Pre-clinical modelling of rectal cancer to develop novel radiotherapy-based treatment strategies

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Abstract

Pre-operative chemoradiotherapy reduces local recurrence rates in locally advanced rectal cancer. 10-20% of patients undergo complete response to chemoradiotherapy, however, many patients show no response. The mechanisms underlying this are poorly understood; identifying molecular and immunological factors underpinning heterogeneous responses to chemoradiotherapy, will promote development of treatment strategies to improve responses and overcome resistance mechanisms. This review describes the advances made in pre-clinical modelling of colorectal cancer, including genetically engineered mouse models, transplantation models, patient derived organoids and radiotherapy platforms to study responses to chemoradiotherapy. Relevant literature was identified through the PubMed and MEDLINE databases, using the following keywords: rectal cancer; mouse models; organoids; neo-adjuvant treatment; radiotherapy; chemotherapy. By delineating the advantages and disadvantages of available models, we discuss how modelling techniques can be utilized to address current research priorities in locally advanced rectal cancer. We provide unique insight into the potential application of pre-clinical models in the development of novel neo-adjuvant treatment strategies, which will hopefully guide future clinical trials.

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Introduction

Improved loco-regional control has been observed in locally advanced rectal cancer (LARC) over the past three decades as a result of refinement of surgical and neo-adjuvant treatment strategies. 'Total Mesorectal Excision' (TME) has been widely adopted as the gold standard surgical treatment, due to significantly improved local recurrence rates.[1] Subsequently, several landmark trials have demonstrated further reduction in recurrence rates when radiotherapy or chemoradiotherapy (CRT) is given pre-operatively. Two neo-adjuvant treatment strategies are now widely accepted - short course radiotherapy (25Gy in 5 fractions) and long course CRT (45 - 50.4Gy in 25-28 fractions, with concurrent fluoropyrimidine based chemotherapy).[2,3] The Dutch Colorectal Cancer Group demonstrated a 2-year local recurrence rate of 2.4% when short-course radiotherapy was administered before TME, compared with 5.3% in patients undergoing TME alone.[4] The MRC CR07/NCIC-CTG C016 multi-center trial also demonstrated the benefit of radiotherapy, with a 3-year local recurrence rate of 4.4% observed with short-course radiotherapy prior to TME, compared with 10.6% following selective post-operative CRT.[5] The German Rectal Cancer Study Group trial demonstrated a 5-year local recurrence rate of 6% when pre-operative CRT was administered for T3/T4 or node positive tumors, compared with 13% following post-operative CRT.[6] Short-course radiotherapy and long-course CRT are widely accepted neo-adjuvant strategies for LARC, facilitating tumor shrinkage and margin free resection. The Trans-Tasman Radiation Oncology Group trial compared short-course radiotherapy with long-course CRT pre-operatively in patients with T3 tumors, demonstrating equivalent 3-year local recurrence rates of 7.5% and 4.4% respectively.[7] Interestingly, long term follow up of the Dutch Colorectal Cancer Group and German Rectal Cancer Study Group trials, failed to show improvement in overall survival or distant metastasis rates in patients treated with neo-adjuvant radiotherapy or CRT.[8,9]

In recent years, organ preservation has emerged as a novel and attractive treatment paradigm in LARC gaining significant sup-

