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REVIEW | *Stem Cells, Tissue Engineering, Development, and Cancer*

CRISPR/Cas 9 genome editing and its applications in organoids

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Driehuis E, Clevers H. CRISPR/Cas 9 genome editing and its applications in organoids. *Am J Physiol Gastrointest Liver Physiol* 312: G257–G265, 2017. First published January 26, 2017; doi:10.1152/ajpgi.00410.2016.—Organoids are three-dimensional (3D) structures derived from adult or embryonic stem cells that maintain many structural and functional features of their respective organ. Recently, genome editing based on the bacterial defense mechanism CRISPR/Cas9 has emerged as an easily applicable and reliable laboratory tool. Combining organoids and CRISPR/Cas9 creates exciting new opportunities to study organ development and human disease in vitro. The potential applications of CRISPR in organoids are only beginning to be explored.

CRISPR/Cas9; gene editing; organoid

SINCE 2012, THE TECHNIQUE of CRISPR/Cas genome engineering has been developing rapidly. This technique, which exploits an innate bacterial defense mechanism against bacteriophages, is currently widely used throughout molecular biology. Applications and variations of this novel technique were published quickly after the first studies that showed the potency of CRISPR in mammalian cells. Discussions concerning the ethical questions raised barely keep up with the speed at which this technique is developing.

The development of organoid culture from adult epithelial stem cells allows researchers to study the multicellular composition of tissue epithelia in a dish. It was shown that the epithelial lining of the human intestine could be expanded indefinitely in vitro when provided with a basic matrix, necessary growth factors, and the stem cell-stimulating molecules Wnt and R-spondin. Since then, additional media requirements were established to allow the in vitro growth of many different epithelia (4, 29, 36, 60). Consequently, there has been a large increase in the number of research groups using organoids as a model system for a diverse range of applications. An organoid is best defined as a three-dimensional (3D) structure grown from stem cells and consisting of organ-specific cell types that self-organizes through cell sorting and spatially restricted lineage commitment (13, 40, 41).

CRISPR technology works well in adult stem cell-derived epithelial organoids. We first applied CRIPSR technology in small intestinal and colon-organoids from cystic fibrosis patients to demonstrate the feasibility of functional CFTR gene correction (61), combining both techniques generates a wide range of fascinating new opportunities. Nevertheless, because both methods were developed only recently, the number of published studies that combines the two is still limited. In addition to giving an overview of this already published work, this review highlights the recent advances made in the field of CRISPR/Cas genome engineering and organoid technology and subsequently explores the potential of combining the two as a research tool.

CRISPR/Cas9

CRISPR (short for Clustered regularly interspaced short palindromic repeats) is a defense mechanism of bacteria and archaea against viral infections. The presence of these tandem repeats in the *Escherichia coli* genome was first described in 1987, and was further characterized two years later (32, 52). The biological function of these repeats was revealed 20 years later by researchers of a dairy company that were studying the bacteriophages that infected their dairy bacteria cultures. In the meantime, CRISPR loci and the genes associated with this genomic region, named *Cas* genes (for CRISPR-associated genes) (33), were found to be present in many species of bacteria, a characteristic that was rapidly exploited in the clinic to identify bacterial strains causing disease (26, 35). It was proposed that the small unique sequences that are interspersed between the repeats in CRISPR regions were derived from bacteriophages, and that, somehow, the CRISPR/Cas system provided protection against these viruses (5, 51, 56). Finally, in 2007, Barrangou and coworkers (25) observed a direct relationship between integrated DNA sequences and resistance to phage infection. The researchers showed that naive strains of *Streptococcus thermophilus* obtained new spacers with sequence similarity to that of the phages that infected the bacterium upon exposure to these pathogens. After this integration, the bacteria became resistant to phage infection. Collectively, these data led the authors to propose a model of nucleic-acid based "immunity" that is heritable due to its stable integration into the bacterial genome.

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Five types of CRISPR systems have been identified so far, of which type II is the most studied. These CRISPR systems function slightly differently and are summarized elsewhere (69). As the type II CRISPR system has been adapted as a genome-editing tool, we will focus here on the specifics of this system. The CRISPR system is characterized by a region in the bacterial genome where fragments of foreign DNA (protospacers) are integrated between repetitive DNA sequences (spacers) that are present in tandem in the host DNA. Transcription of this region results in formation of CRISPR RNA (crRNA), generating transcripts that contain both the spacer and protospacer sequence. The crRNA molecule subsequently hybridizes with the so-called transactivating CRISPR RNA (tracrRNA) to allow it to form a complex with the Cas9 nuclease, encoded by one of the *Cas* genes. After processing of the crRNA and tracrRNA by RNAse III and Cas9, the spacer sequence guides Cas9 to its target: a complementary DNA sequence in the genome of the invading organism. Here, the nuclease introduces a double strand break (DSB) in the DNA. Protospacer-complementary DNA can only be cut by the nuclease if it is followed by a protospacer-adjacent motif (PAM), a consensus sequence that differs between Cas9-nucleases from different bacterial strains. So, as the nuclease cuts the phage DNA, the absence of the PAM in the host genome protects the bacterial DNA from self-destruction. In addition to recruiting Cas9 to potential target sites, the PAM sequence has also been shown to trigger activation of Cas9 nuclease activity (47). A schematic overview of the mechanism of action of the CRISPR system is given in Fig. 1.

Even before thorough mechanical understanding of this system, it was already suggested that CRISPR and its programmable nuclease could be exploited for genome editing in molecular biology (48). The CRISPR system is much more flexible than existing techniques that use proteins such as transcription activator-like effectors (TALEs) and zinc-finger proteins. Although effective for targeting DNA in a sequence-specific manner, these systems utilize proteins that contain a DNA-binding domain (rather than nucleic acids) for their target specificity. Consequently, this protein domain needs to be extensively reengineered for each new target sequence (23). Additionally, as the structure of the targeted protein needs to be maintained, these techniques are limited in the number of DNA sequences that can be targeted. Keeping this in mind, it might not be surprising that the potential of CRISPR as a tool for genome editing was already noted early on, because of the fact that it is based on complementary strand nucleic acid hybridization: Since the CRISPR system consists of a universal endonuclease whose activity is targeted to any desired location by a small RNA molecule, this could potentially make genome editing much faster and easier.

After elucidating the different components and their function, the type II CRISPR system was quickly adapted as a tool for genome editing. The first reports of the design of a CRISPR-based editing system in vitro were published in 2012 (25, 34). As early as January 2013, CRISPR was adapted to function in mammalian cells (8, 46), and soon the first applications of the technique were reported. One of these early studies showed effective genome editing of the zebrafish germline using CRISPR (30). Since then, new applications and improvements of the technique have appeared with ever-increasing frequency in many different fields. One of the most profound consequences of the introduction of CRISPR as a genomic engineering tool is the potential to manipulate genes in the germline. Up to now, genetically modified fish, flies, mice, and primates have been created from CRISPR-edited zygotes (27).

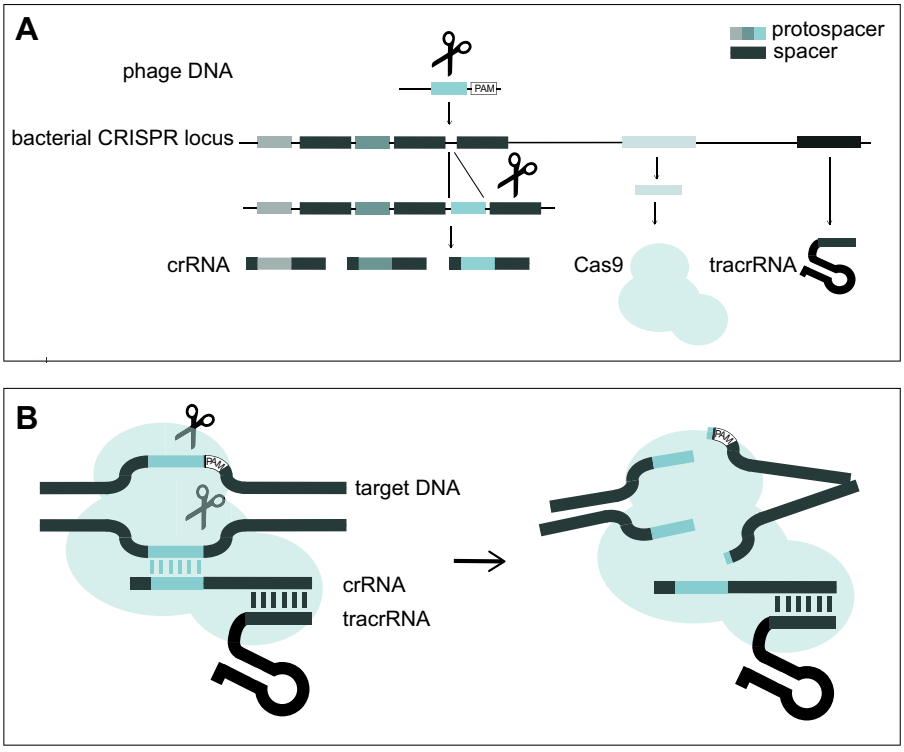

Fig. 1. The mechanism of action of the type II CRISPR system of *Streptococcus pyogenes*. *A*: a region of viral DNA is excised and integrated as protospacer between the repetitive elements (spacers) present in the bacterial CRISPR locus. Trimming of the resulting transcript generates crRNAs that encode both viral and bacterial DNA. In addition to crRNAs, the nuclease Cas9 and tracrRNA molecules are transcribed from nearby regions of the CRISPR locus. *B*: the complex formed by assembly of Cas9, crRNA, and tracrRNA can cut DNA regions that are complementary to the spacer sequence encoded by the crRNA. Importantly, the presence of the PAM is essential for the nuclease activity of the complex. When both spacer and PAM are detected in the phage DNA, Cas9 introduces a DSB into the virus genome. However, as the PAM is only present in the phage genome and absent in the bacterial genome, the nuclease activity is only guided toward the invader's DNA. In this way, the absence of the PAM prevents the host genome from self-destruction.

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CRISPR provides researchers with a tool to introduce a DSB at any desired location in the genome with high specificity. Under physiological condition, a DSB can be repaired via one of two repair pathways: nonhomologous end joining (NHEJ) or homology directed repair (HDR). NHEJ is the predominant repair pathway for DSBs in the DNA and is active throughout the cell cycle. As the name implies, this repair pathway does not require a homologous template to repair a DSB. Therefore, NHEJ-mediated repair is more error-prone than HDR and can result in small "scars" (small insertions or deletions) at the site of the repaired DSB. HDR has a lower capacity for repair than NHEJ and requires a template to repair the DSB. Therefore, HDR can only occur during S/G2-phase of the cell cycle, when a sister chromatid is present to serve as a template. Although more complex, HDR repairs DNA with more precision and has a much higher fidelity than NHEJ. Through homologous recombination of carefully designed DNA templates, virtually any change can be engineered near the DSB.

In the four years since the CRISPR technique has been introduced into the scientific world, adaptations of the system have expanded the "CRISPR toolbox" significantly. The technique can now, among others, be applied to either activate or repress gene expression, cause epigenetic modifications, and detect RNA abundance and localization. Furthermore, additional tools were developed, among which is a mouse strain that constitutively expresses Cas9 (55). Organoids established from these mice constitutively express Cas9 and therefore have a higher efficiency of acquiring desired mutations upon introduction of sgRNAs into the cell (data not shown).

Organoid Culture

Although the term "organoid" has lingered around for much longer to describe aggregates of cells, the first studies describing self-organizing structures grown from stem cells appeared about a decade ago. Sasai and colleagues (14) described the culture of pluripotent stem cells in "balls of neural cells that self-organize." Our laboratory described that single adult $Lgr5^+$ stem cells isolated from mouse intestine could give rise to 3D structures in vitro that could be maintained indefinitely when given the correct growth factors and stimuli (60). In these "mini guts," stem cells give rise to progeny that represents all differentiated cell types present in the gut in vivo.

Organoid cultures can be classified into two subtypes: organoids derived from tissue-restricted adult stem cells (ASC) and those that are derived from embryonic or induced pluripotent stem cells (ESC or iPSC), which are collectively named pluripotent stem cells (PSC). Organoids more closely resemble their tissue of origin than "classical" cell lines, grown in two dimension (2D). While cell lines typically are homogenous and harbor oncogenic mutations, organoids contain multiple cell types and, to a certain extent, recapitulate the 3D organization of the tissue. Importantly, organoids do not require immortalization before in vitro culture and thus allow the study of primary cells over longer periods of time. In line with this rationale, organoid culture can be seen as a missing link between the simpler monolayer cultures and in vivo studies (17).

Stem cells derived from mice and humans have been used to establish ASC-organoid lines of intestine, stomach, salivary gland, esophagus, pancreas, liver, breast, lung, prostate, fallopian tube, and taste bud (3, 10, 28, 29, 36, 37, 44, 53, 58–60, 64). 3D reconstructions of organoids derived from intestinal and liver organoids are depicted in Fig. 2. Organoids derived from ASC generally contain only epithelial cells. However, organoids derived from neonatal intestinal material that consist both of epithelial and mesenchymal cells have been described (54). In addition, organoids derived from PSCs can contain cells derived of all three germ layers, depending on the tissue attempted to mimic and the differentiation protocol applied. Among others, ESC-derived organoids have been described as a model for brain, optic cup, kidney, intestine, stomach, lung, thyroid gland, and liver (12, 42, 45, 50, 63, 65, 66). An overview of the different organoid types and how they are established is given in Fig. 3.

Despite the advantages of organoid technology, there are some characteristics of this culture system that have to be kept in mind. For example, ASC-derived organoids only consist of epithelial cells, which makes the model not suited to study the interaction between epithelial cells and other cell types, such as immune or mesenchymal cells. Nevertheless, adding such cells or their products of interest separately to the organoid cultures is feasible (e.g., Refs, 15, 16), allowing study of the relevant contribution or interactions of these cells with the epithelial cells present in the organoids. Alternatively, PSC-derived organoids can be used, as these have the potential to produce cell types derived from all three germ layers.

