

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

48 responses [12-15]. Single-cell-derived spheroids and multicellular aggregates are widely used [16,
49 17], but they lack tumor cell heterogeneity. Organoids are scaffold-based, self-organized 3D
50 cultures derived from stem cells proliferating in the presence of defined growth factors. They have
51 been established from a broad spectrum of healthy and pathological tissues and represent the most
52 advanced 3D cultures [12, 18-24]. Organoids recapitulate key characteristics of the original tissue,
53 including cellular diversity, spatial organization, and functional behavior. Patient-derived organoids
54 (PDOs) closely reflect the genetic, epigenetic [25] and phenotypic characteristics [26] of the
55 original tumor, including mutational profiles, and inter- and intratumoral heterogeneity [27-30]. In
56 2011, Jung *et al.* established the first CRC-derived PDOs [31]. Subsequently, van de Wetering and
57 colleagues introduced the concept of 'living organoid biobanks' and utilized CRC organoid libraries
58 as a platform for drug screening [32]. While healthy colon-derived organoids recapitulate the
59 cellular composition and differentiation of colon crypts, tumor PDOs retain patient-specific
60 transcriptomic and epigenomic heterogeneity, and primary tumor somatic mutations [33]. CRC-
61 derived PDOs have been used to predict response to various treatment regimens [34-37] and for
62 studying signaling pathways associated with CRC [38-41].

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

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

71

72 **CRISPR/Cas9 as a gene editing tool for PDOs**

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

79 The Cas9 endonuclease induces sequence-specific double-stranded DNA breaks (DSBs), which
80 activate cellular DNA repair mechanisms. CRISPR/Cas9 gene editing relies mainly on non-
81 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

115 genomic integration. The short-time expression supports high editing efficiency with low toxicity
116 [65-67].

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

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

144 **Electroporation** is an efficient method for introducing CRISPR/Cas9 components into target cells
145 [75]. As a physical transfection approach, it utilizes an electric field pulse to induce temporary
146 membrane permeabilization for the entry of external DNA, RNA, or RNP molecules [76]. A wide
147 range of cargoes, from small molecules to large biomacromolecules and nanoparticles, can be
148 delivered via electroporation [77]. Efficiency is determined by the parameters of the applied

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

159 Fujii et al. developed an improved method for electroporation of human intestinal organoids [55],
160 which was later applied by Okamoto et al. to modify colorectal PDOs [53]. They investigated
161 cancer stem cells in CRC PDOs and performed a knock-in of the IRES-EGFP-P2A-iCaspase9
162 reporter cassette into the 3' UTR of the putative stem cell marker OLFM4. The authors published a
163 detailed protocol that enables the visualization and functional assessment of stem cells without
164 disrupting endogenous gene expression. They applied transposon-mediated integration via
165 electroporation using the green fluorescent protein (GFP)-expressing PiggyBac system [55]
166 combined with HITI [52], allowing efficient and precise insertion of donor DNA at the target site.
167 Genetically modified organoids were then selected with puromycin or neomycin, and GFP-positive
168 clones were isolated and cultured in Matrigel-supported ENR medium, allowing continued
169 expansion and functional characterization [53]. This methodology is highly adaptable, providing a
170 robust platform for studying stem cells in various tumor contexts. Using this multi-guide
171 CRISPR/Cas9 RNP approach combined with electroporation, the authors knocked out the FBXW7
172 gene in human colon cells with a knockout efficiency of 75-100%. Multiple Cas9/gRNA complexes
173 targeting three regions of the FBXW7 gene were simultaneously delivered into the cell to ensure a
174 large-scale knockout [80]. Nucleofection is an improved electroporation method for efficiently
175 transfecting DNA directly into the cell nucleus. It was used to assess the risk of CRC development
176 in patients with familial adenomatous polyposis (FAP). The APC mutation correction system based
177 on CRISPR-Cas9 was delivered to FAP-human embryonic stem cell lines. Correction of the APC
178 gene restored normal colon organoid formation, highlighting the role of APC mutations in CRC
179 initiation [58, 75]. Nucleofection of organoids has achieved up to 70% transfection efficiency [62].
180 Furthermore, its combination with an RNP-based approach can improve gene-editing efficiency to
181 98% [65]. A combination of RNPs and electroporation was used to knock down the REG4 gene in a
182 study of immune checkpoint inhibitor resistance in MSS CRC tumors. The gRNA and Cas9-GFP

