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Abstract

Stratification of colorectal cancer (CRC) into subgroups with different response to therapy was initially guided by descriptive associations between specific biomarkers and treatment outcome. Recently, preclinical models based on propagatable patient-derived tumor samples have yielded an improved understanding of disease biology, which has facilitated the functional validation of correlative information and the discovery of novel response determinants, therapeutic targets, and mechanisms of tumor adaptation and drug resistance. We review the contribution of patient-derived models to advancing CRC characterization, discuss their influence on clinical decision-making, and highlight emerging challenges in the interpretation and clinical transferability of results obtainable with such approaches.

Significance

Association studies in patients with CRC have led to the identification of response biomarkers, some of which have been implemented as companion diagnostics for therapeutic decisions. By enabling biological investigation in a clinically relevant experimental context, patient-derived CRC models have proved useful to model the causal role of such biomarkers in dictating drug sensitivity and are providing fresh knowledge on new actionable targets, dynamics of tumor evolution and adaptation, and mechanisms of drug resistance.

Introduction

Colorectal cancer (CRC) is the third most frequent cause of cancer-related deaths in both the United States and Europe (1,2). Around 25% of individuals harbor metastatic disease at the time of diagnosis, while approximately 50% of patients will develop metastases later (3). Although 5-year survival rates experienced by patients with metastatic CRC (mCRC) remain low (14%) (1), therapeutic options developed in the past two decades have prolonged the median overall survival (OS) from 12 to 30 months (4). This survival advantage can be attributed to improved surgical techniques and the use of more effective systemic therapies. At least partially, more informed treatment decisions based on molecular response predictors have also helped increase life expectancy, but biomarker recognition has been slow and often inconclusive due to the difficulty of substantiating correlative observations in patients with functional investigation in clinically relevant model systems. Similarly, while genomic datasets have offered an instructive compendium of the genes that are frequently altered in CRC (5), the question whether the aberrant protein products of such genes represent effective therapeutic targets remains hard to address in the absence of adequate translational tools.

The availability of large collections of patient-derived tumor samples that can be propagated in mice (xenografts) and in three-dimensional cultures (organoids) has spearheaded attempts to afford biomarker-response associations with mechanistic annotation and has facilitated studies aimed to model cancer progression and acquisition of drug resistance (6,7). Herein, we provide an overview of current therapies and related biomarkers, as implemented in patients with mCRC, and discuss how patient-derived xenografts and organoids have been deployed to go beyond correlative descriptions and to illuminate fundamental biological and clinical aspects of CRC, including drug repurposing efforts that have rapidly moved to the clinical space. Further, we consider the practical implications and the limitations of using such models in terms of clinical applicability and predictivity.

Empirical and biomarker-driven treatments: Correlative response predictors in patients

The standard-of-care treatment for mCRC patients includes cytotoxic agents and biological targeted compounds, which are administered cumulatively based on the empirical observation that multi-agent therapeutic cocktails are more effective than monotherapies (8,9). Although some patients receive important clinical benefit from these regimens, responses are typically limited to a fraction of individuals. The polarized distribution of responsive and non-responsive patients likely derives from the genomic and functional heterogeneity of mCRC tumors, which display patient-to-patient molecular differences that influence treatment outcome. While the application of ‘omics’ technologies has been instrumental to enrich for potential responders to targeted therapies, this has been unsuccessful for chemotherapy, in part due to its often incompletely understood and diverse mechanisms of action.

Chemotherapy

The fluoropyrimidine antimetabolite 5-fluorouracil (5-FU) and leucovorin, a biomodulator that enhances 5-FU activity, are most often administered in combination with oxaliplatin, a platinum compound endowed with inter- and intra-strand DNA cross-linking activity (FOLFOX) (10), or irinotecan, a topoisomerase I inhibitor (FOLFIRI) (11). Other therapeutic options include the fluoropyrimidine capecitabine plus oxaliplatin (CAPOX/XELOX) and capecitabine plus irinotecan (XELIRI). CAPOX/XELOX has shown analogous efficacy and safety compared to FOLFOX and it is typically given as first- or second-line therapy in patients refractory to irinotecan-based chemotherapy (12). XELIRI is non-inferior to FOLFIRI in terms of OS and is now recommended as an alternative second-line backbone treatment (13). In patients who have progressed after all standard therapies, a statistically significant (but modest) improvement in OS can be obtained with TAS-102, an agent that combines trifluridine (a nucleoside analog) and tipiracil hydrochloride (an inhibitor of thymidine phosphorylase) (14).

Potential determinants of response to chemotherapy have been brought to the fore based on the mechanism of action and metabolism of the various agents. However, the application of such predictors in clinical practice has been hampered by inconsistent results among different case series and poor

diagnostic sensitivity and specificity. For some chemotherapeutics, in consonance with data from targeted therapies, drug target overexpression may be a positive determinant of sensitivity. For example, high expression of thymidylate synthase (TS), a direct target of 5-FU, has been associated with longer survival in CRC patients treated with adjuvant 5-FU-based therapy in some studies (15,16); however, other reports have not confirmed the positive predictive value of TS overexpression (17,18) (Table 1). Likewise, elevated levels of topoisomerase 1 appear to predict better response to irinotecan (19) (Table 1). The activity of drug metabolic pathways is also thought to affect chemosensitivity. Dihydropyrimidine dehydrogenase (DPD) is a rate-limiting enzyme in fluoropyrimidine catabolism. High expression of DPD has been documented in tumors from patients with reduced sensitivity to capecitabine (20), with or without irinotecan (21), whereas inactivating polymorphisms of the *DYPD* gene (encoding DPD) have been associated with acute toxicity over the course of fluoropyrimidines-based therapy (22-24) (Table 1). In the same vein, deleterious polymorphisms of the *UGT1A1* gene (encoding glucuronosyltransferase, a key enzyme of irinotecan metabolism) are more frequent in patients who experience severe toxicity during treatment with irinotecan-based regimens (25,26) (Table 1).