CRISPR and Organoids

In 2013, it was shown that CRISPR could be applied in mouse and human intestinal organoids either to knock out a gene or to correct a disease-causing mutation (61). We first showed the feasibility of CRISPR targeting in wild-type mouse small intestinal organoids by causing frame shifts in the two Apc alleles, Apc being the most prominent tumor suppressor in intestinal cancer and a negative regulator of Wnt signaling. The resulting organoids indeed grew in a Wnt-independent fashion, while sequencing confirmed the presence of NHEJ-based "scars." We then asked whether HDR could be utilized to repair gene mutations in the gene coding for the cystic fibrosis transmembrane conductor receptor (CFTR) in organoids derived from cystic fibrosis (CF). Mutations in the CFTR gene in CF patients result in an inactive chloride channel protein, causative of the disease. Organoid cultures derived from CF

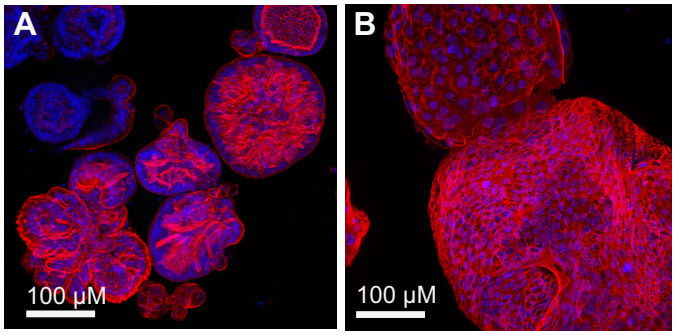

Fig. 2. Three-dimensional (3D) reconstruction of the midsection of ASC-derived colon and liver organoids, stained for the actin cytoskeleton (red) and nuclei (blue) and imaged by confocal microscopy. *A*: human intestinal organoids. *B*: human liver organoids. (Kretzschmar K, Gerhart H, Clevers H, unpublished data).

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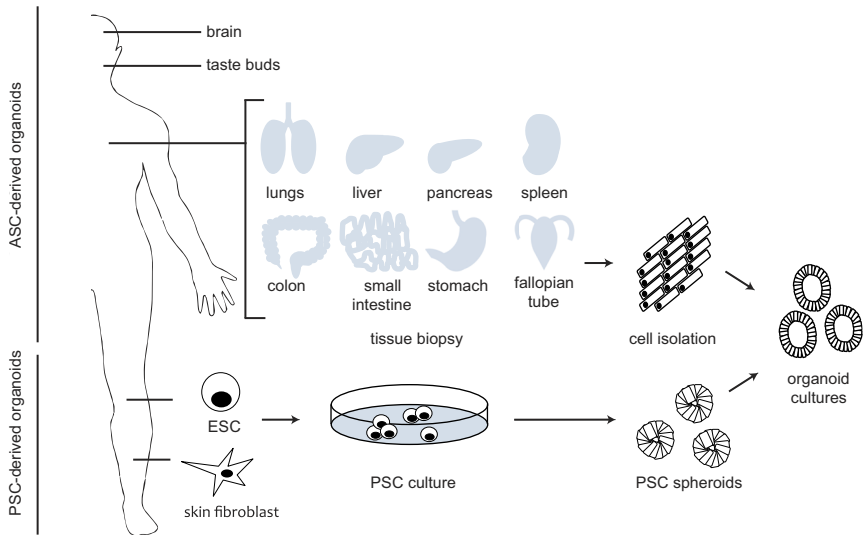

Fig. 3. Establishment of ASC- and PSC-derived organoids. ASC-derived organoids can be established from either digested tissue pieces or specific cell populations (such as Lgr5^{+} stem cells). After isolation of the desired cell population, cells are suspended in Matrigel and kept in medium containing growth factors. The exact composition of the medium depends on the cultured tissue type. PSC-derived organoids can be obtained either from ESCs or iPSC, which are derived from, e.g., skin fibroblasts. Depending on the differentiation protocol applied, stem cells can be differentiated in vitro toward different organoid types. Redrawn from Refs. 7, 40.

patients offer an ideal platform for in vitro research and diagnostics. Previously, it was already shown that colon organoids established from CF patients show reduced or absent swelling upon exposure to forskolin, a cyclic AMP inducing agent (9). When the CFTR mutation was restored using CRISPR, the organoids showed restored swelling in response to forskolin. Moreover, the detected level of swelling was comparable to that of wild-type organoids, confirming that CFTR is the single impaired gene causing this effect. Although this use of CRISPR is not directly amenable for clinical applications yet, this study did reveal the potential of CRISPR for gene correction in monogenetic diseases. Moreover, it showed the potential of combining organoid and CRISPR techniques in research. Taken together, organoids can be modified by CRISPR/Cas9 both by utilizing "simple" NHEJ and by HDR, the latter allowing for more complex genetic modifications. An overview of the applications of CRISPR in organoids that are discussed in this review is given in Fig. 4.

CRISPR in ASC-Derived Organoids

The mutation of Apc[45] suggested that CRISPR could thus be used to modify the genome of organoids to model tumorigenesis. Tumorigenesis is characterized by multistep genetic changes that result in inactivation of tumor suppressor genes and activation of oncogenes, which collectively drive cancer growth. CRISPR enables the introduction of mutations at specific sites in the genome, which makes it possible to specifically modify genes that are commonly found mutated in cancer as a means to mimic tumorigenesis. Inactivation of tumor suppressor genes such as Apc usually only requires the induction of frameshifts by CRISPR-induced NHEJ. Activation of proto-oncogenes typically relies on HDR-mediated knockin of the desired oncogenic mutation.

Following the "Vogelgram" [a sequence of mutations accompanying the adenoma to carcinoma transition in colon cancer as originally suggested by Fearon and Vogelstein (18)], two independent studies showed that CRISPR can be used to efficiently introduce mutations that are found in colon cancer in organoids (11, 49). The genes targeted in these studies were KRAS, APC (see above), p53, and SMAD4. KRAS is an oncogene that is commonly altered in colon cancer, resulting in a constitutively active protein. With the use of a DNA template carrying the KRAS^{G12D} mutation that is commonly found in cancer, this specific genomic alteration was introduced into the Kras gene of the organoids by HDR-directed DNA repair. For the other three genes that were targeted, NHEJ-directed repair of the DSB introduced in the coding region of these tumor suppressors resulted in their inactivation. Indeed, these genetic alterations resulted in biological changes that are also observed in patients, such as chromosomal instability. When subsequently transplanted into mice, mutant organoids grew out as

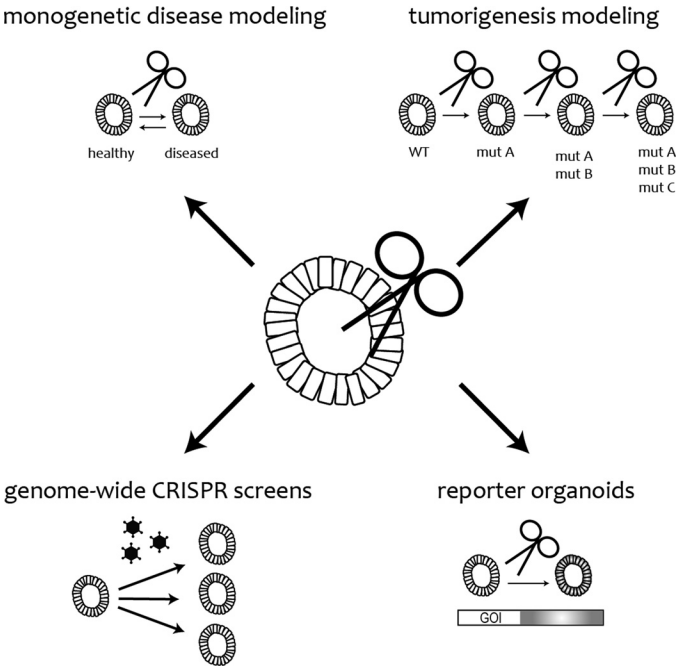

Fig. 4. Applications of CRISPR-based gene editing in organoids that are discussed in this review. CRISPR can be applied to organoid cultures to model monogenetic diseases or tumorigenesis in vitro, to perform genome-wide CRISPR screens or to create reporter organoids. WT, wild-type; MutA; CRISPR-induced mutation in gene A; MutB, CRISPR-induced mutation in gene B.

