Targeting the Microenvironment in Advanced Colorectal Cancer

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Abstract

Colorectal cancer (CRC) diagnosis often occurs at late stages when tumour cells have already disseminated. Current therapies are poorly effective for metastatic disease, the main cause of death in CRC. Despite mounting evidence implicating the tumour microenvironment in CRC progression and metastasis, clinical practice remains predominantly focussed on targeting the epithelial compartment. As CRCs remain largely refractory to current therapies, we are compelled to devise alternative strategies. TGF-β has emerged as a key architect of the microenvironment in poor prognosis cancers. Disseminated tumour cells show a strong dependency on a TGF-β-activated stroma during the establishment and subsequent expansion of metastasis. Here, we will review and discuss the development of integrated approaches focused on targeting the ecosystem of poor prognosis CRCs.

Trends

• In the search for new paradigms on which to base novel therapeutic strategies for advanced CRC, focus has increasingly been centred on the tumour microenvironment.

• There is an emerging notion that interactions between epithelial cancer cells and their environment can be understood applying a conceptual framework similar to that used to study ecosystems.

• In CRC patients, a stromal-expressed gene programme enriched for TGF-β and downstream targets has been linked to poor prognosis and metastasis formation.

• TGF-β signalling plays key roles in instructing the tumour microenvironment of late stage CRCs, yet inhibition of TGF-β signalling as a therapeutic strategy remains scarcely explored in the clinic.
The tumour epithelium as primary target of standard therapies

Colorectal cancer (CRC) originates from benign lesions known as adenomas: localised glandular overgrowths in the epithelial lining of the large bowel. Over time, some adenomas accumulate mutations in signalling pathways critical to stem cell maintenance, cellular proliferation, and tumour suppression [1, 2] and they can evolve into invasive CRCs that may ultimately spread to distant organs. This process called metastasis (see Box 1) occurs in about 40-50% of patients and confers a low probability of survival.

In the clinic, patients are classified in 4 stages (see Glossary). Treatment involves aggressive surgical resection of the primary tumour, which cures a large proportion of early stage patients. However, some stage II and many stage III patients will relapse, frequently in the form of metastasis, as a consequence of tumour cells that disseminated before resection. Unlike the primary tumour, metastases are less frequently removed by surgery unless limited in number and extent [3]. Therefore patients that present with metastases at the time of diagnosis (stage IV) and those at perceived risk of relapse receive cytotoxic chemotherapy: in most cases a combination of folinic acid, 5-fluorouracil, and oxaliplatin or irinotecan. This strategy aims to kill highly proliferative cancer cells and has been a staple in the treatment of solid cancers for decades [4]. Although adjuvant chemotherapy is beneficial to stage III patients and has modest potential for improved survival in stage II [5, 6], it performs poorly in the metastatic setting, almost invariably giving rise to drug resistance and disease progression.

In second-line treatment, chemotherapy is increasingly combined with targeted therapy, designed to intervene with specific signalling pathways or cellular mechanisms [4]. A key example in CRC is the use of antibodies targeting the epidermal growth factor (EGF) receptor,
often exploited by cancer cells to stimulate proliferation. Apart from eventual resistance, the main problem with targeted therapies is the variable responses in patients, requiring a better grip on predictive biomarkers [7]. For instance, mutations that activate the MAPK/ERK pathway downstream of EGFR, such as those frequently occurring in the KRAS and NRAS GTPases or BRAF, can render anti-EGFR therapy ineffective [8].

A better understanding of advanced cancer may lead to more effective therapies for metastatic CRC, but is also highly relevant to improve the management of earlier stages of the disease. Arguably the most important question for stage I-III patients is whether or not to treat at all and which therapeutic strategy will be beneficial to prevent recurrence. Assessment of probability of relapse after therapy on the individual level remains a major challenge [9].