Responsiveness to chemotherapy may also be related to defects in DNA repair mechanisms after chemotherapy-induced DNA damage, leading to abnormalities in DNA replication and/or chromosome segregation that culminate in cancer cell death. Excision repair cross-complementation group 1 (ERCC1) is a key effector of DNA repair mechanisms and influences the tumor DNA-targeting effect of oxaliplatin. Some studies have shown that low transcript expression of the *ERCC1* gene correlates with longer survival of patients treated with FOLFOX (27) (Table 1). Similar findings were reported for an *ERCC1* polymorphism at codon 118, which is expected to result in decreased *ERCC1* gene expression (28) (Table 1). These correlations, however, have not been confirmed in other datasets, especially when ERCC1 protein amounts rather than transcript expression were analyzed (29,30). Functional studies are needed to deepen mechanistic investigation of the relationship between DNA repair deficiency and chemosensitization. More in general, we advocate a revamping of biologically oriented research as a means to disentangle the intricacies behind chemotherapy efficacy (or lack of), including the evaluation of how genetic and epigenetic modifications in components of DNA repair pathways shape response to cytotoxic agents. A clearer understanding of the cellular and molecular underpinnings of chemotherapy activity in clinically relevant experimental models is a necessary step for the nomination of response biomarkers above and beyond descriptive variables in patients.

EGFR monoclonal antibodies

The EGFR monoclonal antibodies cetuximab and panitumumab are currently used in association with FOLXOX or FOLFIRI in the first- or second-line treatment of patients with *KRAS* or *NRAS* wild-type tumors (31). The restricted use of anti-EGFR antibodies to patients with RAS wild-type mCRC is the result of a population-level biomarker-development strategy motivated by the plausible rationale that constitutive activation of signaling pathways downstream from EGFR – such as those triggered by RAS mutations – should bypass EGFR inhibition and therefore obviate sensitivity to EGFR targeted agents. Evidence of the correlation between RAS genetic alterations and lack of response to EGFR blockade was initially limited to tumors with exon 2 *KRAS* mutations (32) and was later extended to *KRAS* exons 3 and 4 and *NRAS* exons 2, 3 and 4 (33) (Table 1). The predictive value of the association was so strong that retrospective studies in patients were deemed sufficiently powered to guide the development of companion diagnostics for the routine assessment of "RAS extended" mutations in mCRC patients, even in the absence of prospective validation.

Although patients with "RAS extended" mutations are currently excluded from therapy with cetuximab or panitumumab, there is still some debate as to whether *KRAS* G13D mutations – different from all other RAS mutations – predict some benefit from EGFR antibody treatment. Based on retrospective pooled analyses of multiple trials, the addition of cetuximab to first-line or salvage chemotherapy was shown to improve the outcome of patients with $KRAS^{G13D}$ mutant tumors (with relative treatment effects similar to those observed in subjects with *KRAS* wild-type tumors, but with lower absolute values) (34,35). This association was not reproduced in another pooled retrospective evaluation of patients treated with panitumumab and chemotherapy (36). Whether this discrepancy is due to the different characteristics of the two antibodies (cetuximab is a human-mouse chimeric antibody that contains the

human IgG1 constant region, whereas panitumumab is a fully humanized IgG2), or to differences in the clinical characteristics of the patient subgroups analyzed, remains to be determined. It is worth noting that the longer survival enjoyed by subjects with *KRAS*^{G13D} mutant tumors treated with cetuximab and salvage chemotherapy was not observed in the cetuximab monotherapy arm (34), a finding confirmed in an independent study in patients who had received single-agent cetuximab (37); hence, the confounding effect of the chemotherapy backbone cannot be excluded. The potentially stronger reliance of cancer cells with *KRAS* G13D mutations on the EGFR pathway has received some experimental support; specifically, *KRAS*^{G13D} mutant cell lines have been demonstrated to be sensitive to the RAS-inhibitory activity of the GTPase-activating protein neurofibromin (NF1), which is possibly unleashed by EGFR blockade; this dependence of KRAS^{G13D} mutant cells on the EGFR-NF1 axis could occur either because KRAS G13D proteins are particularly responsive to NF1-stimulated GTP hydrolysis (38) or because they are incompetent to curb NF1-dependent inactivation of the wild-type *KRAS* allele product, which thus remains modulatable by upstream EGFR signaling (39). However, most cell lines with *KRAS*^{G13D} mutations also harbor NF1 loss-of-function alterations (38), so the proposed regulatory circuit is likely to be valid only in restricted model systems that are hardly representative of clinical reality.

While the use of RAS mutation panels for the exclusion of mCRC patients who will not benefit from anti-EGFR therapy is now commonplace, the quest for positive response predictors has lagged behind. EGFR is hardly ever mutated or amplified in CRC, indicating that tumor dependency on the EGFR pathway does not have an evident genetic basis. Retrospective correlative studies have documented higher tumor expression of the EGFR ligands amphiregulin and epiregulin in patients who respond to EGFR antibodies (40,41) (Table 1). Hence, mCRC tumors that are sensitive to EGFR neutralization appear to rely on EGFR signals owing to ligand-mediated autocrine or paracrine receptor activation. This knowledge has not translated into clinical-grade methods for selection of potential responders due to the difficulty of dichotomizing continuous variables, such as transcript or protein expression, into a digital cut off for univocal allocation of patients to treatment.

Anti-angiogenic therapy

The first-line treatment for patients with mCRCs harboring *KRAS* or *NRAS* mutations comprises either FOLFIRI or FOLFOX plus the anti-VEGF antibody bevacizumab (42,43). Whether the addition of VEGF-targeting agents to chemotherapy provides comparable or superior benefit to anti-EGFR antibodies in the context of *KRAS*/*NRAS* wild-type tumors is still a matter of debate (44). Beyond bevacizumab, two other anti-angiogenic drugs have been proved to positively impact on PFS and response rates when combined with chemotherapeutic agents in late lines of treatment: aflibercept (an anti-VEGF-A, VEGF-B, and placental growth factor) (45) and ramucirumab (an anti-VEGF receptor 2) (46). Finally, heavily treated chemorefractory patients can experience slightly longer OS when treated with the multikinase inhibitor regorafenib, which also targets pro-angiogenic receptors (47).

At present, there are no validated predictors of response to anti-angiogenic agents (48). Clinical reports have shown a correlation between more marked responsiveness to bevacizumab and low baseline levels of VEGF-A splice isoforms (49), VEGF-D (50), HGF (51), interleukin-8 (52) or the VEGF-A coreceptor neuropilin-1 (53), either in plasma or tumors (Table 1). Germline polymorphisms of VEGF-A (54,55), VEGF receptor-1 (56) and inflammation- and endoplasmic reticulum-associated genes (57) also show a significant interaction with bevacizumab effectiveness (Table 1). Other potential biomarkers predicting bevacizumab therapeutic efficacy include loss of chromosome 18q11.2-q12.1 (58) and, more in general, a high degree of chromosomal instability (CIN) (59) (Table 1). In the case of regorafenib, initial studies suggest that low expression of vascular cell adhesion protein 1 (VCAM-1) may be associated with better response (60) (Table 1). Polymorphisms of genes related to the C-C motif chemokine ligand 5/C-C motif chemokine receptor 5 pathway (which regulates VEGF-A expression) also predict efficacy in mCRC patients treated with regorafenib (61) (Table 1).

The fact that the expression levels of angiogenic targets positively associate with sensitivity to anti-angiogenic agents has a pharmacokinetic basis ascribable to the relative stoichiometry of drug-target interactions. The biological significance of other candidate biomarkers, such as copy number alterations, is of difficult interpretation in the absence of functional modeling in adequate experimental systems.

Mechanistic investigation of stromal-directed therapies is complicated by a dearth of preclinical resources, which typically consist of syngeneic transplants, genetically modified mice, or xenografts. All approaches have limitations: on the one hand, "mouse-only" models hardly recapitulate the inter-patient heterogeneity and population diversity of human tumors – a severe drawback for biomarker discovery research; on the other hand, xenografts are by definition a chimeric source of angiogenic factors, which can be concomitantly released by human cells of the tumor and murine stromal and inflammatory cells of the host. Therefore, due to species specificity, the cross-talk between heterologous cellular compartments is biased, and therapeutic antibodies cannot have full capacity at the organismal level because they selectively target either murine or human antigens.

Other targeted therapies

All the above therapies are administered in the absence of positive molecular selection; the only criterion is patient exclusion based on the presence of negative response biomarkers, as epitomized by the established association between *KRAS*/*NRAS* mutations and lack of response to EGFR blockade. Recently, a number of low-frequency aberrations in kinase-encoding genes have been identified in mCRC that result in constitutive activation of the corresponding protein products. Alterations in the *ERRB2* gene (mostly gene amplification) are detected in around 5% of *KRAS*, *NRAS* or *BRAF* wild-type mCRCs and lead to overexpression and hyperactivation of the encoded HER2 tyrosine kinase receptor. Phase 2 studies in patients selected for having HER2-positive mCRC tumors have shown that dual inhibition of HER2 using the anti-HER2 antibody trastuzumab and the EGFR/HER2 small-molecule inhibitor lapatinib (HERACLES trial) or the combination of trastuzumab and the anti-HER2 antibody pertuzumab (MyPathway trial) have considerable clinical efficacy, with around 30% response rates in heavily pretreated patients (62,63) (Table 1). Notably, in both studies responsive patients had tumors with higher *ERBB2* gene copy number than resistant patients, consistent with the assumption that higher *ERBB2* gene dosage translates into stronger kinase activation, hence in more profound tumor dependency on HER2 signaling.

Kinase fusions originating from chromosomal translocations and resulting in constitutive activation of neurotrophic receptor tyrosine kinase 1 (NTRK1), NTRK2, NTRK3, anaplastic lymphoma kinase (ALK), and RET account for approximately 1-2% of *KRAS*, *NRAS* or *BRAF* wild-type mCRCs. These rearrangements are enriched in right-sided RAS wild-type tumors and, while typically portending a dismal prognosis (64), they predict therapeutic benefit of inhibitors such as entrectinib (targeting NTRK, ROS1 and ALK) (65,66) and ponatinib (targeting various tyrosine kinases including RET) (67,68) (Table 1).

BRAF gene alterations (with a dominant prevalence of V600E activating mutations) are found in 7-10% of mCRCs (69,70) and are generally mutually exclusive with *KRAS* and *NRAS* mutations, indicating that a single oncogenic hit on the ERK MAPK pathway is sufficient to sustain tumorigenicity. Selective BRAF targeting with specific inhibitors has proven ineffective in patients with *BRAF* mutant mCRC due to feedback reactivation of EGFR signaling, which substitutes for BRAF blockade in stimulating the MAPK pathway (71,72). This observation has prompted the design of clinical trials aimed at evaluating the efficacy of combined BRAF and EGFR inhibition in patients with *BRAF* mutant mCRC. In a recent phase 3 study testing cetuximab and the BRAF inhibitor encorafenib versus cetuximab and irinotecan (or FOLFIRI), combined EGFR and BRAF blockade significantly improved response rates and OS compared with standard therapy. This superior activity was further enhanced by concomitant MEK inhibition (73) (Table 1).

Despite their high prevalence (approximately 50% of all CRCs), *KRAS* and *NRAS* mutant tumors are still treated with conventional chemotherapy and anti-angiogenic agents. Hopes are now placed on the use of KRAS G12C covalent inhibitors, which are currently tested in patients with $KRAS^{G12C}$ solid tumors. Initial results seem to indicate that response rates to these drugs are relatively high in patients with non-small cell lung cancer but limited in mCRC patients, likely due to retained sensitivity of CRC tumors to upstream EGFR signaling (74) (Table 1). These observations echo findings in *BRAF* mutant tumors (71,72) and strengthen the notion that EGFR signaling needs to be concomitantly neutralized to achieve better responses to drugs targeting the RAS-MAPK pathway in mCRC.

Immunotherapy

About 15% of CRCs display defective functionality of mismatch repair (MMR) proteins, which participate in the correction of base-pair mismatches occurring during DNA replication (especially at the level of repetitive DNA sequences called microsatellites). Deficient-MRR (dMMR) tumors with mutations in 30% or more microsatellites (defined as dMMR/MSI-H, *i.e*. with high microsatellite instability) tend to accumulate nonsynonymous mutations; this increased mutational burden can translate into a higher neoantigen load, which makes some dMMR/MSI-H tumors immunogenic and sensitive to immune checkpoint blockade (75). Accordingly, single-agent therapy with the anti-PD-1 antibodies pembrolizumab or nivolumab and combination therapy with nivolumab and the anti-CTLA-4 antibody ipilimumab have been approved for treatment of patients with chemorefractory dMMR/MSI-H mCRC (76-78) (Table 1Preclinical evidence also suggests that anti-PD-1 immunotherapy may complement the activity of KRAS G12C covalent inhibitors. In a CRC syngeneic mouse xenograft model dependent on the *Kras*^{G12C} allele, KRAS G12C blockade resulted in increased infiltration of $CD8^+$ cytotoxic T cells, macrophages, and dendritic cells (79). This pro-inflammatory phenotype sensitized tumors to immunotherapy: when combined with an anti-PD-1 antibody, the KRAS G12C inhibitor induced complete tumor regressions that persisted also after treatment discontinuation. Interestingly, mice that were "cured" by the combined treatment against KRAS G12C and PD-1 rejected tumor rechallenge, indicating that the combination therapy favored the establishment of tumor-specific T cell responses (79).

The assessment of MSI status is now routinely performed for selecting patients likely to respond to immunotherapy, but only a subgroup of individuals with MSI mCRC receive clinical benefit from this treatment. Not always are the protein products of somatic DNA variants efficiently presented by MHC molecules, which means that tumor mutational burden only partially contributes to neoantigen load. Likewise, although an association between high neoantigen load and pronounced immune cell infiltration has been repeatedly documented, the presence of an active immune microenvironment has not predictive value for immunotherapy sensitivity (80). HLA binding prediction tools and artificial intelligence algorithms for multiparametric imaging of immune cell representation and topography in tumors are expected to yield more reliable molecular biomarkers for effective patient stratification in the immuno-oncology space.

Preclinical models for understanding and predicting therapeutic response in CRC

Biomarkers that predict patient response to treatment are usually identified using population-based association studies, in which clinical outcome is correlated with a statistically significant enrichment for a specific molecular trait (typically, a genetic alteration) in subjects who do or do not respond to a given therapy. Albeit useful for clinical decision making, this approach fails to inform whether therapeutically relevant response predictors causally influence drug sensitivity and does not provide insight into the mechanistic underpinnings of the observed correlations. In a complementary perspective, studies using cancer cell lines enable extracting functional annotations and modeling cause-effect relationships; however, cell lines are by definition limited in number; thus, they do not recapitulate the spectrum of genetic heterogeneity spanned by patient tumors. Recently, patient-derived platforms that reflect the diversity of cancers, while retaining experimental manipulability and clinical fidelity, have been developed with the aim to characterize response biomarkers, investigate tumor adaptation under drug pressure, and understand the evolutionary principles of tumor progression. CRC has been – and still is - a testing arena for such efforts.

Patient-derived xenografts (PDXs) for validation of targeted therapy biomarkers

Surgically derived tumor samples that are implanted in mice (known as patient-derived xenografts, PDXs) retain the inherent features of different tumors from different patients (6,81). Vast PDX collections are therefore expected to capture inter-patient tumor heterogeneity at the population level in a clinically relevant *in vivo* setting (82). CRC is a paradigmatic example of the importance of PDX-based research for large-scale genotype-response associations, predictive biomarker identification, and therapeutic studies (83,84) (Figure 1). In 2011 a systematic survey of *KRAS* and *NRAS* mutations in more than 100 PDXs from metastatic CRC tumors, coupled with annotation of sensitivity to cetuximab,

produced a dataset with both confirmatory and discovery aspects (85). On the one hand, the association between *KRAS* mutations in exon 2 and *de novo* resistance to EGFR blockade – which had emerged from clinical studies some years earlier (32, 86) – was "reverse validated" in PDXs and found to be coherent with patient data (85) (Table 1). On the other hand, results in PDXs were among the first to illustrate that *KRAS* mutations in exons 3 and 4 and *NRAS* mutations predict lack of response to EGFR antibodies (85,87). This finding would receive ultimate clinical recognition only two years later, when a retrospective-prospective analysis concluded that patients with tumors harboring "RAS extended" mutations treated with anti-EGFR antibodies had inferior PFS and OS compared with patients with *KRAS*/*NRAS* wild-type tumors (33).

While PDXs appear to have adequate predictive power for cancer cell-directed treatments, they lose value when dealing with therapies against stromal components – such as cancer-associated fibroblasts, endothelial cells, and inflammatory cells – and cells of the adaptive immune system. Indeed, the host must be immunocompromised to tolerate the graft, and human stromal cells are substituted with murine counterparts over serial passaging (6). But this drawback bears some advantages: the chimeric nature of PDXs has been leveraged to decompose – from bulk tumors – cancer cell-specific and stromal signals using analytical methods that distinguish human *versus* mouse transcripts. This exercise has increased the granularity and informative merit of gene expression classifications. For example, the clinical aggressiveness of a poor-prognosis transcriptional subtype named CMS4 had been initially ascribed to the ability of cancer cells to undergo epithelial-mesenchymal transition (EMT), a phenotypic switch that instigates cell motility and invasion (8,88). With the possibility to discriminate between human and mouse transcripts, it became clear that – together with displaying some cancer cell-autonomous EMT traits – the vast majority of mesenchymal CMS4 tumors are in fact characterized by a heavy content of stromal cells, which likely foster the malignant characteristics of this subtype by conveying mitogenic, pro-invasive and anti-apoptotic cues (89). In the same vein, CRIS, a new CRC classification based only on PDX human transcripts, identified subtypes endowed with prognostic and predictive significance and showing limited overlap with transcriptional classes obtained from whole bulk CRCs (90). Moreover, by focusing on cancer-cell intrinsic gene expression features that are not influenced by stromal abundance in isolated, randomly taken tumor samples, CRIS demonstrated higher accuracy in clustering CRCs by patient-of-origin rather than tumor region-of-origin (91).

PDXs to study the clonal dynamics of CRC tumors under chemotherapy pressure

Tumors are composed of heterogeneous cell subsets that display different proliferation kinetics, susceptibility to apoptosis, and sensitivity to drug insults (92). Some works have used PDX models to investigate the clonal propagation dynamics of CRC subpopulations, both during spontaneous tumor growth and under drug pressure (Figure 1). DNA copy number alteration profiling and deep sequencing of mutational hotspots were combined with lentiviral lineage tracking to follow the progeny of single CRC cells over serial xenografts and to interrogate the relative contribution of genetic and nongenetic mechanisms to the functional heterogeneity of the individual cancer cells (93). While genetically identical clones remained stable upon serial transplantation, lentivirally marked lineages were variable within each clone, with pronounced differences in proliferation rates, ability to persist, and susceptibility to exhaust through passages (93). Likewise, treatment of xenografts with oxaliplatin did not result in a detectable bottleneck or selection for novel genetic clones; rather, chemotherapy shaped a new dominance of previously dormant lineages and culled actively proliferating progeny (93). Together, these results indicate that cancer cells subpopulations can be genetically homogeneous (and stable) but functionally heterogeneous (and plastic) in CRC.

The finding that CRC cancer cells oscillate between periods of dormancy and activity appears to have a positional determination. Using a tamoxifen-inducible labeling system to stochastically mark cancer cells in mouse xenografts of patient-derived spheroids, coupled with computational modeling, Lenos and colleagues documented that CRC grows through surface expansion (94). This peripheral accretion is driven by the local availability of mitogenic gradients secreted by cancer-associated fibroblasts, which are sensed only by cancer cells located in the outermost zone of the tumor (94) (Figure 1). Chemotherapy with 5-FU and oxaliplatin reduced tumor growth rates but did not affect the residual dynamics of surface growth, indicating that microenvironmental influences dictating spatially confined cell proliferation are not interfered by cytotoxic treatment (94). Other studies with multicolor lineage tracing approaches in

xenografts of CRC primary cultures and cell lines confirmed that tumor outgrowth is geometrically orchestrated by large proliferating clones confined at the leading tumor edge, whilst small quiescent clones reside in the center (95,96).

PDXs and genetically modified animal models for target discovery

Besides providing preclinical hints for response biomarker validation, PDXs have also been deployed for testing therapeutic options in newly identified, molecularly circumscribed mCRC subsets (Figure 1). Amplification of the *ERBB2* oncogene was detected in some *KRAS*/*NRAS* wild-type, cetuximab-resistant PDX models, and was found to predict response to HER2 targeted therapies in PDX-based preclinical trials (85,97) (Table 1). Other clinically actionable alterations were shown to be enriched in *KRAS*/*NRAS* wild-type, cetuximab-refractory PDXs and patients, including activating mutations of *ERBB2* and *MAP2K1* (encoding the RAS downstream effector MEK1), amplification of the tyrosine kinase receptors *MET* and *FGFR1*, and outlier overexpression of the survival factor *IGF2* (98-101). In general, the sole inhibition of the hyperactive oncoproteins proved to be ineffective in PDXs, but treated tumors were invariably sensitized to concomitant EGFR blockade. Subsequent clinical studies confirmed that patients with HER2-positive mCRC tend to respond poorly to EGFR antibodies and can benefit from dual treatments against HER2 and EGFR (62,63). Similarly, MEK1 mutations were found to predict resistance to EGFR inhibition and response to a combination of trametinib (a MEK inhibitor) and panitumumab in patients (102).

PDXs have been shown to recapitulate clinical reality also in terms of depth of response. Similar to metastases in patients, mCRC PDXs that respond to EGFR antibodies can experience massive shrinkage but are hardly ever eradicated. The residual cancer cells that withstand upfront drug treatments act as a reservoir for the stochastic acquisition of resistance-conferring mutations, with the ensuing expansion of subclones responsible for tumor relapse (103). Recent evidence indicates that residual mCRC PDXs (and residual tumors in patients) at maximal response to prolonged anti-EGFR therapy relax their dependency on EGFR signals by reducing the expression of genes encoding EGFR-activating ligands and increasing alternate HER2/HER3 pathway activity, while becoming similar to slowly-cycling secretory precursors of the normal intestine (104). The finding that cetuximab-tolerant residual tumors exhibit decreased abundance of EGFR cognate ligands is consistent with the clinical observation that patients with mCRC tumors expressing low levels of amphiregulin and epiregulin tend to respond less to EGFR antibodies (40,41) (Table 1). Pseudodifferentiation into tissue-specific lineages has been documented as a mechanism of therapy resistance in other tumors; for example, the manifestation of neuroendocrine traits is a hallmark of emerging resistance to EGFR inhibitors and anti-androgen treatment in lung and prostate cancer, respectively (105,106). In the context of CRC, cetuximab-induced phenotypic reprogramming towards a secretory fate with high HER2/HER3 signaling makes cancer cells vulnerable to concomitant targeting of EGFR, HER2 and HER3, as shown by reduction of residual disease burden and prolonged time to relapse after treatment discontinuation in PDX trials with a Pan-HER antibody (104).

As noted above, PDXs are inadequate tools for predicting response to therapies against stromal and immune cells. Genetically modified mouse models (GEMMs) develop autochthonous CRC tumors in an immune-competent background, but the artificial introduction of founder oncogenic mutations may result in evolutionary trajectories different from those occurring in spontaneous tumors (107). This limitation has been addressed by engineering the ordered expression of salient mutant oncoproteins along the linear progression sequence that typifies human CRC, with the aim to more faithfully recapitulate the natural history of human tumors. In seminal experiments, individual inactivation of the *Apc* gene (which normally represses intestinal stem cell proliferation by blocking mitogenic signals of the Wnt pathway) caused the formation of adenomas and *in situ* carcinomas (108). In the context of *Apc* deficiency, the concomitant expression of oncogenic *Kras* or the concomitant loss of the tumor-suppressor gene *Trp53* or the *Smad2*/*Smad4* genes (the latter being downstream mediators of TGFβ signaling) accelerated intestinal tumorigenesis and resulted in the development of locally invasive (albeit not metastatic) carcinomas (109-112). Ultimately, the compound assortment of mutations in *Apc*, *Kras*, and *Trp53* or *Apc*, *Kras* and *Tgfbr2* (encoding the type-2 TGFβ receptor) enabled implementation of the full metastatic phenotype (113,114). Likewise, animals with targeted gene recombination of *Apc*, *Kras*,

Tgfbr2, and *Trp53* (AKTP) to intestinal stem cells developed invasive CRC adenocarcinomas with hallmarks of human microsatellite-stable tumors, including low mutational burden and scant T-cell infiltration (115). Moreover, AKTP tumors had an abundant representation of carcinoma-associated fibroblasts engaged in massive deposition of extracellular matrix and profuse secretion of TGF-β. Importantly, increased TGFβ in the tumor microenvironment was found to be a major determinant of T-cell exclusion, and blockade of TGFβ signaling rendered tumors more T cell-inflamed and susceptible to immunotherapy (115) (Figure 2). Another mouse model harboring *Kras* and *Trp53* mutations along with hyperactive Notch signaling in intestinal cells developed metastatic tumors with serrated morphology, extensive stromal content, and gene expression profiles similar to those of poor-prognosis tumors in patients (116). In these mouse tumors, hyperactivation of the Notch pathway resulted in secretion of TGF-β by cancer cells, which prompted neutrophil accumulation in the tumor stroma and neutrophil-dependent metastatic dissemination. Accordingly, targeting neutrophil recruitment or TGF-β signaling reduced metastatic burden (116) (Figure 2). Collectively, these results underscore the value of transgenic mice as investigational models to explore the interplay between genetic alterations and the immune-competent tumor microenvironment and to integrate PDX-based research on cancer cell-intrinsic vulnerabilities.

Patient-derived organoids for mechanistic investigation and pharmacologic studies

PDXs represent more authentic working models than conventional cell-line xenografts to study how cancer cells evolve and react to therapies in a clinically relevant scenario that reflects organismal complexity. However, PDXs are not endowed with sufficient experimental tractability to distill causality from description, nor do they show sufficient scalability to enable high-throughput pharmacogenomic screens. Short-term culture of tumor sections allows for *in vitro* screening at a reasonably large scale (117), but it is constrained by the fact that the proliferative capacity of the cultures dissipates over time. To overcome these limitations, three-dimensional organotypic or "organoid" long-term culture methods have been developed that combine the architectural complexity of tissues with the experimental flexibility of "immortalized" cell-culture systems (118). For colon, normal organoids containing only epithelial cells can be derived by culturing primary nontransformed intestinal tissue in Matrigel – a gelatinous mixture made of laminin-rich extracellular matrix and growth factors – supplemented with additional growth factors that mimic the intestinal niche (119,120) (Figure 3). CRC organoids require less stringent combinations of niche factors than normal intestinal organoids (121,122). Mouse and human organoids are commonly used not only for biological and pharmacologic studies *in vitro* but also as model tools of CRC spontaneous metastatization. GEMM-derived organoids, normal mouse organoids engineered to express oncogenic mutations, and patient-derived human tumor organoids can readily give rise to invasive carcinomas that infiltrate the muscularis propria and colonize the liver after orthotopic engraftment by colonoscopy-guided mucosal injection, enema, or surgical implantation into the submucosa of the caecal wall (123-127).

Patient-derived normal and CRC organoids have been leveraged to advance cancer modeling and decompose mechanisms of CRC tumorigenesis. Using CRISPR/Cas9-based genome editing, Matano et al. sequentially introduced loss-of-function mutations of *APC*, *SMAD4* and *TP53* and gain-of-function mutations of *KRAS* and/or *PIK3CA* in normal human colon organoids, followed by growth selection under customized cell culture conditions (128). Organoids engineered to express all five mutations formed small, highly differentiated tumors with limited local infiltration after implantation under the kidney subcapsule in mice, and were unable to metastasize to the liver after injection into the spleen. Conversely, organoids from patients' advanced tumors that had accumulated spontaneous oncogenic mutations during their evolutionary history displayed robust renal subcapsular growth and produced prominent spleen-to-liver dissemination (128) (Figure 3). These results suggest that the ectopic introduction of canonical driver mutations in normal human intestinal cells results in incipient tumor formation but is not sufficient for a CRC tumor to exhibit an invasive and metastatic behavior. Additional lesions that drive full-blown CRC malignancy may be fueled by epigenetic modifications and CIN; indeed, the engineered organoids largely lacked karyotypic or DNA methylation aberrations, which were instead abundantly present in patient-derived CRC organoids (128). The acquisition of gene copy number alterations after genetic manipulation of normal intestinal organoids appears to be influenced by experimental variables; for example, different from Matano et al., Drost et al. found that

combined loss of *APC* and *TP53* in normal human colon organoids was sufficient for the appearance of CIN and massive aneuploidy (129). In patient-derived CRC organoids, a combination of genetic lineage tracing and ablation systems revealed robust functional plasticity. $LGR5^+$ cells were shown to act as cancer stem cells that constantly fueled tumor growth through self-renewal and at the same time were able to morph into differentiated post-mitotic cells. Selective ablation of $LGR5^+$ cells transiently regressed tumors; however, this shrinkage was followed by tumor regrowth due to the replenishment of the $LGR5^+$ pool by differentiated cells that had reacquired stem-like features (130).

Clonal organoids derived from isolated cells can be considered as proxies for the single cells from which they originate (Figure 3). Phylogenetic trees constructed through deep genomic analysis of CRC clonal organoids revealed that driver mutations commonly found in CRC (such as those in *APC*, *KRAS*, and *TP5*3) were present in all organoids, that is, they were trunk mutations common to all cells of the original tumor. However, many "private" mutations could be detected in the distal branches of the phylogenetic trees, indicating that they had arisen later during tumor progression and had contributed to tumor genetic diversification (131). These results are in line with the "big bang" model of CRC tumorigenesis, according to which genetic variants that confer selective advantages occur early in a cancer's evolution and are followed by the neutral expansion of genetically different but equally fit subclones (132,133). Stable alterations of DNA methylation and transcriptome states were also observed in clonal organoids, with phylogenetic topologies similar to the mutation-based trees (Figure 3). Conversely, response to drugs commonly used in CRC was variable – especially with chemotherapeutic agents – and not linked to the geographical location of the organoid-initiating cells in the original tumor or the genetic distance between clones (131). Similar to that observed in PDX-based lineage tracking experiments (93), these results suggest that diversification in biological behavior has no evident correlation with the extent of mutational diversification.

The application of organoid technology in systematic high-throughput drug screens to validate clinically relevant response biomarkers and nominate new ones is rapidly expanding (Figure 3). A seminal study with a library of 83 compounds tested in 19 organoids from primary CRC tumors confirmed the association between *KRAS* mutations and lack of response to EGFR blockade as well as general refractoriness of *BRAF* mutant tumors to BRAF inhibitors, as observed in the clinic (121). This effort also identified loss-of-function mutations in *RNF43*, resulting in cell hypersensitivity to secreted WNT factors, as predictive biomarkers of CRC susceptibility to neutralization of autocrine/paracrine activation of the WNT pathway (121). Organoids from metastatic samples have been shown to recapitulate the clinical response of the donor patient to cetuximab, regorafenib, and TAS-102 (134). Interestingly, organoids derived from a patient with regorafenib-sensitive liver metastases proved to be resistant to the drug when cultured *ex vivo* as isolated cancer cells; however, liver orthotopic xenografts developed from the same organoids coopted the host's blood vessels and displayed reduced vascularity after regorafenib administration, in keeping with the assumption that response to regorafenib is mainly driven by its antiangiogenic activity (134). A concordance between cancer cell viability and patient response was also found in organoids from metastatic tumors treated with irinotecan monotherapy or FOLFIRI, but not when FOLFOX was used (135). Possibly, stromal and immune components absent in organoid cultures tune sensitivity to oxaliplatin more than they do with other drugs, or reliable response to oxaliplatin requires tailored culture conditions that are less stringent for other chemotherapeutics. Finally, organoids derived from primary rectal cancers have been demonstrated to predict clinical and histopathologic responses to neoadjuvant chemoradiation, as observed in matched donor patients (136,137).

Challenges and emerging opportunities

The utilization of living biobanks of tumor samples holds considerable promise for *in vivo* and *in vitro* interrogation of clinically actionable pathways and for the study of tumor evolution. But the use of patient-derived models should be accompanied by careful appreciation of their real potential not only as platforms for biomarker validation and target discovery but also as reliable proxies of the biological and molecular fingerprints of matched tumors in donor patients. A critical knowledge of the accuracy of

patient-derived models in retaining the characteristics of original tumors is crucial for assessing their ability to predict drug activity in the clinic.

Preservation of genomic architecture in propagated tumor-derived models

An ongoing debate revolves around the question whether serially passaged PDXs and long-cultured organoids preserve the genomic makeup, in terms of copy number alterations (CNAs), of their pre-derivation counterparts. Using gene expression microarray data to infer large-scale CNA profiles, Ben-David et al. reported extensive copy number divergence between the pre-implantation tumor of origin and the corresponding xenograft at the first *in vivo* passage, which was exacerbated along serial propagations (138). This raised concerns that mouse-specific selective pressures may "artificially" influence PDX tumor evolution, with implications for the ability of PDXs to faithfully model patient treatment response. However, expression-based CNA calling only enables assessment of aberrations at the gross scale of chromosomal arms. Recently, a joint effort of the National Cancer Institute PDXNet consortium and the EurOPDX consortium produced a DNA-based enumeration of copy number profiles at high segmental resolution in a large collection of PDX models (139). This analysis did not confirm systematic copy number deviation between patient tumors and PDXs; rather, it documented high CNA retention during PDX engraftment and passaging (both globally and at the level of cancer-related genes) for many tumor types including mCRC. Notably, CNA variations between pre-implantation and xenografted tumors were comparable to differences in multi-region samples of tumors in patients, indicating that the impact of PDX-associated CNA drift is similar to the natural intratumoral evolution that occurs in patients.

Somatic mutations, typically assessed by whole exome sequencing, are largely concordant between original tumors and matched PDXs, even though evolutionary neutral subclonal alterations may arise at low allele frequency during PDX propagation (140). In CRC, mutations in known oncogenic drivers are retained in PDXs when present in the corresponding patient tumors and do not appear *de novo* in mouse-passaged xenografts from either primary (84) or metastatic samples (85, 99), including matched samples of primary tumors and synchronous or metachronous metastases (83, 141). An overall preservation of CNA and mutational landscape, with the caveat that the number of samples analyzed so far is limited, has also been observed in CRC organoids as compared with the corresponding patient tumors (84,121,142). However, CIN CRC organoids tend to tolerate mitotic errors, which results in the accrual of chromosome mis-segregations over time (143). Similarly, an accumulation of synonymous and nonsynonymous mutations has been noted during prolonged culturing of MSI CRC organoids (122).

The hurdles of co-clinical trials

A number of exploratory studies have shown the potential of PDXs for mirroring therapeutic response in the patients who contributed tumor samples; for instance, the clinical outcome of individuals who had received various chemotherapeutic regimens for the treatment of liver or peritoneal metastases reflected the objective response (or lack of it) monitored months or years before in patient-matched PDXs that had been established at the time of primary tumor resection (144). Similarly, when PDX models were generated from pretreatment core biopsies of *BRAF* mutant metastases and tested for their sensitivity to combined BRAF and MEK blockade, the objective response in mice was similar to the radiological response in the biopsied lesions (145).

If patient-derived models are high-fidelity "avatars" of pre-derivation tumor samples, they could be used – in principle – for real-time assessment of drug sensitivity, which may be reverse-exploited to guide treatment decisions in donor patients. Co-clinical trials have been proposed in which PDX mice are treated with panels of drugs – either agents with broad-brush anticancer activity or targeted compounds based on molecular predictors; then, when a positive signal for a specific therapy emerges, the information is transferred back to the donor patient for clinical evaluation (81,146). While intriguing, an approach of this kind requires that therapeutic findings be univocally deciphered and rigorously interpreted. For example, spurious positive signals may arise for treatments that delay tumor growth, resulting in tumors that are smaller than untreated controls at end point, but larger than they were at treatment initiation. This outcome may be indicative of biological sensitivity (*i.e.*, the drug reduces cancer cell proliferation) but has little clinical relevance; indeed, in patients, a lesion that enlarges during

treatment (even to a relatively small extent) denotes tumor progression, and the therapy is usually discontinued due to lack of efficacy.

Another issue with the execution of PDX-based co-clinical trials is the need to cope with quick turnarounds. Results in mice must be promptly returned to donor patients to inform treatment decisions, but research with PDXs notoriously implies long-term and time-consuming experiments. "Cutting corners" in the name of rapidity, for example by reducing the number of animals tested in each treatment cohort, would lead to insufficiently powered studies and scientifically unreliable conclusions. