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Review: US Spelling Colorectal cancer models for novel drug discovery

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Abstract

Introduction—Despite increased screening rates and advances in targeted therapy, colorectal cancer (CRC) remains the third leading cause of cancer-related mortality. CRC models that recapitulate key features of human disease are essential to the development of novel and effective therapeutics. Classic methods of modeling CRC such as human cell lines and xenograft mice, while useful for many applications, carry significant limitations. Recently developed *in vitro* and *in vivo* models overcome some of these deficiencies and thus can be utilized to better model CRC for mechanistic and translational research.

Areas Covered—The authors review established models of *in vitro* cell culture and describe advances in organoid culture for studying normal and malignant intestine. They also discuss key features of classic xenograft models and describe other approaches for *in vivo* CRC research, including patient-derived xenograft, carcinogen-induced, orthotopic transplantation, and transgenic mouse models. We also describe mouse models of metastatic CRC.

Expert opinion—No single model is optimal for drug discovery in CRC. Genetically engineered models overcome many limitations of xenograft models. Three-dimensional organoids can be efficiently derived from both normal and malignant tissue for large-scale *in vitro* and *in vivo* (transplantation) studies, and are thus a significant advance in CRC drug discovery.

Keywords

Colorectal cancer mouse models; intestinal organoid; patient-derived xenograft; transgenic colorectal cancer mouse model

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1. Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide and confers significant morbidity and mortality [1]. Incidence and mortality are decreasing in the United States due to a combination of enhanced screening and improved treatment; however, despite these gains, five-year survival of metastatic (i.e., Stage IV) CRC remains less than 10% [1]. The discovery and preclinical testing of novel therapeutic strategies requires the use of *in vitro* and *in vivo* models of colorectal cancer. However, of candidate therapies selected for clinical trials based on encouraging preclinical data, only approximately 5% percent demonstrate clinical efficacy in Phase III trials [2]. The failure of the drug development process can be at least partially attributed to the use of preclinical models that poorly recapitulate the disease. In this review, we will discuss the limitations and appropriate use of cell and animal models in CRC research.

2. In Vitro Models

Typically, drug discovery begins with mechanistic and efficacy studies in cell-based models. The most common *in vitro* models include human and mouse cancer cell lines. Recently, three-dimensional “organoid” culture systems have been described that model cancer. Practically, robust *in vitro* systems are essential for mechanistic studies that identify pathways or targets for cancer therapy.

2.1 Human Cell Lines

Cell lines have played a significant role in elucidating signaling pathways in cancer since the derivation of the HeLa cervical cancer line in 1951. Primary tumors, upon surgical removal, are digested into individual cells and cultured on plastic dishes. Many human CRC cell lines are commercially available and have been extensively used in drug discovery [3]. Cell lines are relatively easy and inexpensive to use, and provide rapid experimental results. Most of the widely used cell lines have been genomically characterized and represent the genetic landscape of human CRC. Thus, a panel of lines with activating *KRAS* mutations can be easily compared to a panel lines with wild-type *KRAS*. Alternatively, cell lines can be genetically manipulated through homologous recombination, short hairpin RNA (shRNA) gene knockdown, or CRISPR-Cas9 gene editing. Many assays that evaluate antitumor efficacy can be automated, which aids in drug discovery as multiple agents can be concurrently tested against a range of cell lines.

There are several important limitations to cancer cell lines. Cell lines represent a clonal population of tumor cells that are naturally selected to grow in culture plates and media, and thus likely differ substantially from the original tumor. Cell lines do not recapitulate the functional and genetic heterogeneity of human cancers, which is a significant factor in resistance to targeted therapies [4]. Additionally, there have been reports of cross contamination of one cell line into another [5]; a CRC cell line known as WiDr was thought to be unique until chromosome analysis proved it to be the HT-29 line [6]. Modern authentication techniques and use of cells from repositories (e.g., the American Type Culture Collection or ATCC) reduce the likelihood of cell line cross-contamination. Finally, cell lines are difficult to create from individual patient tumors and cannot be derived from

matching normal tissue. Thus, traditional cancer cell lines are not well suited for personalized clinical application.

2.2 Mouse Cell Lines

CRC cell lines are also available from murine sources. Among the commonly used mouse cell lines are MC38, an adenocarcinoma cell line derived from a C57BL/6 mouse [7] and CT26, derived from a BALB/c mice [8]. These cell lines were developed in the 1970s after repeated subcutaneous injection of the carcinogen 1,2-dimethylhydrazine dihydrochloride and repeated rectal administration of the carcinogen N-nitro-N-methylurethane, respectively. They are easy to culture and readily available from repositories. They are most often used for syngeneic subcutaneous transplantation studies (i.e., MC38 cells into C57BL/6 recipient mice and CT26 cells into BALB/c mice) to study tumor growth in the setting of an intact immune system. Our group derived primary cell lines from genetically engineered colorectal tumors. These primary cell lines have the advantage of being low passage, and thus less likely to have acquired additional mutations *in vitro* or been subjected to contamination. The gene expression profiles of these *Kras* mutant and wild-type lines closely reflect the profiles of human *KRAS* mutant and wild-type CRC tumors, which suggests that they are a useful *in vitro* model of human disease [9]. There are important disadvantages to the use of mouse CRC cell lines for drug development. First, there are far fewer murine lines available compared to human lines. Second, mouse cell lines that model less common cancer mutations are generally unavailable. Finally, murine cells are not as well functionally and genetically characterized as human cell lines.