Anatomical and histopathological features of the tumour such as extent of invasion (T), number of lymph node metastases (N), bowel perforation or obstruction, the presence of lymphovascular invasion, and a poorly differentiated histology are used to identify CRC patients at risk of recurrence. However, these parameters only hold a moderate predictive power and do not help select optimal treatment options. Furthermore, with the exception of chromosomal or microsatellite instability (MSI) [10, 11] and mutations in the BRAF oncogene [12], no molecular feature is robustly associated with prognosis and therefore used routinely in clinical practice [9, 13].
Transcriptomics redefining CRC classification

To improve patient stratification and identify potential molecular targets for therapy, the field has generated large collections of transcriptomic datasets from tumour samples, which have enabled the identification of CRC subtypes based on distinctive global gene expression profiles [14-19]. In an attempt to consolidate these data, a recent meta-analysis divided CRCs into 4 defined consensus molecular subtypes (CMS), representing an MSI-like class (CMS1), a canonical WNT/MYC class (CMS2), a metabolically dysregulated class (CMS3), and a mesenchymal class (CMS4) [20]. The latter subtype, to which about 1 in every 4 CRCs belongs, is of particular interest as it relapses with higher frequency than the others.

The elevated expression of mesenchymal genes in an epithelial cancer led to the proposal that epithelial-to-mesenchymal transition (EMT) characterizes poor prognosis in CRC [21]. However, the transcriptome of a tumour sample not only reflects the expression programme of epithelial cancer cells but also the profile of mesenchymal cells present in the tumour microenvironment (TME). Indeed, our group and the group of Enzo Medico recently discovered that expression of mesenchymal genes in transcriptomic CRC data is largely contributed by cells of the TME, mainly by cancer-associated fibroblasts (CAFs), rather than by cancer cells [22, 23]. In agreement with these observations, CMS4 cancers show a higher degree of stromal infiltration than the other subtypes [20]. Furthermore, our analyses indicate that a large proportion of these CAF-expressed genes are strongly associated to cancer relapse and poor prognosis in CRC cohorts [22, 23]. Of note, the fact that the expression of mesenchymal genes, including many EMT master regulators such SNAIL1, TWIST and ZEB1, is contributed by stromal
cells in transcriptomic profiles does not rule out the possibility that subsets of tumour cells may undergo EMT, particularly at invasion fronts.

**Treating Cancer as an Ecosystem**

Focussing on the tumour stroma is not novel. At the end of the 19th century, English physician Stephen Paget – observing preferential patterns of metastatic dissemination to particular organs – proposed the ‘seed and soil’ hypothesis to explain this phenomenon as the combination of the colonizing cancer cells’ adaptability and a congenial microenvironment in the distant organ [24]. He suggested it would be useful to study the properties of the TME, as well as its crosstalk with cancer cells. Indeed, the role of the TME both at the original site and in the distant organ has become a topic of intense investigation in recent decades [25]. Similar to how intestinal crypt stem cells are regulated by a specialized stem cell microenvironment called a *niche*, the TME can foster cancer stem cells and thus drive recurrence and metastasis (Box 1) [26, 27], as well as provide mechanisms for drug resistance [28, 29]. It is now accepted that the TME is an active player in tumour malignization and that it provides a fertile ground to delve for therapeutic targets [30, 31].

The study of complex interactions between cancer cells and their environment inspires parallels with the interrelationships recognized in an ecosystem. The application of ecological concepts to oncology may help explain why a rigid focus on epithelial cancer cells alone has led to a high number of failed new drugs and clinical strategies. Just as ecology is the framework of choice for understanding the mechanisms of organismal evolution through natural selection, ecology working on the level of cellular populations serves to clarify the dynamics of cancer evolution. This includes natural selection due to limited resources as well as the artificial
selection of subclones resistant to therapy. Hence, the fitness of a cellular population (or of a set of cancer mutations) should be considered in the context of its local environment, which consists of cellular interactions, physical parameters (oxygen levels, pH, mechanical pressure), and molecules like extracellular matrix components, growth factors and cytokines.

Moreover, ecological types of interaction can describe various levels of crosstalk in tumour dynamics. Examples of competitive and cooperative interactions have been reported both between different epithelial subclones and between epithelial cancer cells and the stroma [32-34]. Additionally, the immune system can be converted from a predator to an accomplice (see Box 2). Indeed, the stromal environment is not static and co-evolution is a compelling concept: parallel evolution of neoplastic epithelial cancer cells and cells in the microenvironment, subject to epigenetic and gene expression changes [35]. Furthermore, the stromal compartment contributes significantly to intratumoral heterogeneity, with important implications for disease progression and therapeutic responses [36]. Thus, cancer ecology integrates complex interactions between epithelial cancer cell populations and their environment to promise a deeper understanding of the biology of cancer [37, 38].

