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Mouse models of colorectal cancer as preclinical models

Rebecca E. McIntyre1), Simon J.A. Buczacki2), Mark J. Arends3) and David J. Adams1)

In this review, we discuss the application of mouse models to the identification and pre-clinical validation of novel therapeutic targets in colorectal cancer, and to the search for early disease biomarkers. Large-scale genomic, transcriptomic and epigenomic profiling of colorectal carcinomas has led to the identification of many candidate genes whose direct contribution to tumourigenesis is yet to be defined; we discuss the utility of cross-species comparative omics-based approaches to this problem. We highlight recent progress in modelling late-stage disease using mice, and discuss ways in which mouse models could better recapitulate the complexity of human cancers to tackle the problem of therapeutic resistance and recurrence after surgical resection.

Keywords: colorectal cancer; disease biomarker; mouse model; omics and omics; target identification; target validation

Introduction

Colorectal cancer (CRC) accounts for 700,000 deaths and 1.4 million newly diagnosed cases globally per annum, making it the number one cause of non-smoking related cancer deaths [1]. Most CRCs arise in the epithelium, a process driven by genetic and/or epigenetic alterations that result in the formation of premalignant lesions called adenomas (see Table 1 for list of genes most frequently mutated in CRCs). The transition from normal epithelium to benign adenoma is initially driven by alterations that hyperactivate the Wnt pathway (>95% CRCs), and this predominantly occurs through inactivation of the APC gene (>80% CRCs) [2–4]. APC is a negative regulator of the Wnt pathway and hyperactivation of the Wnt pathway is critical to both the initiation and maintenance of the vast majority of CRCs, although pathway activation is finely tuned [2, 5, 6]. For example, alteration of many genes that are known to regulate Wnt signalling, such as AXIN2 (Axis inhibition protein 2) and AMER1 (APC membrane recruitment 1) are found in tumours that also harbour APC mutations [2, 7]. The Wnt pathway is also critical for the maintenance of the intestinal epithelium, which undergoes complete renewal every 4–5 days in humans [8]; therefore, it has not been possible to target this pathway in cancers without disrupting intestinal regeneration [9].

Activation of the Ras-MAPK (Kirsten rat sarcoma viral oncogene homolog and mitogen activated protein kinase) pathway, usually via point mutations that constitutively activate KRAS or BRAF (v-raf murine sarcoma viral oncogene homolog B), and inactivation of the TGFβ (transforming growth factor β) pathway through inactivation of SMAD gene family members or TGFβ receptors, promote the development of advanced adenomas or invasive adenocarcinomas [3]. Over time, a small proportion of advanced adenomas acquire further molecular abnormalities that transform them to invasive and then metastatic carcinomas. Inactivation of TP53 (Tumour protein p53) or IGF2R (Insulin-like growth factor 2 receptor) occurs with greater frequency in established carcinomas that invade submucosal layers than in adenomas [3, 10]. However the molecular alterations that support metastases are poorly understood; at the genomic level at least, it would appear that there is high concordance between primary CRCs and their matched metastatic lesions, which suggests that mechanisms other than gene mutation may be responsible for progression to metastatic disease—for example epigenetic or post-translational modifications [10–12].
Methods, Models & Techniques

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Table 1. Selection of some of the most frequently mutated genes in colorectal cancer

<table>
<thead>
<tr>
<th>Human/mouse gene symbol</th>
<th>Frequency (%)</th>
<th>Full gene name</th>
<th>Role in development of CRC</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC/Apc</td>
<td>&gt;80</td>
<td>Adenomatous Polyposis Coli</td>
<td>- Inactivation of APC is the initiating event in the majority of colorectal cancers [100]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- APC is a negative regulator of the Wnt pathway</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Hyperactivation of the Wnt pathway initiates development of CRC by stabilising the β-catenin transcription factor, increasing expression of transcription factors such as c-Myc and c-Fos, which regulate expression of cell cycle genes</td>
</tr>
<tr>
<td>TP53/Trp53</td>
<td>~65</td>
<td>Tumor protein p53</td>
<td>- Loss of TP53 is associated with disease progression [101]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- TP53 regulates expression of genes that induce cell cycle arrest, apoptosis, senescence, DNA repair, or changes in metabolism</td>
</tr>
<tr>
<td>KRAS/Kras</td>
<td>~45</td>
<td>Kirsten rat sarcoma viral oncogene homolog</td>
<td>- Mutation of KRAS (&gt;75% of mutations are at codon 12) and BRAF cause activation of the Ras-MAPK pathway and mutations in these genes are mutually exclusive [2]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Activation of Ras-MAPK pathway is associated with disease progression and poor prognosis [102]</td>
</tr>
<tr>
<td>BRAF/Braf</td>
<td>~8</td>
<td>v-raf murine sarcoma viral oncogene homolog B</td>
<td>- Activation of the Ras-MAPK pathway leads to activation of transcription factors such as c-Myc, and c-Fos, which in turn regulate expression of cell cycle genes</td>
</tr>
<tr>
<td>FBXW7/Fbxw7</td>
<td>~20</td>
<td>F-Box And WD Repeat Domain Containing 7</td>
<td>- E3 ubiquitin ligase that targets cyclin E and c-Myc (cell cycle regulators) for ubiquitin-mediated degradation [103]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Low expression of FBXW7 is associated with poor prognosis [104]</td>
</tr>
<tr>
<td>TGFB2/Tgfb2</td>
<td>~12</td>
<td>Transforming growth factor, beta receptor 2</td>
<td>- Alterations in TGFβ pathway e.g. mutation of TGFB2, SMAD2, SMAD3 and SMAD4, are associated with disease progression [93, 105]</td>
</tr>
<tr>
<td>SMAD2/Smad2</td>
<td>~7</td>
<td>SMAD family member 2, 3, 4</td>
<td>- The SMAD family are intracellular signal transducers that are activated by TGFβ receptors and act as transcriptional modulators.</td>
</tr>
<tr>
<td>SMAD3/Smad3</td>
<td>~5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SMAD4/Smad4</td>
<td>~12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTEN/Pten</td>
<td>~10</td>
<td>Phosphatase and tensin homolog</td>
<td>- PTEN dephosphorylates phosphoinositide substrates and negatively regulates intracellular levels of phosphatidylinositol-3,4,5-trisphosphate (PIP3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Negatively regulates AKT/PKB signaling pathway</td>
</tr>
<tr>
<td>CDK8/Cdk8</td>
<td>~12</td>
<td>Cyclin dependent kinase 8</td>
<td>- CDK8 regulates the cell cycle by acting as a co-activator for transcription of nearly all RNA Polymerase II-dependent genes</td>
</tr>
</tbody>
</table>

