INTRODUCTION

Carcinoma arises from epithelial cells through accumulation of genetic aberrations and the tissue-specific microenvironment could further promote this process. More than 3 decades ago, rats served as a major animal model of human carcinogenesis, particularly for those types of carcinogenesis induced by chemical carcinogens. For example, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), a food-borne carcinogen in well-cooked meat, induced tumor development in colon, prostate and mammary glands in rats, which was significantly promoted by a high-fat diet, while it preferentially induced lymphoma in mice. Considering the sharp increase in deaths from these types of cancers, coinciding with the Westernization of diet in Japan, PhIP-induced carcinogenesis models in rats, indeed, phenocopied many aspects of the pathogenesis of the human disease.

Ever since genetically engineered mice (GEM) became available as a result of gene-targeting technology, mice have been the gold standard in modeling cancer. GEM with conditional alleles could also be generated by using the Cre-loxP system to achieve recombination in a tempo-spatially controlled manner, paving the way for reconstitution of common genetic aberrations in organoids resulted in development of various lesions, ranging from aberrant crypt foci to full-blown cancer, recapitulating multi-step colorectal tumorigenesis. Due to its simplicity and utility, similar organoid-based approaches have been applied to both murine and human cells in many investigations, to gain mechanistic insight into tumorigenesis, to validate putative tumor suppressor genes or oncogenes, and to establish preclinical models for drug discovery. In this review article, we provide a multifaceted overview of these types of approaches that will likely accelerate and advance research on colon cancer.

KEYWORDS
Apc, colon cancer, lentivirus, model, organoid
for organ-specific or adult-onset carcinogenesis studies. Despite these benefits, critical drawbacks of GEM might include that its generation could take a long time and be laborious. This is particularly true if conditional gene targeting and multiple intercrossing are required. In addition, modifier genes could become problematic when intercrossing between different strains. Indeed, cooperation between Apc inactivation and Trp53 loss towards intestinal tumorigenesis became evident only in the C57BL/6J background after backcrossing for many generations.

Matrigel-based organoid culture has recently emerged as a technique that essentially recapitulates tissue homeostasis in vitro exclusively with epithelial cells. It is likely that laminin, abundantly contained in Matrigel, and defined factors can reconstitute the intestinal stem cell niche. Both self-renewal and differentiation of stem cells are maintained, thereby enabling infinite proliferation of stem cells without forced immortalization or transformation. By using this technique, we demonstrated that transformation of murine normal intestinal cells was feasible, through in vitro gene transduction and inoculation in nude mice. Importantly, induced carcinogenesis was highly concordant with earlier studies using GEM-based in vivo models. Due to its simple and rapid nature, genetic engineering of organoids might be established as a next generation model of carcinogenesis. In this article, we illustrate the technical basics of this new approach, and review studies by others with similar techniques.

2 | CONVENTIONAL MOUSE MODELS FOR INTESTINAL TUMOR DEVELOPMENT

In human colorectal cancer (CRC), mutations in the Adenomatous polyposis coli (APC) gene and the CTNNB1 gene encoding β-catenin are found in > 80% and about 10%, respectively. Both mutations result in nuclear accumulation of β-catenin, which cooperates with TCF4 to establish constitutive transcriptional activation of the Wnt pathway. Reflecting its critical role in tumor initiation, mouse models for intestinal carcinogenesis are based on the genetic aberrations in either gene. For any candidate gene related to CRC, GEM are usually generated first and intercrossed with these models to evaluate the impact on tumor progression by examining the changes in the incidence, size, multiplicity and histology of the tumors (Figure 1A).

The APC gene is typically inactivated by truncating mutations. ApcMin/+ mice carrying the truncated allele were generated through N-ethyl-N-nitosourea (ENU)-based mutagenesis. While homozygous ablation of Apc results in embryonic lethality, heterozygously mutant mice spontaneously develop numerous adenomatous polyps after the second hit in Apc resembling CRC-prone hereditary syndrome familial adenomatous polyposis (FAP), except that tumors were predominantly observed in the small intestine. Mice with a conditional allele of mutant Apc (eg, Apcflox/+ intercrossed with villin-Cre mice) also developed polyps preferentially in the small intestine. These observations strongly suggest that small intestinal cells are more susceptible to transformation by Apc inactivation than colonic cells in mice. To develop cancer in the colon, Apcflox/+ mice were intercrossed with transgenic mice that preferentially expressed Cre in the large intestine, such as Cdx2-Cre or CAC-Cre, or an adenovirus encoding Cre (adeno-Cre) was directly infused into the rectum of Apcflox/flox. Colon carcinogenesis can be alternatively initiated by treatment with the carcinogen azoxymethane (AOM), which efficiently introduces an activating mutation in the Ctnnb1 gene in the murine colon, and promoted by dextran sulphate sodium (DSS) to induce colitis.