port from patients and clinicians alike, with potential to avoid the morbidity and mortality risks associated with surgery.[10] Non-operative management following CRT was first reported by Habr-Gamr *et al.* in 2004, where clinical complete response (cCR) was observed in 26.8% of patients (n=265) with resectable T2-4 distal rectal adenocarcinoma.[11] Recently, the OnCoRe project (Oncological Outcomes after Clinical Complete Response in Patients with Rectal Cancer) studied 129 patients managed by a 'watch and wait' strategy.[12] Although 44 patients (34%) developed local regrowth within 3-years, the majority (82%) successfully underwent salvage surgery. Similarly, retrospective analysis of 113 patients with cCR treated conservatively at the Memorial Sloan Kettering Cancer Centre was carried out; 22 patients (19%) required salvage surgery, with pelvic control achieved in 91%.[13] Approximately 20% of patients are potentially suitable for non-operative management, and a recent systematic review reported a 22.4% incidence of cCR following neo-adjuvant CRT across 17 studies comprising 692 patients.[14] However, concern remains over local regrowth and distant metastasis, and current evidence is not robust enough to support surveillance as a standard approach out with the setting of a well-designed clinical trial.[15] The International Watch and Wait Database (IWWD) study, a registry of pooled individual patient data, detailed the oncological outcomes of surveillance strategies following cCR to neo-adjuvant treatment.[16] A 2-year local recurrence rate of 25.2% and 3-year distant metastasis rate of 8.1% (n=880) were described, further highlighting that surveillance strategies would require careful patient selection, rigorous follow-up and dedicated centers.

Refinement of neo-adjuvant strategies may expand the potential for organ preservation by achieving cCR in more patients. Novel drug-radiotherapy combinations or extended regimens may help to achieve this. For example, total neo-adjuvant therapy (TNT) strategies are being developed, with additional systemic chemotherapy (induction or consolidation) and CRT administered prior to surgery, to optimize administration of systemic therapy and potentially target occult micro-metastases at an early stage.[17] Cercek *et al.* demonstrated significant benefit with the addition of induction chemotherapy to standard CRT, importantly showing a combined clinical and pathological response rate of 36%, compared with 21% in the standard CRT group.[18] However, recent meta-analysis of treatment outcomes from TNT reveals modest improvement, with a pooled pathological complete response (pCR) rate of 22.4% reported.[17] Longer follow-up periods will determine whether TNT regimens result in improved disease-free survival and distant metastasis rates.

Future clinical trials must better address the need to optimize complete cCR rates, promote organ preservation strategies and reduce distant recurrence. By identifying immunological and molecular factors associated with sensitivity and resistance to CRT, novel therapeutic agents can be developed to augment treatment effects and overcome resistance mechanisms. Treatment resistance is seen in a significant proportion of patients, with recent multi-center observational data reporting no tumor regression in 53.8% of patients (n=649) following CRT.[19] Robust markers to predict response to CRT are lacking; developing methods to predict response to neo-adjuvant CRT will aid treatment planning, patient consent and facilitate individualized treatment.[20] Pre-clinical models have been invaluable tools to improve our understanding of the molecular mechanisms underpinning colorectal cancer (CRC) initiation and progression. Until recently, models have poorly recapitulated locally advanced and metastatic disease. Here we describe recently developed disease models and irradiation platforms for advanced CRC research, and discuss their potential utilization in addressing current research priorities in LARC.

Studying the effects of radiotherapy in pre-clinical models

Most pre-clinical irradiation studies have involved whole body irradiation (WBI), however, radiation-induced gastrointestinal syndrome (RIGS) and hematopoietic syndrome are a cause of significant toxicity associated with such platforms.[21] Whole body irradiation studies have provided useful insight into radiation-induced intestinal regeneration, for instance, demonstrating that Lgr5+ intestinal stem cells are crucial for robust intestinal regeneration following radiation exposure.[22] However, newly developed platforms to deliver targeted radiotherapy to animal models offer significant advantages over WBI, by allowing image-guided irradiation of targeted tissues, with repeated fractions and minimal radiotherapy related side effects. Development of the Small Animal Radiation Research Platform (SARRP; XStrahl) now allows delivery of high-precision radiotherapy in the pre-clinical setting, and more closely recapitulates the targeted and fractionated regimens administered in the clinical setting.[23,24] This platform has been tested in other disease models including glioblastoma and lung cancer to evaluate radiotherapy techniques and novel radiotherapy-drug combinations.[25,26] A recent study by Grapin *et al.*, used a SARRP in a murine subcutaneous CRC transplant model, to assess different radiotherapy fractionation regimens in combination with anti-PD-L1 (programmed death ligand 1) and anti-TIGIT (T-cell immunoreceptor with Ig and ITIM domains).[27] The study demonstrated that different lymphoid and myeloid responses were induced by different radiotherapy fractionation regimens, and highlights the ability to study novel drug-radiotherapy combinations and determine optimal scheduling in a pre-clinical setting. Furthermore, murine colonoscopy systems represent a simple means to assess treatment response in site-specific rectal cancer models.[28] In the context of pre-clinical studies to improve neo-adjuvant treatment strategies for rectal cancer, technological advances in the delivery of radiotherapy to small animals must be coupled with recent developments in disease modelling, so that irradiation studies are carried out in anatomically accurate models which closely represent the human condition.