Endoscopic or surgical resection is routinely used for patients with early premalignant adenomas, as well as for the treatment of most early stage carcinomas and selected patients with late stage or advanced metastatic disease, for whom chemotherapy (or radiotherapy/chemoradiotherapy for rectal cancers) is also a key treatment modality [13]. Liver metastases of colorectal carcinomas occur in about 50% of patients, either at the time of diagnosis or at recurrence, and this is a major cause of CRC-related deaths [13]. Long-term survival of CRC patients is correlated with disease stage at diagnosis, and the 5-year survival rate for patients with metastatic CRC is less than 10% [14].

Aside from our lack of understanding of the molecular events that drive metastases, there are several other plausible explanations for our limited success in treating CRC. Firstly, there are currently few biomarkers that are predictive of early disease, the likelihood of favourable treatment response, recurrence or the development of metastatic disease [10]. Secondly, therapeutic resistance, be it acquired or intrinsic, to several licensed colorectal cancer therapies is a major problem [15]. For example, overexpression of the epidermal growth factor (EGF) receptor is associated with increased metastatic potential and poor prognosis in CRC. In these subgroups of cancer patients, monoclonal antibodies to the EGF receptor e.g. Cetuximab and Panitumumab, block EGF binding and result in tumour regression [16, 17]. Resistance to therapy eventually develops through a variety of mechanisms including point mutations in the EGF receptor that inhibit Cetuximab binding but not EGF, or activating mutations in KRAS, an intracellular signalling pathway that converges with the EGF receptor pathway. Finally, the drug discovery pipeline attrition rate for oncology investigational compounds is extremely high. Only 5% of compounds that show promise in pre-clinical studies are eventually approved for clinical use as the majority fail in Phase I/II trials [18]. One of the reasons for this may be the extensive use of CRC cell lines and xenograft models derived from them in pre-clinical anticancer drug testing (reviewed by [18, 19]). Cell culture does not model the interaction of primary tumour cells with surrounding cells (the tumour microenvironment or stroma), the process of organ colonisation by metastatic cancer cells (the ‘seed and soil’ hypothesis), and the recruitment and activation of the adaptive and innate immune systems during tumorigenesis, which are known to exert selective pressures on cancer cells [20–22]. Several cell culture systems have been developed to make allowances for reciprocal cell communication; examples include organoid cultures, the co-culture of tumour epithelial cells and stromal cells or three-dimensional...
cultures [23–25]. While these systems provide important insights into disease biology, they are limited in their ability to model the dynamic interplay between tumour and host. In this review, we summarise the strengths and weaknesses of xenograft models derived from CRC cell lines and primary patient derived material, and genetically modified mouse models of colorectal cancer. We discuss several novel applications of these models in cancer gene validation, biomarker discovery and target validation, and discuss their future roles in cross-species comparative ‘omics’ approaches to understanding metastasis and therapeutic resistance.

**Cell line xenografts for reductionist target validation**

Xenografting involves the injection of cells or the surgical transplantation of primary tissue into immune-deficient mice such as the nude mouse, which is athymic and does not produce T-lymphocytes, or the severe combined immune-deficient (SCID) mouse, which has altered adaptive immunity but normal innate immunity, to prevent rejection. It was thought that the large number of commercial colorectal cell lines available would represent the inter-tumour heterogeneity of the human disease, but in reality, few cell lines lead to reliable primary tumour growth, and fewer still to naturally metastatic CRC [26]. Success rates appear to be highest when using the colorectal carcinoma cell lines HCT-116 or HT-29, but it is not yet clear why this is. Xenograft models are useful for rapid, reductionist, target validation studies, including validation of the many candidate cancer genes identified by The Cancer Genome Atlas (TCGA), however the results must be interpreted with caution (Table 2, Fig. 1 [27, 28]). For example, the environment of subcutaneous tumours is very different from that of autochthonous colorectal carcinomas (i.e. tumours that originate in the location where they are found) and liver metastases; and the extent to which the species mismatch in tumour (human) and stromal (mouse) cells influence tumour growth in vivo is uncertain. CRC stroma is composed of macrophages that secrete matrix-degrading factors such as metalloproteinases, myofibroblasts that secrete Wnt ligands, T-cells that secrete proinflammatory cytokines, and endothelial cells and bone-marrow derived cells, all of which contribute greatly to the growth and progression of this tumour type [20, 22, 29].

**Orthotopic xenografts of CRC cell lines improve tumour-stromal cell cross-talk and the reproducibility of liver metastases**

Microenvironmental differences likely explain why the morphology of subcutaneous CRC cell line xenografts, especially the stromal elements, are often markedly different from the human disease and may explain why they have a low

<table>
<thead>
<tr>
<th>Table 2. Transplantation models of colorectal cancer (CRC)</th>
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<tbody>
<tr>
<td><strong>Transplantation model</strong></td>
</tr>
</tbody>
</table>
| Subcutaneous CRC cell line xenograft: injection of cells e.g. HCT116 and HT29 to immune deficient mouse | • Low cost  
• Rapid tumour growth (2 weeks)  
• Well characterized cell-lines: gene mutation status, transcriptome and drug response data available [73, 106, 107]  
• Easy to genetically manipulate prior to transplantation e.g. with inducible shRNA or by CRISPR/Cas9 [108]  
• Model accessible to the majority of research labs |
| Orthotopic xenografts of CRC cell lines: injection of cells into intestinal serosa of immune deficient mouse | • As above  
• More natural microenvironment for CRC cells  
• Some metastasise to liver e.g. from HCT116 or HT29 |
| Patient-derived xenografts (PDXs): Suturing of 1–2 mm fragments of fresh surgical specimens of CRC, to intestine of immune deficient mouse | • Reproducible liver metastasis  
• Avoid selection of dominant clones during long-term cell culture  
• Temporary preservation of species-specific tumour-stromal cell cross talk  
• More natural microenvironment for CRC cells |
| Syngraft/Isograft: Suturing 1–2 mm mouse tumour fragments or mouse tumour cell lines e.g. MC38 cells to a genetically identical inbred, immune competent mouse | • No species mismatch between tumour and stromal cells  
• Host has intact immune system that enables testing of immunomodulatory anti-cancer agents |
| | **Weaknesses**                                                                 |
| | • Representative of advanced disease  
• Have undergone significant clonal selection [73]  
• Microenvironmental differences (cells derived from colorectum injected under skin)  
• Species mismatch in tumour (human) and stromal (mouse) cell may limit cross-talk  
• Immune deficient host  
• Rarely metastasise |
| | • As above (except for ‘rarely metastasise’)  
• May require surgery to implant cells |
| | • Not readily scalable  
• Host (mouse) stromal cells replace human stromal cells within a few weeks (species mismatch)  
• Immune deficient host  
• Limited by availability of surgical specimens  
• Expensive (labour intensive and time consuming) |
| | • Expensive (labour intensive and time consuming)  
• The model is not human |
metastatic potential [30]. In an attempt to improve the utility of xenograft models, several investigators have systematically tested different variables and found that CRC cell line, site of injection, mouse age and the genetic background of the mouse all markedly affect the frequency of lymph-node or liver metastases [27, 31, 32]. Orthotopic xenografts involve the injection of differentiated cells to a more natural microenvironment i.e. the serosa of the intestine for CRC cell lines, and appears to result in more reproducible liver metastasis [31, 33]. In addition, orthotopic and subcutaneous xenograft models show differing sensitivities to chemotherapeutic agents [30, 31], suggesting that the microenvironment is important for both disease progression and therapeutic response. Recent refinements to the caecal pouch xenograft method include the nonsurgical trans-anal injection of cells into the distal rectum or the microinjection of cell lines to the caecum and engineering cells to express β-human chorionic gonadotropin to support growth [31, 34].

**Patient-derived orthotopic xenografts temporarily preserve tumour-stromal cell cross-talk and improves reproducibility of metastasis**

Patient derived xenografts (or ‘PDXs’) avoid the natural selection of dominant clones and epigenetic and genetic alterations that occur during long-term cell culture as well as temporarily preserving the original, species-specific, tumour-stromal cell interactions. The suturing of small fragments of fresh surgical specimens of CRC liver metastases to the caecum of immune-deficient mice has been reproducible in generating models of metastatic CRC [35, 36]. It was thought that PDXs could be used to test therapies and development of resistance ahead of patient treatment to help personalize cancer therapy, however the approach requires subculture or serial xenografting which currently takes too long [37]. A number of studies have also now reported that host cells replace the human stroma and vasculature of patient derived xenografts, and in the case of CRCs, this appears to occur relatively rapidly (3 weeks vs. 9 weeks for mesothelioma; [38, 39]).

**‘Syngraft’ or ‘Isograft’ models retain species-specific cross-talk between tumour, stroma and immune cells**

The two major limitations of all xenograft mouse models of human CRC cell lines are the species mismatch between tumour and stroma, which likely affects cell communication, and the use of immune-deficient hosts. The role of the immune system appears to be particularly important for the development of CRC; T cell immune infiltrate is an important predictive criterion for patient survival [40] and mice with deletion of Smad4 in T-cells excessively secrete proinflammatory cytokines and develop gastrointestinal tumours [41]. The use of immune-deficient hosts allows development of tumours in the absence of an immune infiltrate, which is critical for CRC tumour progression, and also precludes the testing of immunomodulatory anti-cancer agents. Grafting tumour fragments or the cell lines derived from them, for example MC38 cells (derived from a mouse colon adenocarcinoma), to a genetically identical inbred, immune competent mouse (‘syngraft’ or ‘isograft’) is a way of overcoming both of these problems [19].

---

**Figure 1.** Mouse models of advanced colorectal cancer (CRC) as research and preclinical tools. The development of genetically engineered mouse models (GEMMs) carrying gene mutations that closely match those found by deep-sequencing of human colorectal carcinomas provide information on the homeostatic and tumorigenic role of a gene in the intestine. Orthotopic transplantation of select colorectal carcinoma cell lines to the caecum of immune-deficient mice results in primary tumour growth and metastases to lymph nodes or liver. Prior to transplantation, colorectal carcinoma cell lines can be genetically manipulated to overexpress (cDNA vector) or inhibit (shRNA or CRISPR-Cas9 vector) a gene of interest to rapidly assess its biological role or to validate potential therapeutic targets. Introducing a reporter gene, such as green fluorescent protein (GFP), to cells prior to transplantation allows in vivo monitoring of primary tumour or metastases growth, which is extremely useful when assessing responses to pharmacological agents or inducible genetic manipulation. Single-cell suspensions from carcinomas can be flow-sorted using cell-specific markers to identify cells of interest, for example stem-like cells for growth of organoids, or to gain further insight into the tumour cells of origin and their interactions with the microenvironment. Cultured cells can be utilised for drug sensitivity screens. Mouse models of advanced CRC can be used to screen for cancer driver genes and disease biomarkers.
Genetically modified mouse models of colorectal cancer retain species-specific cross-talk between tumour, stroma and immune cells

Genetically engineered mouse models (GEMM) offer several advantages over cancer cell lines as research tools in cancer research. Despite their evolutionary distance, the gene content of the mouse and human genomes has been largely conserved through evolution [42], and most cancer pathways are operative in both species [43]. The genomes of inbred laboratory mouse strains are well-characterised and mice offer a ‘clean’ system in which to test the biological effects of genetic alterations or environmental effects; they are essentially homozygous at every locus and are housed in a controlled environment.