3 | AN ORGANOID-BASED CARCINOGENESIS MODEL FOR MURINE INTESTINE

The NIH3T3-based transformation assay in nude mice (Figure 1B) has contributed to the validation of oncogenic potential of many genes. Given the development of the organoid culture for murine small intestinal cells, we reasoned that similar approaches with epithelial cells might become feasible. As a proof-of-concept experiment, knockdown of Apc and other tumor suppressor genes were achieved by lentiviral delivery of corresponding short-hairpin RNA (shRNA), either alone or in combination, into primary intestinal organoids from wildtype mice with the C57BL/6J background (Figure 1C). Transduced organoids were inoculated in the subcutis of nude mice and monitored for tumor development. We here illustrate the key technical features of this model.
stem-like cell populations on Matrigel, without conducting cell sorting by stem cell markers.

3.2 Re-defining organoid-derived tumors in the subcutis of nude mice

As readouts for the tumorigenicity in mice models, it is common to take the incidence, multiplicity, size and histology of the tumors into account. To evaluate the results from organoid-derived carcinogenesis models, however, it was necessary to newly determine criteria for the diagnosis of “tumors.” Transduced intestinal organoids mixed with Matrigel formed subcutaneous nodules in nude mice, which fell into 4 categories based on their macroscopic and microscopic features. The former 2 were regarded as non-tumors and the latter 2 as tumors (Figure 3).

3.2.1 Matrigel plug

Unless Apc is silenced in inoculated organoids, no nodule can be detected, yet flat white semi-transparent materials occasionally remained in the injection sites. Microscopically, no epithelial cells were observed, while fibroblasts, immune cells or calcified remains of organoids could be detected. These materials without viable epithelial glands are defined as Matrigel plugs.

3.2.2 Non-tumorous nodule

Matrigel plug-like nodules could contain a tiny population of epithelial glands. Histologically, a few non-dysplastic tubular glands are lined up in a monolayer. Despite the presence of epithelial glands in the subcutis, these lesions were classified as non-tumorous nodules, as bona fide tumors would have potently outgrown Matrigel. No viable organoids were, in fact, recovered from these nodules, denying the presence of tumor-initiating cells. It is possible that organoids acquired a growth advantage as a result of random genomic integration of lentiviral vectors.

Organoids expressing KrasG12D can be generated by delivery of Cre into organoids from conditional knock-in mice heterozygous for the LSL-KrasG12D allele. Upon inoculation, a few enlarged glands could be transiently formed in non-tumorous nodules, but eventually...
disappeared to form Matrigel plugs, consistent with the low tumorigenic potential of Kras alone in vivo. Similar histology and disappearance have been observed in chemically induced aberrant crypt foci (ACF), putative early lesions in colon carcinogenesis. Whereas dysplastic ACF represent a pre-neoplastic lesion, hyperplastic ACF are associated with Kras mutation but do not progress into tumors. Thus, hyperplastic ACF might also be present, at least temporarily, in non-tumorous nodules.

3.2.3 | Solid tumor

A solid tumor is defined as a palpable round-shaped nodule. It typically has a similar color to white muscle, and consists of tubular glands accompanied by stromal infiltration. Co-injected Matrigel is no longer observed, indicating that the tumor outgrew and substituted Matrigel with newly developed tumor stroma. In approximately 70% of the cases tested, organoids transduced with shRNA against Apc (shApc) developed small but solid tumors over several weeks, while no tumor developed without Apc knockdown. With the co-introduction of shPten or shTrp53, the development rate for solid tumors reached 100% and the size became larger, consistent with earlier in vivo studies. Interestingly, shApc-driven tumors contained prominent mucus pools in the stroma, due to the intratumoral disruption of glands. Similarly, simultaneous augmentation of proliferation and apoptosis has been observed upon acute inactivation of Apc in the murine intestine. As it proved to be mediated by the major Wnt-target gene myc, these observations might be a reflection of myc’s dual roles.

3.2.4 | Large solid tumor with cysts

LSL-KrasG12D organoids co-infected with lentiviruses encoding Cre and shApc rapidly gave rise to significantly large tumors with both cystic and solid components, consistent with a strong synergy between Kras mutation and Apc inactivation in vivo. Reflecting active angiogenesis, the surface of these tumors tended to be red and contained a huge amount of serous or hematogenous fluid inside cysts. Histologically, tumor glands were densely packed, while disrupted glands were rarely observed, suggestive of anti-apoptotic effects by Kras. Our experience in organoid-based tumorigenesis for the lung, pancreas (Matsuura et al, submitted) and hepatobiliary tract (Ochiai et al, submitted) suggests that emergence of a
cystic component is closely associated with \( \text{Kras}^{G12D} \). Accordingly, future classification of the nodules might as well include "cyst" in the category of non-tumorous nodules.

