitor with CRISPR-HOT for effortless gene tagging.

Another Cas9 nickase-based system is prime editing (Anzalone et al.,

2019). This technique also reduces CRISPR-Cas9 indel frequency due to the lack of double-stranded breaks. It utilizes prime editing guide RNA, which harbors both the guide and a small template, to enable substitutions, small insertions, or deletions (Fig. 2). This highly accurate tool was developed in 2019 and has already been applied in various organoid systems. Several studies highlight how patient organoids can be subjected to prime editing to recover disease-causing mutations. Notably, patient AdSC-derived intestinal organoids with DGAT-1 deficiency, liver organoids of Wilson disease, and in both rectal and hepatocyte organoids with cystic fibrosis-causing mutations (Bulcaen et al., 2024; Geurts et al., 2021; Schene et al., 2020). Furthermore, iPSCs were also subjected to CFTR correcting mutations and subsequently differentiated to airway epithelial cells and lung organoids (Chao Li et al., 2023).

2.5. Screening

CRISPR-Cas9 is also famous for being used in screening methods through sgRNA libraries. A major example was shown in combination with CRISPR-Cas9 for mutagenesis screening. In 2018, Bian et al. established their neoplastic cerebral organoid (neoCOR) model (Bian et al., 2018). It utilizes healthy human PSC-derived cortical organoids with integrated Cas9 and GFP to quickly mutate various target genes and highlight which cells had Cas9 activity. They modeled combinations of high-incidence mutations and/or insertions, which are important for glioblastoma initiation in cerebral organoids. The transposon system *Sleeping Beauty* mediated the insertions, which will be discussed in the next section. This model is limited by the Cas9 efficiency as not all GFP-tagged Cas9-expressing cells will harbor a mutation following the addition of guide RNAs. A more consistent and advanced screening method was later introduced by Li et al., which is their CRISPR-human-organoids-single-cell RNA sequencing (CHOOSE) system (Li et al., 2023). They revealed how autism-related genes affect cell fate through loss-of-function screening of barcoded mosaic organoids. Another screening method within brain organoids utilized a loss-of-function-based cell enrichment after fluorescent cell sorting as a readout, named CRISPR-LICHT (Esk et al., 2020). This setup was developed to assess microcephaly-associated pathways and revealed the importance of an endoplasmic reticulum regulator for the development of the disease. Additionally, various screening methods that have already been developed in iPSCs can also easily be adapted for use in organoid systems, for possible new hits that were missed in their 2D screenings due to a lack of cell-type distribution and interaction.

AdSC-derived organoid screening has been employed for various goal-ends, of which some will be discussed. Colon-organoids have been genetically engineered to mimic oncogenic KRAS mutations and were screened for their drug resistance (Cortina et al., 2017). Intestinal organoids rely heavily on WNT signaling for its renewal. A study by Andersson-Rolf et al. proved how simultaneous knock-outs of paralogue genes allowed for WNT independence (Andersson-Rolf et al., 2016). They did so by utilizing multiplexed gRNAs within a single vector for simultaneous knockouts in single cells (Andersson-Rolf et al., 2016). Through pooled CRISPR screening, tumor-suppressive genes were identified in both intestinal and colon organoids (Michels et al., 2020; Ringel et al., 2020). CRISPR screens with libraries of gRNAs were applied to mutant intestinal or colon organoids to identify genes that confer TGF-β resistance (Michels et al., 2020; Ringel et al., 2020). In the future, more genome-wide, paralogue-inclusive screening methods are of great importance to reveal overlooked genes of interest with strong genetic redundancy among paralogues. Moreover, screening methods performed in 2D systems could potentially have missed hits within their experiments due to the lack of cell-type variety and architectural interactions. Additionally, many patient-derived organoid libraries have been utilized for efficient and accurate drug screening (Driehuis et al., 2020; Jacob et al., 2020; Vlachogiannis et al., 2018; Zhang et al., 2021). These studies have all highlighted that patient-derived organoids can recapitulate drug responses and predict their efficacies. Notably, organoids from patients can also quickly be established from resected tumors and utilized for research (Jacob et al., 2020). However, conducting these screens through patient-derived organoids is challenging. This is attributed to requiring many cells for screening. Furthermore, performing all these experiments within time to have value for the patient also poses a significant challenge. These issues can be overcome in time as screening techniques improve in required quantity and error rates.

Screening has been a powerful way to identify interesting novel genes. However, considerable limitations are yet to be overcome. There are many challenges remaining in whole-genome screening as the sheer size of the genome is the primary obstacle. Moreover, applying whole-genome screening to organoids poses another big challenge due to limitations in scalability of organoid cultures. This issue will be resolved when large scale floating cultures become available for 3D organoids. Higher-resolution clonal approaches are desired for 3D organoids as recently introduced (Uijttewaal et al., 2024), but it will require further adaptations to be actively used in organoids. Overall, some of the challenges are currently being solved or will be solved soon, which will unlock the full potential of the organoid system in CRISPR screening.