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

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

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

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

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

206 The antibiotic selection (e.g., hygromycin [57, 89], puromycin, or Blasticidin [89, 90] relies on the
207 expression of resistance genes encoded within the editing construct. Since drug sensitivity varies
208 between organoids, optimization of the applied concentration is needed prior to the selection of the
209 engineered cells [90-92]. Another strategy employs fluorescent reporter genes, enabling enrichment
210 of edited cells by fluorescence-activated cell sorting [53, 93]. Dual-fluorescent reporter systems, in
211 which the expression of one fluorescent marker depends on CRISPR/Cas9 activity, enable the
212 distinction between transduced cells and cells undergoing active genome editing, as reported by Liu
213 et al. The reporter construct contained a dual fluorochrome system, consisting of a constitutively
214 expressed red fluorescent protein iRFP-720 under the control of the EF1 α promoter, serving as an
215 indicator of cell transduction, and GFP encoded out-of-frame, whose expression depended on the
216 activity of the CRISPR/Cas9 system. Target sequences of the genes of interest were inserted

217 upstream of the GFP gene and recognized by the corresponding sgRNAs. Cas9 cleaves both the
218 genomic target sequence and the identical sequence within the reporter construct. Subsequent repair
219 via NHEJ introduced INDELs that restored GFP's correct reading frame, leading to its expression
220 [94].

221 A functional selection based on culture conditions or growth factor withdrawal can also be used.
222 For instance, APC-mutant organoids can survive in the absence of Wnt signaling components [95-
223 97], KRAS-mutant organoids can be selected by EGF withdrawal [97, 98], and TP53-mutant
224 organoids can be enriched using Nutlin-3a [79, 97, 98].

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

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

231

232 **Insights into CRC carcinogenesis using CRISPR-edited organoids**

233 Approximately 70% of CRCs develop through the conventional adenoma-carcinoma sequence,
234 whereas 30% of cases arise through the serrated neoplasia pathway, characterized by BRAF
235 mutations, microsatellite instability (MSI), and the CpG island methylator phenotype (CIMP) [100-
236 102]. In the adenoma-carcinoma sequence, the most common initial event is a loss-of-function
237 mutation in the APC gene [103-105]. In the next stage, point mutations in the KRAS gene appear.
238 G12D is the most common KRAS mutation in CRC, leading to constitutive activation of the KRAS
239 protein [106, 107]. Chromosomal instability (CIN) accounts for around 85% of sporadic CRC [108,
240 109]. Late-stage CRC is characterized by loss of chromosomal regions, particularly in 17q and 18q.
241 Loss of the 18q has been detected in up to 70% of advanced adenocarcinomas. These changes lead
242 to the inactivation of key tumor suppressor genes, such as DCC/SMAD2/SMAD4 (18q) and TP53
243 (17p), promoting progression to the malignant stage [110, 111]. Approximately 10-15% of CRCs
244 exhibit MSI, which arises from a defect in the DNA mismatch repair (MMR). The key MMR
245 genes, including MLH1, MSH2, MSH6, and PMS2, are mutated, leading to the accumulation of
246 INDELs and a hypermutated phenotype [112-114].

247 An MSI CRC model was established using CRISPR/Cas9-modified intestinal organoids. MLH1
248 knockout led to a defect in DNA repair, allowing the development of a mutational profile typical of
249 MSI tumors [115]. This work is followed by a study investigating the development of tumor cells
250 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,

285 were selected for functional validation in mouse intestinal tumor organoids, carrying $Apc^{\Delta 716}$ and
286 $Kras^{G12D}$ mutations and subsequently in human CRC-derived organoids. Engineered organoid
287 clones stably expressing Cas9 were prepared by transducing the Cas9-GFP lentivirus. Subsequently,
288 the CRISPR pooled screening was performed, where a mixture of multiple guide RNAs was
289 introduced into Cas9-expressing cells, each targeting a different gene. This approach allowed
290 parallel testing of a large number of candidate genes in a single experiment and the identification of
291 those contributing to tumor growth. CRC organoids transduced with sgRNAs targeting key tumor
292 suppressor genes developed liver metastases following intrasplenic application. Activin receptors
293 $Acvr1b$, $Acvr2a$, and $Arid2$ were identified as tumor suppressors, whereas simultaneous mutations
294 in Activin and TGF- β receptors synergistically enhanced tumor progression [104]. A CRISPR/Cas9
295 screening platform was employed for combined *in vitro* and *in vivo* functional genomics studies
296 using engineered premalignant $APC^{-/-}$; $KRAS^{G12D}$ colon organoids and organoid-derived
297 xenografts, with a pan-cancer gRNA library targeting multiple (n=85) tumor suppressor genes.
298 Xenotransplantation into NSG mice enabled *in vivo* identification of genes that confer a growth
299 advantage in the complex microenvironment. Loss of $TGFBR2$ resulted in the strongest clonal
300 proliferation *in vivo*, underscoring the growth-suppressive role of TGF- β signaling. In addition,
301 known CRC tumour suppressors, including $SMAD4$, $FBXW7$, $STK11$, and $PIK3R1$, as well as
302 potential novel candidate growth regulators, such as $CDKN2A$, $PBRM1$, and $ZFP36L1$, were
303 identified [99].