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tumors that, depending on the number of genes altered by CRISPR, could be characterized by varying levels of differentiation and invasive behavior.

In recent years, it has become clear that colorectal tumors are molecularly heterogeneous, and that this diversity is reflected in the premalignant precursor lesions (31). In a study published in 2015, $Braf^{V600E}$ mutant organoids were used as a model for the precursor lesions of sessile serrated adenomas (SSA), a tumor type that is both histologically and molecularly distinct from the "classical" APC^{min} tumors and their precursor lesions (20). The researchers studied the effect of TGFβ on these two different tumor precursor lesions, to see whether there was a difference. As it had not been possible to culture SSAs or their corresponding precursor lesions in vitro, the researchers used CRISPR-modified organoids to model these tumor precursor lesions in vitro. SSAs are characterized by activating mutations in the proto-oncogene BRAF, and the commonly found V600E mutation was introduced in wild-type organoids using CRISPR technology. Interestingly, while exposure to TGFβ induced apoptosis in APC^{min} organoids, the $BRAF^{V600E}$ organoids did not show this response. Moreover, in response to TGFβ, the $BRAF^{V600E}$ organoids underwent epithelial-to-mesenchymal transition (EMT), a phenotype associated with poor prognosis. This shows that TGFβ can have opposing effects on different tumor precursors types, inducing cell death in one and invasive behavior in another.

An alternative way in which genetically modified organoids can contribute to research is illustrated by a recent study (67a). In this study, EGFR and MEK inhibitors were tested on a panel of colorectal organoids lines. The goal was to determine the effect of Ras-mutation status on the sensitivity to these drugs. The authors found that activation of the oncogene RAS decreased sensitivity to combined EGFR/MEK inhibitory treatment. To confirm that the difference in sensitivity was indeed a direct result of RAS status, CRISPR was used. In a KRAS wild-type colon tumor line that was sensitive to the combinational therapy, the introduction of a $KRAS^{G12D}$ mutation via CRISPR resulted in a loss of drug sensitivity. As the genetic makeup of these isogenic lines is otherwise identical, this finding proved that it was indeed the RAS status of the tumor that was responsible for the loss of drug sensitivity. In this way, CRISPR-guided genetic modification of organoids can serve to strengthen conclusions based on large drug screens. More specifically, if a correlation between gene status and drug response is detected in a large cohort of tumors or cell lines, CRISPR can be used to validate these results and show a direct relation between the genetic alterations of interest and the drug sensitivity.

Other epithelial organoid types have also been successfully targeted using CRISPR. Organoids derived from Rosa26-CreERT2; Pik3CA* mouse mammary ducts have been used to introduce inactivating mutation in Mll3, a histone methyltransferase commonly mutated in breast cancers (70). Hyperplasia could be observed upon transplantation of $Mll3^{-/-}$ but not $Mll3^{+/+}$ organoids. Moreover, when tumor formation was induced by tamoxifen administration (resulting in PIK3CA overexpression), $Mll3^{-/-}$ developed much faster than their Mll3 wild-type counterparts. Also, mouse tracheal cells have been successfully modified using CRISPR. To study the role of the transcription factor Grainyhead-like 2 (Grhl2) in airway epithelium, a knockout of this gene was created in organoids using CRISPR technology (24). The authors found that loss of this transcription factor prevents proper differentiation into ciliated cells and establishment of barrier function.

In summary, these studies underscore that CRISPR/Cas provides researchers with the possibility to rapidly create combinations of multiple genetic changes in human cells, something that was previously restricted to animal models.

CRISPR in PSC-Derived Organoids

CRISPR technology has also been applied in PSC-derived organoids. PSC-derived organoids were used to study the disease dyskeratotis congenita (DC). This disease, caused by a failure to maintain telomere length, is characterized by degeneration of highly proliferative tissues such as the hematopoietic system, epidermis, and gastrointestinal tract (19). One of the most commonly affected genes in DC is DKC1, the gene encoding Dyskerin. Dyskerin is a protein that is critical for telomere maintenance (2). In this particular study, the researchers created isogenic pairs of iPSCs. They did this either by establishing these cells from a healthy individual and subsequently introducing the disease causing mutation, or, reversibly, by establishing iPSCs from an affected individual and restoring the mutation to wild-type. Next, the stem cells were differentiated toward an intestinal phenotype and cultured as human intestinal organoids. Indeed, in DCK1 defective organoids, telemores were shorter when compared with isogenic DKC1 wild-type controls. Furthermore, while wild-type organoids matured into gutlike tubes containing budding crypts, the mutant organoids failed to form these cryptlike structures in vitro. These results agree with clinical findings, where DC patients present with mucosal ulceration and malabsorption. Interestingly, the authors found that restoration of Wnt-signaling in these diseased organoids could partially rescue the phenotype, a finding that might have important implications for the clinic.

The other study that applies CRISPR in PSC-derived organoids was published in 2015. Freedman et al. (21) show that CRISPR can be used to model disease phenotypes in hPSC-derived organoids. These researchers created an in vitro model for polycystic kidney disease (PKD), a monogenetic disease. As the name implies, patients affected by PKD present with intrarenal cystic structures that eventually disrupt the architecture of the kidney. CRISPR was used to introduce biallelic truncating mutations in either PKD1 or PKD2, the genes causative for PKD. The authors subsequently described a protocol to differentiate hPSCs into kidney organoids. Indeed, after differentiation, organoids derived from mutated hPSCs displayed a different phenotype compared with isogenic wild-type organoids. Although the initial stages of differentiation appeared identical between PKD mutant and control organoids, PKD-mutant organoids behave differently when differentiated toward more mature kidney cells. In addition to the tubular structures that develop in wild-type hPS-derived organoids, maturation of PKD organoids results in the formation of large cystic structures. These in vitro findings resemble observations from the clinic, where PKD patients present with large renal cysts. Two important conclusions can be drawn from these findings. First, in addition to ASC-derived organoids, PSC-derived organoids are amenable to genome editing by CRISPR. Second, PSC-based organoids create the unique opportunity to

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introduce pathogenic mutations in a precursor cell-state in which the mutation does not necessarily result in a phenotype. This allows us to study the molecular effects of mutations that would normally prevent the affected cell type from growing in culture. In theory, an in vivo cell type-specific phenotype will only appear in vitro when organoids are differentiated toward that specific cell type. Therefore, when kept in an undifferentiated state, mutant cells can be expanded and studied.