Modulating the CRC ecosystem for therapeutic purposes

The composition of the TME during colorectal tumorigenesis and in advanced cancers is subject to intensifying inquiry (for a recent overview see refs: [39, 40]) and is found to be different from normal intestinal stroma (see Figure 1, Key Figure). As recently reported, the majority of genes that predict cancer recurrence in CRC patients are expressed in CAFs and not by the cancer cells [22, 41]. The striking prognostic power of the TME raises the opportunity to more accurately estimate the risk of relapse of any given stage I-III patient. It also paves the
way for the development of additionally refined molecular classifications based on the contents of the ecosystem. A relevant early example of such an ecological classification of CRC was established by Galon and others [42-44], who observed that infiltration of particular subsets of T cells in the tumour mass predicts longer disease free survival intervals after therapy.

Moreover, novel CRC classifications based on the type and features of stromal cells will be key to stratify patients for treatments that target the TME. Based on the idea that CRC cells depend on particular stromal factors to effectively disseminate, therapeutics that modulate the CRC ecosystem may be effective in treating or preventing metastasis. An additional advantage of targeting the TME is its genetic stability, making drug resistance less likely to occur.

Furthermore, the commonality of most elements in the stroma across cancer types suggests a broad applicability of effective therapies. A handful of therapies based on the above concept have already been developed and are currently being applied. For instance, blood vessel formation and endothelial cells have been successfully targeted by anti-angiogenic therapy [45]. Indeed, a monoclonal antibody against vascular endothelial growth factor (VEGF, i.e. bevacizumab) inhibits angiogenesis and has been shown to improve survival for stage IV patients [46, 47]. Additionally, metronomic chemotherapy – aiming to forestall drug resistance and suppress neo-angiogenesis – has shown promise for prolonging survival and limiting metastasis in preclinical models, and is being tested in clinical trials [48, 49].

Another prime example of therapy directed to the TME is immunotherapy (Box 2). It has been proposed that several (chemo-) therapies that do not directly target immunity, such as 5-fluorouracil and platinum compounds, in fact do rely on immune components. These
conventional treatments are effective at least in part by reinforcing tumour immunosurveillance, especially when they induce immunogenic cancer cell death [50].

**TGF-β as a main organizer of the metastatic CRC ecosystem**

TGF-β has a dual role in CRC, with tumour suppressive functions in early stages and promoting disease progression in advanced disease (see Box 3). Our group demonstrated that elevated TGF-β signalling in the TME links to poor prognosis in CRC [22, 41]. The stromal TGF-β response encodes a gene programme that includes a plethora of cytokines, growth factors and extracellular matrix proteins, many of which have been shown to play key roles during disease progression and metastasis in several cancer types. Among these potential therapeutic targets is interleukin-11 (IL-11), a cytokine secreted by TGF-β-stimulated fibroblasts that induces pro-survival response in CRC cells during cancer progression and metastatic colonization [41, 51].

The picture that emerges is that key functions required to complete the formation of metastasis are provided by the TGF-β-activated microenvironment (Figure 1).

Because disseminated tumour cells thrive on a TGF-β-activated environment, this dependency could be exploited in the clinical setting to improve patient treatment; our group showed for the first time that pharmacological inhibition of stromal TGF-β signalling is effective in blocking metastatic colonization in mice [22, 41]. Besides activating a pro-metastatic programme in CAFs [52], stromal TGF-β signalling induces a pro-tumorigenic phenotype in neutrophils and macrophages [53-55]. In addition, TGF-β directly suppresses the anticancer response of the adaptive immune system, including the deregulation of cytotoxic T lymphocytes [56-58]. Also, neutralizing the TGF-β pathway in T cells led to immune-mediated
eradication of tumours in a model of melanoma [59]. That the dual role of TGF-β signalling in instructing immune cells and CAFs might be more related than is immediately obvious, is suggested by report in which CAFs were shown to have immune suppressive effects [60, 61].

Thus, it will be interesting to assess the efficacy of pharmacological intervention in this pathway on the CRC ecosystem. Several therapeutic strategies targeting TGF-β are in clinical development (see Box 4) [62-64]. This spurs the hope that if we can block TGF-β pharmacologically, we may prevent a vicious cycle of stroma activation, expression of protumorigenic secreted factors, and the generation of an immunosuppressive environment (Figure 1). Indeed, TGF-β inhibition may work as (or synergize with other types of) immunotherapy (Box 2).

Caveats about the dual role of TGF-β signalling in CRC

Due to the tumour suppressive function of TGF-β signalling in epithelial component of CRC (see Box 3), elevated TGF-β levels necessary to instruct the poor prognosis-associated microenvironment might not be compatible with tumour growth. We speculate that the loss of TGF-β response in the epithelial component of the cancer may facilitate the elevation of TGF-β levels during tumour progression. A functional link between these two events has been observed in experimental models [65]. In a biobank of CRC organoids, independence of TGF-β-mediated growth inhibition was associated with advanced stages of the disease [66], whereas loss of SMAD4 in epithelial CRC cells correlated with poor prognosis in several studies [67, 68]. Yet, abrogation of the cytostatic TGF-β response in CRC cells is not exclusively explained by loss-of-function mutation in pathway components suggesting additional mechanisms of TGF-β
resistance [66]. Also, a proportion of TGF-β-reactive CRCs may respond to the cytokine by undergoing EMT while bypassing the cytostatic effect. Apparently, this effect is relevant to trigger invasion of a particular subset of early lesions named sessile serrated adenomas [69], which might therefore benefit from anti-TGF-β therapy. Conversely, it remains unclear whether anti-TGF-β therapy would be safe for patients with cancer cells that have an intact TGF-β pathway – even if they represent a subclone – and are kept in check by its cytostatic effects.

Although the TGF-β pathway has emerged as a powerful architect of the pro-metastatic poor prognosis TME, its effect on individual stromal cell types is pleiotropic and incompletely charted. Accordingly, we propose that, to treat the cancer ecosystem with more integrated approaches, we need to study and understand its complexity in greater detail. As such, manipulating a master regulator like TGF-β in sufficiently complex model systems of metastatic CRC should not only provide the needed rationale for clinical translation, but would additionally give us relevant parameters to help map the crosstalk in the cancer ecosystem.

Need for better preclinical models moving forward

To study the efficacy of above-mentioned and other stroma-directed therapies in the preclinical setting, there is a demand for more comprehensive, predictive models. Although the field has moved from subcutaneously injecting 2D-cultured human CRC cell lines to exploiting 3D organoid culture and patient-derived xenografts (PDX) – approaches that promise to retain tumour heterogeneity in genetics, architecture and drug responses [70-72] – these in vivo models lack an intact TME. This is due to a mismatch between some murine and human signalling molecules and because of the absent or abrogated immune system required for the
engraftment of human tumour tissue in murine hosts. To address these problems, mouse models are being generated that correct known signalling deficits as well as feature humanized immune system components, potentially leading to patient-specific immune environments. These advances promise to strongly enhance current models for cancer biology and preclinical, personalized oncology.

An alternative approach to study the CRC TME and cancer immunity involves genetically modified mouse models (GEMMs), which feature an intact, immunocompetent TME yet typically fail to capture advanced CRC. This leaves a gap: to test current ideas and discover and develop novel therapeutic strategies we are in need of CRC models that are humanlike in molecular characteristics, genetics and histopathology, and that are metastatic in a fully immunocompetent environment. The ideal scenario would include a transplantable (organoid) system in the commonly used C57BL/6 strain, so that it can be leveraged across a wealth of existing genetic models to dissect specific functions of stromal pathways. Furthermore, such a system would be critical to testing novel therapies that target the tumour ecosystem.

Concluding remarks

The CRC oncology field has evolved in the past decades. From histological analyses to molecular subtyping, from cytotoxic chemotherapy to personalized combinations with targeted therapy. What has not changed, unfortunately, is that besides surgery there is very little we can do to improve overall survival for advanced disease.

However, the paradigm shift that points to the TME as a critical factor in determining metastatic success opens the door to a more integrated understanding of the biology of
metastasis. One that invites us to consider cancer as a complex ecosystem rather than as a tenacious weed. In this context, therapeutic targeting of stromal TGF-signalling might be expected to reprogramme the poor prognosis cancer ecosystem (Figure 1), with the potential to make metastatic lesions unsustainable or to prevent metastatic initiation altogether. We hope that this emerging concept will find a timely translation to the clinic. Acknowledging the many challenges ahead (see also Outstanding Questions), we need more sophisticated and predictive CRC models to show proof-of-principle results that may catalyse the effort of targeting the TME.

**Outstanding Questions**

- What triggers the upregulation of TGF-β in some tumours but not others?
- What cell type is (or which cell types are) the source of secreted and activated TGF-β that reprograms the CRC ecosystem?
- As not all CRCs have known mutations in the TGF-β pathway, and some tumours may have co-existing mutant and sensitive subclones, to what extent is the responsiveness of epithelial cells to TGF-β (mainly associated to early disease stages) still relevant in advanced CRC?
- And within this context, is TGF-β therapy safe for all patients?
- Given the dependency of metastatic initiation on a TGF-β-activated TME, is the TGF-β therapeutic window limited to adjuvant therapy – to inhibit metastatic colonization – or could TGF-β therapy also treat established metastases, e.g. as immunotherapy?
Box 1. CRC metastasis: biology and therapeutic scope

Initial transformation of the colon epithelium and subsequent transitions through the adenoma-carcinoma sequence are thought to require successive genetic alterations in 4 signalling pathways: WNT, EGFR, TGF-β and P53 [1, 2]. In the healthy colonic mucosa, these signalling pathways regulate the behaviour of intestinal stem cells (ISCs). Therefore, during CRC progression, tumour cells become independent from these crypt niche signals [66]. About 40% of all CRC disseminate to other organs. Metastasis can either be present during diagnosis or reveal itself months or years after surgery as a recurrence. In many cases, distant recurrence targets the liver, at least in part because intestinal blood vessels drain into the portal vein, which connects to the liver. Metastasis is a multistep process that requires cells to detach from their epithelial neighbours, invade the submucosa, intravasate and survive in the vasculature (blood or lymphatic vessels), extravasate and survive in an alien organ, to eventually reinitiate a thriving tumour [73, 74]. There is much of the biology of metastasis that we don't understand, but from what we know, it is a very harsh and inefficient process [75, 76]. Arguably, the process selects for the fiercest and most resilient cancer (stem) cells and discovering what makes them successful might uncover therapeutic targets. Importantly, the metastatic process has not been linked to specific genetic alterations within epithelial CRC cells [77]. Therefore, studies have shifted focus onto the role of the TME and the mechanism by which the metastatic cell exploits the TME for survival, immune evasion, and growth stimuli [78, 79]. Perhaps, success in metastasis is defined as who can best remodel their microenvironment.
Box 2. Cancer inflammation, immunity and immunotherapy

Inflammation is a well-described risk factor in gastrointestinal tumorigenesis [80], and encompasses disparate signalling pathways by a variety of immune cells that impact every step of cancer progression and metastasis [81]. Although the immune system can be a powerful tumour suppressor by the coordinated elimination of aberrant cells, tumours find a way to survive in spite of immunosurveillance. There is evidence for immunoselection in many cancers, including CRC – where the number of observed neoantigens (in remaining subclones) is lower than expected based on mutation rates – and cancer cells can acquire resistance to immunity by e.g. abrogating antigen presentation [82, 83].

Moreover, it has become increasingly clear that inflammatory mechanisms in tumours can also help drive cancer progression [81]. By studying how progressing cancers suppress the anti-tumour response and subvert immune regulation to foster immune suppressor cells and produce pro-tumorigenic factors that support cancer cells, the expansive field of cancer immunology has introduced a multitude of novel therapeutic strategies [84, 85]. On the conceptual level, many such therapies aim to enhance endogenous anticancer immunity that is somehow insufficient or dysfunctional [86]. To achieve this effect, a therapy can reduce cellular or molecular crosstalk mechanisms of immune suppression and/or increase T cell survival, proliferation, infiltration, and activation in the TME [87].

The promise of immuno-oncology has recently been demonstrated by long-lasting responses to immune checkpoint inhibitors. These proved beneficial in various types of cancer by unleashing T cells that were crippled by signalling either from immune suppressor cells or cancer cells hijacking anti-inflammatory mechanisms [88, 89]. It is still unclear whether these
therapies will broadly benefit CRC patients. However, one study on mismatch repair-deficient (MSI) cancers, including of the colon, did show therapeutic responses [90]. This supports the concept that tumour cells with higher frequency of neoantigens, such as in microsatellite instable cancers, are generally more vulnerable to T cell-mediated anti-tumour immunity, possibly explaining their relatively good prognosis [91, 92]. If current immunotherapies turn out not to have a broad applicability as single agents in CRC, there is still hope that a combination of treatments that both enhances intratumoral immune infiltration as well as activation will fare better [93, 94].

Box 3. The dual, biphasic role of TGF-β in CRC

The pro-metastatic effects of TGF-β signalling on the tumour microenvironment can occur independently of signalling in epithelial cancer cells, as many cancers silence the epithelial pathway so as to progress. TGF-β signalling is considered a tumour suppressor pathway in colon carcinogenesis because it triggers a potent cytostatic response in epithelial cells [22, 65, 95-97]. Indeed, about 40% of CRCs display loss-of-function mutations in TGF-β pathway components, which are acquired around the adenoma carcinoma transition [2, 98]. An early clue for a positive role for TGF-β signalling in disease progression was found by Kawata and colleagues who reported that serum levels of TGF-β1 predict relapse in CRC patients [99]. Later, our group showed that TGFβ1, TGFβ2 and TGFβ3 mRNA levels associate with shorter disease free survival intervals after therapy in stage I-III patients [41]. Elevated TGFβ1-3 expression correlates with upregulation of TGF-β response gene signatures (TBRS) in cells of the TME, specifically in T cells, macrophages, endothelial cells and most prominently CAFs. These TBRSs hold a striking
predictive power for cancer recurrence in CRC [41] and detection by immunohistochemistry of
several proteins upregulated in TGF-β-activated CAFs identifies patients at high risk of relapse
[22]. Furthermore, comparison of the molecular subtypes revealed that the main signalling axis
deregulated in the stromal/mesenchymal subtype (CMS4) is the TGF-β pathway [20]. A
significant proportion of the genes differentially expressed in CMS4 cancers that predict a
higher probability of recurrence are included in the TBRSs and correspond to genes induced by
TGF-β in CAFs. The role of a TGF-β-activated ecosystem in disease progression has been
confirmed in experimental models of advanced CRC, where enforced TGF-β signalling in the
TME strongly increases metastatic burden [41].

Box 4. TGF-β therapeutics in clinical development

Several methods of targeting the TGF-β pathway have been described. These include
antisense oligonucleotides to TGF-β ligand mRNA, small molecule inhibitors of the receptor
kinase domains, and monoclonal antibodies against ligands or receptors. In addition, there are
vaccines and adoptive cell transfer strategies that target TGF-β signalling as part of their
mechanism. For an overview of all (pre-) clinical strategies, see references [62-64]. Here, we
selected TGF-β targeting therapies that are currently in clinical trials for CRC (Table I).
Table I. TGF-β targeted therapies in clinical trials for CRC

<table>
<thead>
<tr>
<th>Type</th>
<th>Drug</th>
<th>MoA</th>
<th>Setting (Phase)</th>
<th>Trial Identifier</th>
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<tr>
<td><strong>Antisense oligos</strong></td>
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<td></td>
<td>Trebedersen (AP12009 Antisense Pharma)</td>
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<tr>
<td>Kinase inhibitors</td>
<td>Galunisertib (LY2157299 Eli Lilly)</td>
<td>TβRI (ALK5) kinase inhibitor</td>
<td>Rectal cancer (II) Study cancer immunology in solid tumours (I)</td>
<td>NCT02688712</td>
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<td>NTT02304419</td>
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<td>Checkpoint inhibitor combination in solid tumours (I/II)</td>
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<td>TβRI (ALK5) kinase inhibitor</td>
<td>Solid tumours (I)</td>
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<td>TβRII neutralizing MAb</td>
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<td></td>
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<td><strong>Immunotherapy</strong></td>
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MoA: Mode of action. ASO: antisense oligo. ALK1: Activin receptor-like kinase 1 (non-canonical TGF-β receptor type I in endothelial cells). *As part of its MoA, autologous tumour cells carry bifunctional shRNA blocking furin, leading to lower TGF-β1 and TGF-β2 levels.
**Glossary**

**Adjuvant chemotherapy:** chemotherapy given after primary treatment (surgery in CRC) to lower the risk of the cancer coming back.