304 The RAS oncogene plays a key role in CRC by regulating the RAS/MAPK signaling pathway
305 [121]. RAS activity is tightly regulated by RAS GTPase-activating proteins (RASGAPs), such as
306 $NF1$, $RASA1$, and $GAP1$ family members, which catalyze its inactivation [122]. Mutations in RAS
307 or loss-of-function mutation in $NF1$ lead to constitutive activation of the RAS/MAPK pathway and
308 uncontrolled cell proliferation. These alterations are associated with resistance to anti-EGFR
309 therapies [123, 124]. CRISPR knockout of a panel of RASGAP genes was performed in patient-
310 derived CRC organoids (RAS wild-type/ APC and $TP53$ mutant) to elucidate their roles in
311 tumorigenesis and anti-EGFR therapy resistance. Among the analyzed RASGAP genes, only $NF1$
312 inactivation led to significant RAS activation and organoid growth after temporary EGFR
313 inhibition. Conversely, long-term EGFR inhibition led to decreased RAS-GTP levels in $NF1$
314 knockout organoids, suggesting that $NF1$ -loss effects can be detected only in the presence of
315 upstream RAS-activating signals. Overall, $NF1$ deficiency may be associated with reduced
316 sensitivity to anti-EGFR therapy and could serve as a biomarker in CRC [12].

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

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

326 Tumor cells often harbor loss-of-function mutations in key regulatory genes, creating a genetic
327 dependency on alternative pathways required for their survival. Identification of such specific
328 dependencies enables the exploitation of synthetic lethality. For example, MMR deficiency in CRC
329 cells creates a dependency on the WRN helicase [129, 130]. Genome-wide CRISPR/Cas9 knockout
330 screening was performed to identify genetic dependencies and potential therapeutic targets via
331 synthetic lethality. The TP53 wild-type F147T organoids with an MSI-H phenotype, harboring
332 mutations in MMR genes (MLH3, MSH3) and in RNF43, BRAF, and PTEN, were used as the
333 primary model. To avoid TP53-mediated apoptosis triggered by Cas9-induced DNA damage, TP53-
334 KO F147T organoid counterparts were generated. Mutations in the tumor suppressor gene RNF43
335 led to increased dependence on components of the Wnt signaling pathway, including CTNNB1,
336 WNT7B, and PORCN. PTEN mutation was linked with PIK3CA and PIK3CB dependence.
337 Moreover, PIK3CB dependence was higher in TP53-deficient organoids, suggesting a potential
338 therapeutic strategy for PTEN/TP53 double-mutant cells. TP53 loss was associated with increased
339 sensitivity to inhibition of topoisomerase II β (TOP2B). Dependence of MSI tumors on the Werner
340 syndrome (WRN) helicase and Essential Meiotic Structure-Specific Endonuclease 1 (EME1) was
341 also detected. Knockout of PPP6C was lethal for both MSS and MSI organoid models. These
342 findings help identify tumor-specific vulnerabilities with targeted therapeutic potential in CRC
343 [131].

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

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

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

377

378 **Limitations and ethical concerns**

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

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

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

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

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

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

431 Beyond the limitations of genome editing itself, PDO-based models also pose several technical and
432 practical challenges. One of the main issues is the complexity of handling and the cultivation costs.
433 Moreover, not every patient sample grows *in vitro*. In a large retrospective analysis, the overall
434 success rate in establishing CRC organoid cultures was 76.3% (939/1231). The efficacy of organoid
435 establishment depends on the type of starting material, with the highest rate observed in malignant
436 ascites obtained by abdominal puncture (96.4%, 27/28), followed by surgical resection specimens
437 (76.5%, 864/1130) and endoscopic biopsies (65.8%, 48/73) [166]. Another challenge in establishing
438 PDOs is preventing outgrowth or contamination by non-tumor cells [167]. Furthermore, slight shifts
439 in clonal composition, loss of tumor heterogeneity, and potential genetic drift can occur during
440 extended passaging or under selective culture conditions, potentially reducing their ability to
441 recapitulate patients' tumor characteristics [168].

