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Running title: Engineered organoids in cancer research

CRISPR/Cas9-edited organoids as a platform for tailored research of colorectal cancer

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Colorectal cancer is one of the most commonly diagnosed cancers worldwide. Mortality rates and limited therapeutic options justify the development of reliable preclinical research models, as their translational value remains limited. Simple *in vitro* models do not recapitulate tumor heterogeneity, while *in vivo* research faces ethical concerns and interspecies differences. Patient-derived organoids offer a physiologically more relevant platform that retains the genetic, epigenetic, and phenotypic characteristics of the original tumor. Tumor-derived organoids enable precise investigation of novel treatments, functional genomics, and modeling of cancer development. Integration with CRISPR/Cas9 gene editing further enables accurate manipulation of specific genes to study carcinogenesis and therapeutic resistance. This review highlights recent advances in the use of organoids for colorectal cancer research and explores the potential of gene-edited organoids to discover the genetic and molecular mechanisms underlying colorectal cancer development, progression, and treatment resistance.

Keywords: colorectal cancer; patient-derived organoids; CRISPR/Cas9; gene editing

Colorectal cancer (CRC) is the second leading cause of cancer-related mortality [1, 2]. More than 1.9 million patients with CRC were diagnosed in 2022 [3], and epidemiological projections indicate that incidence will rise to 3 million cases by 2040 [1]. To address the unfavorable state, more effective diagnostic and therapeutic strategies are needed [4, 5]. *In vitro* models represent an irreplaceable tool in cancer research. Adherent (2-dimensional) cell cultures have many limitations, including a lack of physiological relevance and a lack of three-dimensional architecture [5]. The use of laboratory animals raises ethical concerns [6-8]. Moreover, there are significant interspecies and genotypic differences that limit their translational relevance [9, 10].

Three-dimensional cell cultures bridge the gap between conventional adherent cell lines and *in vivo* models [11]. They offer a physiologically more relevant environment that better reflects tissue architecture and cell-cell interactions, and provide deeper insight into tumor biology and treatment

responses [12-15]. Single-cell-derived spheroids and multicellular aggregates are widely used [16, 17], but they lack tumor cell heterogeneity. Organoids are scaffold-based, self-organized 3D cultures derived from stem cells proliferating in the presence of defined growth factors. They have been established from a broad spectrum of healthy and pathological tissues and represent the most advanced 3D cultures [12, 18-24]. Organoids recapitulate key characteristics of the original tissue, including cellular diversity, spatial organization, and functional behavior. Patient-derived organoids (PDOs) closely reflect the genetic, epigenetic [25] and phenotypic characteristics [26] of the original tumor, including mutational profiles, and inter- and intratumoral heterogeneity [27-30]. In 2011, Jung *et al.* established the first CRC-derived PDOs [31]. Subsequently, van de Wetering and colleagues introduced the concept of 'living organoid biobanks' and utilized CRC organoid libraries as a platform for drug screening [32]. While healthy colon-derived organoids recapitulate the cellular composition and differentiation of colon crypts, tumor PDOs retain patient-specific transcriptomic and epigenomic heterogeneity, and primary tumor somatic mutations [33]. CRC-derived PDOs have been used to predict response to various treatment regimens [34-37] and for studying signaling pathways associated with CRC [38-41].

The integration of CRISPR/Cas9 gene editing into organoid-based disease modelling has expanded the experimental and translational applications of 3D cultures. Gene-edited PDOs serve as a versatile platform linking clinical observations with molecular mechanisms and functional genomics. They enable high-throughput screening of tumor-associated genes, and investigate their roles in growth, progression, and therapeutic resistance in a patient-specific context [42, 43].

In this review, we summarize the options for CRISPR/Cas9 editing of CRC-derived organoids to prepare precise research tools to reveal mechanisms of CRC pathogenesis and identify reliable biomarkers and novel therapeutic targets.

CRISPR/Cas9 as a gene editing tool for PDOs

To study the functional consequences of specific mutations or gene inactivation in organoids, a range of genome editing approaches has been applied. Among these, CRISPR/Cas9 has emerged in recent years as a powerful and versatile tool, enabling precise modification of organoid genomes and expanding the possibilities for modelling disease and testing therapeutic strategies [43]. In the following sections, we describe methods for editing organoids using CRISPR/Cas9 and discuss their contributions to understanding CRC biology.

The Cas9 endonuclease induces sequence-specific double-stranded DNA breaks (DSBs), which activate cellular DNA repair mechanisms. CRISPR/Cas9 gene editing relies mainly on non-homology end joining (NHEJ) and homology-directed repair (HDR) [44-46]. NHEJ is a template-

independent, rapid mechanism active throughout the cell cycle. However, it is error-prone and leads to insertions and/or deletions (INDELs) near DSBs [47].

The HDR is a template-dependent, high-fidelity repair pathway that enables precise genetic changes and is preferred for gene editing [45]. However, it has several limitations, including its slowness and its restriction to the S/G2 phase of the cell cycle [46]. Organoids contain cells at various stages of the differentiation hierarchy, including stem-like, progenitor, and differentiated epithelial cell states [48]. Adult stem cells are predominantly quiescent, typically in the G0/G1 phase of the cell cycle [49], where the NHEJ repair mechanism predominates [50].