Adapting models for radiotherapy studies: genetically engineered mouse models of colorectal cancer

Several disease modelling systems for CRC exist, and we will discuss genetically engineered mouse models (GEMMS), transplant models and patient derived organoids, while summarizing the key advantages and disadvantages of each system (Table 1). GEMMs have become increasingly sophisticated over the past three decades, however, the majority of CRC models do not specifically recapitulate the clinical scenario of rectal cancer, where tumors are situated in the pelvis at a short distance from the anal verge. Development of GEMMs of intestinal cancer began in the early 1990s, following identification of the Adenomatous polyposis coli (APC) tumor suppressor gene in 70-80% of sporadic CRC.[29] Early GEMMs contributed to our understanding that loss-of-function APC mutations drive pre-cancerous adenoma formation, and that malignant CRC progression occurs through subsequent mutations in other key driver genes *e.g.* KRAS, TP53, SMAD4 and PIK3CA, and through activation of the Wnt signaling pathway.[30] The first Apc mutant mouse model was generated by introducing the germ line mutagen N-ethyl-nitrosurea (ENU) to cause a loss of function Apc gene mutation.[31,32] Spontaneous loss of Apc heterozygosity, resulted in mice developing multiple small intestinal adenomas and a small number of colonic polyps. This

early MIN (multiple intestinal neoplasia) or $Apc^{Min/+}$ model is a significant departure from the human condition, as polyps predominantly develop in the small intestine and fail to progress to invasive carcinoma. However, the $Apc^{Min/+}$ model facilitated discovery of molecular mechanisms in early CRC and enabled functional testing of other genes driving progression. For instance, Sansom *et al.* demonstrated that acute activation of Wnt signaling follows Apc loss in murine small intestinal epithelium, leading to several phenotypic changes associated with early colorectal lesions.[33] In addition, driver mutations have been modelled to develop our understanding of the molecular basis of the adenoma-carcinoma-metastasis sequence, including Kras mutation, p53 loss, Smad2 loss, and Smad4 loss.[34-38]

GEMMs have also been used to investigate the serrated colorectal neoplasia pathway, which accounts for ~20% of CRC cases.[39] Rad *et al.* illustrated the role of BRAF mutations as a driver of this alternative pathway, with sustained intestinal hyperplasia observed in Braf knock-in mice; the authors showed that consequent MAPK (mitogen-activated protein kinase) signaling intensification drove tumor progression and conversely had a role in the activation of intrinsic tumor suppression.[40] Early CRC models predominantly develop small intestinal tumors, with high tumor burden (typically 30-100 polyps) and tumor progression limited beyond early adenoma formation.

Technological advances have overcome many of the drawbacks of early GEMMS, and helped development of clinically relevant CRC models. Cre-Lox technology has allowed the generation of conditional Apc mutant mouse models, with selective mutational expression in tissues of interest.[41] Furthermore, Cre-Lox technology enables mouse models to be developed with mutations which are constitutively active or expressed selectively on induction. The Cre recombinase enzyme in bacteriophage P1 effects recombination between pairs of loxP gene recognition sites, which are inserted to flank a genomic segment of interest; Cre recombinase then induces deletion, inversion or translocation of the 'floxed' locus in Cre-expressing cells.[42] Shibata *et al.* demonstrated colonic adenoma formation within 4 weeks of delivery of Cre-recombinase via an adenovirus vector injected through the anus of a mouse carrying the mutant Apc^{580S} allele.[43] Furthermore, Cre-expressing transgenic mice expressing homozygous Apc deletion can be generated to develop colonic adenomas without requiring surgical manipulation.[44] Ligand-dependent Cre-recombinase systems have been developed, such as tamoxifen-dependent Cre recombination, whereby recombination occurs throughout the digestive epithelium under the control of the Villin promoter (vil-Cre-ER^{T2}) following tamoxifen injection.[45,46] Cre-lox technology has enabled GEMMs with multiple CRC signature mutations to be developed in a time- and tissue-specific manner, which are more anatomically and histologically representative of the human condition, enabling the clinical scenario to be replicated more readily.