Although effective, there are some limitations to using organoids in combination with CRISPR. Methods for the lentiviral transduction (39), liposomal transfection (11, 61), and electroporation (49) of organoids have been described. A detailed protocol describing the method of electroporation for genetic engineering of human intestinal organoids was recently published (22). However, the efficiency of transfection using any of these approaches is relatively low compared with other culture systems. In addition, not all cells that are transfected with the required CRISPR components acquire the desired mutation. Transfected stem cells must be expanded clonally and screened to confirm the clonal presence of mutations and their mono- or biallelic nature. When introducing a specific mutation using a homology cassette, validation of the resulting genetic alterations in one or both alleles is essential. Generally, when one allele is targeted via HDR, the other allele contains a frameshift introduced by NHEJ. Introduction of homozygous nonsynonymous mutations occurs with an even lower efficiency. In summary, the number of cells that are required to establish an organoid line with the desired genomic alteration is relatively high and dependent on the cell origin, cell type, and desired genetic alteration. A method to select cells with the desired mutation is therefore essential when performing CRISPR experiments in organoids. Such selections can be based on growth factor withdrawals when the targeted gene is known to be directly involved in growth factor dependence [e.g., selection of Kras-mutant organoids by EGF withdrawal, and Apc mutants by Wnt withdrawal in the work of Drost et al. (11)]. Other selection methods include the use of Cas9 or sgRNA constructs that additionally encode fluorescent proteins. After transfection, efficiently transfected cells can be enriched using FACS. A third approach involves the introduction of antibiotic resistance cassettes using HR-directed repair. As HDR-directed repair occurs with a lower frequency than NHEJ-directed repair, this approach does decrease efficiency.

Second, the number of cells that can be kept in culture as organoids is restricted by the cost of growth factors and Matrigel as well as by the growth rate of organoids, which, depending on tissue type, can be slow. It is not unlikely that some of these potential limitations might be overcome in the future, as new techniques that can be used to transfect, culture, and select organoids are still being developed. Furthermore, recent publications of the studies discussed above showed that most of these limitations are surmountable. For instance, a synthetic gel has now been described which can replace Matrigel (25a).

Future Directions

Genome-wide CRISPR screens. CRISPR was quickly applied to genome-wide genetic screens after the initial reports on the technique in 2013. Before the introduction of CRISPR, reverse genetic screens were performed by using mutagens that damage the DNA in an random fashion. Subsequent identification of the causative mutations (that could be located anywhere in the genome) was required, a time-consuming and laborious process (1). Later on, this technique was largely replaced by RNA interference (RNAi) studies, where mRNA molecules are targeted for degradation when small complementary RNA oligos are introduced into the cell. Nevertheless, RNAi in general leads to incomplete gene silencing and only affects the transcriptome, not the genome. From this perspective, the technique never was the ideal model system to mimic the genetic changes that underlie biological processes such as tumorigenesis. CRISPR allows us to truly mimic the genetic alterations underlying such events. The first studies using CRISPR in genome-wide screens were already published early in 2014 (38, 55, 62, 68). In these reports, sgRNA libraries were used to find genes involved in resistance to drugs and bacterial toxins in an unbiased manner. For reasons that were already mentioned previously, organoids represent a more suitable model system for tissue composition and structure than the 2D cell lines that were used in the studies described above.

To our knowledge, there have not been reports yet of genome-wide CRISPR screens in organoids. However, in a recent study, organoid cultures were used to validate the results obtained by performing such a screen in cell lines (67). This particular study aimed to identify targets of *Clostridium difficile* toxins by using a genome-wide sgRNA library in HeLa cells that stably expressed Cas9. By using increasing concentrations of *C. difficile* toxins, the authors could select for cells that lost sensitivity to these molecules. FZD2, the gene encoding the membranous Wnt-receptor Frizzled2, was found to be one of the top hits of the screen. Cells with inactive FZD2 (caused by infection with FZD2 targeting sgRNAs from the library) were enriched in the toxin-resistant cell population. Because HeLa cells are not the optimal in vitro model to mimic the colonic infection that is observed in infected patients, the authors used human colon organoids to study the effect of FZD2 knockout in vitro. They found that $FZD2^{-/-}$ organoids were not affected by the presence of *C. difficile* toxin B, whereas wild-type organoids showed decreased viability upon exposure to this compound. Furthermore, the authors showed that the toxin B-FZD interaction prevented FZD from binding to Wnt molecules. Wnt signaling, initiated by this Wnt-FZD interaction, is essential for stem cells to maintain their undifferentiated state, both in vivo and in vitro. Therefore, it was expected that exposure to inactivated toxin B would still inhibit organoid growth, as long as the interaction between the toxin and FZD was not disturbed. Indeed, this is what the authors found. These findings suggest that, by disrupting Wnt signaling, the binding of *C. difficile* toxin may directly contribute to the disruption of the colon epithelium upon infection. Indeed, this effect could be rescued by treatment with CHIR99021, a downstream activator of the Wnt pathway.

This work underscores the added value of organoid cultures over 2D cell lines. In this particular case, the biological effect of the toxin-FZD interaction could not have been understood by merely working with 2D cell lines, as these do not mimic the stem cell-based maintenance of the intestinal epithelial sheet. The authors of this work elegantly combined the use of 2D cell lines for high-throughput screening with organoid culture to validate their results, as this was the more suited in vitro model system to model intestinal epithelium. We expect

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that high-throughput screens such as CRISPR-based whole genome library screens become feasible to perform in organoids, both cost- and timewise. As DNA delivery approaches are improving constantly and less expensive alternatives for Matrigel are being developed, these limitations can be overcome in the future.

Reporter organoids. Apart from mimicking the genetic alterations that occur during tumor formation, the CRISPR system can also be applied to introduce reporter sequences into organoid DNA. Prior to CRISPR technology, introduction of a reporter gene often included the expression of a transgene under the regulation of the promoter from the gene of interest (GOI). Although valuable, there are obvious limitations to this technique. Most importantly, regulation of gene expression is highly dependent on the genomic context, and thus placing a reporter gene outside its genetic context will impair this regulation. With the aid of CRISPR, we can now introduce reporter proteins in the endogenous site of virtually any GOI, either by inserting the reporter sequence downstream of the gene product or by replacing it. For example, if a fluorescent reporter is introduced under the endogenous regulation of a cell type-specific marker, this will create the opportunity to set up a large-scale screen to test a wide range of differentiation conditions. In such a scenario, the readout will simply be fluorescence, a characteristic that can be studied in real time and is much quicker and easier than other readouts such as gene expression analysis or immunohistochemistry. Such a tool will greatly aid the establishment of differentiation protocols that push organoids into a range of developmental fates.

Concluding Remarks

In this review, we discuss the combined use of CRISPR-based genome editing and organoid technology. Organoids derived from pluripotent stem cells are the model of choice to study developmental processes "in a dish" or to generate tissues (such as the brain) that cannot be grown from ASCs. Organoids derived from ASCs can be used to directly model hereditary diseases such as CF or cancer. While we discuss only CRISPR-based gene editing, other interesting CRISPR tools have been developed, for instance to modify gene expression using CRISPRi or CRISPRa (6, 43, 57). The coming years should witness the generation of many more organoid- and CRISPR-based technologies. It will be exciting to watch progress in both research fields and the merging of the two.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

E.D. prepared figures; E.D. drafted manuscript; H.C. edited and revised manuscript.