An alternative DNA repair mechanism, homology-independent targeted integration (HITI), was developed to enable precise DNA insertion into the genome of even non-dividing cells. Simultaneous Cas9-mediated cleavage of both genomic and donor DNA results in blunt ends, allowing integration of the donor sequence via NHEJ, thereby bypassing the requirement for homologous recombination. Sequence analysis showed that most junctions contained no INDELs. The system also allows control over the correct orientation of the inserted DNA segment, ensuring reliable unidirectional gene insertion [51, 52]. HITI has also been shown to be suitable for CRC-derived PDOs engineering [53].

Gene-edited PDOs in CRC research

The complexity of organoids represents a challenge for their gene editing. To achieve high transduction efficiency, dissociation into a single-cell suspension is required [54, 55], because remaining cell clumps result in low transduction efficiency, mosaicism, and the need for prolonged passages to establish a homogeneous organoid culture [56]. A combination of mechanical and enzymatic dissociation (TrypLE [53, 57], trypsin [54], or Accutase [58]) is a common approach to obtain small cell clusters and to support isogenic organoid culture [57]. A chemical dissociation using the enzyme-free Gentle Cell Dissociation Reagent was utilized by Bergin and Benoit [59].

CRISPR/Cas9 delivery strategies. CRISPR/Cas9 cargo can be delivered into organoids by several approaches, including viral vectors [60], physical methods (electroporation [61], nucleofection [62] or mechanical forces (nanopuncturing) [63]. Figure 1 summarizes the methods of CRISPR/Cas9 delivery into organoids.

The CRISPR/Cas9 components can be delivered as an extrachromosomal plasmid or viral DNA encoding Cas9 and a guide RNA (gRNA), or as mRNA for Cas9 paired with gRNA [64]. Novel approaches rely on the Cas9/gRNA ribonucleoprotein (RNP) complex, which delivers the pre-assembled Cas9 protein with gRNA. This approach provides transient expression and avoids

genomic integration. The short-time expression supports high editing efficiency with low toxicity [65-67].

Viral vectors. Viral vectors are widely used delivery systems for CRISPR/Cas9 into tumor cells due to their high transduction efficiency. The most commonly used viral vectors include lentiviral, adenoviral, and adeno-associated viruses. Lentiviral vectors can integrate into the host genome, leading to stable transgene expression in daughter cells after cell division [68]. For a genome-scale CRISPR-Cas9 knockout library, a single-vector system (lentiCRISPRv1) expressing both Cas9 and single guide RNAs (sgRNAs) has been constructed [69]. To optimize viral titer, the initial LentiCRISPRv1 was modified to lentiCRISPRv2 by repositioning the U6-driven sgRNA cassette and optimizing the nuclear localization signal and the P2A bicistronic linker sequences [70]. This ´all-in-one´ system is widely used in lentivirus-mediated CRISPR/Cas9 gene editing, including in organoids. With an optimized protocol for lentiCRISPRv2-based knockout of human colonic organoids, an efficiency exceeding 90% can be achieved within 1-2 weeks. Compared to inducible Cas9, constitutive Cas9 expression showed higher efficiency; however, off-targets were not completely monitored [71]. LentiCRISPRv2 has been used to deliver both Cas9 and sgRNAs targeting FUT2 or STAT1 genes in human intestinal epithelial organoids to determine their role in norovirus infection [72]. To increase viral titer, a dual-vector lentiviral system was developed in which Cas9 and sgRNA are delivered separately, enabling the establishment of Cas9-expressing stable cell lines. However, this approach allows for subsequent transduction with different sgRNAs for functional screening. A single-vector system may be more suitable for *in vivo* applications or primary cells, where a single transduction step is preferable [70].

Nanoblades are virus-like particles derived from retroviruses used to deliver CRISPR components into target cells. Unlike conventional viral vectors, nanoblades deliver pre-assembled, active RNP complexes. Their viral envelope enables them to enter cells via membrane fusion, making them suitable for genome editing in cells that are difficult to transfect [73]. Organoids derived from mouse and human tissues, including colonic and rectal PDOs, were used to assess the efficacy and toxicity of the nanoblade-based delivery strategy. Editing efficiency reached up to 50% without affecting cell viability. Nanoblades enabled rapid generation of engineered organoids without selection, INDELs, or off-target effects by transient delivering Cas9 in RNP complexes [74].

Electroporation is an efficient method for introducing CRISPR/Cas9 components into target cells [75]. As a physical transfection approach, it utilizes an electric field pulse to induce temporary membrane permeabilization for the entry of external DNA, RNA, or RNP molecules [76]. A wide range of cargoes, from small molecules to large biomacromolecules and nanoparticles, can be delivered via electroporation [77]. Efficiency is determined by the parameters of the applied

electrical pulse (strength, duration, and number of pulses), cellular properties, and the size, structure, and charge of the delivered cargo [78]. In a pioneering study, a plasmid-based CRISPR/Cas9 system was used to modulate gene expression in human organoids derived from healthy intestinal epithelium or adenomas to investigate the potential cooperation among major signaling pathways (Wnt, MAPK, PI3K, TGF-β, TP53) in CRC. A bicistronic plasmid, px330, encoding Cas9 nuclease and gene-specific sgRNAs targeting tumor suppressor genes APC, TP53, and SMAD4, was used. Next, activating mutations into the KRAS and PIK3CA oncogenes were introduced to ensure their constitutive expression. Findings obtained by introducing driver mutations with CRISPR/Cas9 were verified by engineering organoids with a self-inactivating lentiviral vector encoding shRNA or transgenes [79].