2.3 Mouse Organoids

A key limitation of traditional monolayer cell culture is that normal intestine cannot be efficiently cultured. The Clevers lab recently described a three-dimensional culture model in which murine intestinal crypts (which contain self-renewing stem cells and adjacent niche Paneth cells) are cultured in three-dimensional collagen gel and specialized media containing Wnt3, the Wnt activator R-spondin1, the BMP inhibitor Noggin, and other growth factors. The crypts grow into “mini-intestines” or organoid structures that contain stem cells and the differentiated cell types of the intestine. Alternatively, FACS-sorted stem cells from *Lgr5-EGFP-creERT2* mice (in which *Lgr5*⁺ stem cells are labeled with GFP) can be cultured as organoids. While single *Lgr5*⁺ stem cells can form organoids, culture efficiency is markedly increased in the presence of niche Paneth cells that provide endogenous Wnt3 ligand to support stem cell function [10]. Primary intestinal organoids have been maintained in culture for greater than 1.5 years [11]. Murine colon organoids have also been reported by the Clevers lab [12]. Intestinal organoids have been extensively used as *in vitro* models of normal intestinal function. An alternative approach to generating organoids involves culture of minced neonatal intestinal tissue within a collagen gel with an air-liquid interface; stromal cells in the tissue provide Wnt and other supportive signals [13].

The majority of CRCs are initiated by truncating *APC* mutations in intestinal stem cells, which lead to activation of Wnt signaling. Colorectal cancer organoids have been derived from murine *Apc*-deficient intestinal adenomas. Unlike normal intestinal organoids, cancer organoids exhibit endogenous Wnt activation, and thus do not require supplemental Wnt or

R-spondin1 ligand to grow. *Apc* can also be deleted *in vitro* in *Apc^{flx/flx} villin-CreER* mice with 4-OH-Tamoxifen, or in organoids derived from wild-type mice by shRNA knockdown or CRISPR/Cas9 editing; selection of cells with *Apc* loss requires only removal of Wnt and R-spondin1 from culture media [14–16]. Additional mutations associated with CRC have been modeled by infection with retrovirus encoding mutant *Kras^{G12D}* or shRNA-mediated deletion of the tumor suppressor genes *Tp53* or *Smad4* [17]. A distinct advantage over conventional cell culture for drug discovery is that normal and cancer organoids can be compared side-by-side with candidate pharmacologic agents.

2.4 Human Organoids

Human intestinal and colon tissue can be cultured as three-dimensional organoids from colonoscopy biopsy specimens, surgical resections, or even single human EphB2+ stem cells [11, 18]. Human intestinal crypts are more difficult to grow than murine crypts and require supplementation with inhibitors of the Alk and p38 pathways for long-term culture, in addition to Wnt, R-spondin1, and Noggin. Organoids derived from human colon biopsies can undergo at least 100 population doublings in culture, thus allowing for adequate numbers of organoids for experimental study [11]. The development of a cell line that produces Wnt3, R-spondin1, and Noggin may substantially reduce the cost of large-scale genetic loss of function or small molecule screens of human intestinal organoids [19].

Organoid cultures are used to model CRC, either directly from cancer tissue or from genetic manipulation of normal intestinal tissue. Similar to murine *Apc*-deficient organoids, human cancer organoids exhibit activated Wnt signaling and thus do not require Wnt for growth and long-term passage [11]. CRISPR/Cas9-mediated editing of *APC*, followed by deletion of *SMAD4*, and *TP53*, and introduction of activating mutations in *KRAS* and *PIK3CA* results in isogenic human organoids that model the adenoma-carcinoma sequence in human *CRC* [20, 21]. The Clevers lab has developed a library of organoids grown from human CRCs and matching adjacent normal colon for *in vitro* drug testing; agents with efficacy in the tumor organoid that are not toxic to the normal organoid can be selected for further study [22]. For example, one patient's CRC organoid was exquisitely sensitive to Wnt inhibition, which was predicted by a mutation in the Wnt negative feedback regulator RNF43. Organoids were successfully derived from approximately 90% of tumor specimens. Thus, organoid culture of malignant and normal colorectal tissue is a novel platform for translating tumor genetic data into personalized therapy. Organoids could also conceivably be cultured from different sections of tumors to model tumor heterogeneity, or from primary and metastatic sites to identify mechanisms of cancer metastasis.

3. *In Vivo* Transplant Models

Despite the many advantages of *in vitro* cancer models, *in vivo* systems are essential to assess the role of the tumor microenvironment, host immune system, and angiogenesis in tumor response to therapy. The most common *in vivo* model is the murine xenograft. Recently, patient derived xenografts and orthotopic transplant models have been developed to overcome deficiencies of the xenograft.

3.1 Cell Line Xenografts

Injection of human colorectal cancer cells subcutaneously into an immunodeficient mouse will typically result in growth of tumor at the injection site. Commonly used mouse strains include classically nude (athymic) and severe combined immunodeficient (SCID) mice, which are devoid of T lymphocytes [23] or both B and T lymphocytes, respectively [24]. NOD/SCID mice, unlike SCID mice, also have deficient NK cells. This model is commonly used for cancer drug discovery due to the lack of significant technical skill required to implant tumors, the straightforward monitoring of tumor growth by the naked eye, and the reasonable cost of maintaining colonies, and reasonable tumor latency of a few weeks [25].

Xenograft models have significant disadvantages. Injection of a large, homogenous cell population in order to form tumor does not reflect the cellular heterogeneity seen in most human CRCs (Figure 1a). The use of immunodeficient animals eliminates the important role of the host immune system in tumorigenesis. Given the difference in species, interactions between cancer cells and stromal cells cannot be studied. In addition, cells in xenografts are typically injected into the subcutaneous space, a microenvironment that is very different than the intestine. Finally, tumors derived from transplanted CRC cell lines do not recapitulate the histological features of human cancer, although transplanted cancer organoids partially overcome this problem. Perhaps due to these factors, drug response in xenograft models correlate poorly with drug response in clinical trials [26]. Recently, transplant models have been developed that attempt to overcome some of these limitations.