Sequencing of DNA from several tumour types that have developed in mouse or man has recently revealed that human tumours are more heterogeneous than their GEMM-derived counterparts [44, 45]. The difference in complexity of mouse and human tumours is likely to be a consequence of factors. Humans have a more varied diet and a different microbiome compared to the laboratory mouse, and so their intestinal cells are exposed to more exogenous genotoxins [46]. In addition, these gene-environment interactions occur over a longer period of time in man; the development of tumours in GEMM is limited by the short lifespan of the mouse or by local research ethics; for example the UK Home Office enforces a tumour size limit of around 1 cm². Furthermore, there is a lack of inter-tumour heterogeneity of GEMM tumours because laboratory mice are inbred and therefore lack the genetic heterogeneity present in the outbred human population. Moreover, GEMM tumours are often initiated and maintained by the same genetic mutation of a strong cancer driver gene, which may limit the number of pathways to tumourigenesis. While the difference in complexity of tumours is advantageous when applying a cross-species oncogenomics approach to cancer gene validation [45], the lack of complexity of mouse tumours poses a problem when trying to faithfully recapitulate the human disease for pre-clinical studies. Some tumour types can be treated in mouse models of cancer, but it is thought that the heterogeneity of human tumours results in a greater potential for resistance to therapy to develop, or for recurrence after surgical resection. Nonetheless, GEMMs of CRC that develop autochthonous intestinal tumours can better model the dynamic interplay between intestinal tumour cells, stroma and the immune system, as well as the response to therapy, when compared with xenograft models.

Genetically modified mouse models of late stage CRC—25 years in the making

Table 3 summarises some of the genetically modified mouse models (GEMM) of CRC that have been developed over the last 25 years. The first key mouse model of CRC was the multiple intestinal neoplasia (Min) mouse, which arose from a random ethylnitrosourea (ENU) mutagenesis screen [47, 48]. The 

**Table 3**

<table>
<thead>
<tr>
<th>Model</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Apc</strong>&lt;sup&gt;Min/⁺&lt;/sup&gt;</td>
<td>Mouse was subsequently recognised as a paralog for human familial adenomatous polyposis (FAP) syndrome and provided confirmation of the causal gene mutation in sporadic CRCs with 5q21 deletions [49]; later studies showed that somatic inactivation of Apc is observed in &gt;80% of sporadic CRCs [2].</td>
</tr>
</tbody>
</table>
| **Hereditary non-polyposis CRC (HNPPC) or Lynch Syndrome** | syndrome, is the most common inherited, autosomal dominant CRC syndrome and accounts for around 3% of all CRCs. HNPPC is most frequently caused by mutations in MLH1, PMS1, PMS2, MSH2 or MSH6, (MutH Homolog 1, Post-Meiotic Segregation Increased 1 and 2 and MutS Homolog 2 and 6, respectively) that encode the enzymatic machinery of DNA mismatch repair. As with sporadic cancers, intestinal cancers from HNPPC patients have a high frequency of mutations in APC, as well as KRAS, TP53 and TGFBR2 [50–52]. Further, approximately 15% of sporadic CRCs display microsatellite instability (MSI) secondary to impaired DNA mismatch repair, frequently because of hypermethylation of the MLH1 promoter [53]. One of the earliest models that had the potential to recapitulate HNPPC was a homozygous Msh2<sup>−/−</sup> deficient mouse [54] (Table 3). Unfortunately, these mice were predisposed to lymphomas, which limited their use as a model of HNPPC. One way to restrict genetic modifications to tissues of interest is to use a Cre-recombinase driven by a tissue-specific promoter. Cre-recombinases catalyse recombinase between two loxP sites that are situated in the introns surrounding a critical exon(s) and result in excision of the DNA between the sites. Using a conditional strategy that results in expression of Cre recombinase from the Villin promoter (intestinal specific gene) a Villin-Cre/Msh2<sup>−/−</sup> mouse was generated that resulted in intestinal adenosomas and adenocarcinomas in the absence of lymphoma [55] (Table 3).
| The short lifespan of the Apc<sup>Min/⁺</sup> mouse limits the utility of the model and it was thought that mice with fewer tumours would live longer, and that if tumours were restricted to the colon, they might progress to carcinoma and metastasise [56, 57]. A number of different Apc mutant mice with germline or inducible cell-type specific conditional alleles of Apc were developed (see Table 3 for examples and for an extensive review, see [58]). Despite evidence of malignant transformation and colonic invasion, aged Apc mutant mice did not develop metastases, suggesting that to better model the later stages of disease in the mouse, additional genetic mutations would be required (Table 3). Apc mutant mice have been crossed to mice with gene mutations found in later stage disease, including TP53, KRAS, TGFBR2, FBXW7 and SMAD2-4, in an attempt to generate more useful pre-clinical models. At best, double mutants displayed more adenocarcinomas, but the mice did not model the metastatic disease (see Table 3). The many different Apc mutant mice that have been created have lead to greater understanding of the biology of Apc, for example, to ascertain which protein domains and functions of Apc are responsible for intestinal tumour initiation and maintenance; it is widely accepted that Apc’s role in activation of the Wnt pathway is the major contributor to this process [59]. Apc mutant mice have also been used to probe the effect of environmental factors and drugs on intestinal tumourigenesis and stem cell specific Apc deletion
### Table 3. Selection of genetically modified mouse models of colorectal cancer

<table>
<thead>
<tr>
<th>Mouse allele</th>
<th>Rationale</th>
<th>Strategy</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apc&lt;sup&gt;Min+&lt;/sup&gt; [47, 48]</td>
<td>• N/A • Ethylnitrosourea (ENU) mutagenesis screen • Tumour multiplicity easily quantifiable</td>
<td>• Germline truncating mutation (N terminus) • Loss of heterozygosity occurs at wildtype allele in tumours</td>
<td>• Model of human familial adenomatous polyposis (FAP) syndrome and &gt;80% of sporadic CRCs contain mutations [2, 4] • Short-lived for rapid studies</td>
<td>• Developed hundreds of low-grade adenomas • Short-lived as tumour burden causes obstruction, prolapse and bleeding • Short life-span means that adenomas do not acquire sufficient mutations to progress to adenocarcinoma and metastasis [56, 57] • Tumours predominantly located in small rather than large intestine</td>
</tr>
<tr>
<td>Msh2&lt;sup&gt;-/-&lt;/sup&gt; [54]</td>
<td>• Msh2 mutations common in CRC</td>
<td>• Germline knock-out</td>
<td>• Model of Hereditary non-polyposis CRC (HNPCC) or Lynch Syndrome (3% of all CRCs)</td>
<td>• Msh2 mutation in all cells of body and mice are predisposed to lymphomas</td>
</tr>
<tr>
<td>Villin-Cre/ Msh2&lt;sup&gt;loxP&lt;/sup&gt;/ [55]</td>
<td>• To restrict malignancy to intestine to prevent lymphoma</td>
<td>• Conditional • Cre recombinase&lt;sup&gt;a&lt;/sup&gt; expressed from promoter of intestinal specific gene (Villin)</td>
<td>• Developed intestinal adenomas and adenocarcinomas • No deaths from lymphoma</td>
<td>• Do not develop metastases</td>
</tr>
<tr>
<td>Apc&lt;sup&gt;580S/580S&lt;/sup&gt; [64]</td>
<td>• To model advanced CRC by restricting tumours to colon and reducing tumour burden</td>
<td>• Conditional Adenoviral Cre recombinase&lt;sup&gt;a&lt;/sup&gt; is administered through the anus to inactivate Apc</td>
<td>• Live &gt;1 year. • Developed two or three intestinal adenomas • Some mice developed adenocarcinomas</td>
<td>• Do not develop metastases • Conditional allele (unactivated) reduces Apc expression in all cells, similar to Apc&lt;sup&gt;Min+&lt;/sup&gt; [10], which results in development of life-limiting hepatocellular carcinomas by 14 months (REM unpublished observations of Apc&lt;sup&gt;Min/+&lt;/sup&gt;Cre&lt;sup&gt;-/&lt;/sup&gt;)</td>
</tr>
<tr>
<td>Apc&lt;sup&gt;lox14/lox14&lt;/sup&gt;, Fabpl-Cre [109]</td>
<td>• To model advanced CRC by restricting tumours to the colon and reducing tumour burden</td>
<td>• Germline mutations in Apc and Trp53</td>
<td>• Live &gt;1 year. • Developed two or three intestinal adenomas • Some mice developed adenocarcinomas</td>
<td>• Do not develop metastases</td>
</tr>
<tr>
<td>Apc&lt;sup&gt;lox5/lox5&lt;/sup&gt;, Trp53&lt;sup&gt;-/-&lt;/sup&gt; [110]</td>
<td>• To model advanced CRC through addition of cooperating gene mutations</td>
<td>• Conditional • Cre recombinase&lt;sup&gt;a&lt;/sup&gt; is expressed in the distal small intestinal and colonic epithelia to inactivate Apc</td>
<td></td>
<td>• Do not develop metastases</td>
</tr>
<tr>
<td>Apc&lt;sup&gt;2lox14/lox14&lt;/sup&gt;, Kras&lt;sup&gt;LSL-G12D&lt;/sup&gt; and Fabpl-Cre [111]</td>
<td>• To model advanced CRC through addition of cooperating gene mutations</td>
<td>• Conditional • Cre recombinase&lt;sup&gt;a&lt;/sup&gt; is expressed in the distal small intestine/colon/epithelia and results in constitutive activation of K-ras and inactivation of Apc</td>
<td>• Developed more adenocarcinomas than single mutant (Apc or Kras alone) control mice</td>
<td>• Do not develop metastases</td>
</tr>
<tr>
<td>Apc mutant with disruption of Tgfb2, Smad2 or Smad4. [112–114]</td>
<td>• To model advanced CRC through addition of cooperating gene mutations</td>
<td></td>
<td>• Developed more adenocarcinomas than single mutant control mice.</td>
<td>• Do not develop metastases</td>
</tr>
<tr>
<td>Apc&lt;sup&gt;CRCkre&lt;/sup&gt;/ Villin Cre Fbxw7&lt;sup&gt;+/loxp&lt;/sup&gt; [115]</td>
<td>• To model advanced CRC through addition of cooperating gene mutations</td>
<td>• Germline mutation of Apc • Conditional mutation of Fbxw7 • Cre recombinase&lt;sup&gt;a&lt;/sup&gt; expressed from promoter of intestinal specific gene (Villin)</td>
<td>• Decreased lifespan • Increased tumour burden • Fbxw7 null control mice developed adenomas by 9-10 months of age</td>
<td>• Do not develop adenocarcinomas or metastases</td>
</tr>
<tr>
<td>Apc&lt;sup&gt;CRCkre&lt;/sup&gt;/ LSL-G12D; Kras&lt;sup&gt;LSL-G12D&lt;/sup&gt; [82]</td>
<td>• To model advanced CRC through addition of cooperating mutations. To restrict tumours to the colon and reduce tumour burden</td>
<td>• Adenoviral Cre solution administered via the anus to simultaneously disrupt Apc and activate K-ras</td>
<td>• Developed adenocarcinomas after five months • Developed metastases to distant organs after six months e.g. liver • In vivo monitoring via colonoscopy</td>
<td>• Labour intensive mouse surgery required to clamp a section of colon to deliver Adenoviral Cre solution to the colon via the anus</td>
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</table>

CRC, Colorectal carcinoma.

<sup>a</sup>Cre recombinases catalyse recombination between two lox<sup>P</sup> sites that are situated in the introns surrounding a critical exon(s) and result in excision of the DNA between the sites.
models lent support to the so-called ‘bottom-up’ model of adenoma formation [60, 61].

One of the most promising models for both the elucidation of the molecular events that support the formation of distant metastases of colorectal cancer and for preclinical investigations is a mouse that has simultaneous, inducible inactivation of Apc and activation of Kras in the adult colon (Table 3). Six months after surgery 20% of Apc<sup>CKO/CKO</sup> LSL-Kras mice (G12D; Kras<sup>m4tv</sup>/+) allele mice had developed metastases to distant organs such as the liver [62]. It is important to note that expression of wild-type Apc may be disrupted by introduction of conditional alleles, which may elevate Wnt signalling levels in tissues other than the intestine, and which appears to drive the formation of life-limiting hepatocellular tumourigenesis before the onset of intestinal tumourigenesis. For example Apc<sup>B−/−</sup> and Apc<sup>SROS/SROS</sup> mice show reduced expression of Apc protein throughout their tissues (in the absence of Cre-mediated recombination of alleles), and develop hepatocellular carcinomas between 9 and 15 months (our unpublished observations of Apc<sup>SROS/SROS</sup> mice; [63, 64]). These findings may preclude the use of some mouse models in studies that aim to understand the events that drive the metastasis of CRC to the liver.

**Mouse models of inflammatory bowel disease and colorectal cancer**

Inflammatory bowel diseases (IBD) such as ulcerative colitis predispose patients to developing colorectal cancer as a result of chronic inflammation. It is becoming apparent that these tumours may develop through alternative TP53 dependent routes compared with classical APC-driven tumours [65]. In an attempt to model this disease process mice have been generated that are predisposed to intestinal specific inflammation, including II-10−/−, II-2<sup>null</sup> and Muc2−/− deficient strains [66, 67]. Colitis can also be experimentally induced by the administration of dextran sulfate sodium (DSS) to the drinking water of mice. In addition to mimicking IBD, mice treated this way also produce intestinal tumours [68]. Azoxymethane (AOM) is another commonly used chemical that predisposes to cancer experimentally. AOM is thought to enable base mispairings, and when administered alone or in combination with DSS, AOM reliably produces tumourigenesis in wild type mice [69].

**Genetic screens in mice for validation of cancer ‘driver’ genes**

TCGA has identified genes that are frequently mutated in many different types of malignancy (frequently referred to as ‘pan-cancer’ genes) suggesting that they are likely to accelerate the process [70]. While these genes are obvious targets for therapy, many are considered intractable drug targets, including MYC and RAS. Within different cancer sub-types there are cancer drivers that could be more amenable to therapy, however it is difficult to make the distinction between the ‘driver’ and ‘passenger’ genes (mutations that drive disease progression and mutations that do not) in patient specimens because of genetic, epigenetic and environmental heterogeneity. In contrast, mouse models, with their homogeneous genetic background and living environment, can be an efficient tool for validating candidate cancer genes (Figs. 1 and 2). Forward genetic screens in mice using insertional mutagenesis have aided the identification or validation of many drivers of cancer, the relevance of which is assessed by comparing the candidates identified through screening with mutations found in the human cancer subtype [45].

One of the first insertional mutagenesis studies to identify novel genetic drivers of intestinal tumourigenesis involved restricting transposition to epithelial cells of the gastrointestinal tract using a Villin-Cre recombinase system [71]. Analysis of the common insertion sites for transposon in neoplasms, adenomas and adenocarcinomas revealed that 80% of insertions fell in known CRC genes including Apc, FBXW7 (F-box and WD repeat domain containing 7), PTEN (phosphatase and tensin homolog), and SMAD4, thus validating the approach. The remainder, including RSPO2 (R-spondin 2), had not been previously implicated in the disease. R Spondin is a ligand for LGR4-6 receptors (LGR, leucine-rich repeat containing G protein-coupled receptor), which stimulate the canonical Wnt signalling pathway. We now know that RSPO2 fusion genes occur in a subset of colorectal carcinomas and are mutually exclusive with APC mutations [7].

To identify mutations that cooperate with inactivation of Apc and hyperactivation of the Wnt pathway, two independent teams of researchers mobilised the Sleeping Beauty transposon in Apc<sup>Min/+</sup> mice or conditional Apc<sup>B−/−</sup> mice [6, 72]. The most frequently mutated gene (~85%) in both studies was Apc, consistent with the requirement for loss of heterozygosity of Apc for intestinal tumour induction. Interestingly, all three mutagenesis screens identified a high number of insertions in the WW domain-containing adaptor with coiled-coil gene (Wac), which is also mutated in human cancers [6, 71–73]. WAC has recently been shown to be required for activation of the cell-cycle checkpoint in response to DNA damage suggesting that inactivation may cause genomic instability [74]. Both insertional mutagenesis studies of Apc mutant mice identified common insertions in the voltage-gated potassium channel subunit Kcnq1 [6, 72]. Mutations in voltage-gated ion channel subunits have been identified in a variety of human cancers, including colon, and mounting evidence suggests that tumour cells have a depolarised membrane voltage relative to surrounding tissue, which could be exploited for therapy [75]. In support of this finding, Apc<sup>Min/+</sup> Kcnq1<sup>−/−</sup> double mutant mice developed more intestinal adenomas than Apc<sup>Min/+</sup> control mice, and progression to aggressive adenocarcinomas was observed. The authors went on to show that reduced KCNQ1 expression correlated with poor survival in patients; however, the exact mechanism of tumour suppression remains unknown [76]. These examples prove the utility of transposon-mediated insertional mutagenesis for rapid validation of colorectal cancer genes and for colorectal cancer gene discovery.
**Apc<sup>Min/+</sup>** mice for early disease biomarker discovery

Most screening strategies aimed at the early detection of adenomas and CRC involve endoscopy, and thus have limited applicability. Early detection of CRC is critical for disease management, and so the identification of reliable, early disease biomarkers would improve the outcome for many patients. Mouse models that are predisposed to colorectal tumour formation can be an efficient tool for early disease biomarker discovery (Fig. 2). Since early detection of CRC is critical for disease management, investigators have used mice with germine Apc mutations, which model the initiation of intestinal tumourigenesis, to screen for early disease biomarkers. Clusterin was a ‘proof-of-principle’ for the cross-species approach to biomarker discovery [77]. Uregulation of clusterin was reported in adenomas from Apc<sup>Min/+</sup> mice and human tumours, and was recently shown to be highly-expressed in intestinal stem cells [77, 78]. Secreted clusterin is cytoprotective and its pro-survival function forms the basis of the current Phase I/II clinical trials against prostate, lung, and breast cancers [79].

**Cross-species proteomics identifies early disease biomarkers**

Recent improvements in proteomic profiling methods, based primarily on mass spectrometry, have allowed the detection and identification of peptides in the blood and biological fluids of cancer patients (Fig. 2). The plasma of tumour-bearing Apc<sup>Fabs</sup>/A mouse was screened by mass spectrometry, and cathepsin B and D were found to be elevated compared with control mice [80]. Cathepsins are involved in the degradation of the basement membrane and extracellular matrix, a key step in tumour invasion and metastasis. Cathepsin B and cathepsin D are thought to associate with tumour grade of CRC and survival suggesting they may be useful biomarkers of the disease [80, 81]. Similarly, proximal fluid proteome profiling of colon tumours from aged Apc<sup>580S</sup>/C<sup>223</sup> Fabpl-Cre mice resulted in the identification of 192 proteins that were more highly excreted by tumours relative to control, including Chitinase 3-like 1 (Chi3l1) [82]. CHI3L1 is thought to be involved in processes such as inflammation and tissue remodeling, and was shown to be significantly elevated in the sera of patients with adenomas and advanced adenomas compared with control individuals [83].

**Cross-species epigenomics identifies epigenetic signatures of early disease**

Epigenomic profiling of adenomas from Apc<sup>Min/+</sup> mice has also been useful in the identification of patient relevant, early disease biomarkers (Fig. 2). Immunoprecipitation of methylated DNA followed by sequencing (known as ‘MeDIP-seq’) identified regions of the genome were differentially methylated [84]. These regions were mainly enriched for targets of the Polycomb Repressive Complex, the subunits of which, together with those for several DNA methyltransferase complexes, were up-regulated in adenomas. This genome-wide pattern was shown to be partly conserved in human colon carcinomas, suggesting that an epigenetic signature is established early and is retained during progression from adenoma to carcinoma.

**Target identification and validation requires cancer cell lines, xenograft models and GEMM**

Not all cancer genes are essential for the sustained growth of established tumours. In addition, tumours may have intrinsic therapeutic resistance, which is believed to arise through natural selection of a latent subclone within a tumour, or acquired therapeutic resistance, which arises through acquisition of a genetic mutation that allows the cancer cell to survive in the presence of the therapeutic agent [15]. It is therefore important to understand both the contribution of a
disease target to tumourigenesis and the mechanisms by which cancers evade targeted therapies. Here, we highlight several recent approaches to the identification and validation of novel therapeutic targets using mouse models of CRC.

Although genetic ablation or overexpression of potential therapeutic targets in CRC cell lines before xenotransplantation does not necessarily mimic pharmacological intervention, nor does it mimic the biology of autochthonous tumours, it may provide a shortcut to target validation (Fig. 1). This approach has recently been useful for the validation of potential therapeutic targets within the Wnt and TGFβ pathways.

**Targeting the Wnt pathway—reality or drug discovery pipe dream?**

Since the Wnt–β-catenin signalling pathway is hyperactivated in 93% of colorectal tumours and high Wnt activity defines colon cancer stem cells, the Wnt pathway is the most attractive therapeutic target for colorectal cancer [2, 29, 85]. Despite this, therapeutic agents that specifically target the Wnt pathway have only recently entered clinical trials, and none has yet been approved. The Wnt inhibitor XAV939 inhibits the poly(ADP)-ribosylating (PARP) enzymes tankyrase 1 and tankyrase 2, which interacts with AXIN (acts with APC to destruct β-catenin and damper Wnt pathway activity) to promote its ubiquitylation and degradation [86]. The development of more selective and potent second-generation tankyrase inhibitors is in progress, however their anti-tumour potential may not be fully realised owing to their intestinal toxicity in pre-clinical mouse models [9]. Rosenbluh and colleagues took a more targeted approach to inhibition of the Wnt pathway: they screened 85 cancer cell lines and found that β-catenin active cancers are dependent on a signalling pathway involving the transcriptional regulator YAP1 (Yes-associated protein 1) [28]. YAP1 and the transcription factor TBX5 were shown to form a complex with β-catenin, and phosphorylation of YAP1 by the tyrosine kinase YES1 lead to localisation of the complex to the promoters of anti-apoptotic genes such as BIRC5. To determine whether YAP1 was required for HCT116 xenograft growth they transfected cells with a doxycycline-inducible shRNA to YAP1. Three weeks after injection, tumour growth was restricted to 80% of control animals, suggesting that the YAP1 pathway might be an attractive therapeutic target [28]. Further studies will be required to confirm whether this effect occurs in vivo, where many endogenous ligands that are secreted by stromal cells are known to modify Wnt pathway activity [22].

**Pharmacological inhibition of TGFBR1 inhibits metastasis formation**

A large proportion of CRCs display mutational inactivation of the TGFβ pathway. However, paradoxically, they are characterised by elevated TGFβ production. In other cancer types, such as breast or prostate, which retain a functional TGFβ signalling pathway, TGFβ induces a variety of prometastatic programmes that range from induction of epithelial-to-mesenchymal-like transition (or ‘EMT’) to expression of genes that allow colonisation of foreign organs [93]. During 10 years of follow-up after surgical resection of colorectal tumours, only patients with medium or high TGFβ expression in the primary tumour suffered cancer recurrence, whereas all patients bearing TGFβ-low stage I, II and III tumours remained disease-free [94]. When Nude mice were inoculated with KM12L4a cells that overexpressed TGFβ, nearly all developed lung and/or liver metastasis, whereas only one third of control mice developed metastases. It is thought that the metastatic KM12L4a-TGFβ cells were conferred a survival advantage by suppressing apoptotic stimuli encountered during organ colonization. Moreover, inhibition of TGFBR1 has been shown to inhibit metastasis formation in multiple xenograft models [94, 95]. Since the mechanism is thought to involve cross-talk between tumour and stromal cells, it will be important to show that this regulation occurs in autochthonously occurring liver metastases of CRC, such as those that arise in the conditional Apc<sup>CKO/CKO/KrasG12D</sup> colon cancer model [62].

**Targeting MYC—drugging the undruggable**

The transcription factor Myc proto-oncogene protein (Myc) is thought to be a target of the β-catenin–Tcf4 transcription complex in CRC cells in vitro, in normal crypts in vivo and in intestinal epithelial cells acutely transformed on in vivo deletion of the Apc gene [59, 87, 88]. Array expression analysis revealed that Myc is required for the majority of β-catenin–Tcf4 transcription complex gene activation following Apc loss in mice. While Myc is required for the formation of intestinal crypts, it is dispensable for homeostasis of the adult intestinal epithelium [89]. Using a combination of conditional mouse models, it was shown that simultaneous inactivation of Myc and Apc completely ablated tumour development in mice despite high levels of nuclear β-catenin in the intestinal epithelium [90]. Multiple cancer-associated single-nucleotide polymorphisms (SNPs) have been mapped to conserved sequences within a 500-kilobase region upstream of the MYC oncogene on human chromosome 8q24, and mice lacking a Myc enhancer (Myc-335) that includes human SNP rs6983267 are resistant to intestinal tumours induced by the ApcMin mutation [91]. While these studies have established Myc as a critical mediator of the early stages of intestinal neoplasia following Apc loss, it has not yet been possible to pharmacologically regulate Myc. Instead, a selective small-molecule BET bromodomain inhibitor, JQ1, inhibits proteins that are regulatory factors for Myc [92]. Treatment with JQ1 resulted in genome-wide downregulation of Myc-dependent target genes, reduced proliferation and had anti-cancer properties in a mouse model of multiple myeloma, a Myc-dependent hematologic malignancy, suggesting that JQ1 may perform similarly in mouse models of colorectal cancer.

**Isograft models for validating the role of the immune system in CRC**

In a recent study, Bindea and colleagues examined the spatio-temporal dynamics of 28 different immune cell types (the
"immunome") that were infiltrating colorectal tumours were analysed by whole-genome expression, PCR arrays and multicolored immunohistochemistry [96]. The analysis revealed that patients with elevations in genes related to MHC-II, B cell co-stimulation, T cells and Th cells experience increased survival. One chemokine in particular, CXCL13, and one cytokine, IL-21, positively correlated with disease-free survival. Bindea and colleagues showed growth acceleration of MC38 isografts in Cxcr5−/− mice (CXC5R is the receptor for CXCL13) compared to control, and rejection of tumour cells when recombinant CXCL13 was injected into the colonic submucosa of wild-type mice before transplantation [96]. These data confirmed the prognostic value of CXCL13 in assessing CRC tumour burden, and prove the utility of mouse models in the validation of disease biomarkers.

Conclusions and outlook

In combination with studies of CRC cell lines and organoid models, mouse models of late-stage CRC will help us to build a comprehensive picture of the molecular alterations that drive tumour formation and disease progression, through cross-species analysis of the genomes, epigenomes, transcriptomes, proteomes and even immunomes of cancers of mice and men. Mouse models will be critical for reductionist approaches that aim to validate the cancer drivers from mutations identified by the TCGA.

Human CRC is the result of interactions between genes, including those involved in host immunity, and the environment, including diet and the microbiome [97, 98]. The mouse affords the ability to investigate each of these factors independently of each other, which is simply not possible in man. However, recent studies have shown that 'pound for pound’, the average mouse tumour is less complex than the average human tumour. These sequencing studies, together with the knowledge that lifestyle factors strongly influence the chances of getting cancer, suggest that the relative simplicity of GEMM tumours is due to the lack of gene-environment interactions in the controlled environment of the laboratory mouse. There is a reasonable body of evidence to suggest that high red meat intake and alcohol consumption are risk factors for CRC, but we are probably only just beginning to understand how our environment affects the course of disease [99]. Modelling the full heterogeneity of human tumours will be required to help tackle the problems of therapeutically resistant recurrence after surgical resection in CRC; this might be better achieved by generating GEMM that combine a genetic predisposition to CRC with a diet rich in foods associated with increased risk of CRC.

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References