4 | OTHER ORGANOID-BASED CARCINOGENESIS MODELS FOR THE INTESTINE AND COLON

Following our previous study, \(^{17}\) several studies reported modeling colon carcinogenesis with similar approaches. \(^{56-60}\) Although based on analogous concepts, they varied in many respects, including the methods for cell culture, genetic engineering and inoculation, as well as which organs or species were used. These studies are listed in Table 1.

| Organoid-based carcinogenesis | Normal | Aberrant crypt foci | Adenoma | Adenocarcinoma |
|-------------------------------|--------|---------------------|---------|---------------|
| Control                       |        |                     |         |               |
| \( \text{Kras}^{G12D^*} \)    |        |                     |         |               |
| \( \text{shPten} \)           |        |                     |         |               |
| \( \text{shApc} \)            |        |                     |         |               |
| \( \text{shApc + Kras}^{G12D^*} \) |        |                     |         |               |
| \( \text{shApc + shPten} \)   |        |                     |         |               |
| \( \text{shApc + shTrp53} \)  |        |                     |         |               |
| Corresponding in vivo lesions |        |                     |         |               |

4.1 | Liquid-air interface culture-based heterotopic carcinogenesis in nude mice

Minced tissue fragments were embedded in collagen gel, set in a culture insert, and exposed to serum-containing media on the bottom and to free air on the top. \(^{61}\) This setting, referred to as liquid-air interface culture, enables the generation of gradients in concentration of nutrients and oxygen, regenerating tissue structure in a configuration close to the intestinal mucosa. As both epithelial cells and stromal cells are maintained in culture, histological analysis can be directly conducted. Gene transduction was basically conducted in an inducible manner with tamoxifen or acute introduction of Cre. Notably, delivery of Cre worked efficiently for adenovirus, but not for retrovirus, through brief exposure (approximately 30 minutes) to dissociated cells. Then, direct microinjection of the viral particles encoding shRNA or cDNA into the organoids was conducted, which successfully validated the pro-tumorigenic effects by 11p15.5 amplicon in Apc-null colon organoids. \(^{56}\)

4.2 | Colon organoid-based orthotopic carcinogenesis models in severe immunodeficient or syngenic mice

Colon organoids isolated from GEM harboring doxycycline (Dox)-inducible shApc were treated with Dox to knockdown Apc. To generate a niche for engraftment in the colon of host mice, mucosal damage had been induced in advance through oral administration of DSS. This procedure enabled development of benign adenomas in nude mice by Apc knockdown alone. \(^{60}\) When further interbred with \( \text{Kras}^{G12D^+} ; \text{Trp53}^{f/f} \) mice, their colon organoids, infected with adenovirus-Cre and treated with Dox to achieve triple mutations, gave rise to invasive carcinoma. Intriguingly, similar results were obtained for orthotopic inoculation in syngenic C57BL/6J mice, demonstrating significant augmentation of tumor engraftment by DSS. Moreover, metastases to the liver and the lung were also observed following injection to the spleen and tail vein, respectively. \(^{60}\) In another study, organoids derived from colon adenoma in Apc\text{min/+}; \( \text{Kras}^{G12D^+} \) mice were subject to further deletion of Trp53 and Smad4 through genome editing with CRISPR/Cas9. Orthotopic inoculation of these quadruple mutant organoids in severe immunodeficient NSG/NOG mice resulted in adenocarcinoma development with

![FIGURE 3](attachment:image-url) Classification of subcutaneous nodules developed in nude mice. Tumors and non-tumors developed in the subcutis of nude mice are classified based on their macroscopic and microscopic features. The results from organoid-based carcinogenesis in murine intestinal cells (Ref. 17) are mapped in the lower row.
## Table 1: A list of representative studies on modeling colon carcinogenesis using organoid-based approaches