3.2 Organoid Xenografts

Xenograft transplantation of *Apc*-deficient murine colon organoids with additional driver mutations has been reported. Combinatorial *Apc*, *Tp53*, *Kras*^{G12D}, and *Smad4* mutations result in tumors with histology similar to invasive adenocarcinoma, although adenoma organoids deficient in *Apc* alone do not successfully engraft [17]. Xenograft transplantation can also be performed under the kidney capsule, which permits metastasis to the liver. The Sato laboratory compared kidney capsule engraftment and liver metastasis of CRISPR/Cas9-engineered human CRC organoids (i.e., editing of the *APC*, *TP53*, *SMAD4*, *KRAS*, and/or *PIK3CA* genes from normal colonic organoids) and organoids derived from metastatic CRC. Benign engineered adenomas (i.e., *APC* deficient) did not engraft, whereas organoids with additional driver mutations successfully formed tumors. In addition, only metastatic CRC organoids, not engineered organoids, efficiently metastasized to the liver [20]. In a related study, the Clevers lab found that CRISPR-mediated deletion of *APC*, *TP53*, and *SMAD4*, and activation of *KRAS*^{G12D}, was required for efficient tumor formation in human small intestinal organoid xenografts. All colon organoids with mutations in *APC*, *KRAS*, and *TP53* (with or without *SMAD4* deletion) formed adenomas upon transplantation. Histologically, only xenografts with all four mutations exhibited small areas of invasive carcinoma; xenografts with fewer mutations formed cystic structures that lacked important features of colon adenocarcinoma [21]. These studies demonstrate the potential application of CRC organoid xenografts for research and personalized therapy. However, the clinical utility of CRC xenograft engraftment may be limited by low engraftment rates for less advanced cancers. Further research is required to evaluate the transplantation efficiency and histological features of patient-derived CRC organoid xenografts.

3.3 Patient Derived Xenografts

In the Patient Derived Xenograft (PDX) model, portions of patient tumor tissue are obtained during surgery and implanted into an immunodeficient mouse (P0). Once the tumor grows it is surgically removed and implanted into other mice (P1). This is repeated until enough animals are obtained for the experiment (i.e., P2, P3, etc.). Unlike traditional xenografts, in PDXs tumor stroma will grow with the tumor cells, thus allowing for tumor-stroma cross-talk (Figure 1c) [27]. Both cellular and molecular tumor heterogeneity of tumor is more pronounced compared with monoclonal xenografts. PDXs conserve important aspects of tumor histology, vascularity and architecture of primary CRCs. Important driver mutations appear to be consistent along passages of PDXs, such as *KRAS* and *PIK3CA* [28] and establishment of PDX models that reflect the heterogeneity of CRC is feasible [29, 30]. PDX models predict clinical response to therapy better than traditional xenografts [26, 31, 32]. An important application of PDX is personalized cancer treatment. Following surgery, an individual patient's cancer can, following surgery, be implanted into a mouse, passaged, and studied with various chemotherapy agents to determine a clinical approach to treat the patient [32]. PDXs may have an important role in the care of patients with rare tumor types or combination of driver mutations where there is no established treatment regimen, or for patients with resistance to established therapy [33].

Despite excitement about their potential, PDX models carry important disadvantages. Like traditional xenografts, PDX models require the use of immunodeficient host animals. As with cell line xenografts, a "more" immunodeficient mouse (e.g., NOD/SCID) may be better suited for engraftment than a "lesser" immunodeficient mouse (e.g., nude). While most cell lines implanted into xenografts are well defined, primary tumors used in PDXs require additional molecular characterization. It has been shown that human tumor stroma is initially preserved after transplantation but is slowly replaced by murine stroma with time; the significance of this is somewhat unclear [27]. Despite the promise of PDX for personalized medicine, the reported tumor initiation rate for PDX is approximately 70%, which may limit clinical utility [34]. Additionally, tumor implantation and screening potential therapies may require six or more months. Finally, PDX models are expensive for clinical application and are typically not covered by private or national health plans.

3.4 Orthotopic Transplant Models

A major disadvantage of traditional and patient-derived xenografts is that the microenvironment of the subcutaneous space differs greatly from that of the colon. Thus, orthotopic transplant models have been developed in which CRC cells are directly implanted into the colon. Implantation of tumor cells can occur via a laparoscopy directly into the serosal surface of the cecum. This approach results in both a primary tumor and possible metastasis to local lymphatics, lung, and liver [35]. Orthotopic transplantation of as few as 1,000 cells into the distal colon, via enema or colonoscopy-guided mucosal injection, results in tumors that can be monitored by optical colonoscopy (Figure 1e) [9, 36]. With colonoscopy, tumor growth can be longitudinally determined by the degree of luminal obstruction and response to therapy assessed with serial biopsy. Human colon cancer cell lines are implanted into immunodeficient mice, while mouse cell lines are transplanted into syngeneic recipient mice. Intestinal organoids can also be transplanted into the colon;

administration of dextran sulfate sodium (DSS) to induce colitis allows successful rectal delivery and engraftment of normal organoids [37]. This approach could be used to orthotopically transplant malignant murine or human intestinal organoids for validation of drug targets or study of mechanisms of carcinogenesis.