REFERENCES

1. **Agrotis A, Ketteler R.** A new age in functional genomics using CRISPR/Cas9 in arrayed library screening. *Front Genet* 6: 300, 2015. https://doi.org/10.3389/fgene.2015.00300.
2. **Armanios M, Blackburn EH.** The telomere syndromes. *Nat Rev Genet* 13: 693–704, 2012. doi:10.1038/nrg3246.
3. **Barker N, Huch M, Kujala P, van de Wetering M, Snippert HJ, van Es JH, Sato T, Stange DE, Begthel H, van den Born M, Danenberg E, van den Brink S, Korving J, Abo A, Peters PJ, Wright N, Poulsom R, Clevers H.** Lgr5+ve stem cells drive self-renewal in the stomach and build long-lived gastric units in vitro. *Cell Stem Cell* 6: 25–36, 2010. doi:10.1016/j.stem.2009.11.013.
4. **Boj SF, Hwang C, Baker LA, Chio IIC, Engle DD, Corbo V, Jager M, Ponz-Sarvise M, Tiriac H, Spector MS, Gracanin A, Oni T, Yu KH, van Boxtel R, Huch M, Rivera KD, Wilson JP, Feigin ME, Öhlund D, Handly-Santana A, Ardito-Abraham CM, Ludwig M, Elyada E, Alagesan B, Biffi G, Yordanov GN, Delcuze B, Creighton B, Wright K, Park Y, Morsink FHM, Molenaar IQ, Borel Rinkes IH, Cuppen E, Hao Y, Jin Y, Nijman IJ, Iacobuzio-Donahue C, Leach SD, Pappin DJ, Hammell M, Klimstra DS, Basturk O, Hruban RH, Offerhaus GJ, Vries RGJ, Clevers H, Tuveson DA.** Organoid models of human and mouse ductal pancreatic cancer. *Cell* 160: 324–338, 2015. doi:10.1016/j.cell.2014.12.021.
5. **Bolotin A, Quinquis B, Sorokin A, Ehrlich SD.** Clustered regularly interspaced short palindrome repeats (CRISPRs) have spacers of extrachromosomal origin. *Microbiology* 151: 2551–2561, 2005. doi:10.1099/mic.0.28048-0.
6. **Cheng AW, Wang H, Yang H, Shi L, Katz Y, Theunissen TW, Rangarajan S, Shivalila CS, Dadon DB, Jaenisch R.** Multiplexed activation of endogenous genes by CRISPR-on, an RNA-guided transcriptional activator system. *Cell Res* 23: 1163–1171, 2013. doi:10.1038/cr.2013.122.
7. **Clevers H.** Modeling development and disease with organoids. *Cell* 165: 1586–1597, 2016. doi:10.1016/j.cell.2016.05.082.
8. **Cong L, Ran FA, Cox D, Lin S, Barretto R, Hsu PD, Wu X, Jiang W, Marraffini LA.** Multiplex genome engineering using CRISPR/Cas systems. *Science* 339: 819–823, 2013.
9. **Dekkers JF, Wiegerinck CL, de Jonge HR, de Jong NWM, Bijvelds MJC, Nieuwenhuis EES, van den Brink S, Clevers H, van der Ent CK, Middendorp S.** A functional CFTR assay using primary cystic fibrosis intestinal organoids. *J Cyst Fibros* 11: S32, 2012. doi:10.1016/S1569-1993(12)60101-5.
10. **DeWard AD, Cramer J, Lagasse E.** Cellular heterogeneity in the mouse esophagus implicates the presence of a nonquiescent epithelial stem cell population. *Cell Reports* 9: 701–711, 2014. doi:10.1016/j.celrep.2014.09.027.
11. **Drost J, van Jaarsveld RH, Ponsioen B, Zimberlin C, van Boxtel R, Buijs A, Sachs N, Overmeer RM, Offerhaus GJ, Begthel H, Korving J, van de Wetering M, Schwank G, Logtenberg M, Cuppen E, Snippert HJ, Medema JP, Kops GJPL, Clevers H.** Sequential cancer mutations in cultured human intestinal stem cells. *Nature* 521: 43–47, 2015. doi:10.1038/nature14415.
12. **Dye BR, Hill DR, Ferguson MA, Tsai Y-H, Nagy MS, Dyal R, Wells JM, Mayhew CN, Nattiv R, Klein OD, White ES, Deutsch GH, Spence JR.** In vitro generation of human pluripotent stem cell derived lung organoids. *eLife* 4: e05098, 2015. doi:10.7554/eLife.05098.
13. **Eiraku M, Sasai Y.** Mouse embryonic stem cell culture for generation of three-dimensional retinal and cortical tissues. *Nat Protoc* 7: 69–79, 2012. doi:10.1038/nprot.2011.429.
14. **Eiraku M, Watanabe K, Matsuo-Takasaki M, Kawada M, Yonemura S, Matsumura M, Wataya T, Nishiyama A, Muguruma K, Sasai Y.** Self-organized formation of polarized cortical tissues from ESCs and its active manipulation by extrinsic signals. *Cell Stem Cell* 3: 519–532, 2008. doi:10.1016/j.stem.2008.09.002.
15. **Farin HF, Van Es JH, Clevers H.** Redundant sources of Wnt regulate intestinal stem cells and promote formation of Paneth cells. *Gastroenterology* 143: 1518–1529.e7, 2012. doi:10.1053/j.gastro.2012.08.031.
16. **Farin HF, Karthaus WR, Kujala P, Rakhshandehroo M, Schwank G, Vries RGJ, Kalkhoven E, Nieuwenhuis EES, Clevers H.** Paneth cell extrusion and release of antimicrobial products is directly controlled by immune cell-derived IFN-γ. *J Exp Med* 211: 1393–1405, 2014. doi:10.1084/jem.20130753.

Downloaded from journals.physiology.org/journal/ajpgi (065.021.193.086) on February 24, 2022.