Table 1. Key features of tumor model systems.

Model system	Advantages	Disadvantages
Genetically engineered mouse models		
Transgenic oncogene expression	Enables mechanistic studies of genetic mutations of interest	- Typically demonstrate small intestinal polyps/adenomas - Limited ability to demonstrate progression beyond adenoma - Long latency to tumor development - Do not model genomic heterogeneity seen in clinical practice
Recombinase systems (Cre-Lox)	- Enables induction of multiple genetic mutations - Time and tissue-specific - Tumors develop at the relevant site and tissue layer	- Limited ability to demonstrate metastasis - Long latency to tumor development - Expensive - Low throughput
CRISPR/Cas-9 genome editing	- Enables manipulation of the entire genome - Time and tissue-specific - Tumors develop at the relevant site and tissue layer - Capacity to reverse genetic mutations - Can be utilized *ex vivo* (*e.g.* organoid cultures) - Lower cost than conventional GEMMs	- Limited ability to demonstrate metastasis - Long latency to tumor development - Low throughput
Transplant models		
Surgical transplant	- Time efficient models - Able to recapitulate invasiveness and metastasis	- Failure of cell lines to recapitulate colorectal cancer histology - Tumors not anatomically representative - Commonly use immunocompromised mice
Colonoscopy guided injection of organoids	- Tumors develop at correct anatomical location - Can be utilized in immunocompetent mice - Easily reproducible - Time efficient model enabling high throughput - Organoids can be genetically manipulated	- Tumors do not arise in mucosal layer - Low penetrance of metastatic disease
Non-animal models		
Patient derived organoids	- Avoids animal studies - Tissue easily obtained through biopsy before treatment - Time effective - Potential utility as a predictive tool	- Lack of host stroma and immune system - Labor intensive to employ in routine clinical practice

More recent development of CRISPR/Cas9 technology has allowed specific genome editing.[47] Hsu *et al.* used multiple RNA guide sequences encoded into a single CRISPR (clustered regularly interspaced short palindromic repeats) array to enable editing of several sites within the mammalian genome, and showed applicability to targeting genes in mouse models.[48] CRISPR-Cas9 genome editing involves transfection of a cell with Cas9 protein and a guide RNA, directing Cas9 nuclease localization to specific DNA sequences. Double strand breaks occur in the target sequence, with gene knockout or modification due to the error prone non-homologous end joining repair process, or following homologous repair if a template is provided. Using this system, GEMMs can be generated quickly with multiple genes edited.[49] Dow *et al.* exploited CRISPR/Cas9 technology to develop GEMMs with conditional and reversible control of Apc expression.[50] In this model, doxycycline was used to initiate Apc silencing, with expression restored upon withdrawal of doxycycline, resulting in sustained regression of established tumors. CRISPR/Cas9 technology has further advanced the capabilities of GEMMs, with application in embryo providing a more rapid and cost-effective method than other genome editing technologies.

Despite technological advances, GEMMs have been limited in their capacity to recapitulate the entire adenoma-carcinoma-metastasis sequence. Using Cre-Lox technology, Boutin *et al.* developed a GEMM of metastatic CRC with an inducible oncogenic Kras allele and conditional null alleles of Apc and Trp53 ('iKAP model').[51] This study demonstrated that oncogenic Kras signaling was essential for progression to metastasis in this model, which was observed in 25% of mice. However, other studies exhibiting metastasis in autochthonous models show low penetrance, long latency and lack of Apc mutation.[52] Recently, Jackstadt *et al.* established that activation of epithelial Notch1 signaling in a Kras-driven serrated cancer GEMM resulted in a highly penetrant metastatic model of CRC.[53] The paucity of Apc-driven metastatic GEMMs deviates from the human condition of metastatic CRC, and represents a significant limitation of autochthonous CRC models. In addition, application to pre-clinical studies is impinged by slow tumor growth (typically 4-6 months), and their inability to reliably recapitulate the entire adenoma-carcinoma-metastasis sequence.[54] However, GEMMs can accurately replicate the anatomy and histology of CRC, and enable investigators to manipulate the entire genome to study relevant combinations of CRC signature mutations. These valuable tools have advanced our understanding of the molecular basis of CRC; we have discussed the key developments in genetic engineering techniques, however, many other GEMMs of CRC have been described and are described in other detailed reviews.[55-57]

Transplant models

Transplant models of CRC are widely used in pre-clinical research, with early attempts involving implantation of human colon cancer cell lines into immunocompromised mice. Fidler *et al* injected cells via laparotomy into the mouse caecum or spleen, resulting in hepatic metastases.[58] However, this method deviates from the human condition as splenic injection allows the complex molecular events driving metastasis to be bypassed. Kashtan *et al*. specifically attempted to model rectal cancer, injecting either human or murine cancer cell lines directly into the rectal submucosa, using a rectal prolapse and needle injection technique.[59] This study established large rectal carcinomas, as well as locally aggressive tumors with lymph node metastases. A similar submucosal distal colonic injection method described by Donigan *et al.*, demonstrated a 65% tumor engraftment rate without metastasis.[60] Modelling of rectal cancer can be aided by inducing colitis using dextran sulfate sodium treatment. Takahashi *et al.* employed this method, with 94% of mice developing submucosal rectal adenocarcinoma 4 weeks after intraluminal instillation of murine CRC cells.[61] A recent rectal transplant model developed distant metastasis; Enquist *et al.* showed liver and lung metastases at 7 weeks, when human CRC cell lines were sutured to the rectal mucosa using a prolapse technique.[62] These transplant models successfully recapitulate rectal cancer; however, immunocompromised mice are used to overcome cell rejection. Significant alteration to the tumor immune micro-environment limits the application of these models to pre-clinical treatment studies. Furthermore, immortalised cell lines are not representative of normal cell biology and human disease; extensive in vitro selection enables cell lines to divide indefinitely as a result of aberrant gene expression, and genetic heterogeneity between strains predisposes to experimental variability.[63]

CRC modelling has benefited from developments in organoid systems, whereby human and murine intestinal epithelium and adenocarcinoma cells can be cultured as 3D organoids replicating their tissue of origin.[64] Drost *et al.* showed that human intestinal stem cells can be genetically modified in culture using CRISPR/Cas9 technology to induce mutations in APC, p53, KRAS and SMAD4.[65] On subcutaneous organoid transplantation into immunodeficient mice, tumors with features of invasive adenocarcinoma developed. Similarly, subcapsular kidney transplant of mutant organoids derived from normal human intestinal epithelium has demonstrated tumor formation.[66] Subcutaneous organoid transplant models have also shown a role for Lgr5+ cancer stem cells in metastasis.[67] Thus, these models have proven their value in driving our understanding of the mechanisms of tumor growth and metastasis. Developments in organoid systems have advanced preclinical modelling abilities, as organoids can be genetically engineered to mimic tumors harboring mutations of interest. However, these subcutaneous models fail to recapitulate the anatomical and tissue layer of origin, and the use of immune-deficient mice limits their value by excluding the significant tumor-host immune interactions involved in the metastatic process.

Recently more anatomically accurate CRC organoid transplant models have been developed with delivery of organoids to the colon or rectum. Transplant of APC, KRAS, p53 and SMAD4 mutant human colon organoids onto the caecal wall in a study by Fumagalli *et al.*, showed lung and liver metastases at 6-8 weeks in 44% of mice.[68] A similar caecal wall transplant method employed by Tauriello *et al.*, illustrated that increased TGF-β (Transforming growth factor beta) signaling promotes CRC metastasis, and identified TGF-β blockade as a therapeutic target to prevent metastasis by enhancing cytotoxic T-cell responses.[69] This highlights the utility of these models in identifying novel treatment targets. O'Rourke *et al* described injection of Apc/Kras/p53 mutant murine derived organoids by pipette injection into the rectum of immunocompetent C57BL/6 mice after induction of colitis.[70] Adenocarcinoma was typically observed at 6 weeks (62% success rate), local disseminated disease at 11-12 weeks, and metastasis seen at >20 weeks in 1/6 of mice.

Further developments have been made through colonoscopic submucosal injection techniques.[71] Replicating the conventional pathway to CRC, Roper *et al* generated Apc/Kras/p53 mutant murine colon derived organoids, which were orthotopically injected into the colonic submucosa of both C57Bl/6 and immunocompromised NSG (NOD Scid gamma) mice under colonoscopic guidance. A high penetrance of invasive tumors was observed at 12 weeks, with liver metastasis seen in 33% of NSG mice; similar findings were observed with transplantation of human CRC

derived organoids. Lannagan *et al*, utilized CRISPR/Cas9 technology to develop a panel of serrated CRC organoids that exhibited increasing penetrance with additional gene hits when injected orthotopically into the colon under colonoscopic guidance.[72] This method robustly allows the use of mouse and patient-derived organoids to model the different types of CRC in the native colon, with local invasion and distant metastasis observed. Colonoscopy guided injection is an efficient and easily performed technique, which also allows a simple method for tumor surveillance.

Early transplantation models fail to recapitulate the anatomical location, native stroma, originating tissue layer, tumor histology and mutational burden of human CRC. Recent techniques involving colonoscopy guided submucosal injection of organoids carrying known driver mutations, have successfully modelled anatomically accurate colonic adenocarcinoma with the capacity to metastasize. Application of this technique to immunocompetent mice holds promise to study tumor-host immune interactions and perform treatment studies. Advances in organoid systems and transplant techniques are directly applicable to generating robust rectal cancer models, which holds great potential to study the immune and molecular mechanisms underlying the heterogeneous responses to neo-adjuvant CRT, and to identify novel treatment strategies.

Patient derived organoids

Clinical need for predictive tools to determine which patients might respond to neo-adjuvant CRT in LARC has generated interest in patient-derived organoids (PDOs). Ganesh *et al* developed a biobank of human rectal cancer organoids from biopsied or resected rectal tumours.[73] Of the 65 organoid lines established, 49 were obtained using endoscopic biopsy forceps, demonstrating the feasibility of collecting pre-treatment samples. When organoids were irradiated ex vivo, varying sensitivity to radiation was observed which correlated with the response seen in the corresponding patient. Similar concordance in response was seen when organoids were treated with chemotherapy. Although some organoids were derived from biopsies after treatment initiation, results suggest that treatment sensitivity determined *ex vivo* in PDOs, has potential as a predictive tool for patient treatment. Similarly, Yao *et al* generated an organoid biobank from LARC patients prior to treatment with neo-adjuvant CRT.[74] 80 PDO lines were treated individually with irradiation, 5-Fluorouracil and irinotecan, then compared with the clinical response in the corresponding patients. The study demonstrated that organoids closely recapitulated the molecular profiles and pathological features of the corresponding tumors, and that response to CRT matched that of the patient with 84% accuracy, 78% sensitivity and 92% specificity. Although lacking an intact tumor micro-environment, PDO systems may predict the epithelial response to combined therapy regimens, which in turn may determine patient sensitivity to radiotherapy and individual chemotherapy agents, acting as a useful adjunct (or patient 'avatar') to aid treatment planning. Crucially, treatment response tests were completed in less than 4 weeks by Yao *et al.*, representing a clinically applicable time-frame.

Future application of pre-clinical models

Pre-clinical models are becoming an essential part of the development and optimization of novel neo-adjuvant treatment strategies in rectal cancer, through identification and testing of novel therapeutics. Interest has recently developed in combining radiotherapy with immune-checkpoint inhibition to enhance tumor immunogenicity. Dovedi *et al.* studied the effects of fractionated radiotherapy in combination with PD-1 (Programmed cell death protein 1) blockade in a subcutaneous transplant model of CRC, with results suggesting PD-1/PD-L1 pathway blockade stimulates systemic anti-cancer immune responses.[75] TGF-β signaling inhibits the function of many components of the immune system, playing a key role in tumor immune evasion.[76] Furthermore, increased TGF-β signaling, stromal activation and abundant cancer-associated fibroblasts (CAFs) are associated with poor prognosis sub-types of CRC.[77,78] Tauriello *et al.* demonstrated that elevated TGF-β in CAFs was associated with CD4+ and CD8+ T-cell exclusion from the tumor center, which is associated with poor prognosis.[69,79] Upon treatment of mice with Galunisertib (TGFBR1 inhibitor), T-cell infiltration was triggered. Dual treatment with Galunisertib and PD-L1 inhibition induced a potent immune response, which eradicated metastasis in the model. Similarly, Nakanishi *et al*. demonstrated that dual treatment with Galunisertib/PD-L1 inhibition in a GEMM of serrated CRC, resulted in mice having reduced number, size and aggressiveness of tumours.[80] By limiting anti-tumor T-cell responses, it is hypothesized that TGF-β activity represents a resistance mechanism to radiotherapy. In pre-clinical breast cancer models, TGF-β inhibition alongside radiation therapy generated CD8+ T-cell responses to multiple tumor antigens, resulting in regression of irradiated tumors and non-irradiated metastases.[81,82] Recently, Rodriguez-Ruiz *et al.* studied the effects of radiotherapy, TGF-β and immunotherapy in combination (anti-PD-1 and anti-CD137 monoclonal antibodies), utilizing a bilateral subcutaneous transplant model with murine CRC and breast cancer cell lines.[83] Treatment with irradiation, TGF-β blockade and immunotherapy, resulted in contralateral non-irradiated tumor volume reduction and increased CD8+ infiltration, with results suggesting that TGF-β blockade enhances the abscopal and systemic efficacy of radiotherapy-immunotherapy combinations. These studies did not employ the newly developed small animal radiotherapy platforms discussed previously, and image-guided precision radiotherapy is now possible in pre-clinical studies. Research platforms to deliver clinically relevant image-guided radiotherapy to anatomically accurate murine models, will enable more sensitive and selective study of the influence of radiotherapy on the tumor micro-environment, by allowing study of dosing, targeting and scheduling. Furthermore, they will enable more robust pre-clinical testing of CRT regimens and radiotherapy combined with novel immune and stromal-targeting therapies.

Conclusions

Multi-modality treatment of LARC requires an optimal combination of chemotherapy, radiotherapy and surgery. Recent advances in CRC modelling and novel small animal radiotherapy research platforms can be utilized to faithfully recapitulate the clinical and anatomical aspects of rectal cancer, with tumors subjected to targeted pelvic irradiation. Such models will help to establish the immune and molecular basis underlying the heterogeneous responses to CRT. Identification and pre-clinical testing of novel immune and stromal targeting agents, will inform future clinical trials aimed at improving neo-adjuvant CRT strategies. PDOs are a promising diagnostic tool, which may assist treatment planning by rationalizing effective agents. As outlined, mouse models have been invaluable tools to improve understanding of the genetic events underpinning initiation and progression of CRC. Although management and local recurrence rates for LARC have improved significantly, 5-year survival and distant metastasis rates

have not improved significantly and should be a focus of future research.[84] Optimization of 'complete response' to CRT and development of non-surgical management strategies should also be a research priority, so that patients can benefit from organ preservation. The current pre-clinical models and tools described here will allow researchers to answer fundamental questions regarding the mechanisms underlying treatment response to CRT, and represent an exciting opportunity to identify and test novel therapeutic agents in combination with radiotherapy, to ultimately improve and personalize neo-adjuvant treatment strategies for LARC patients.

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