Fujii et al. developed an improved method for electroporation of human intestinal organoids [55], which was later applied by Okamoto et al. to modify colorectal PDOs [53]. They investigated cancer stem cells in CRC PDOs and performed a knock-in of the IRES-EGFP-P2A-iCaspase9 reporter cassette into the 3′ UTR of the putative stem cell marker OLFM4. The authors published a detailed protocol that enables the visualization and functional assessment of stem cells without disrupting endogenous gene expression. They applied transposon-mediated integration via electroporation using the green fluorescent protein (GFP)- expressing PiggyBac system [55] combined with HITI [52], allowing efficient and precise insertion of donor DNA at the target site. Genetically modified organoids were then selected with puromycin or neomycin, and GFP-positive clones were isolated and cultured in Matrigel-supported ENR medium, allowing continued expansion and functional characterization [53]. This methodology is highly adaptable, providing a robust platform for studying stem cells in various tumor contexts. Using this multi-guide CRISPR/Cas9 RNP approach combined with electroporation, the authors knocked out the FBXW7 gene in human colon cells with a knockout efficiency of 75-100%. Multiple Cas9/gRNA complexes targeting three regions of the FBXW7 gene were simultaneously delivered into the cell to ensure a large-scale knockout [80]. Nucleofection is an improved electroporation method for efficiently transfecting DNA directly into the cell nucleus. It was used to assess the risk of CRC development in patients with familial adenomatous polyposis (FAP). The APC mutation correction system based on CRISPR-Cas9 was delivered to FAP-human embryonic stem cell lines. Correction of the APC gene restored normal colon organoid formation, highlighting the role of APC mutations in CRC initiation [58, 75]. Nucleofection of organoids has achieved up to 70% transfection efficiency [62]. Furthermore, its combination with an RNP-based approach can improve gene-editing efficiency to 98% [65]. A combination of RNPs and electroporation was used to knock down the REG4 gene in a study of immune checkpoint inhibitor resistance in MSS CRC tumors. The gRNA and Cas9-GFP

molecules were mixed to form RNPs, where GFP served to monitor successful internalization of the RNP complexes [81].

A recent study showed that introducing Cas9-sgRNA RNP constructs into intact organoids via calcium shock resulted in remarkably high efficiency. Calcium deprivation is associated with temporary disruption of cell-cell junctions and increased endocytosis. Although the study used liver- and iPS cell-derived organoids, it may have potential for application with intact colon or CRC-derived PDOs [82].

Nanopuncturing is a novel, promising mechanical method for high-throughput delivery of CRISPR/Cas9 constructs utilizing nanoneedles that temporarily perforate the cell membrane, forming micropores without causing significant cell damage. This allows high transfection efficiency even in difficult-to-transduce cells, reaching approximately 45 %, as shown in human induced pluripotent stem cells-derived organoids [63].

In addition to viral vectors and electroporation-based approaches, ***nanocarrier-mediated systems*** have emerged as a promising platform for targeted CRISPR/Cas9 delivery [83]. Among them, stimuli-responsive nanocarriers have emerged to overcome the limitations in complex environments. These smart nanocarriers are capable of controlled CRISPR/Cas9 delivery and release in response to tumor-specific or externally applied stimuli, such as pH, redox conditions, enzymes, temperature, light, or ultrasound [84, 85]. Since organoids better recapitulate tissue architecture, extracellular matrix interactions, and biochemical gradients [86]. They represent physiologically relevant preclinical models for evaluating the penetration, release kinetics, and genome-editing efficacy of these nanoformulations [87, 88].

Selection and validation of edited organoids. Introducing CRISPR/Cas9 into organoids is typically followed by the selection of engineered cells [57].

The antibiotic selection (e.g., hygromycin [57, 89], puromycin, or Blasticidin [89, 90] relies on the expression of resistance genes encoded within the editing construct. Since drug sensitivity varies between organoids, optimization of the applied concentration is needed prior to the selection of the engineered cells [90-92]. Another strategy employs fluorescent reporter genes, enabling enrichment of edited cells by fluorescence-activated cell sorting [53, 93]. Dual-fluorescent reporter systems, in which the expression of one fluorescent marker depends on CRISPR/Cas9 activity, enable the distinction between transduced cells and cells undergoing active genome editing, as reported by Liu et al. The reporter construct contained a dual fluorochrome system, consisting of a constitutively expressed red fluorescent protein iRFP-720 under the control of the EF1α promoter, serving as an indicator of cell transduction, and GFP encoded out-of-frame, whose expression depended on the activity of the CRISPR/Cas9 system. Target sequences of the genes of interest were inserted

upstream of the GFP gene and recognized by the corresponding sgRNAs. Cas9 cleaves both the genomic target sequence and the identical sequence within the reporter construct. Subsequent repair via NHEJ introduced INDELs that restored GFP's correct reading frame, leading to its expression [94].

A functional selection based on culture conditions or growth factor withdrawal can also be used. For instance, APC-mutant organoids can survive in the absence of Wnt signaling components [95-97], KRAS-mutant organoids can be selected by EGF withdrawal [97, 98], and TP53-mutant organoids can be enriched using Nutlin-3a [79, 97, 98].

After transduction, a heterogeneous cell population is obtained, comprising cells with different edits. To generate organoid cultures with uniform genetic alterations, single-cell clonal expansion is used to isolate a genetically identical cell line [63]. Combining clonal expansion with single-cell sequencing enables genotyping of the resulting organoid populations, thereby confirming the genetic uniformity of the organoid culture [99].

The overall workflow of genetic editing of organoids is illustrated in Figure 2.