2.6. Transposon systems

Genetic engineering in organoids without the use of CRISPR-Cas9 comes with their own advantages and disadvantages. Firstly, they are primarily transposon-based and allow for easy random knock-ins but not precise knock-ins and knock-outs. The transposons-based systems like *piggyBac* and *Sleeping Beauty* allow for integrating Inverse Terminal Repeat (ITR) flanked regions into the genome (Cary et al., 1989; Ivics et al., 1997). This easy incorporation of genes is random and thus can result in unintended disruption of crucial genes. However, thanks to the abundance of junk DNA within mammalian genomes, the chance is relatively low, and incorporation sites can be easily verified through sequencing. *piggyBac* is the go-to cut-and-paste mechanism in organoids as it can be used to integrate a large DNA fragment. It has been utilized in various organoid models such as gastric and intestinal organoids to reveal the functions of genes important for tissue homeostasis, repair, and tumorigenesis (Ahn et al., 2024; Lee et al., 2022). *Sleeping Beauty* is a simpler version with a smaller payload capacity which is redeemed by its ease of use (Sandoval-Villegas et al., 2021). Importantly, the aforementioned transposon systems, *Sleeping Beauty* and *piggyBac*, are reversible through excision. *Sleeping Beauty* leaves a footprint but *piggyBac* can be excised from the genome without a trace. This approach has been particularly advantageous for iPSC generation, especially when removing reprogramming factors or a targeting construct from the genome (Sandoval-Villegas et al., 2021).

2.7. Fused organoid models

Unlike the previous examples, organoids can be studied through modifications in alternative ways. One such emerging method is through organoid mixing. Fusing organoids of different genetic backgrounds within a controlled environment allows for research into competition and even invasion, especially when cancer-like organoids are used. Garcia et al. described a method to fuse tumor-like fluorescently labeled organoids, harboring various tumorigenic mutations, with fluorescently labeled wild-type organoids (Krotenberg Garcia et al., 2021). These mixed organoids revealed how cell competition enhanced intestinal cancer cells and how a fetal-like state induced by JNK activation disadvantages wild-type cells, which lead to their elimination. Furthermore, in a recent preprint of their continuation of mixed organoid experiments, normal liver organoids were mixed with tumor-like intestinal organoids (Krotenberg García et al., 2023). They found that metastatic competition is different from the competition observed within fused organoids derived from one organ. The study highlighted tissue-specific competition mechanisms, including reduced fitness and

forced differentiation.

For PSC-derived organoids, useful fused organoid models have also been generated. Notably, glial cerebral organoids (GLICO), which mix wild-type cerebral organoids with fluorescently tagged patient-derived glioblastoma multiforme (GBM) organoids (Linkous et al., 2019). The system can not only model primary human GBM *ex vivo* but can also deepen the understanding of invasion and tumor microenvironment interaction. Moreover, it can efficiently be utilized for high-throughput drug screening to resolve invasiveness. Taken together, mixing wild-type organoids with genetically edited or tumorigenic organoids allows close monitoring of normal cell-tumor interactions. Furthermore, metastatic capabilities and molecular changes can also be studied through fused organoids models.

2.8. Transplantation and xenografting

Another way to study metastatic capacities is xenografting, which mimics the tumor microenvironment. Transplantation of organoids to *in vivo* models allows for deeper analyses of cell-cell interactions. One major limitation of organoids is their lack of surrounding cell types found in the original tissues, which in part has been circumvented for some organoids through co-culture with endothelial cells and other cell types (Hofer and Lutolf, 2021). However, a lack of immune system interactions and other intra-tissue communications persist. Therefore, xenografting is a powerful way to further utilize human organoids in a more accurate *in vivo* context. PSC-derived organoids, primarily human cortical organoids, have been readily xenografted into mouse brains to assess invasiveness (Bian et al., 2018; Zhang et al., 2021). Xenografting of patient-derived GBM organoids has proven invaluable as they allow for understanding what mutations enhance invasiveness and aggressiveness. The other major limitation of PSC-derived organoids is their lack of maturation. Transplanting or xenografting to *in vivo* models also enhances their maturation through vascularization and inputs from the existing microenvironment (Revah et al., 2022). Furthermore, general interactions with host-tissues, formation of neural circuits, and seeing how the immune system reacts are key points that can be addressed with xenografting. Transplantation of AdSC-derived organoids has been a hot topic and has shown promising results for various means. Watanabe et al. highlighted that donor organoids can aid in rebuilding an injured colon epithelium (Yui et al., 2012). Furthermore, organoids from the human small intestine have been shown to repair a damaged mucosal epithelial layer (Zhang et al., 2023), and hPSC-derived liver organoids have been shown as promising tools for treating liver fibrosis (Tadokoro et al., 2024). Additionally, insulin-producing islet organoids have been transplanted in diabetic mice, reversing their disease phenotype (Huang et al., 2023). Even colonic organoids have been utilized to treat ulcers, highlighting that organoids can be utilized for regenerative medicine (Zhang et al., 2023).

