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Review

Intestinal multicellular organoids to study colorectal cancer

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ABSTRACT

Modeling colorectal cancer (CRC) using organoids has burgeoned in the last decade, providing enhanced *in vitro* models to study the development and possible treatment options for this type of cancer. In this review, we describe both normal and CRC intestinal organoid models and their utility in the cancer research field. Besides highlighting studies that develop epithelial CRC organoid models, *i.e.* organoids without tumor microenvironment (TME) cellular components, we emphasize on the need for TME in CRC modeling, to help reduce translational disparities in this area. Also, we discuss the utilization of CRC organoids derived from pluripotent stem cells, as well as their potential to be used in cancer research. Finally, limitations and challenges in the current CRC organoids field, are discussed.

1. Introduction

Even though CRC is one of the most studied cancer types, and several genome-wide studies have successfully identified the major driver genes for CRC [1], improving early detection and therapy is still needed to win the battle against one of the deadliest cancers worldwide [2]. This is partly due to the huge molecular diversity between and within colorectal cancers, which complicates the search for treatments. An important contribution to this tumor heterogeneity comes from the cells and components surrounding the tumor cells, the tumor microenvironment (TME) [3].

Cell lines have long been used to improve our understanding of CRC origin, as well as to study new treatment options for the disease. However, the translation to humans, *e.g.* drug response, is often limited, as cell lines do not represent the original tumor characteristics: i) cell lines lack the heterogeneity of clinical tumors [4], ii) cell lines are isolated from the microenvironment in which cancer originates and develops, and iii) 2D-cultured cells lose their polarity, and have equal access to various compounds in the medium in disparity with *in vivo* circumstances [5]. Although the use of animal models has overcome some of these limitations, the rate of failure of new cancer drugs in clinical trials remains very high [6], likely because animal models do not mirror the human physiology [7]. Alternatively, patient-derived xenografts in animal models have gained attention as they are usually accompanied with their TME, and better resemble the original tumor in growth, progression, and metastatic potential [8]. However, production of patient-derived xenografts is a time-consuming and expensive procedure, which may cause them to lose the race with the recently invented and rapidly enhanced human organoid models [9,10].

During the last ten years, improved intestinal organoid models have been built and used for different applications, including the study of different diseases such as, genetic diseases, inflammatory diseases, host pathogen interactions and CRC [13]. The invention of organoid technology has played a crucial role in understanding gastrointestinal cancer-related mechanisms as well as in improving treatment outcomes, as recently reviewed by Lau et al. [14] Even though the gut consists of different cell types, and the importance of the TME has been well established, most intestinal CRC organoid models developed so far consist only of epithelial cellular lineages [11,14]. Nonetheless, organoids are being complexified to mimic the gut microenvironment by incorporating different cellular elements, such as fibroblasts, immune cells, endothelial cells, and enteric neurons and glia, as well as other

Abbreviations: ALI, air-liquid interface; CRC, colorectal cancer; DCs, dendritic cells; ECM, extracellular matrix; ENS, enteric nervous system; FAP, familial adenomatous polyposis; ISCs, intestinal stem cells; NKs, natural killers; PSC, pluripotent stem cell; PSC-HCOs, PSC-derived colonic organoids; PSC-HIOs, PSC-derived intestinal organoids; TME, tumor microenvironment; vHIOs, vHCOs, vascularized HIOs or HCOs.

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Table 1
Terms currently used to describe organoids and our suggestion to use in this review.

	Currently used	The term used in this review
ISCs-derived epithelial organoids	Small intestine: • Enteroids • Human intestinal enteroids (hIE) • Small intestinal epithelial organoids (sIEOs) • Intestinal organoids (IOs) and human IOs (HIOs) Large intestine: • Colonoids • Colonic organoids (COs) and human CO (HCOs)	Small intestine: • Enteroids Large intestine: • Colonoids
PSCs-derived multicellular organoids	Small intestine: • Intestinal organoids (IOs) and human IOs (HIOs) Large intestine • Colonic organoids (COs) and human CO (HCOs)	Small intestine: • PSC-IOs and PSC-HIOs Large intestine • PSC-COs and PSC-HCOs
Tissue-derived multicellular organoids	• Organoids • Organoid units (OUs)	Tissue-derived multicellular organoids.

inherent internal factors of the intestine like, microbiota and peristalsis [12,15–22].

In this review, we summarize the different multicellular models of intestinal organoids and describe how this added complexity can be used to model CRC. Moreover, we discuss the potential of combining cancer organoids and pluripotent stem cells (PSCs) technologies to help bridge the aforementioned translational gap. Finally, limitations and challenges in the current CRC organoids field are discussed.