17. **Fatehullah A, Tan SH, Barker N.** Organoids as an in vitro model of human development and disease. *Nat Cell Biol* 18: 246–254, 2016. doi:10.1038/ncb3312.
18. **Fearon ER, Vogelstein B.** A genetic model for colorectal tumorigenesis. *Cell* 61: 759–767, 1990. doi:10.1016/0092-8674(90)90186-I.
19. **Fernández García MS, Teruya-Feldstein J.** The diagnosis and treatment of dyskeratosis congenita: a review. *J Blood Med* 5: 157–167, 2014.
20. **Fessler E, Drost J, van Hooff SR, Linnekamp JF, Wang X, Jansen M, De Sousa E, Melo F, Prasetyanti PR, IJspeert JE, Franitza M, Nürnberg P, van Noesel CJ, Dekker E, Vermeulen L, Clevers H, Medema JP.** TGFβ signaling directs serrated adenomas to the mesenchymal colorectal cancer subtype. *EMBO Mol Med* 8: 685–829, 2016. doi:10.15252/emmm.201606184.
21. **Freedman BS, Brooks CR, Lam AQ, Fu H, Morizane R, Agrawal V, Saad AF, Li MK, Hughes MR, Vander Werff R, Peters DT, Lu J, Baccei A, Siedlecki AM, Valerius MT, Musunuru K, McNagny KM, Steinman TI, Zhou J, Lerou PH, Bonventre JV.** Modelling kidney disease with CRISPR-mutant kidney organoids derived from human pluripotent epiblast spheroids. *Nat Commun* 6: 8715, 2015. doi:10.1038/ncomms9715.
22. **Fujii M, Matano M, Nanki K, Sato T.** Efficient genetic engineering of human intestinal organoids using electroporation. *Nat Protoc* 10: 1474–1485, 2015. doi:10.1038/nprot.2015.088.
23. **Gaj T, Gersbach CA, Barbas CF III.** ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends Biotechnol* 31: 397–405, 2013. doi:10.1016/j.tibtech.2013.04.004.
24. **Gao X, Bali AS, Randell SH, Hogan BLM.** GRHL2 coordinates regeneration of a polarized mucociliary epithelium from basal stem cells. *J Cell Biol* 211: 669–682, 2015. doi:10.1083/jcb.201506014.
25. **Gasiunas G, Barrangou R, Horvath P, Siksnys V.** Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. *Proc Natl Acad Sci USA* 109: 2579–2586, 2012. doi:10.1073/pnas.1208507109.
25a. **Gjorevski N, Sachs N, Manfrin A, Giger S, Bragina ME, Ordóñez-Morán P, Clevers H, Lutolf MP.** Designer matrices for intestinal stem cell and organoid culture. *Nature* 539: 560–564, 2016. doi:110.1038/nature20168.
26. **Groenen PMA, Bunschoten AE, Van Soolingen D, Van Embden JD.** Nature of DNA polymorphism in the direct repeat cluster of Mycobacterium tuberculosis; application for strain differentiation by a novel typing method. *Mol Microbiol* 10: 1057–1065, 1993. doi:10.1111/j.1365-2958.1993.tb00976.x.
27. **Hockemeyer D, Jaenisch R.** Induced pluripotent stem cells meet genome editing. *Cell Stem Cell* 18: 573–586, 2016. doi:10.1016/j.stem.2016.04.013.
28. **Huch M, Bonfanti P, Boj SF, Sato T, Loomans CJM, van de Wetering M, Sojoodi M, Li VSW, Schuijers J, Gracanin A, Ringnalda F, Begthel H, Hamer K, Mulder J, van Es JH, de Koning E, Vries RGJ, Heimberg H, Clevers H.** Unlimited in vitro expansion of adult bi-potent pancreas progenitors through the Lgr5/R-spondin axis. *EMBO J* 32: 2708–2721, 2013. doi:10.1038/emboj.2013.204.
29. **Huch M, Dorrell C, Boj SF, van Es JH, Li VSW, van de Wetering M, Sato T, Hamer K, Sasaki N, Finegold MJ, Haft A, Vries RRG, Grompe M, Clevers H.** In vitro expansion of single Lgr5+ liver stem cells induced by Wnt-driven regeneration. *Nature* 494: 247–250, 2013. doi:10.1038/nature11826.
30. **Hwang WY, Fu Y, Reyon D, Maeder ML, Tsai SQ, Sander JD, Peterson RT, Yeh JR, Joung JK.** Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nat Biotechnol* 31: 227–229, 2013. doi:10.1038/nbt.2501.
31. **IJspeert JEG, Vermeulen L, Meijer GA, Dekker E.** Serrated neoplasia—role in colorectal carcinogenesis and clinical implications. *Nat Rev Gastroenterol Hepatol* 12: 401–409, 2015. doi:10.1038/nrgastro.2015.73.
32. **Ishino Y, Shinagawa H, Makino K, Amemura M, Nakata A.** Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in Escherichia coli, and identification of the gene product. *J Bacteriol* 169: 5429–5433, 1987.
33. **Jansen R, Van Embden JDA, Gaastra W, Schouls LM.** Identification of genes that are associated with DNA repeats in prokaryotes. *Mol Microbiol* 43: 1565–1575, 2002.
34. **Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E.** A programmable dual-RNA-guided DNA endunuclease in adaptive bacterial immunity. *Science* 337: 816–822, 2012. doi:10.1126/science.1225829.
35. **Kamerbeek J, Schouls L, Kolk A, Van Agterveld M, Van Soolingen D, Kuijper S, Bunschoten A, Molhuizen H, Shaw R, Goyal M.** Simultaneous detection and strain differentiation of mycobacterium tuberculosis for diagnosis and epidemiology. *J Clin Microbiol* 35: 907–914, 1997.
36. **Karthaus WR, Iaquinta PJ, Drost J, Gracanin A, Van Boxtel R, Wongvipat J, Dowling CM, Gao D, Begthel H, Sachs N, Vries RGJ, Cuppen E, Chen Y, Sawyers CL, Clevers HC.** Identification of multipotent luminal progenitor cells in human prostate organoid cultures. *Cell* 159: 163–175, 2014. doi:10.1016/j.cell.2014.08.017.
37. **Kessler M, Hoffmann K, Brinkmann V, Thieck O, Jackisch S, Toelle B, Berger H, Mollenkopf H-J, Mangler M, Sehouli J, Fotopoulou C, Meyer TF.** The Notch and Wnt pathways regulate stemness and differentiation in human fallopian tube organoids. *Nat Commun* 6: 8989, 2015. doi:10.1038/ncomms9989.
38. **Koike-Yusa H, Li Y, Tan E-P, Velasco-Herrera MDC, Yusa K.** Genome-wide recessive genetic screening in mammalian cells with a lentiviral CRISPR-guide RNA library. *Nat Biotechnol* 32: 267–273, 2014. doi:10.1038/nbt.2800.
39. **Koo B-K, Stange DE, Sato T, Karthaus W, Farin HF, Huch M, van Es JH, Clevers H.** Controlled gene expression in primary Lgr5 organoid cultures. *Nat Methods* 9: 81–83, 2012. doi:10.1038/nmeth.1802.
40. **Kretzschmar K, Clevers H.** Organoids: modeling development and the stem cell niche in a dish. *Dev Cell* 38: 590–600, 2016. doi:10.1016/j.devcel.2016.08.014.
41. **Lancaster MA, Knoblich JA.** Organogenesis in a dish: modeling development and disease using organoid technologies. *Science* 345: 1247125, 2014. doi:10.1126/science.1247125.
42. **Lancaster MA, Renner M, Martin C-A, Wenzel D, Bicknell LS, Hurles ME, Homfray T, Penninger JM, Jackson AP, Knoblich JA.** Cerebral organoids model human brain development and microcephaly. *Nature* 501: 373–379, 2013. doi:10.1038/nature12517.
43. **Larson MH, Gilbert LA, Wang X, Lim WA, Weissman JS, Qi LS.** CRISPR interference (CRISPRi) for sequence-specific control of gene expression. *Nat Protoc* 8: 2180–2196, 2013. doi:10.1038/nprot.2013.132.
44. **Linnemann JR, Miura H, Meixner LK, Irmler M, Kloos UJ, Hirschi B, Bartsch HS, Sass S, Beckers J, Theis FJ, Gabka C, Sotlar K, Scheel CH.** Quantification of regenerative potential in primary human mammary epithelial cells. *Development* 142: 3239–3251, 2015. doi:10.1242/dev.123554.
45. **Longmire TA, Ikonomou L, Hawkins F, Christodoulou C, Cao Y, Jean JC, Kwok LW, Mou H, Rajagopal J, Shen SS, Dowton AA, Serra M, Weiss DJ, Green MD, Snoeck HW, Ramirez MI, Kotton DN.** Efficient derivation of purified lung and thyroid progenitors from embryonic stem cells. *Cell Stem Cell* 10: 398–411, 2012. doi:10.1016/j.stem.2012.01.019.
46. **Mali P, Yang L, Esvelt KM, Aach J, Guell M, Dicarlo JE, Norville JE, Church GM.** RNA-guided human genome engineering via Cas9. *Science* 339: 823-826, 2013.
47. **Marraffini LA, Sontheimer EJ.** Self versus non-self discrimination during CRISPR RNA-directed immunity. *Nature* 463: 568–571, 2010. doi:10.1038/nature08703.
48. **Marrafini LA, Sontheimer EJ.** CRISPR interference limits horizontal gene transfer in staphylococci by targeting DNA. *Science* 322: 1843–1845, 2009. doi:10.1126/science.1165771.
49. **Matano M, Date S, Shimokawa M, Takano A, Fujii M, Ohta Y, Watanabe T, Kanai T, Sato T.** Modeling colorectal cancer using CRISPR-Cas9–mediated engineering of human intestinal organoids. *Nat Med* 21: 256–262, 2015.
50. **McCracken KW, Catá EM, Crawford CM, Sinagoga KL, Schumacher M, Rockich BE, Tsai Y-H, Mayhew CN, Spence JR, Zavros Y, Wells JM.** Modelling human development and disease in pluripotent stem-cell-derived gastric organoids. *Nature* 516: 400–404, 2014. doi:10.1038/nature13863.
51. **Mojica FJM.** Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. *J Mol Evol* 60: 174–182, 2005.
52. **Nakata A, Amemura M, Makino K.** Unusual nucleotide arrangement with repeated sequences in the Escherichia coli K-12 chromosome. *J Bacteriol* 171: 3553–3556, 1989.
53. **Nanduri LSY, Baanstra M, Faber H, Rocchi C, Zwart E, De Haan G, Van Os R, Coppes RP.** Purification and Ex vivo expansion of fully functional salivary gland stem cells. *Stem Cell Reports* 3: 957–964, 2014. doi:10.1016/j.stemcr.2014.09.015.
54. **Ootani A, Li X, Sangiorgi E, Ho QT, Ueno H, Toda S, Sugihara H, Fujimoto K, Weissman IL, Capecchi MR, Kuo CJ.** Sustained in vitro