Specifically for brain organoids, another technique gaining popularity is called patch-sequencing (Patch-seq). Patch-seq is a technique that combines electrophysiology, morphology, and single-cell RNA sequencing (scRNA-seq). It starts by recording the electrical activity of a cell using patch-clamp electrophysiology. Then, the cytoplasmic content is extracted for RNA sequencing to analyse gene expression, often alongside imaging to capture the cell's morphology. This enables a comprehensive understanding of each cell's function, structure, and molecular identity simultaneously (Cadwell et al., 2016; Lipovsek et al., 2021). Though not readily applied in organoids, it has been used to identify different maturity states in neuronal cultures (Bardy et al., 2016), and to dissect islet dysfunction in diabetic pancreas samples (Camunas-Soler et al., 2020). This technique could be applied to disease-mimicking organoids derived from patients with neurological disorders to assess cellular behavior and molecular identity simultaneously.

Regardless of the many tools available for organoid manipulation, numerous techniques can still be applied to organoid systems. These include novel spatial and single-cell multi-omics, which can reveal cell-type interactions, advanced live imaging systems, innovative biochip-based technologies to more accurately recapitulate the microenvironment, and higher-throughput screening systems for broader genome-wide coverage. Moreover, as organoids offer unique characteristics unattainable in traditional culture systems, novel strategies can open up previously inconceivable avenues of research.

3. Future perspective

It has become evident from this review that organoid genetics and associated tools have been rapidly evolving alongside organoid techniques themselves. Logically, this is easy to grasp as both PSC-derived and AdSC-derived organoids are both highly malleable with readily established tools for different reasons. PSC-derived organoids come from 2D cell cultures, which have been studied for decades, leading to the development of numerous engineering tools specifically for PSCs. Since the predecessors of the PSC-derived organoids can easily be genetically manipulated, adapting them to utilize many existing strategies was straightforward.

However, some research requires alternative approaches where the 3D cultures themselves are the primary targets. For example, Bian et al. performed electroporation directly on whole 3D embryoid bodies. Nevertheless, transfection efficiencies within the interior of structures are generally low to nonexistent. As a potential solution, localized transfection could be performed through needle injections in mature, structurally organized organoids to address various aims in the future.

The advantages of tool adaptations to AdSC-derived organoids stem from the fact that many tools and techniques already well-established for mouse models and human PSCs. In addition, AdSC-derived organoids can be isolated directly from genetically engineered mice or human patients, making genetically altered organoid models more accessible. This also means that additional genetic engineering can be applied to both isolated mouse and human models for advancing organoid genetics. With a plethora of optimized strategies currently available for different aims, researchers can start with either normal control or genetically altered organoids to create desired organoid models. In general, it is faster to directly establish a genetically engineered organoid line from normal organoids using CRISPR-Cas9 based gene editing tools than to generate a mouse model with subsequent organoid isolation. Likewise, creating human organoid models using gene editing tools is faster for rare genetic mutations than searching and waiting for a patient with specific mutations. Notably, base- and prime-editing makes it feasible to introduce various substitutions and short deletions or insertion mutations in organoids (Fig. 2).

Most of the aforementioned developments have progressed rapidly due to advancements and years of research in 2D cell systems and mouse modeling. As a result, a substantial toolbox has been established for organoids, which largely overlaps with tools used for their 2D or *in vivo* counterparts. However, as each model system comes with its own set of advantages to be exploited, it is important to recognize the specific nature of the organoid system. It is an *in vitro* culture system with primary cells in 3D, where various cell types co-exist. It is notable that additional cell types from different sources can be incorporated into the culture, like vascularized brain organoids (Cakir et al., 2019). This allows not only the co-existence of different cell types but also the co-culture of different genotypes.

3.1. Fused organoid models

One can model the interaction between mutant immune cells and wild-type epithelial cells or vice versa. Organoid fusion experiments are another way of co-culturing different genotypes, which is also possible *in vivo* using mosaic genetics, but it is much easier to model it using fusion organoids. If we need to assess the interactions between many different genotypes at once, mixing organoids for all the possible combinations is

the way to go. Here, gene editing in organoids can provide models with various genotypes. As highlighted before, both GLICO and fusion organoids have shown the potential of organoid fusions. Not only can patient specific tumors be assessed for their metastatic capability within their organ but also across different tissues. These techniques are likely to become more refined, which will lead to new insights into how cancer spreads and metastasizes due to the extremely controlled environment.