2. Normal intestinal organoids

Several definitions have been used to define organoids. A consensus defines organoids as long-term self-organizing and self-assembling 3D cell cultures that are grown *in vitro*. They mirror the original tissue both structurally and functionally, despite the material that they originate from: pluripotent stem cells(PSCs) or intestinal stem cells (ISCs) [23,24]. Therefore, the term 'intestinal organoids' includes three main categories: ISCs-derived epithelial organoids, PSCs-derived multicellular organoids, and tissue-derived multicellular organoids (Table 1, Fig. 1).

ISCs-derived intestinal organoids are budding, sphere-like structures also called enteroids or colonoids [25]. Enteroids and colonoids harbor the same types of cells that are usually present in the epithelium. In addition, depending on the culturing protocols, they largely preserve their regional identity, differentiation trajectories, gene expression, microRNA expression, epigenetic footprint, physiology, and pathophysiology [26–31]. This indicates their usefulness in modeling the intestine and shows their potential use in the clinic to regenerate the intestinal epithelium.

Enteroids and colonoids lack any stromal components which physiologically provide ligands and gradients of growth factors that preserve the stemness of ISCs in their niche [32]. Two more complex intestinal organoid systems, namely PSC-derived intestinal organoids and tissue derived multicellular organoids, have been developed that can include mesenchyme, enteric nervous cells, vasculature, and/or immune cells [15,20,33]. The presence of such components helps improve the representability of this system and allows a better study of interactions between epithelial and other cell types, not only in physiological settings but also during pathophysiological conditions.

On one hand, the group of C. Kuo developed a robust method using an air-liquid interface (ALI) setting for long-term growing of intestinal fragments *in vitro*. The resulting organoids contained epithelia, as well as other intestinal cell types, such as fibroblasts, immune cells, muscle fibers and enteric neurons and glia, while the collagen matrix mimicked the extracellular matrix (ECM) of the intestinal tissue [12] [.33,34]. However, these organoids cannot multiply as a whole, and serial passaging eventually leads to loss of stromal components [33]. In addition, mincing the tissue arbitrarily results in fragments that differ in size, composition ratios, and subsequently, growth rate, which may cause reproducibility issues.

On the other hand, PSCs have been used to generate intestinal organoids that harbor cell types that originated from different germ layers, and therefore, organize themselves in several layers, as they would do in the native intestine. Spence et al. guided PSCs through a developmental trajectory to form complex intestinal organoids [16] Later, two approaches have been applied to bias these organoids towards colonic fate (PSC-HCOs), by manipulating BMP and WNT signaling pathways [35,36]. The resulting organoids resembled a developing fetal gut with an epithelium layer, and mesenchymal cells [16,36]. PSC-derived organoids have since been further developed to include enteric nervous system components as well as endothelial cells [15,20,37]. Organoids are currently used for numerous applications to answer basic scientific questions and to study the developmental processes giving rise to tissues and organs. Moreover, it is a rapidly evolving field to study cancer disease mechanism, as they conserve the characteristic of primary cancers, enabling the screening of therapeutic drugs and improving personalized medicine. Up to date, several CRC models have been developed.

3. CRC organoids

3.1. Tumor colonoids

3.1.1. Primary tumor colonoids

As established for the healthy tissue, Sato et al. described the feasibility to grow colonoids from intestinal adenoma or adenocarcinoma tissue samples [25]. These tumor colonoids faithfully recapitulate the genetic diversity of the tumor of origin [38,39]. In addition, proteomic profiling showed similarities between tumor colonoids and the tumor they are derived from, reflecting CRC individual-specific features. Keeping in mind the heterogeneity of CRC and the continuous genetic changes, primary tumor colonoids can be implemented in personalized medicine [40]. The development of automated high-throughput platforms for drug screening paved the way to use tumor-colonoids for testing drug efficacy and toxicity [41], as well as for screening potential medications, replacing conventional *in vitro* cell lines and even primary patient-derived tumor xenografts [42,43]. Moreover, the ability to cryopreserve them and later retrieve organoids with high genetic fidelity has opened the door to establish CRC biobanks, making patient materials available for CRC research even when collected several years before [44].

Within the CRC organoid field, tumor-colonoids are the most common used organoid model to study CRC, as they are relatively easy to maintain compared to other organoid models. Due to increasing demand, CRC-colonoid lines have become commercially available. However, as tumor-colonoids are derived from established CRC tumors, they are not the most optimal model to study the first steps in carcinogenesis. In the next section, we describe two well-defined methods to develop tumor colonoids with specific CRC mutations, which are more suitable for such studies.