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intestinal epithelial culture within a Wnt-dependent stem cell niche. *Nat Med* 15: 701–706, 2009. doi:10.1038/nm.1951.

55. **Platt RJ, Chen S, Zhou Y, Yim MJ, Swiech L, Kempton HR, Dahlman JE, Parnas O, Eisenhaure TM, Jovanovic M, Graham DB, Jhunjhunwala S, Heidenreich M, Xavier RJ, Langer R, Anderson DG, Hacohen N, Regev A, Feng G, Sharp PA, Zhang F.** CRISPR-Cas9 knockin mice for genome editing and cancer modeling. *Cell* 159: 440–455, 2014. doi:10.1016/j.cell.2014.09.014.
56. **Pourcel C, Salvignol G, Vergnaud G.** CRISPR elements in Yersinia pestis acquire new repeats by preferential uptake of bacteriophage DNA, and provide additional tools for evolutionary studies. *Microbiology* 151: 653-663, 2005. doi: 10.1099/mic.0.27437-0.
57. **Qi LS, Larson MH, Gilbert LA, Doudna JA, Weissman JS, Arkin AP, Lim WA.** Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell* 152: 1173–1183, 2013. doi:10.1016/j.cell.2013.02.022.
58. **Ren W, Lewandowski BC, Watson J, Aihara E, Iwatsuki K, Bachmanov AA, Margolskee RF, Jiang P.** Single Lgr5- or Lgr6-expressing taste stem/progenitor cells generate taste bud cells ex vivo. *Proc Natl Acad Sci USA* 111: 16401–16406, 2014. doi:10.1073/pnas.1409064111.
59. **Rock JR, Onaitis MW, Rawlins EL, Lu Y, Clark CP, Xue Y, Randell SH, Hogan BLM.** Basal cells as stem cells of the mouse trachea and human airway epithelium. *Proc Natl Acad Sci USA* 106: 12771–12775, 2009. doi:10.1073/pnas.0906850106.
60. **Sato T, Vries RG, Snippert HJ, van de Wetering M, Barker N, Stange DE, van Es JH, Abo A, Kujala P, Peters PJ, Clevers H.** Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature* 459: 262–265, 2009. doi:10.1038/nature07935.
61. **Schwank G, Koo BK, Sasselli V, Dekkers JF, Heo I, Demircan T, Sasaki N, Boymans S, Cuppen E, Van Der Ent CK, Nieuwenhuis EES, Beekman JM, Clevers H.** Functional repair of CFTR by CRISPR/Cas9 in intestinal stem cell organoids of cystic fibrosis patients. *Cell Stem Cell* 13: 653–658, 2013. doi:10.1016/j.stem.2013.11.002.
62. **Shalem O, Sanjana NE, Zhang F.** High-throughput functional genomics using CRISPR-Cas9. *Nat Rev Genet* 16: 299–311, 2015. doi:10.1038/nrg3899.
63. **Spence JR, Mayhew CN, Rankin SA, Kuhar MF, Vallance JE, Tolle K, Hoskins EE, Kalinichenko VV, Wells SI, Zorn AM, Shroyer NF, Wells JM.** Directed differentiation of human pluripotent stem cells into intestinal tissue in vitro. *Nature* 470: 105–109, 2011. doi:10.1038/nature09691.
64. **Tadokoro T, Wang Y, Barak LS, Bai Y, Randell SH, Hogan BLM.** IL-6/STAT3 promotes regeneration of airway ciliated cells from basal stem cells. *Proc Natl Acad Sci USA* 111: E3641–E3649, 2014. doi:10.1073/pnas.1409781111.
65. **Takasato M, Er PX, Becroft M, Vanslambrouck JM, Stanley EG, Elefanty AG, Little MH.** Directing human embryonic stem cell differentiation towards a renal lineage generates a self-organizing kidney. *Nat Cell Biol* 16: 118–126, 2014. doi:10.1038/ncb2894.
66. **Takebe T, Zhang R-R, Koike H, Kimura M, Yoshizawa E, Enomura M, Koike N, Sekine K, Taniguchi H.** Generation of a vascularized and functional human liver from an iPSC-derived organ bud transplant. *Nat Protoc* 9: 396–409, 2014. doi:10.1038/nprot.2014.020.
67. **Tao L, Zhang J, Meraner P, Tovaglieri A, Wu X, Gerhard R, Zhang X, Stallcup WB, Miao J, He X, Hurdle JG, Breault DT, Brass AL, Dong M.** Frizzled proteins are colonic epithelial receptors for *C. difficile* toxin B. *Nature* 538: 350–355, 2016. doi:10.1038/nature19799.

67a. **Verissimo CS, Overmeer RM, Ponsioen B, Drost J, Mertens S, Verlaan-Klink I, Gerwen BV, van der Ven M, Wetering MV, Egan DA, Bernards R, Clevers H, Bos JL, Snippert HJ.** Targeting mutant RAS in patient-derived colorectal cancer organoids by combinatorial drug screening. *Elife* 2016 Nov 15;5. pii: e18489. doi:10.7554/eLife.18489.

68. **Wang T, Wei JJ, Sabatini DM, Lander ES.** Genetic screens in human cells using the CRISPR-Cas9 system. *Science* 343: 80–84, 2014. doi:10.1126/science.1246981.
69. **Wiedenheft B, Sternberg SH, Doudna JA.** RNA-guided genetic silencing systems in bacteria and archaea. *Nature* 482: 331–338, 2012. doi:10.1038/nature10886.
70. **Zhang Z, Christin JR, Wang C, Ge K, Oktay MH, Guo W.** Mammary-stem-cell-based somatic mouse models reveal breast cancer drivers causing cell fate dysregulation. *Cell Reports* 16: 3146–3156, 2016. doi:10.1016/j.celrep.2016.08.048.

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