4. Carcinogen Induced Mouse Models

Colonic tumors can be induced in mice with 1,2-dimethylhydrazine (DMH), or its metabolite, azoxymethane (AOM). AOM is N-oxidated and hydroxylated in the liver, then excreted in the bile. In the colon, microbial flora then metabolize the agent into a form that promotes carcinogenesis [38]. AOM administration results in tumors that often have mutations in *Kras* and *Ctnnb1* (encoding beta-catenin) [39]. However, mucosal invasion and distant metastasis is rarely seen in this model. A commonly used mouse CRC cell line, MC38, was obtained from a DMH-induced tumor [7]. A limitation of AOM is that a latency period of up to 30 weeks may be required for tumor formation. An alternative method is the administration of AOM together with dextran sulfate sodium (DSS), an agent that causes colitis [40]. DSS alone can cause tumorigenesis; however, the incidence is low if not combined with AOM. This combination lowers latency time to 10 weeks and is used to model inflammatory bowel disease-associated CRC with a tumor incidence of close to 100% [41]. Typically, DSS is administered orally via drinking water and AOM via sequential intraperitoneal injection [42]. Other carcinogens are used either alone or with DSS to model CRC, including heterocyclic amines, aromatic amines, and alkylnitrosamide compounds; however, DMH and AOM are the best characterized cancer-inducing agents [7, 43]. Carcinogen induced models have been used for chemoprevention studies in CRC, including research on COX-2 inhibitors and Peroxisome Proliferator-Activated Receptor (PPAR) ligands such as pioglitazone and rosiglitazone [44, 45].

Advantages to carcinogen-induced models include high reproducibility, straightforward and inexpensive tumor initiation, low multiplicity, the ability to monitor tumors in the distal colon with optical colonoscopy, and the ability to induce tumors in mice of different genetic backgrounds. Thus, whereas xenograft and genetically engineered models are genetically homogeneous, tumorigenesis in multiple murine strains, including wild mice, can be studied using carcinogens to model the genetic diversity of human cancer. However, the AOM/DSS model may be more suited as a model of inflammatory bowel disease-associated CRC, which constitutes a minority of CRCs, rather than sporadic CRC.

5. Genetically Engineered Mouse Models

Genetically engineered mice are powerful models, as they recapitulate adenoma or carcinoma formation in the native, immunocompetent colon microenvironment. Since the initial discovery of the *Apc^{Min}* mouse in 1990, numerous additional genetically engineered models have been added to the armamentarium of colon cancer modeling.

5.1 Germline *Apc* Mutant Models

The *Apc^{Min}* mouse was discovered as a result of N-ethyl-N-nitrosourea (ENU) mutagenesis screening [46]. During the mutagenesis screen, mice were subjected to ENU, an alkylating

agent. One phenotype was anemia that was associated with a heterozygous nonsense mutation at codon 850 in the *Apc* gene, the mouse equivalent of human *APC*, and acquired homozygous mutations in intestinal polyps [47]. This mouse was named Min (Multiple intestinal neoplasia) as it spontaneously develops 30 or more polyps by 4–6 months of age (Figure 1b). Thus, the mouse is an excellent model of familial adenomatous polyposis (FAP), which is caused by a germline heterozygous truncating *APC* mutations and early onset polyposis from subsequent loss of the second *APC* allele [48]. Almost all tumors in the *Apc^{Min}* mouse are adenomas by histology; carcinoma and metastasis are rare. Additionally, as the polyps develop predominantly in the small intestine, tumors cannot be monitored via colonoscopy. The *Apc^{Min}* mouse led to the critical discovery of the central role of *APC* in intestinal tumorigenesis: inactivation of *APC* results in nuclear localization of beta-catenin and transcription of Wnt target genes, in particular *MYC* [49, 50]. The *Apc^{Min}* mouse has been used in hundreds of chemoprevention, therapeutic and mechanistic studies [51]. Since the development of the *Apc^{Min}* mouse, other mice bearing targeted, truncating germline mutations in *Apc* have been created which vary in the number and location of intestinal lesions but produce polyps with similar histological features [52–62].

5.2 Other Germline Genetically Engineered Models of CRC

Mutations in DNA mismatch repair genes, predominantly in *MSH2* and *MLH1*, cause hereditary nonpolyposis colorectal cancer (HNPCC), also known as Lynch Syndrome, and are characterized by microsatellite instability. Mice deficient in *Mlh1*, *Msh2*, *Msh3*, *Msh6* or *Pms2* develop tumors in the small bowel as well as lymphomas and skin tumors [63–66]. These models may be helpful when dealing with cancer subtypes of human disease such as medullary, mucinous, undifferentiated and signet-ring carcinomas, as they display high levels of microsatellite instability [52, 67]. Mismatch repair-deficiency increases adenoma formation in an *Apc* deficient background [68, 69].

TGF- β activates the SMAD4 pathway and is mutated in 10–35% of colorectal cancers, usually late in cancer progression. Germline *SMAD4* deletion is responsible for familial juvenile polyposis, an inherited disease characterized by early onset gastrointestinal polyps and cancers; *Smad4^{+/-} Apc^{Min}* mice exhibit greater invasion and transplant efficiency compared to *Apc^{Min}* controls [70, 71]. *Tgfb1* deletion in the immunocompromised *Rag2* strain results in cecal and colon neoplasm without *Apc* loss [72, 73]. Knockout of *Smad3*, a downstream protein from TGF- β , results in mucinous carcinoma in the colon [74].