Insights into CRC carcinogenesis using CRISPR-edited organoids

Approximately 70% of CRCs develop through the conventional adenoma-carcinoma sequence, whereas 30% of cases arise through the serrated neoplasia pathway, characterized by BRAF mutations, microsatellite instability (MSI), and the CpG island methylator phenotype (CIMP) [100-102]. In the adenoma-carcinoma sequence, the most common initial event is a loss-of-function mutation in the APC gene [103-105]. In the next stage, point mutations in the KRAS gene appear. G12D is the most common KRAS mutation in CRC, leading to constitutive activation of the KRAS protein [106, 107]. Chromosomal instability (CIN) accounts for around 85% of sporadic CRC [108, 109]. Late-stage CRC is characterized by loss of chromosomal regions, particularly in 17q and 18q. Loss of the 18q has been detected in up to 70% of advanced adenocarcinomas. These changes lead to the inactivation of key tumor suppressor genes, such as DCC/SMAD2/SMAD4 (18q) and TP53 (17p), promoting progression to the malignant stage [110, 111]. Approximately 10-15% of CRCs exhibit MSI, which arises from a defect in the DNA mismatch repair (MMR). The key MMR genes, including MLH1, MSH2, MSH6, and PMS2, are mutated, leading to the accumulation of INDELs and a hypermutated phenotype [112-114].

An MSI CRC model was established using CRISPR/Cas9-modified intestinal organoids. MLH1 knockout led to a defect in DNA repair, allowing the development of a mutational profile typical of MSI tumors [115]. This work is followed by a study investigating the development of tumor cells from MLH1-deficient cells under hostile culture conditions (growth factor removal). MLH1-

deficient organoids showed a mutational profile typical of MSI tumors, including the accumulation of point mutations and INDELs. During long-term cultivation, mutations in the AXIN1 and AXIN2 genes were the first to appear, leading to activation of the Wnt signaling pathway, representing the initial step of cell transformation. Subsequently, mutations in the ACVR2A, BMPR2, and NRAS genes were identified. The combination of mutations in the four main pathways (Wnt, BMP, p53, and RAS/MAPK) led to complete malignant transformation of the organoids. These organoids were independent of growth factors and formed tumors after transplantation into mice, confirming their tumorigenic potential [116].

Commercially available human colon AKP (APC^{KO}, $KRAS^{G12D}$, $TP53^{KO}$) and AKPS (APC^{KO}, $KRAS^{G12D}$, $TP53^{KO}$, $SMAD4^{KO}$) organoids prepared with the CRISPR/Cas9 method were used to study the effect of SMAD4 inactivation. The SMAD4 inactivation disrupted the oncogenic TGF- β, Wnt, and VEGF pathways, leading to increased proliferation and migration, and decreased differentiation. Overexpression of DKK3, which decreases NK cell antitumor function, was also detected [117].

Intestinal organoids are widely used to model cancer initiation, progression, or to recapitulate the adenoma-carcinoma sequence. CRISPR/Cas9 was used to alter the expression of key CRC driver genes APC, KRAS, SMAD4, TP53, and PIK3CA of organoids derived from normal colonic epithelia or CIN adenoma. Engineered organoids were compared with CRC-derived organoids to investigate the possible cooperation of major signaling pathways Wnt, MAPK, PI3K, TGF-β, and TP53 in cancer development and progression. Culture conditions were designed to selectively support the growth of cells with specific mutations in individual signaling pathways, allowing the introduction of combinations of mutations without the need for antibiotic selection. Genome editing and organoid selection resulted in a significant reduction in the organoid dependence on exogenous niche factors. This approach generated AKSTP organoids ($APC^{-/-}$, $KRAS^{G12V}$, $SMAD4^{-/-}$, $TP53^{-/-}$, $PIK3CA^{E545K}$) that were completely independent of niche factors. Intrasplenic injection of AKSTP organoids led to the development of micrometastatic lesions containing dormant tumor-initiating cells. Organoids derived from metastatic CRC and engineered PDOs from CIN adenomas colonized the liver. These findings suggest that the combination of five driver mutations enables cancer stem cells to survive in a hostile TME. Still, metastasis development likely requires additional genetic aberrations associated with CIN [79].

In recent years, large-scale genome sequencing studies of CRC have identified numerous additional, less frequently mutated genes, indicating significant genetic intra- and inter-heterogeneity [118-120]. Takeda and colleagues used mouse intestinal tumor organoids for CRISPR/Cas9 screening [104]. Twenty-nine candidate CRC tumor-suppressor genes, including Pten, Smad4, and Trp53,

were selected for functional validation in mouse intestinal tumor organoids, carrying $Apc^{\Delta 716}$ and $Kras^{G12D}$ mutations and subsequently in human CRC-derived organoids. Engineered organoid clones stably expressing Cas9 were prepared by transducing the Cas9-GFP lentivirus. Subsequently, the CRISPR pooled screening was performed, where a mixture of multiple guide RNAs was introduced into Cas9-expressing cells, each targeting a different gene. This approach allowed parallel testing of a large number of candidate genes in a single experiment and the identification of those contributing to tumor growth. CRC organoids transduced with sgRNAs targeting key tumor suppressor genes developed liver metastases following intrasplenic application. Activin receptors Acvr1b, Acvr2a, and Arid2 were identified as tumor suppressors, whereas simultaneous mutations in Activin and TGF-β receptors synergistically enhanced tumor progression [104]. A CRISPR/Cas9 screening platform was employed for combined *in vitro* and *in vivo* functional genomics studies using engineered premalignant $APC^{-/-}$; $KRAS^{G12D}$ colon organoids and organoid-derived xenografts, with a pan-cancer gRNA library targeting multiple (n=85) tumor suppressor genes. Xenotransplantation into NSG mice enabled *in vivo* identification of genes that confer a growth advantage in the complex microenvironment. Loss of TGFBR2 resulted in the strongest clonal proliferation *in vivo*, underscoring the growth-suppressive role of TGF-β signaling. In addition, known CRC tumour suppressors, including SMAD4, FBXW7, STK11, and PIK3R1, as well as potential novel candidate growth regulators, such as CDKN2A, PBRM1, and ZFP36L1, were identified [99].