3.2. Lineage tracing

Next, techniques expected to gain popularity within the organoid field will be discussed. One strategy that has recently come to light is lineage tracing, nicely reviewed elsewhere (Betjes et al., 2021). In a paper dissecting the stem cell properties in human intestinal organoids, a simple lineage tracing cassette was used to mark cells stemming from ones that are or have previously expressed AVIL, a recently discovered tuft cell marker (Huang et al., 2024). Within PSC-derived organoid systems, some lineage tracing has also been done. The lineage and fate establishment within cerebral organoid formation was determined through combination of barcoding and CRISPR-Cas9 induced scarring (He et al., 2021). Furthermore, barcoding was also used in another study to dissect tissue plasticity within cerebral organoids (Lindenhofer et al., 2024). These techniques, and the previously discussed CHOOSE system allow lineage tracing after sequencing (Chong Li et al., 2023). However, it lacks spatial resolution and visualization capabilities which would provide unique insights into spatial dynamics and cell behavior. A system that could be used and is already popular for lineage tracing is Brainbow, a multicolor reporter. Though popular *in vivo*, organoids have yet to be combined with this technique. The employment of multicolor reporters will enable researchers to visualize and track multiple cell types simultaneously within complex organoid structures. To further advance this, it could be combined with an oncogene, like the Red2Onco system (Yum et al., 2021). This way of doing mosaic genetics where different genetic modifications are applied to distinct cell populations within the same organoid will further enhance our ability to dissect cellular interactions at a smaller level. This approach will allow for precise mapping of cell lineage relationships, signaling pathways, and the role of individual cells in tissue development and disease in human models. As mentioned, the humanized model aspect of organoids enables research and pathway dissection that simply could not be realized before.

3.3. Xenografts

It is also possible to reintroduce organoids back to *in vivo* e.g., human organoids to a mouse tissue. This provides another exciting opportunity as gene editing can be applied *in vitro* before the transplantation (mouse organoids to mouse) or xenografting (human organoids to mouse). Transplants of intestinal organoids have proven to be a functional remedy for a damaged intestinal epithelium. This can also be utilized for genetic studies as we can observe whether mutant epithelium forms glands with a different morphology. If transplantation efficiency improves, many different mutants can be reintroduced back to the *in vivo* context for phenotypical screening, followed by genotyping. This could be a novel way of forward genetics in the gut. Genetically engineered iPSC-derived brain organoids can also be xenografted in the mouse brain with a monitoring window (Revah et al., 2022; Wilson et al., 2022). Neuronal activities and surrounding cell reactions can be monitored in real-time *in vivo*. If combined with fluorescent protein sensors and optogenetics tools, this could allow a detailed analysis of human brain organoids *in vivo*. These systems can also be used with other organoid types as *in vivo* or intravital imaging is rapidly improving (Entenberg et al., 2022).

3.4. Optogenetics

Fluorescent protein sensors have not been readily utilized in organoids. Within this review many ways of which CRISPR-Cas9 has been applied to organoids have been discussed. Now, one such technique that has seen limited use but holds interesting potential is the light-activated CRISPR-Cas9 effector (LACE) system (Polstein and Gersbach, 2015). In short, this light-inducible CRISPR set-up only permits the dCas9 to function when blue light is present. LACE has decreased off-target effects, confined dynamic spatial control, and highly precise temporal transcriptional control. CPTS2.0, an improved version of LACE, has significantly increased the magnitude of activation (Nihongaki et al., 2017). The system was recently utilized in combination with spatial transcriptomics to study tissue patterning in organoids (Legnini et al., 2023). The latter highlights how optogenetics can be exploited to study complex tissue behavior within organoids. Combining the aforementioned optogenetic tools with conditional knock-out systems such as SCON would enable precise genetic engineering with high spatiotemporal resolution.

Organoid models, of course, are not yet perfect for all the applications that are desired. As previously mentioned, inaccuracies remain in their structures, cell-type compositions, and their functionalities. However, as improvements to the model system continue, the genetic tools available for organoids advance alongside it. Therefore, it is crucial to understand the possibilities and limits of the given system and available research tools. In short, the future of organoid technology is expected to play a major role in deciphering cellular interactions through advanced genetic tools. As the aforementioned techniques evolve and get incorporated into organoid studies, new avenues will be created for studying diseases, drug responses, and regenerative medicine in previously impossible ways. All in all, this progress will lead to more accurate and comprehensive models of human biology.

CRediT authorship contribution statement

Koo Bon-Kyoung: Writing – review & editing, Supervision. **Klompstra Thomas Maarten:** Writing – original draft, Project administration, Investigation, Conceptualization. **Yoon Ki-Jun:** Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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