5.3 Conditional or inducible *Apc* deletion Models

The *Cre* recombinase system is used to conditionally target gene deletion or activation to the intestine, thus avoiding lethality and extra intestinal manifestations that may be present with germline mutations. This can be achieved by crossing mice with an intestine-specific cre with *Apc* floxed mice. Constitutive and tamoxifen-inducible Villin-cre mice are widely used to limit deletion of *Apc* and other genes to the intestinal epithelium and model intestinal tumorigenesis [75]. The Lgr5-EGFP-creERT2 mouse marks Lgr5+ stem cells, which are located at the base of intestinal crypts and are the cells of origin of intestinal cancer; conditional deletion of *Apc* in Lgr5+ stem cells leads to small intestinal polyposis [14, 76]. *Apc* loss in a quiescent intestinal stem cell marker, *Lrig1*, induces colorectal adenomas with

high-grade dysplasia that are seen on colonoscopy [77]. Other cre mouse lines that are used to model CRC (with varying polyp burden in small intestine vs. colon) include Fabp-Cre [59], Ah-Cre (Cyp1A promoter) [78], CDX2P-NLS-Cre [79], and CAC-Cre (carbonic anhydrase I gene promoter, expressed only in the large intestine) [80]. The addition of *LSL-Kras^{G12D/+}* to *Apc^{loxp14/+}* CAC-Cre results in an average of 4.3 colon adenomas (but not carcinomas) per mouse [81].

Another colon-specific CRC model involves exogenous delivery of adeno-cre to the colons of *Apc^{loxp14/loxp14}* mice. This can be achieved by rectal enema [82] or, more efficiently, by restricting adeno-cre infection to the distal colon with colonic clips placed during laparotomy [83]. Surgical delivery of adeno-cre results in reproducible formation of 1–4 adenomas in the distal colon. *Apc*-null tumors are monitored longitudinally by optical colonoscopy to assess therapeutic response; tumor biopsy before and after treatment is used to determine tumor histology, genotype, mRNA or protein expression. *Tp53* loss or *Kras* or *Braf* mutation in the setting of *Apc* deficiency results in invasive adenocarcinoma in a subset of tumors (Figure 1d) [9, 83–85]. The *Kras* mutant murine model recapitulates the gene expression patterns and prognosis of *Kras* mutant CRC patient cohorts, which suggests that it is an excellent resource for drug discovery [86].

A novel approach to studying gene function during defined time periods is to use a mouse with cre-dependent expression of rtTA3, providing induction of TRE-controlled transgenes in the presence of doxycycline [87]. For example, inducible expression of Cas9 permits CRISPR-mediated editing of *Apc* and subsequent tumorigenesis. This model can be used to assess the role of up to six genes in tumorigenesis, perhaps most efficiently tumor suppressors, with limited off-target effects [88].

6. Metastatic models

Given that the majority of CRC deaths are from metastatic disease, a great deal of effort has gone into development of mouse models that mimic the metastatic spread of tumor cells. Colonic delivery of adeno-Cre to *Apc^{loxp14/loxp14} LSL-Kras^{G12D/+}* mice (described above) is reported to result in liver metastases that are high-grade carcinomas in 20% of mice 24 weeks following adeno-cre injection [67, 83]. *VillinCre LSL-Kras^{G12D} Tgfbr2^{E2flx/E2flx}* mice develop lymph node and lung metastases from primary colon tumors via a beta-catenin-independent mechanism [89]. However, CRC metastases in these models are however infrequent and thus may not be practical for drug testing or large-scale experiments.

The major site of CRC metastasis is the liver [1]. One method of assessing the growth of colon cancer in the liver is direct inoculation of tumor cells into the liver parenchyma [90]. This is the most direct method of studying the metastatic progression of colorectal cancer in the liver as it directly assesses colonization competence of tumor cells [91]. Hematogenous seeding of tumors via the portal vein through splenic injection is an alternative route (Figure 1f). In such models, tumor cells are injected into the spleen via laparotomy (the spleen can be removed, partially removed, or left in place after injection), after which growth of liver metastasis follows within a few weeks [92]. The splenic injection approach can be performed with human CRC cells into immunodeficient mice or with murine cell lines into

syngeneic mice; metastasis rate is affected by the tumor cell line and recipient mouse strain [93]. This model relies heavily on the latter events of the metastatic cascade (mainly seeding and extravasation); however, it does so at very high efficiency and thus can reliably be used for experiments with multiple therapeutic conditions or where the number of animals used in a study is limited. Alternatively, tumor cells can be directly injected into the portal vein [93].

Orthotopic transplantation models of CRC metastasis have also been developed. Tumors injected into the serosal surface of the cecum develop metastasis at relatively low rate, but passage of tumor cells through a xenograft host increases lymph node and lung metastases formation rate to approximately 10% in Kras wild-type cells [94, 95]. Orthotopic injection of murine tumor cells into the rectal mucosa of syngeneic recipient mice produces a liver metastasis rate of only 3.3% 50 days after transplantation [96]. Metastasis formation in cell line models can be confirmed and monitored with the use of fluorescence and/or luciferase-tagged cells [97]. The Lipkin lab recently reported a novel approach to establishing orthotopic CRC metastatic cancers by hijacking the ability of chemokine receptor 9 (CCR) to target lymphocytes to the intestine and colon [98]. Human CRC cell lines engineered to conditionally express CCR9 form intestinal tumors following intravenous injection in immunodeficient mice. Silencing of CCR9 expression results in liver metastasis formation. In summary, many CRC metastasis models have been reported and are potentially useful for preclinical drug discovery research.

7. Conclusion

In the past few decades, many CRC models have been developed to aid research and drug discovery. Cell lines, xenografts, organoid culture, and genetically engineered mice offer the ability to model the genetic, histologic, and molecular features of human CRC. While no single model is optimal, each has advantages and disadvantages that must be considered by the investigator.

8. Expert Opinion

The historically poor correlation between drug efficacy in animal models and clinical trials suggests that innovative *in vitro* and *in vivo* models are needed to develop novel colorectal cancer (CRC) treatments. Advantages and disadvantages of each model are described in Table 1. Key historical developments in CRC modeling include: the development of the HeLa cancer cell line (1951) followed by derivation of many human CRC cell lines; discovery of the *Apc^{Min}* genetically engineered model (1990); discovery that loss of *Apc* in intestinal stem cells causes carcinogenesis (2009); first report of murine intestinal organoids (2009), successful organoid culture from murine and human CRCs (2011); development of a cell line based orthotopic transplant CRC model (2011); and report of a tissue “biobank” of human CRC organoids for drug discovery (2015). Here, we offer our recommendations on the use of these models in drug development.

Human cell line and xenograft models are the mainstay of therapeutic studies due to low cost and ease of use, but poorly reproduce the heterogeneity of human cancer. Since human cell lines have been extensively studied as *in vitro* and *in vivo* models, they will continue to

play an important role in cancer biology and drug discovery research. Orthotopic transplantation of human cell lines into the cecum is not widely used because of technical challenges and difficulty in tumor monitoring. Cell line transplantation into the colonic mucosa is a promising new approach that will require further optimization before it can be applied in CRC research. We believe that cell line xenografts will gradually be replaced with patient-derived xenografts or patient-derived organoid xenografts for personalized medicine applications.

The *Apc^{Min}* genetically engineered model of CRC and related *Apc*-deficiency models have been extensively used in drug discovery and mechanistic studies. However, the key limitations that have prevented genetically engineered models from replacing xenografts include long tumor latency and need for expensive and time consuming gene targeting and breeding to study the effects of genetic mutations on tumorigenesis. Therefore, while genetically engineered models will continue to be essential for mechanistic studies, they will not replace the low cost and ease of use of xenograft models. Carcinogen-induced models share similar advantages and disadvantages with genetically engineered models, with the additional limitation that the initiating event is a chemical insult instead of loss of *Apc* function (as in most human CRCs). CRISPR/Cas9-based gene targeting in the germline may reduce the time and expense of studying tumor suppressor gene function *in vivo*.

A major recent innovation in CRC modeling is the ability to recapitulate the ultrastructure of normal and malignant intestine in three-dimensional culture using defined media conditions (i.e., organoid or mini-intestine culture). Small intestinal or colonic organoids can be derived from essentially any mouse model or patient, and therefore have far-reaching implications for drug discovery applications. In particular, human organoids are ideal for personalized medicine because individual patient cancers and control normal tissues can be propagated indefinitely for genetic, functional, and drug response studies in culture and, potentially, xenograft transplantation assays. Thus, we believe that as the costs of organoid research decrease, organoid culture will gradually replace other mouse CRC models for many applications.

The ideal CRC model recapitulates essential aspects of human cancer: 1) tumorigenesis in the colon; 2) sequential mutagenesis (i.e., loss of *APC*, followed by activating *KRAS* mutations and loss of *TP53*); 3) histological features of adenoma, invasive adenocarcinoma, and metastasis; 4) immune system interactions between intestinal stroma and tumor; and 5) genetic and functional heterogeneity. The model should also have high tumor penetrance, short tumor latency, and be relatively easy to use. We believe that much of the failure of the drug discovery pipeline can be attributed to deficiencies with commonly used cell line and traditional xenograft models. While no single currently available model achieves all of these goals, we believe that genetically engineered mice should be preferred as *in vivo* models of tumor initiation and stroma-tumor interaction. A reproducible, efficient genetically engineered model of CRC metastasis is unfortunately not available. For most research and clinical applications, we recommend use of murine and patient-derived organoid cultures, which have been shown to meet many of the above requirements of an ideal CRC model. The CRISPR/Cas9 gene editing system is an important advance for rapid study of gene function in organoids. Human cancer organoids hold great promise for studying tumor

heterogeneity in drug response and for selecting targeted therapies for cancer patients. In addition, we anticipate that CRC metastases will soon be modeled with organoid cultures. Further research is needed to define the complementary roles of in vitro patient-derived organoids, organoid xenografts, and patient-derived xenografts in CRC modeling, drug development, and clinical application.

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Highlights

- Classic colorectal cancer models for drug discovery include cell lines and xenograft animal models. While widely utilized, these models have significant limitations that limit their ability to predict drug response in clinical trials.
- Three-dimensional organoid culture systems can be applied for mechanistic studies, candidate drug screening, and cancer therapy selection.
- Patient derived xenografts can be used for personalized cancer treatment.
- Genetically engineered mouse models are employed in mechanistic studies that may result in novel targets for cancer therapy.
- Carcinogen-induced models are useful for studying inflammatory bowel disease-associated colorectal cancer.
- CRC *in vivo* metastasis models are available but not yet widely used in drug discovery.

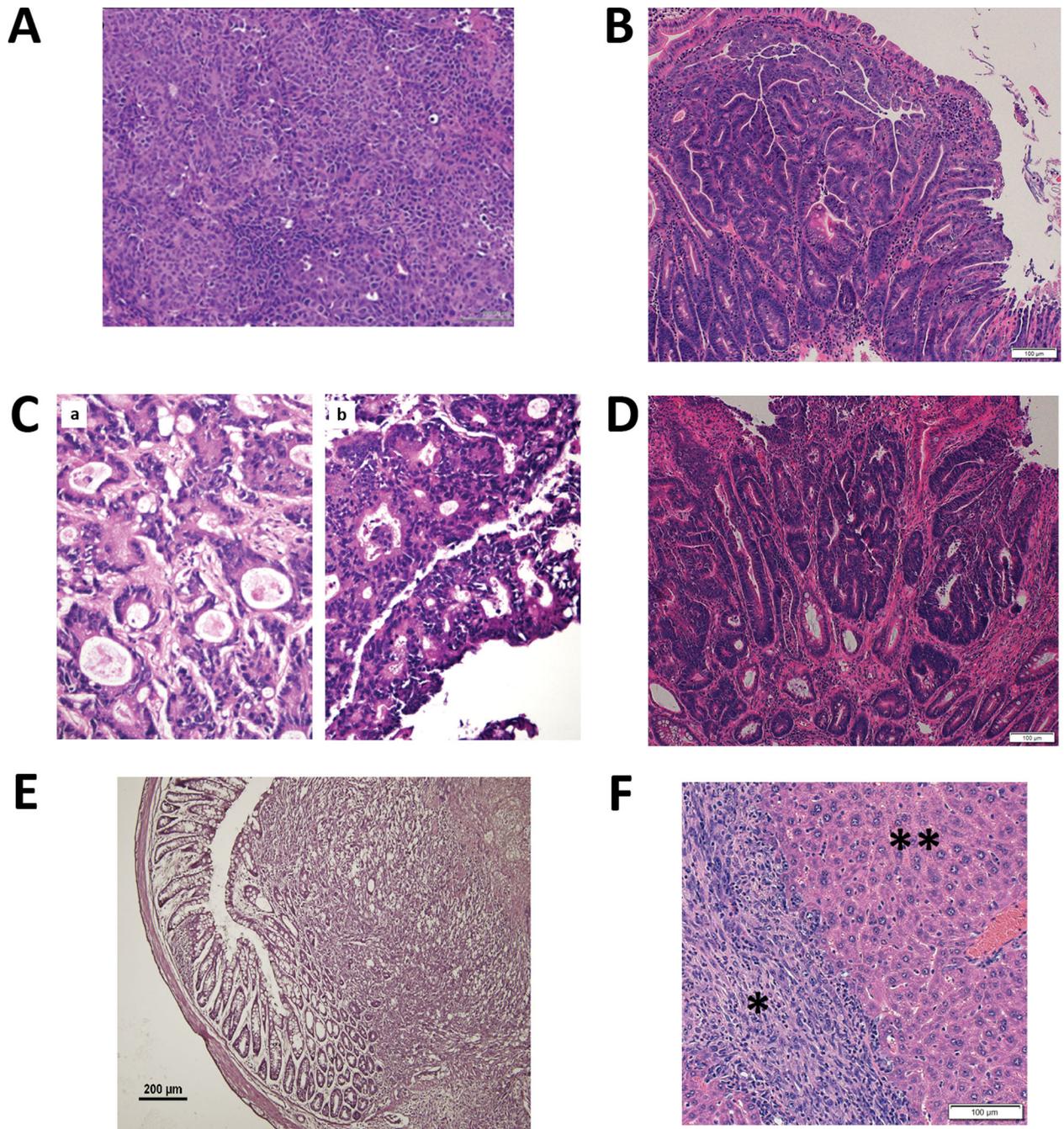


Figure 1. Histology of in vivo tumors of commonly used in vivo tumor models

A – Hemotoxylin & Eosin (H&E) stain of xenograft tumor formation after injection of human SW480 colorectal cells into an immunodeficient mouse. Note the homogenous cell population. Reprinted from [99] with permission of Nature Publishing Group **B** – H&E stain of an adenoma seen in the small bowel of an Apc^{Min} mouse. **C** – H&E stains of a patient derived xenograft. a is the primary, patient tumor and b is the tumor after 11 generations of passage in mouse. Note the preservation of tumor structure after passaging. From [100] with permission of PLoS One. **D** – H&E stain of an Apc and $Tp53$ -deficient murine colonic

tumor with features of high-grade dysplasia. **E** – H&E stain of an murine CRC cell line orthotopic transplant tumor. Murine MC38 colon cancer cells were injected via mouse colonoscope. From [36] with permission of PLoS One. **F** – H&E stain of liver metastasis. A murine cell line derived from an *Apc*, *Kras* and *Tp53*-mutant transgenic mouse were injected into the spleen of a wild type C57BL/6J mouse. Four weeks later, liver was explanted. * metastatic tumor, ** surrounding healthy liver.

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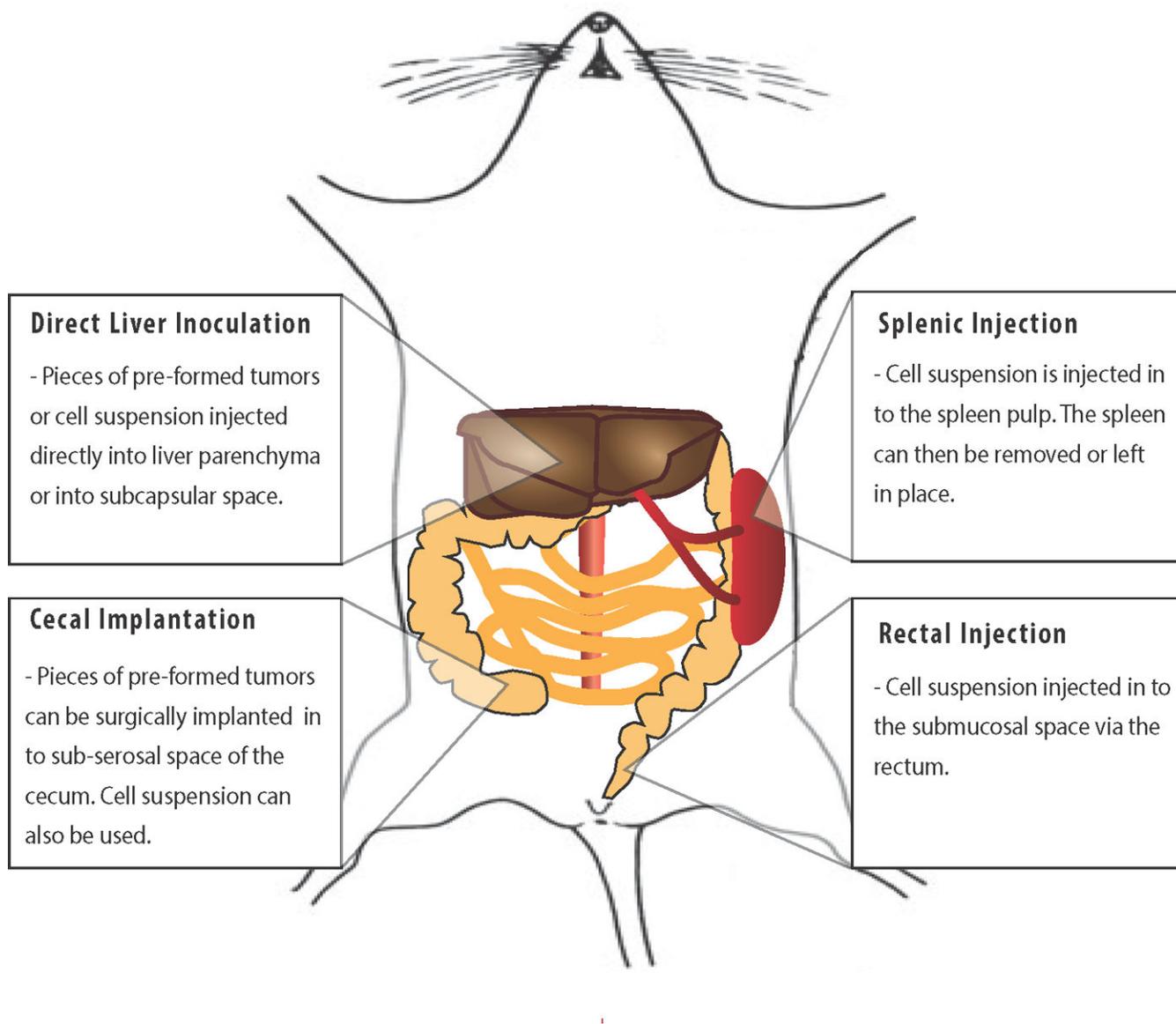


Figure 2. Schematic of the common routes of forming metastasis in modeling metastatic colorectal cancer

Tumor cells can be directly injected into the liver [90] or into the spleen [92] resulting in efficient metastasis in the liver. Alternatively, cells can be implanted into the cecum [94] or rectum [96], although this is associated with less frequent liver metastasis formation.

Table 1

Overview of the advantages and disadvantages of *in vitro* and *in vivo* colorectal cancer models.

Model type	Model	Advantages	Disadvantages
<i>In vitro</i>	Cell lines	<ul style="list-style-type: none"> • Easy to culture • Many well-characterized lines available • Easy to model multiple genetic mutations 	<ul style="list-style-type: none"> • Monoclonal cells poorly represent heterogeneity of tumor
	Organoids	<ul style="list-style-type: none"> • Possibility for personalized treatment • Ability to model normal intestinal tissue • Easy to model multiple genetic mutations 	<ul style="list-style-type: none"> • Can be costly to culture
<i>In vivo</i>	Xenografts Organoid xenograft Patient-derived xenograft	<ul style="list-style-type: none"> • Easy to establish, rapid tumor onset • Can be used with most human cell lines • Recapitulates histology of human CRC • Can be derived from patient tumor or from genetically altered normal tissue for personalized therapy • Therapy tailored for individual patients • Tumor histology and genetic heterogeneity is similar to original tumor 	<ul style="list-style-type: none"> • Requires immunodeficient host • Non-colon microenvironment • Homogeneous cell population does not reflect tumor heterogeneity • Requires immunodeficient host • Can be used with most human cell lines • Requires immunodeficient host • Potentially long tumor latency
	Orthotopic transplantation	<ul style="list-style-type: none"> • Uses the same microenvironment (colon) as human cancer • If transplanted into distal colon, optical colonoscopy can be used to monitor growth and response to therapy 	<ul style="list-style-type: none"> • Technically challenging to perform • If human cells are used, requires immunodeficient animal
	Carcinogen-induced	<ul style="list-style-type: none"> • Near 100% efficiency in tumor formation • Ability to induce tumors in most mouse strains • Correct tumor microenvironment • Recapitulates human adenoma histology 	<ul style="list-style-type: none"> • Models inflammatory bowel disease-mediated CRC rather than sporadic CRC • Long tumor latency
<i>In vivo</i> metastasis	Genetically engineered Genetically engineered (adeno-cre model) Splenic transplantation CCR9-mediated liver metastasis	<ul style="list-style-type: none"> • Correct tumor microenvironment • Recapitulates human adenoma histology • Histologically accurate liver metastases • Reproducible liver metastasis or seeding • Liver metastasis form from primary intestinal tumors 	<ul style="list-style-type: none"> • Long tumor latency • Time consuming and expensive to model multiple mutations • Most tumors are adenomas, not carcinomas • Long tumor latency and poor penetrance make this model impractical for large-scale study • Requires technically challenging surgery in <i>Apc^{loxP14/loxP14}LSL-</i> <i>Kras^{G12D/+}</i> mice [83] • Does not model

Model type	Model	Advantages	Disadvantages
			true metastasis from primary tumor • Requires genetically manipulated cell lines

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