442 **Ethical concerns.** There are some ethical concerns associated with the derivation, long-term
443 cultivation, and sharing of PDOs. Informed consent must be obtained from donors and must include
444 the potential long-term use, genetic characterization, and distribution of organoids between
445 laboratories. Particular care is required when PDOs are derived from donors with rare genotypes, as
446 the high scientific value of such samples may put implicit pressure on individuals to contribute their
447 cells. Further ethical concerns are associated with the ownership and commercialization of patient-
448 derived material, including potential patenting and profit-making [169, 170]. The application of
449 CRISPR/Cas9 will raise further ethical and safety concerns. While engineered PDOs offer
450 unprecedented opportunities for research and personalized medicine, their application must be
451 guided by strict ethical oversight. Genetically modified organoids offer unique opportunities for
452 disease modelling, but off-target effects and unintended genome alterations remain major concerns.
453 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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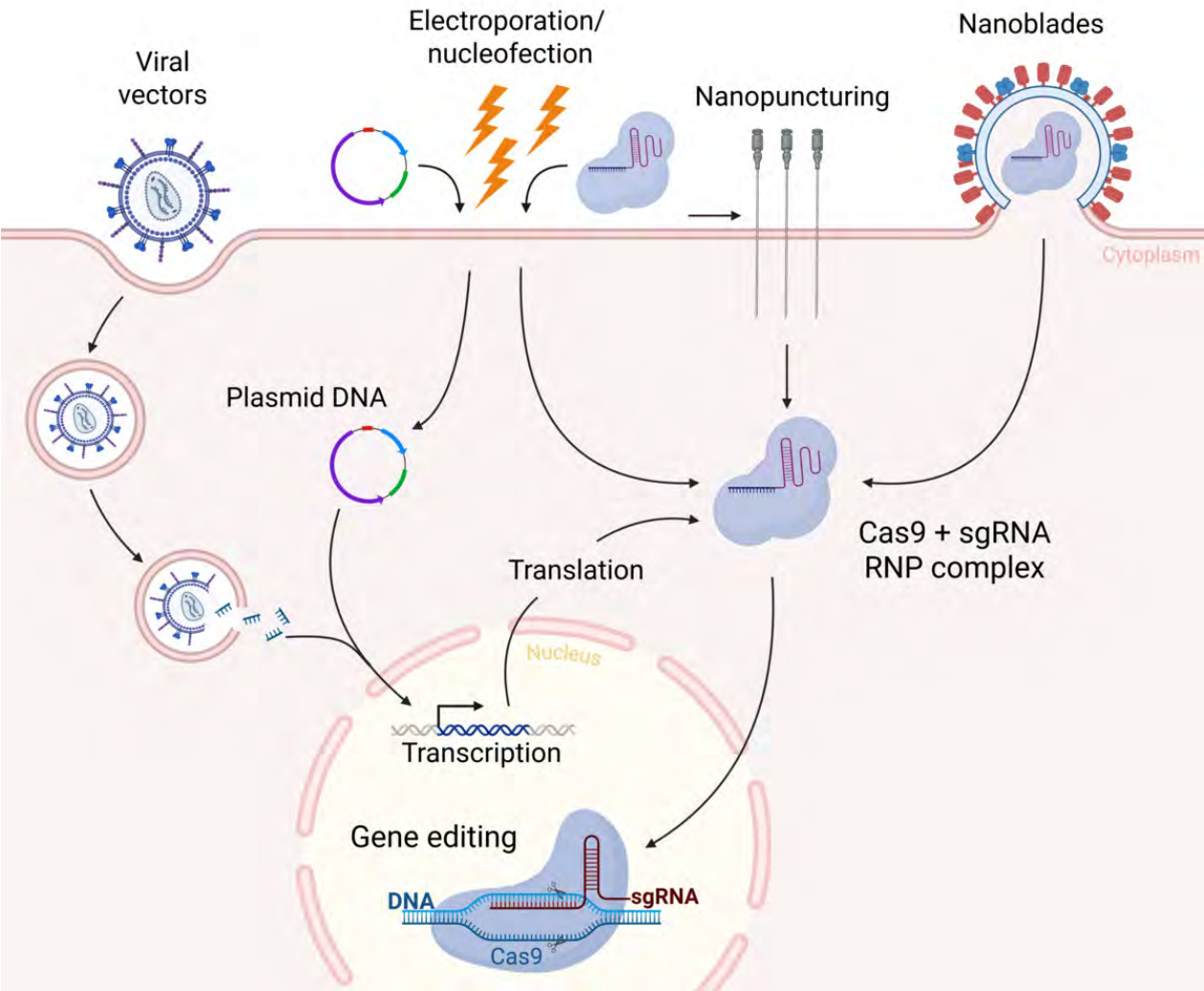