**Cancer evolution:** the theory that within a tumour, accumulating mutations and/or localized selective pressure leads to the generation of subclones that compete with each other for limited resources. Furthermore, at the time of diagnosis a (minority) subclone may already carry a mutation that renders it (partially) resistant to chemotherapy. Both this concept and the realization that this theory helps explain the unique nature of each individual tumour make cancer evolution a highly relevant concept that forms the basis for more integrated (ecological) therapeutic strategies.

**CRC staging:** The American Joint Committee on Cancer (AJCC) has established a general cancer staging protocol called the TNM Staging System. *T* stands for tumour and evaluates the original (primary) tumour in grades of aggressiveness; *N* stands for lymph Node involvement and qualifies the presence of cancer cells spreading to nearby lymph nodes. *M* addresses the presence or absence of distant metastasis (spreading of cancer to distant organs). Based on these 3 categories, patients are grouped into four stages indicated by Roman numerals (I, II, III and IV). While statistically representing significantly different risk groups for recurrence and cancer-related death, the staging system does not accurately predict on the level of individual patients.

**Cytokines:** large class of small secreted signalling proteins involved in cellular crosstalk, especially relevant for the orchestration of immune responses and cancer.

**Ecology:** the comprehensive study of the distribution, abundance and dynamics of organisms, their interactions with others and with their physical environment. It is most frequently applied on the organism level, where populations and their environment form an ecosystem and the largest such system studied is the ecosphere earth, yet it can also be applied to cells in a tissue or in a cancer. Central concepts in ecology include cooperation and competition, parasitism and predation, and evolution.

**Immunosurveillance:** the process by which malignant cells are kept in check by anticancer immunity. Cancer immunity is understood to have an initial elimination phase before the
balance is tipped and tumour cells start escaping from immunosurveillance. Between elimination and escape, there may be a prolonged state of equilibrium between cancer growth and immunity-related killing.

**Relapse/Recurrence:** the return of a tumour after surgical removal of the original tumour (which often entailed removal of parts or the whole of the colon). The tumour can reappear, often as a distant metastasis.

**Metronomic chemotherapy:** frequent or continuous administration of low non-toxic doses with no interruptions for long periods to prevent resistance and target both epithelial cancer cells and the growing tumour vasculature.

**Microenvironment or Stroma:** supportive connective tissue with structural and functional roles both during homeostasis and in disruption such as wounding or disease. The stroma includes fibroblasts, blood and lymphatic vessels, immune cells, and the extracellular matrix. In the context of cancer, the stroma is increasingly understood as a complex, dynamic entity that is transformed by and co-evolves with cancer cells and can drive malignant progression.

**Microsatellite instability (MSI):** due to a deficiency in DNA mismatch-repair genes, MSI patients accumulate spontaneous errors in regions of their genome that are repetitive and thereby challenging to replicate.
Figure legend

Figure 1 (Key Figure). The CRC ecosystem and the progression-driving role of TGF-β.

Besides fibroblasts (CAF), endothelial cells, pericytes and mesenchymal stem cells (MSC), colorectal cancers are infiltrated by a variety of innate and adaptive immune cells, including lymphocytes (T cells, B cells, natural killer cells), monocytes, macrophages, dendritic cells, granulocytes (neutrophils, basophils, eosinophils, and mast cells), and myeloid-derived suppressor cells (MDSC).

TGF-β has emerged as a central architect that drives malignization of the cancer ecosystem, activating stromal cells towards pro-tumorigenic differentiation, inducing the expression of secreted factors, and facilitating the accumulation of immune suppressive and drug resistance functionalities. Effective ecological treatment might include a combination of TGF-β inhibition with other stroma-directed therapies, such as chemotherapy and targeted (immuno-) therapy.

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Cancer activates Stroma

TGF-β inhibition

Immunotherapy

Stroma secretes Protumorigenic factors

TME drives Immune Evasion & Drug Resistance

Stromal therapy

Epithelial Cancer cell

Endothelial cell

CAF

MDSC

Dendritic Cell

Monocyte

Granulocyte

MSC

Lymphocytes

Granulocyte

Extracellular Matrix

Pericyte