3.1.2. Genetically induced tumor colonoids

Instead of isolating cancer stem cells from established tumors,

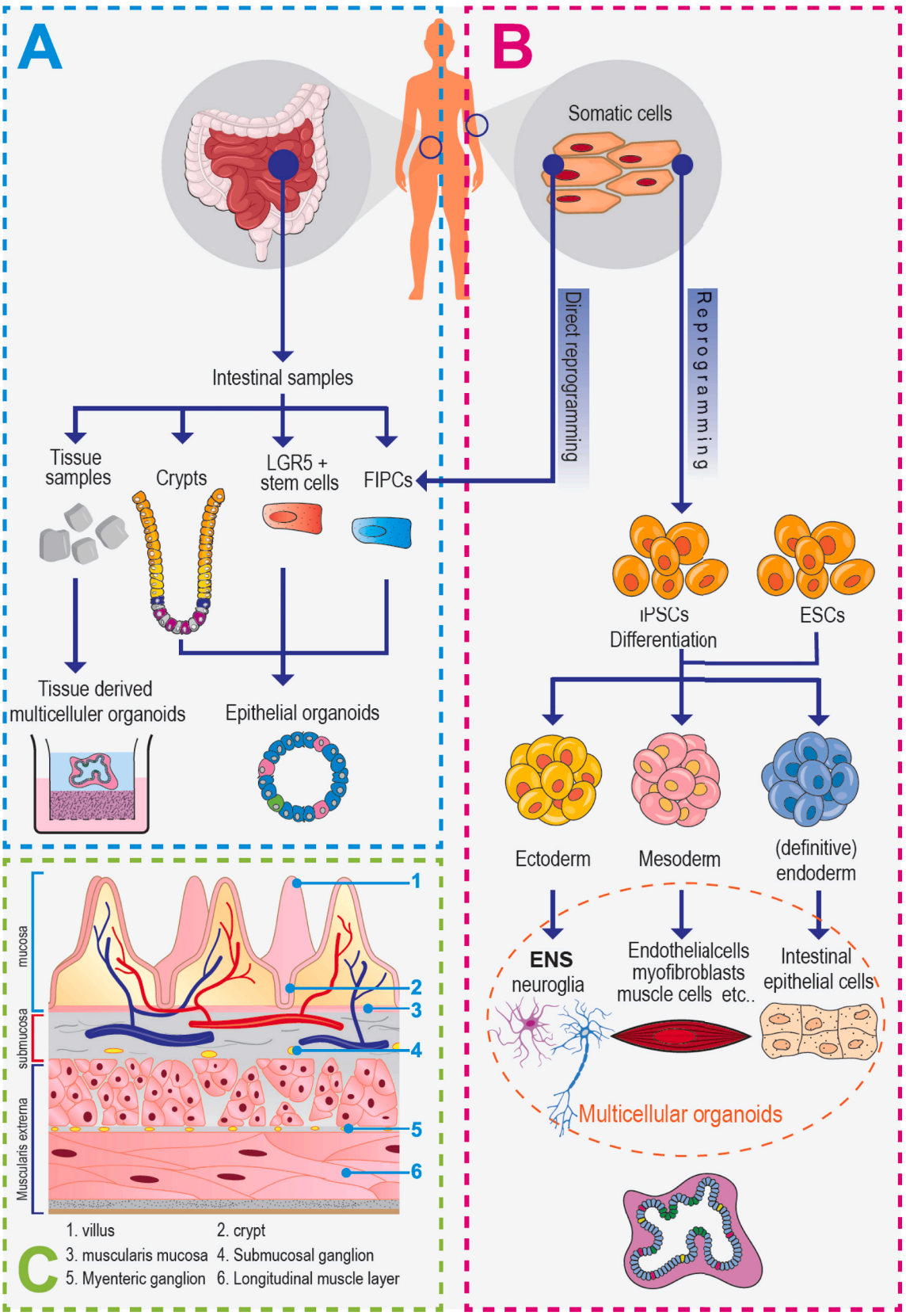

Fig. 1. Different types of intestinal organoids and their origin.
A. Enteroids and colonoids from fetal or adult intestinal tissue samples: LGR5+ cells and crypts containing LGR5+ cells as well as fetal intestinal progenitor cells (FIPCs) have the potential to generate enteroids or colonoids. In the meanwhile, fragments of intestinal tissue can be the starting material to develop tissue-derived multicellular organoids.
B. Somatic cells can be reprogrammed back to pluripotency. From these cells or from embryonic stem cells, PSC-organoids can be formed.
C. Scheme of the intestinal wall.

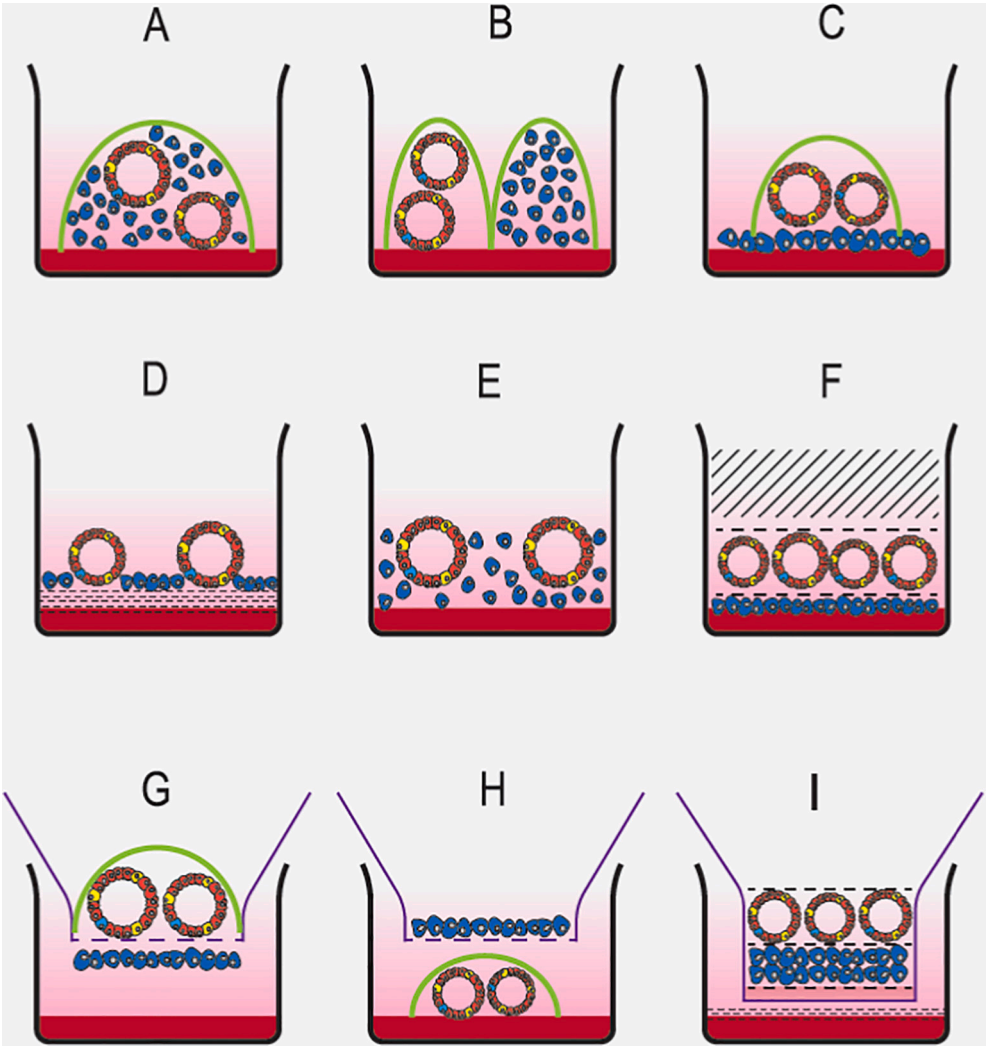

Fig. 2. various setups of co-culturing possibilities that are utilized with intestinal organoids.
A: Organoids and co-cultured cells are mixed in one ECM compartment [56].
B: Organoids and co-cultured cells are mixed in adjacent ECM compartments in the same vessel surrounded by the same medium [57].
C: Organoids reside in ECM while co-cultured cells attach to the plate surface [58].
D: Organoids and co-cultured cells are mixed and seeded on a thin layer of ECM [18].
E: Organoids and co-cultured cells are mixed in a suspension [18].
F: A layer of ECM containing organoids lays over another layer of ECM containing co-cultured cells [59].
G, H: Organoids reside in ECM either on an insert [57] or on the bottom [60] of the well, while the co-cultured cells occupy the other surface.
I: using ALI setup, a layer of co-cultured cells reside under a layer of ECM containing organoids [61].

genetic manipulation has been used to introduce mutations in cancer driver genes, either directly into organoids or indirectly by manipulating the germline of animal models. CRISPR/Cas9, viral transduction, and/or Cre technologies in the CRC field, have been implemented to mutate, silence, and/or induce gene-sets, specifically involved in four pathways: TGFβ1, WNT, P53, and RAS-RAF axis, which are frequently associated with various types of CRC.

Sakai et al. introduced combinatorial mutations into *APC, KRAS, TGFβR2, TRP53,* and *FBXW7* genes to create CRC mouse models, from which intestinal cancer organoids could be obtained [45]. Onuma et al., by contrast, sequentially silenced *TP53, APC, and PTEN* in cultured enteroids, in the first *in vitro* attempt to malignantly transform intestinal organoids. The resulting tumors were studied after transplantation into nude mice and showed the ability to induce tumor development in the absence of the intestinal microenvironment [46]. Later, the CRISPR/Cas9 technology was used to introduce mutations in *TP53, APC, KRAS, and SMAD4* in normal colonoids *in vitro*, which led to genomic instability and the accumulation of further *de novo* mutations, allowing clonal expansion to resemble the conventional adenoma to carcinoma CRC pathway [47,48]. Similarly, CRISPR/Cas9 was used to knock-out key DNA mismatch repair genes. The resulting colonoids had mutation profiles that accurately resembled those observed in DNA mismatch repair–deficient CRC [49].

Similar approaches were also used to develop models for the atypic, serrated pathway of CRC. $BRAF^{V600E}$ mutation was introduced in human colonoids by homologous recombination. The resulting colonoids responded to TGF-β1 treatment by undergoing epithelial-mesenchymal transition, whereas classical CRC colonoids underwent apoptosis [50]. Later in 2019, Tao et al. established proximal colon adenocarcinomas by only mutating *BRAF* and prolonged expansion, allowing the study of slow changes in DNA methylation during carcinogenesis, compared to senescence [51]. More recently, the lab of T. Sato managed to constitutively activate *RSPO2* expression using CRISPR/Cas9, by inducing chromosomal rearrangement in *TP53* knock-out colonoids. The subsequent engineering of *BRAF* and *GREM1* activating mutations resulted in colonoids that mimic traditional serrated adenoma, a precursor of CRC. Implanting these colonoids in immune-deficient mice, allowed the formation of tumors that macroscopically and histologically resemble traditional serrated adenomas [52].

By combining the two aforementioned approaches, Lannagan et al. introduced a conditional *BRAF* mutation into the mice germline to obtain a pre-model mouse for serrated CRC, from which intestinal organoids were grown. After using CRISPR/Cas9 *in vitro,* they sequentially introduced mutations into *TGFβR2*, *RNF43/ZNRF3*, *p16INK4a,* and *MLH1* genes, known to contribute to serrated CRC development and microsatellite instability. They aimed to define the responsible contributors in regulating the stem cell niche and senescence, during this type of CRC [53]. In parallel, Melo et al. grew enteroids from genetically modified mice with *APC* and *KRAS* mutations, in their intestinal epithelium. In this case, they used CRISPR/Cas9 to silence *TRP53* and *SMAD4* in the resulting enteroids, which were later transplanted into wild-type mice. Depleting LGR5+ cancer stem cells, by specifically

expressing diphtheria toxin, restricted the growth of enteroids and the metastatic burden in the liver, but failed to eradicate the tumor, highlighting the importance of LGR5+ cells in carcinogenesis and metastasis, as well as their potential in cancer therapy [54].

All these studies show that tumor colonoids indeed hold great promise for cancer research, and personalized medicine. However, the absence of the TME in these models could influence the outcomes when using them and must be considered when interpreting results. To this end, many efforts have been made to build more complex multicellular organoids, better recapitulating the tumor microenvironment.

3.1.3. Tumor colonoids co-cultures

Organoids are proven to be a flexible model with a huge possibility for manipulation and development. Depending on the research question, one or more types of cells can be grown in the culture dish in the vicinity of the tumor organoids, as in the physiological setting. Although considered a simplistic model, co-culturing improves the complexity of the *in vitro* model. It also enables the study of possible cellular interactions with organoids, which can be 1) mediated by direct contact between cells or 2) soluble communication.

Some co-culturing setups contain only one compartment where all cells in the culture have a direct cell-to-cell contact (*i.e.*, conventional mixture of cells or setups that allow cells to migrate or develop direct contact through side-processes). Other setups contain two or more compartments where organoids can only communicate by secretion of soluble factors, with other co-cultured cells (*e.g.*, Transwell inserts). Also, intestinal co-culture approaches are further under development using microfluidic technologies, to comply with the requirements of high-throughput assays [55]. Different co-culture setups utilized in intestinal organoids are depicted in Fig. 2. Tumor colonoids have also been utilized to study the effect of TME components in CRC, by establishing co-cultures with immune cells, fibroblasts, or adipocytes.

Immune cells are the most extensively studied members of the TME that contribute to colorectal carcinogenesis. Tumors are inflammation sites where different types of immune cells are recruited. Not only tumor-associated macrophages, but also dendritic cells (DCs), mast cells, monocytes, neutrophils, myeloid-derived suppressor cells, T-cells, and natural killers (NKs) are known to play a part in the promotion of malignant lesions [62]. Few studies have generated organoid co-cultures with immune cells, to study the role of these cells and their potential use as immune therapies in CRC. Knowing that cancer therapies based on cytolytic T or NK cells are getting more attention, they can greatly benefit from organoid technology to test them in personalized medicine. In 2019, Schnalzger et al. applied three different setups to co-culture tumor colonoids with chimeric antigen receptors-engineered NKs. These cells were primed against the cancer neoantigen EGFRvIII or the WNT-receptor FRIZZLED, to target tumor cells in a specific and efficient manner. For this purpose, cells and organoids were grown together 1) in a suspension, 2) on a Matrigel coating layer, or 3) the organoids were embedded in a Matrigel and separated from NKs, which were added to the medium. NKs failed to show cell-lysis function in suspension co-cultures or in the separated setup. Only in the presence of the extracellular matrix (Matrigel) and in direct contact with colonoids, cell-lysis was evident. In cytotoxicity assays, the bottom of the wells also contained fibroblasts to test targeting-specificity against tumor cells [18]. Recently, Dijkstra et al. published a protocol to produce patient-derived tumor-activated T cells, by co-culturing CRC colonoids with human peripheral blood mononuclear cells for 2 weeks, in a T-cell enriching culture medium. The T-cells obtained (~90%) could be tested for tumor-killing activity and introduced again in the patient to help eradicate the tumor [19]. These studies and their outcomes emphasize the capacity of combining colonoids with immune effectors, promoting immunotherapies for CRC.

Fibroblasts regulate intestinal homeostasis by controlling the rhythm of cell division in the epithelial layer [63]. In CRC, fibroblasts transform phenotypically and are known as cancer-associated fibroblasts. These cells support the development of cancer through the secretion of many signals that stimulate cellular stemness, promoting proliferation, transition, and invasion, while suppressing the immune response and promoting resistance to chemical drugs [63]. Chen et al. used a decellularized human colon as a natural ECM to grow human colonic epithelial cells, myofibroblasts, and microvascular endothelial cells together. The colonic epithelial cells were *APC*-silenced and *KRAS* constitutively activated, while the TGFβ pathway was pharmacologically inhibited. This resulted in the formation of large adenomas that invaded the submucosa within four weeks. This model was used in combination with the bi-functional Sleeping Beauty transposon mutagenesis, to randomly activate or deactivate genes that promote or inhibit tumor invasion. This screening for effector genes identified 21 novel genes that may contribute to CRC pathogenesis [64].

Finally, adipose tissue contributes to the homeostasis of the intestinal tract. However, during colorectal carcinogenesis, adipocytes secrete factors that dysregulate the inflammatory and angiogenic response systems [65]. Wen et al. co-cultured *APC*- and *KRAS*-mutated murine intestinal organoids together with human adipocytes in Matrigel domes, which increased proliferation and dedifferentiation of tumor cells, as well as enhanced aggressiveness, was observed [59].

Even though the use of co-culture systems helped to answer many questions regarding the role of TME in CRC, some concerns have been described using these models. First, the setup used in co-culture experiments can affect the behavior of cells and organoids [57]. Ihara et al. compared three different co-culture setups for enteroids and DCs: 1) a direct co-culture model where DCs and enteroids are grown together in the same domes of Matrigel, 2) a Transwell model where enteroids are grown in Matrigel on filter-bottomed inserts located above a 2D culture of DCs, and 3) a separated model where enteroids thrive in a dome of Matrigel separated from an adjacent Matrigel dome where DCs grow [57]. Enteroids behavior and morphology from the direct co-culture model were different (large and cyst-like) from those cultured on Transwell or in the model with separated domes of a hydrogel, showing the importance of direct adhesion in intestinal models [57]. Second, co-culturing cells should be done in similar ratios to the *in vivo* environment [66]. Also, missing cells and tissue architecture can have an impact on the conclusions derived from these models. Therefore, more sophisticated models such as tissue-derived and PSCs-derived organoid models with native arrangement and proportions would help to overcome some of these obstacles.

3.2. Tissue-derived multicellular intestinal tumor organoids

Patient-derived CRC tissue can be minced into small fragments and grown in ALI setup, to obtain multilayered cancerous organoids. As these organoids are fragments of the whole tumor, they preserve the original epithelial and mesenchymal structures, as well as the accompanied neurons, glia, and endothelial cells. In addition, these organoids contained immune elements such as T-cells, B-cells and macrophages which better survived after 30 days of cultivation if IL-2 was added to the culture medium. Remarkably, these organoids accurately preserved the T-cell receptor repertoire of the original tumor, which allowed Neal et al. to study the toxicity of tumor-antigen-specific lymphocytes against tumor cells, following pharmacological inhibition of lymphocytic apoptosis [33]. Considering that these organoids possess both the tumor and its microenvironment, including their load of immune cells, they are potentially suitable for personalized therapy prediction, especially after resection surgery.

Similar to what we described in the colonoids part, wild-type tissue-derived multicellular organoids can be genetically engineered *in vitro* by introducing mutations in cancer-driving genes. Alternatively, the germline of animal models can be engineered by introducing inducible mutations in known CRC genes, that can be triggered after the establishment of the organoids culture. Li et al. have sequentially introduced CRC driving mutations in their organoids according to the adenoma-to-