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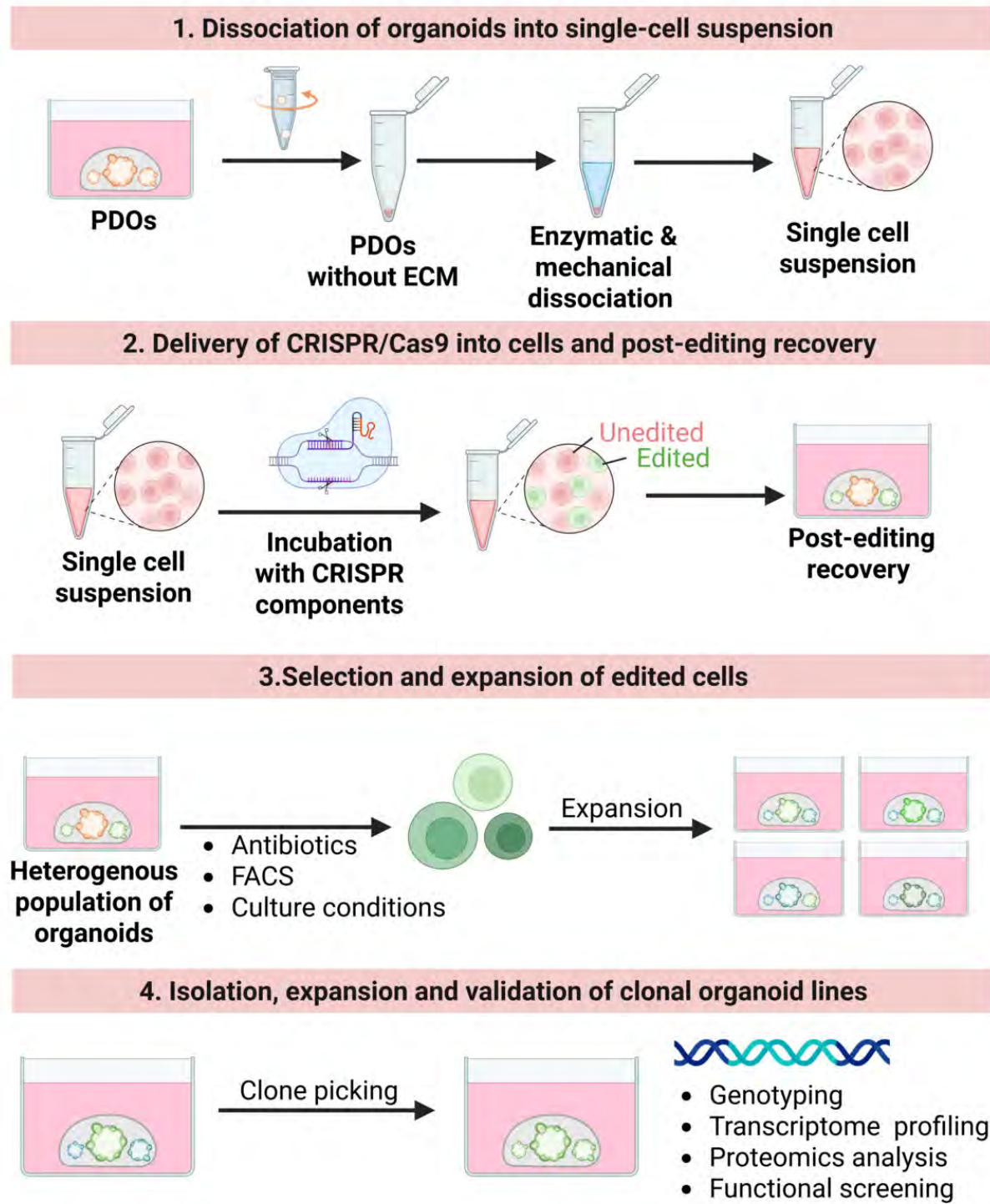


Fig. 3 [Download full resolution image](#)