The RAS oncogene plays a key role in CRC by regulating the RAS/MAPK signaling pathway [121]. RAS activity is tightly regulated by RAS GTPase-activating proteins (RASGAPs), such as NF1, RASA1, and GAP1 family members, which catalyze its inactivation [122]. Mutations in RAS or loss-of-function mutation in NF1 lead to constitutive activation of the RAS/MAPK pathway and uncontrolled cell proliferation. These alterations are associated with resistance to anti-EGFR therapies [123, 124]. CRISPR knockout of a panel of RASGAP genes was performed in patient-derived CRC organoids (RAS wild-type/APC and TP53 mutant) to elucidate their roles in tumorigenesis and anti-EGFR therapy resistance. Among the analyzed RASGAP genes, only NF1 inactivation led to significant RAS activation and organoid growth after temporary EGFR inhibition. Conversely, long-term EGFR inhibition led to decreased RAS-GTP levels in NF1 knockout organoids, suggesting that NF1-loss effects can be detected only in the presence of upstream RAS-activating signals. Overall, NF1 deficiency may be associated with reduced sensitivity to anti-EGFR therapy and could serve as a biomarker in CRC [12].

Many studies focus on investigating resistance to platinum derivatives in CRC [126-128]. Resistance to cisplatin is very common and is mainly associated with deregulation of DNA damage

signaling (TP53 mutations) and/or defects in the cellular MMR mechanism [113]. A high-throughput CRISPR/Cas9 knockout screening was used to identify genes that restore cisplatin sensitivity in TP53-KO CRC organoids. The lentiviral CRISPR library of 76,441 gRNAs was used for the screening. Knockout of genes participating in the DNA repair, including the Fanconi Anemia pathway, led to desensitization of TP53-KO organoids to cisplatin. Inhibition of ERCC6, FANCL, and BRIP1 increased cisplatin-induced apoptosis, suggesting their potential as therapeutic targets to enhance cisplatin efficacy [60].

Tumor cells often harbor loss-of-function mutations in key regulatory genes, creating a genetic dependency on alternative pathways required for their survival. Identification of such specific dependencies enables the exploitation of synthetic lethality. For example, MMR deficiency in CRC cells creates a dependency on the WRN helicase [129, 130]. Genome-wide CRISPR/Cas9 knockout screening was performed to identify genetic dependencies and potential therapeutic targets via synthetic lethality. The TP53 wild-type F147T organoids with an MSI-H phenotype, harboring mutations in MMR genes (MLH3, MSH3) and in RNF43, BRAF, and PTEN, were used as the primary model. To avoid TP53-mediated apoptosis triggered by Cas9-induced DNA damage, TP53-KO F147T organoid counterparts were generated. Mutations in the tumor suppressor gene RNF43 led to increased dependence on components of the Wnt signaling pathway, including CTNNB1, WNT7B, and PORCN. PTEN mutation was linked with PIK3CA and PIK3CB dependence. Moreover, PIK3CB dependence was higher in TP53-deficient organoids, suggesting a potential therapeutic strategy for PTEN/TP53 double-mutant cells. TP53 loss was associated with increased sensitivity to inhibition of topoisomerase IIβ (TOP2B). Dependence of MSI tumors on the Werner syndrome (WRN) helicase and Essential Meiotic Structure-Specific Endonuclease 1 (EME1) was also detected. Knockout of PPP6C was lethal for both MSS and MSI organoid models. These findings help identify tumor-specific vulnerabilities with targeted therapeutic potential in CRC [131].

In recent years, immune checkpoint inhibitors have demonstrated significant clinical efficacy in MSI-high tumors [132, 133], while MSS CRC appear to be resistant due to low mutational burden and an immunosuppressive TME [134]. To better explore these microenvironment-dependent responses, organoid co-culture models have been developed. A combination of PDOs with cancer-associated fibroblasts (CAFs), immune cells [135], endothelial cells [136], or mesenchymal stromal cells [137] enables more accurate recapitulation of the complex interactions within the TME and the investigation of mechanisms of immune evasion and drug resistance. Co-cultivation of PDOs with CAFs identified differentially expressed genes, particularly those associated with IFNα/β signaling and MHC class II protein complex assembly, suggesting their involvement in resistance to

chemotherapy [138]. In another study, a co-culture of REG4 knockout CRC organoids with autologous immune cells (T cells and myeloid-derived suppressor cells) was used to explore its role in immune evasion and in the resistance of MSS CRC tumors to immune checkpoint inhibitors. Knockout of REG4 restored CD8+ T-cell recognition of tumor cells and increased sensitivity to the immunotherapy. Moreover, reduced expression of tolerogenic molecules, galectins (KGALS1, KGALS3, KGALS9), and mucins (MUC1, MUC2), was also observed, suggesting a role for REG4 in immune evasion [81].

In addition to genetic alterations, epigenetic deregulation also plays a significant role in colorectal carcinogenesis. DNA methylation abnormalities [139], chromatin remodeling, histone modifications [140, 141] and altered non-coding RNA expression [142, 143] collectively contribute to tumor initiation, progression and intratumoral heterogeneity in CRC. Aberrant DNA methylation represents a key mechanism involved in CRC pathogenesis. A subgroup of CRCs displays the CIMP phenotype, characterized by widespread hypermethylation of multiple CpG islands, commonly associated with tumors arising through the serrated neoplasia pathway [101, 144]. Several studies have utilized CRISPR-edited organoids to investigate the role of BRAF mutation in CIMP-driven epigenetic reprogramming [145-147]. Engineered colonic organoids recapitulating key molecular alterations in BRAF-mutant serrated CRC were established [145]. Tao and colleagues showed that ageing-associated CIMP development and epigenetic silencing of tumor-suppressive genes sensitize colon organoids to $BRAF^{V600E}$-driven transformation [146]. It was also described that mutant BRAF contributes to the maintenance of CIMP-associated epigenetic changes by regulating DNA methylation, PRC2-mediated histone modifications, and chromatin remodeling. Correction of the $BRAF^{V600E}$ mutation led to demethylation and reexpression of CIMP and PRC2 target genes, thereby reducing the aggressive phenotype [147]. Figure 3 summarizes applications of engineered PDOs in cancer research.

Limitations and ethical concerns

Although CRISPR/Cas9 technology holds significant promise for advancing precision cancer research, investigating potential biomarkers, and developing novel therapeutic strategies, its applications in PDO-based research are accompanied by several challenges, limitations, and ethical concerns related to the use of patient-derived materials.

One of the major limitations of CRISPR/Cas9-based genome editing is the risk of unintended off-target effects. These arise from CRISPR/Cas9 recognition and cleavage of a genomic locus with high sequence similarity to the target site. Off-target sites are primarily determined by the sgRNA, as Cas9 has been shown to tolerate up to three mismatches between the sgRNA and the target

genomic DNA. As a result, Cas9 cleaves several nucleotides upstream or downstream, leading to adverse editing outcomes [148, 149]. Various strategies have been developed to reduce off-target effects, including optimizing sgRNA length [150] or secondary structure [151], using high-fidelity Cas9 variants [152], and shortening the genome's exposure time to active Cas9 nuclease (RNP-based approach) [75]. The technique using RNP complexes of Cas9 and sgRNA has been successfully used in intestinal organoids [65, 153]. and its application was recently further optimized by refining electroporation parameters and culture conditions [75]. Gene editing using a Cas9/sgRNA RNP complex in human intestinal organoids achieved up to 98% efficiency, with no off-target effects [65].

The limitations of conventional CRISPR/Cas9, particularly the genotoxicity associated with DSBs and the low efficiency, have led to the development of next-generation editing technologies. In classical CRISPR/Cas9, efficiency and target sequence range are limited by the requirement for a protospacer adjacent motif and by the CRISPR/Cas9 system's specificity [154]. Improved next-generation editing technologies include base editing, prime editing, and transcriptional and epigenetic modulation [148, 155]. The Cas9 endonuclease variants were developed to enhance Cas9 target specificity and expand its target range. One of the major advancements was the development of modified Cas9 proteins with one or both endonuclease domains deactivated by mutation [156]. The Cas9 variants with nickase activity (nCas9) are produced by deactivating one of the catalytic domains. The nCa9 generates single-strand breaks. Inactivation of the HNH motif allows the RuvC domain to cut the non-complementary strand, whereas inactivation of RuvC allows HNH to cut the complementary strand [157, 158]. Inactivation of both catalytic domains results in a ‘dead’ Cas9 (dCas9), which lacks endonuclease activity but retains DNA-binding specificity, allowing recognition of target sequences. Fusion of dCas9 with transcriptional activators or repressors modulates gene expression through CRISPR activation (CRISPRa) or CRISPR interference (CRISPRi) [154, 159].

Base editing uses the fusion of deaminases to the Cas9 nickase, enabling precise single-nucleotide conversions without inducing DSBs [160]. Cytosine and adenine editors (CBE/ABE) enabled highly efficient multiplex editing of adult stem cell–derived colon organoids. Four sgRNAs were simultaneously co-transfected, targeting APC^{Q1406*}, $PIK3CA^{E545K}$, $SMAD4^{R361H}$, and $TP53^{W53*}$. Following selection in a medium lacking Wnt-3A/R-spondin, only APC-mutant organoids survived. Individual clones were subsequently isolated and genotyped. By a single transfection step, organoids harboring single, double, triple, or quadruple mutations were established with minimal off-target effect and without significant INDELs [89].

Prime editing utilizes a Cas9 nickase fused to an engineered reverse transcriptase, along with a prime editing guide RNA (pegRNA) that identifies the target sequence and provides a template for introducing precise DNA modifications, including insertions, deletions, or point mutations. Unlike conventional CRISPR/Cas9, this approach allows gene editing without DSBs and a donor DNA template [154, 161, 162]. Prime editing was applied in patient-derived intestinal and liver organoids to introduce defined in-frame deletions in CTNNB1 and to correct disease-linked mutations in DGAT1 and ATP7B. Efficient editing levels of 30-50% were achieved, with no detectable off-target mutations, highlighting the high specificity of prime editing in organoid systems [163]. Recent studies have shown that MMR deficiency can enhance prime editing efficiency without detectable microsatellite instability [164, 165], suggesting that genome editing outcomes may differ in MMR-deficient CRC models.

Beyond the limitations of genome editing itself, PDO-based models also pose several technical and practical challenges. One of the main issues is the complexity of handling and the cultivation costs. Moreover, not every patient sample grows *in vitro*. In a large retrospective analysis, the overall success rate in establishing CRC organoid cultures was 76.3% (939/1231). The efficacy of organoid establishment depends on the type of starting material, with the highest rate observed in malignant ascites obtained by abdominal puncture (96.4%, 27/28), followed by surgical resection specimens (76.5%, 864/1130) and endoscopic biopsies (65.8%, 48/73) [166]. Another challenge in establishing PDOs is preventing outgrowth or contamination by non-tumor cells [167]. Furthermore, slight shifts in clonal composition, loss of tumor heterogeneity, and potential genetic drift can occur during extended passaging or under selective culture conditions, potentially reducing their ability to recapitulate patients' tumor characteristics [168].

Ethical concerns. There are some ethical concerns associated with the derivation, long-term cultivation, and sharing of PDOs. Informed consent must be obtained from donors and must include the potential long-term use, genetic characterization, and distribution of organoids between laboratories. Particular care is required when PDOs are derived from donors with rare genotypes, as the high scientific value of such samples may put implicit pressure on individuals to contribute their cells. Further ethical concerns are associated with the ownership and commercialization of patient-derived material, including potential patenting and profit-making [169, 170]. The application of CRISPR/Cas9 will raise further ethical and safety concerns. While engineered PDOs offer unprecedented opportunities for research and personalized medicine, their application must be guided by strict ethical oversight. Genetically modified organoids offer unique opportunities for disease modelling, but off-target effects and unintended genome alterations remain major concerns. Such effects may compromise experimental validity. The translational relevance of gene-edited

PDOs requires rigorous validation and the application of existing ethical and regulatory frameworks for gene therapy [171]. A consensus should be reached within the research community to define the ethical boundaries of gene editing while protecting donors and respecting human dignity.

Conclusion and future directions

The discovery of PDOs has revolutionized CRC research by providing physiologically relevant 3D models that reliably recapitulate tumor heterogeneity and microenvironmental interactions. When integrated with CRISPR/Cas9 genome-editing technology, these models allow precise functional studies of gene alterations, mechanisms of therapeutic resistance, and the discovery of novel treatment targets. Despite their tremendous potential, several challenges remain, including standardising organoid culture protocols, scaling for high-throughput applications, and integrating immune and stromal components to mimic the tumor ecosystem better. Looking forward, the combination of PDOs with novel technologies such as artificial intelligence, single-cell omics, and high-content drug screening is expected to advance personalized oncology and reduce reliance on animal models. Machine learning algorithms are expected to facilitate automated image-based phenotyping, predictive modelling of drug responses, and integration of multi-omics datasets, enhancing the accuracy and scalability of organoid-based platforms. Interdisciplinary collaboration across computational, biological, and clinical sciences will be crucial to unlock the full translational potential of CRISPR-edited organoid-based cancer research.

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Figure Legends

Figure 1. Methods of CRISPR/Cas9 delivery into organoids.

Figure 2. The overall workflow of CRISPR/Cas9 genome editing of organoids.

Figure 3. Possible applications of CRISPR/Cas9 edited PDOs in cancer research.

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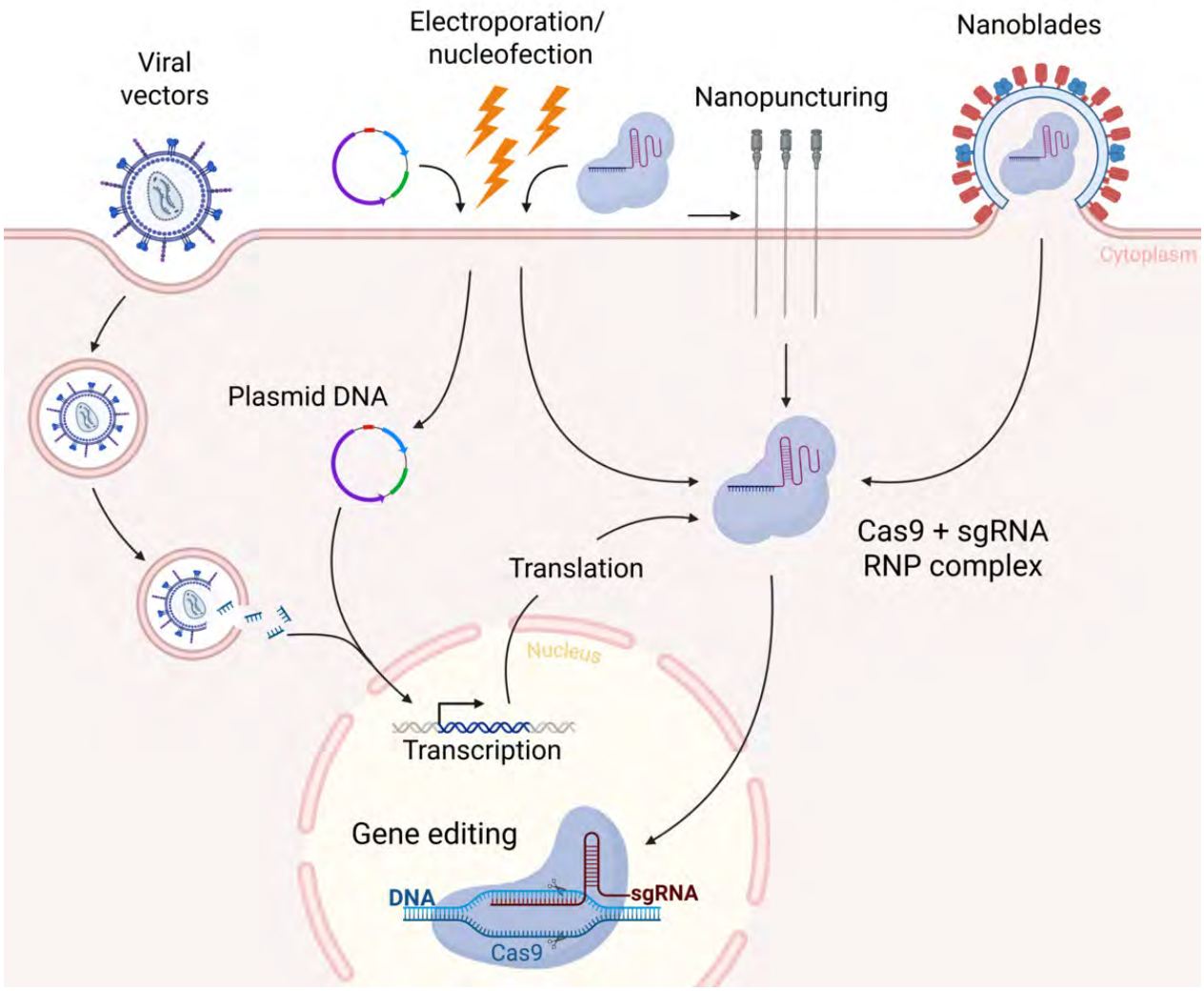

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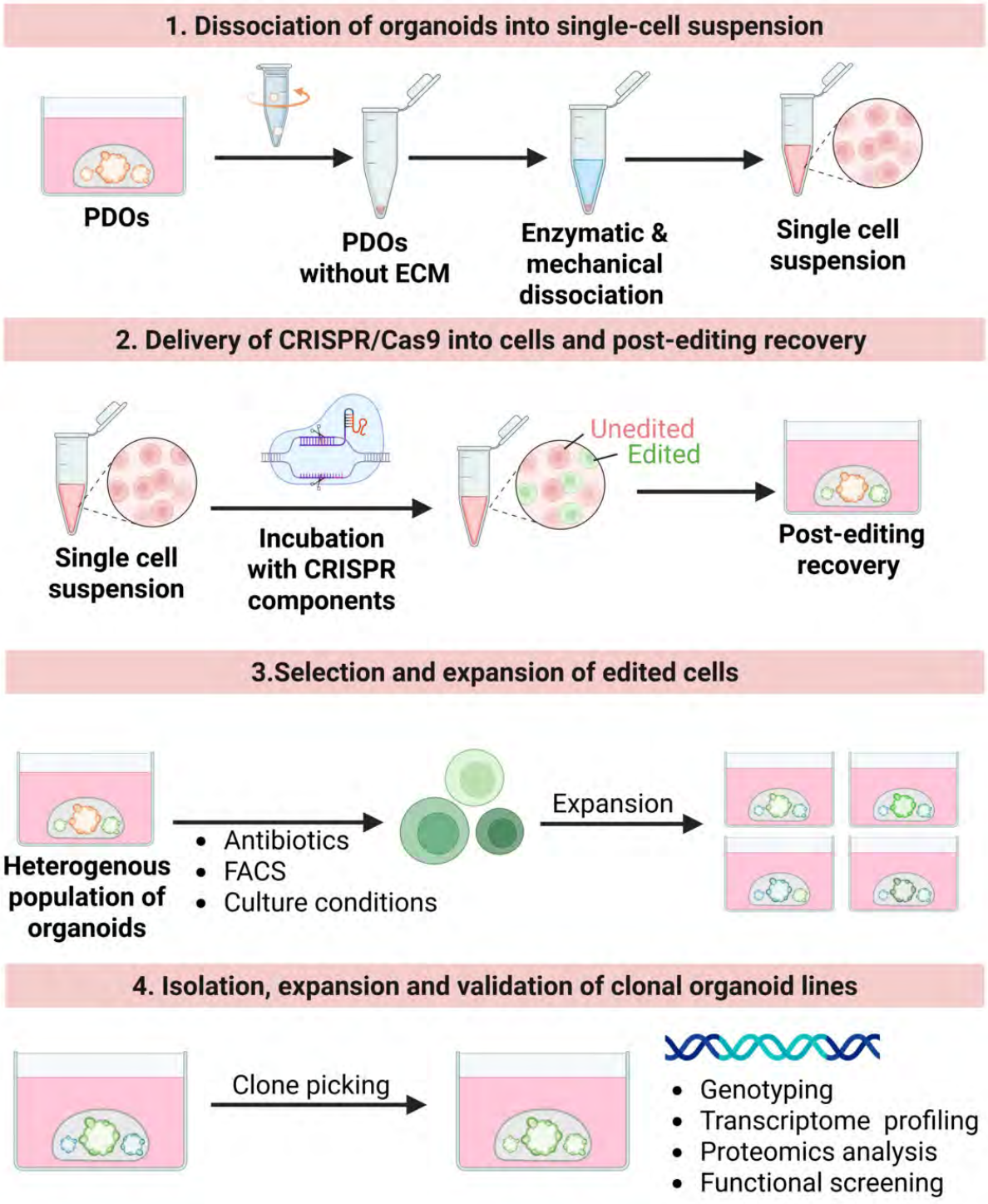

Fig. 3 [Download full resolution image]