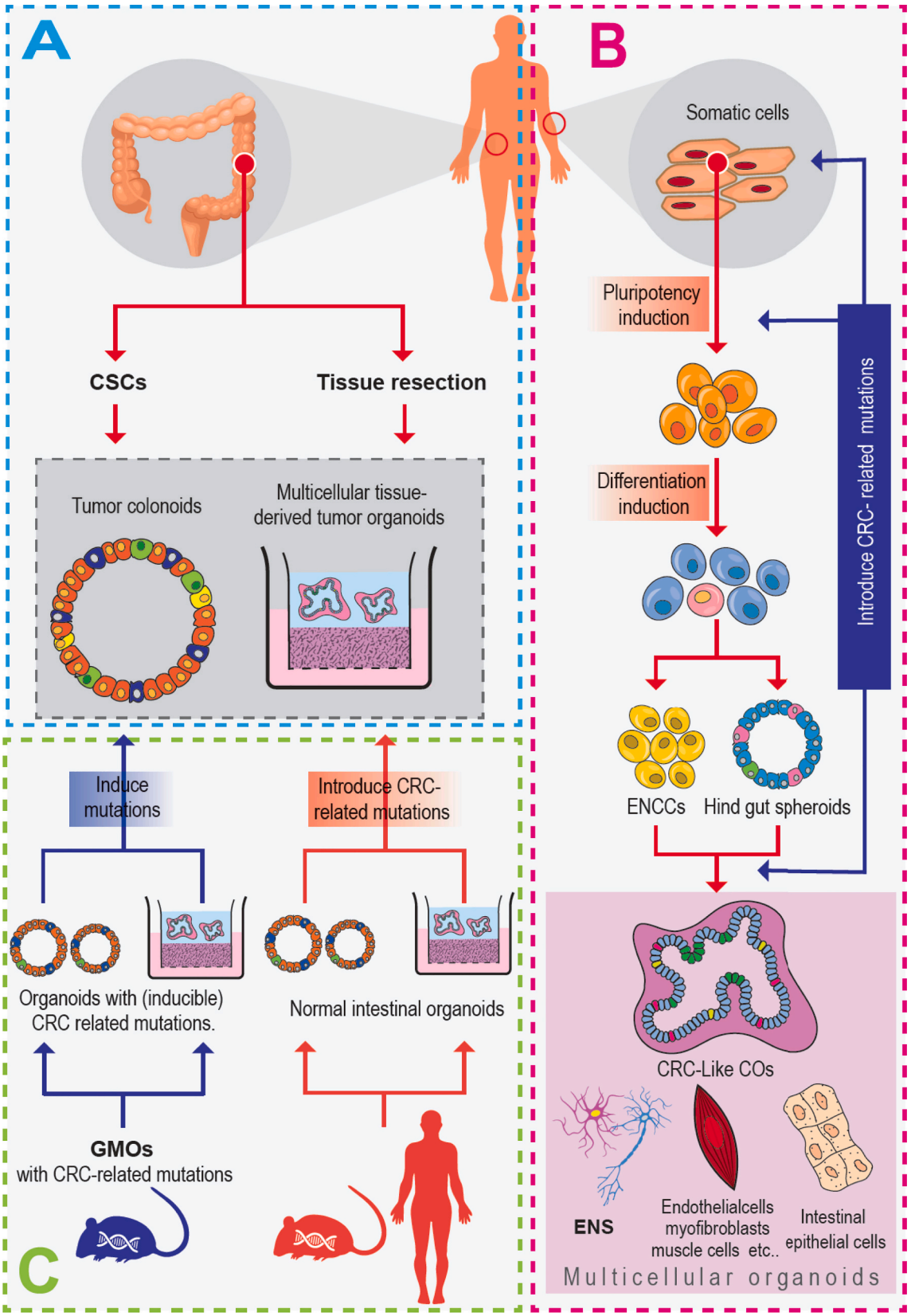

Fig. 3. methods to produce CRC organoids.
A. Tumor biopsies or resection material can be used to isolate cancer stem cells to start CRC-colonoids, while fragments of the tumor can be used to propagate multicellular tissue-derived CRC organoids.
B. CRC-related (inducible) mutations can be introduced to the organoid model either to the somatic cells before reprogramming, to PSCs or to the organoids after being established.
C. CRC-related (inducible) mutations can be introduced into the germline of animal models, or to the established organoids.

adenocarcinoma multi-hit theory. Firstly, they created murine *APC*$^{flox/flox}$*; villin-CreER organoids*. Subsequently, they silenced *TRP53* and *SMAD4*, and introduced a continuously active *KRAS* gene copy, using ecotropic retroviruses. The quadruple mutant organoids obtained showed excessive proliferation and histologically resembled invasive CRC tumors. They also compared mutant organoids from small and large intestinal segments, showing that small intestinal murine organoids are more prone to carcinogenesis compared to the colonic ones [67]. In parallel, Rosenbluh et al. grew fragments of *APC*$^{flox/flox}$; villin-CreER mice organoids in ALI setting, as an inducible model for adenomas and to study the efficacy of Dasatinib, a Src kinase inhibitor used in the treatment of several malignancies [68].

3.3. PSC-derived tumor organoids

Even though PSC-derived CRC organoids are an attractive tool to study TME, as they can include major cell types important for tumor development and progression, there are only a few models developed in this field. In principle, it is possible to reprogram CRC stem cells to become pluripotent [69]. However, generating intestinal organoids from these PSCs would be challenging, as cancer genetic and epigenetic abnormalities might interfere with the differentiation trajectories. On the other hand, traditional bioengineering of PSCs using CRISPR/Cas9 or the transcription activator-like effector nucleases (TALENs) to obtain inducible epithelial-specific mutations (*e.g. APC*, *KRAS*, *SMAD,* and *P53*), faces major obstacles, as PSCs are fragile cells that are not easily manipulated [70]. Moreover, it has to be recognized that HIOs are considered fetal-like tissues, and researchers are still trying to improve protocols to obtain more mature HIOs [71].

By using PSCs derived from patients with familial adenomatous polyposis (FAP), Crespo et al. developed hyper-proliferative HCOs [35]. Patients with FAP carry a hereditary defect in one copy of the *APC* gene, which is also typically mutated in CRC, and develop CRC lesions around their fourth decade of life [72]. By creating FAP-PSC-HCOs, Crespo et al. showed the similarities between FAP-PSC-HCOs and FAP-derived adenomas compared to the normal colonic mucosa, on the transcriptional level, and proliferation capacity. They further used this adenoma model to test potential anticancer drugs on the hyper-proliferating epithelium.

Sommer et al. also produced PSCs with a truncated copy of the *APC* gene, using TALENs. These cells were used to develop HIOs, which showed no enhanced proliferation compared to their wild-type counterparts. This could be attributed to the fact that both mutant and wild-type HIOs were cultured in the presence of R-SPO1, which could mask the effect of the *APC* mutation in regard to proliferation capacity [73]. However, the truncated *APC* copy induced notable changes in cellular behavior, motility, and polarity, as well as in genetic features, such as chromosomal instability and tumorigenesis. Therefore, *APC* mutant models can be considered as a starting point for modeling CRC using PSC-HCOs, but more advanced tumors are yet to be modeled in the future. The development of these organoids is summarized in Fig. 3.

4. Current limitations and challenges in the field of multicellular CRC organoids

Intestinal organoids have the potential to improve the study of CRC both in cancer development and progression and drug prediction. Even though a great promise has been portrayed using tumor organoids as preclinical models for cancer research compared to *e.g. in vivo* models, different shortcomings remain. It is recognized that carcinogenesis occurs due to a bidirectional communication of tumor cells and all members of the TME, including the tumor extracellular matrix. Efforts have been made to mimic the TME by adding immune cells, fibroblasts, adipocytes, and neuronal cells to the culture. However, even consisting of several members of the tumor microenvironment, the cell types and also the matrix always had to be previously defined, thereby missing a holistic view of the *in vivo* situation. Moreover, future research is necessary to fully understand the differentiation state and contributions of the different cell types generated within these organoids. On the other hand organoids lack biological cues and processes provided by other organs in a living organism. For example, many CRC treatments currently used in the clinic are pro-drugs (*e.g.* Capecitabine) and need to be metabolized in the liver and the intestine, to become active [74]. Moreover, even though some studies describe the use of matching intestinal organoids from healthy tissue to investigate individual toxic margins, (liver) toxicity is still a great concern in cancer chemotherapy [75]. Combining normal liver and intestinal organoids with CRC organoids derived from patients in one organoid-on-the-chip system, could provide better insights into the kinetics, efficacy, and toxicity of such therapies. In addition, it could account for inter-individual variability, a concept that is tested with pancreatic cancer organoids [76] and that can be equally transferred to the CRC organoids field.

The tumor vasculature also plays a critical role in the TME, where angiogenesis and tumor hypoxia are important determinants of response to therapy [77]. In the meanwhile, the role of the enteric nervous system (ENS) in CRC progression and metastasis is recently being studied. We speculate that combining tumor PSC-HCOs, ENS + PSC-HCOs, and the recently developed PSC-vHIOs and vHCOs would provide an innervated vascularized tumor HCOs that could better mimic the microenvironment of CRC.

5. Conclusion

Intestinal organoids range from simple epithelial enteroids and colonoids, to multicellular structures that incorporate other stromal cells. Colonoids are relatively easy to handle and do not need special training. Patient-derived CRC-colonoids biobanks are paving the way for development in drug discovery and precision medicine. However, the use of multicellular intestinal tumor organoids to investigate CRC is still in its infancy and needs more attention before it can be used in personalized medicine. Even though tumor colonoids can be co-cultured with other TME cellular components to upgrade their complexity, PSC-derived COs and tissue-derived multicellular organoids can provide an alternative that inherently contains several TME components. Lastly, all types of colonic organoids are valuable tools for researchers studying CRC. Not only do they offer better representability and enhanced translatability, but they also recapitulate the major aspects of tumor initiation and progression.

Contributors

All authors contributed in the content of, and read the manuscript.

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Declaration of Competing Interest

None.

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