| Organoids | Species | Organ | Type | 3-D Culture | Genetic alterations | Transduction | shRNA/cDNA/Gene editing | Inoculation | Mouse | Site | Diagnosis | Reference |
|-----------|---------|-------|------|-------------|--------------------|--------------|------------------------|-------------|-------|------|-----------|-----------|
| Mouse SI | Normal  | Matrigel (bilayer) | WT | Kras<sup>G12D</sup> | Lentivirus | shApc, shTrp53, shPten | Nude | Subcutis | Adenoma-adenocarcinoma | Onuma et al<sup>17</sup> |
| Mouse SI | Normal  | Collagen (air-liquid interface) | Apc<sup>lox/lox</sup>; Villin-Cre<sup>Cre</sup> | Tamoxifen + retrovirus | shTrp53, shSmad4, Kras<sup>25,20</sup> | (in vitro) | | Tubular adenomatous poly-p-adenocarcinoma | Li et al<sup>20</sup> |
| Human SI | Normal  | Matrigel (dome) | WT | | CRISPR/Cas9 (lipofection) | NOG | Subcutis | Adenoma | Drost et al<sup>7</sup> |
| Human LI | Normal  | Matrigel (dome) | WT | | CRISPR/Cas9 (electroporation) | APC, TP53, Kras<sup>G12D</sup> | C57BL/6J | Kidney subcapsule | Adenocarcinoma | Matano et al<sup>50</sup> |
| Human LI | Normal  | Matrigel (dome) | shApc | CRISPR/Cas9 (electroporation) | APC, TP53, Kras<sup>G12V</sup>, SMAD4, PIK3CA<sup>E545K</sup> | | | None | Adenocarcinoma with liver metastasis | De Sousa e Melo et al<sup>59</sup> |
| Mouse LI | Normal  | Matrigel (dome) | shApc | D Roxycycline + adenovirus | C57BL/6J | Colon (+DSS) | Benign tubular adenomas | O’Rourke et al<sup>61</sup> |
| Human CRC | Tumor (T3) | ND | | CRISPR/Cas9 (lipofection) | TP53, Smad4 | NSG | Colon (+DSS) | Liver metastasis | De Sousa e Melo et al<sup>59</sup> |
| Mouse LI | Adenoma | Matrigel (dome) | APC<sup>Cre+</sup>, Kras<sup>G12D</sup>; Villin<sup>Cre+</sup>; Lgr5<sup>Cre+</sup> | | Crisper/Cas9 (lipofection) | Tp53, Smad4 | NSG | Colon | Tumor development and liver metastasis | De Sousa e Melo et al<sup>59</sup> |

CRC, colorectal cancer; DSS, dextran sulfate sodium; LI, large intestine; NSG/NOG, NOD scid gamma mouse (severe immunodeficient mouse); SI, small intestine; WT, wildtype.
spontaneous liver metastasis, which was inhibited by ablation of Lgr5 cells, demonstrating critical roles of cancer stem cells in establishing metastasis. These studies highlight the potential of the organoid-based adoptive transplant approaches in extending the research field, from tumorigenesis to metastatic progression and tumor immunity.

4.3 Human organoid-based carcinogenesis models in severe immunodeficient mice

Organoid culture is also feasible for human intestinal cells. Normal small intestinal cells and colon cells were genetically engineered by CRISPR/Cas9 to reconstitute triple mutations in APC, TP53 and KRAS, followed by depletion of factors or addition of inhibitors to enrich cells that acquired dependency of the stem cell niches through introduced mutations. Engineered organoids from intestine and colon developed adenomas and well-differentiated adenocarcinomas, respectively. Additional inactivation of SMAD4 resulted in development of invasive carcinomas and poorly differentiated adenocarcinomas, respectively. In contrast, patient-derived colon adenoma organoids or APC inactivation in the colon organoids were not tumorigenic in the kidney subcapsule of NSG mice. These results suggest that APC inactivation alone in human cells is not sufficient to induce neoplastic changes even in NSG/NOG mice. Not surprisingly, CRISPR/Cas9-mediated introduction of mutations in TP53, KRAS and PIK3CA cooperated with APC inactivation to generate full-blown tumors and metastatic tumors from normal organoids and adenomas, respectively. Taken together, these observations strongly suggest that human colon organoids can develop adenocarcinomas if only multiple mutations are introduced but not develop premalignant lesions, including ACF or adenomas.

5 CONCLUSION

Each organoid-based colon carcinogenesis model overviewed herein has unique features. Accordingly, researchers will want to select the right model for their research, depending on the aim of their study, as well as taking into consideration that the time and cost that can be afforded. For example, models with murine colon organoids largely depend on the use of GEM with multiple mutations and place more emphasis on generating tumors or phenomena in the advanced stages. Although multiple intercrossing is required, these models might be most suitable for studies on tumor progression and metastasis and for preclinical trials. However, models with murine small intestinal organoids could detect genetic cooperation towards tumorigenesis with the highest sensitivity as preneoplastic lesions or tumors. Hence, validation of candidate genes, whether identified by a murine forward genetic screen or in human CRC samples, is warranted.

Whereas organoids from inbred young laboratory mice of the C57BL/6 strain might be regarded as “clean” in terms of the homogenous nature of genomes and epigenomes, human organoids may not be ideal in this regard because they might harbor genetic polymorphisms and accumulate mutations from environmental carcinogens and aging. Nonetheless, it is noteworthy that they for the first time enabled human models of carcinogenesis, which will provide valuable information not only for basic science but also for future practice of personalized medicine.

Taken together, organoid-based carcinogenesis models are promising tools in cancer research, which would likely substitute and complement in vivo mouse models to various degrees. Therefore, efforts toward establishing similar models for other types of cancer driven by different genetic aberrations are warranted.

CONFLICT OF INTEREST

The authors declare no conflict of interests.

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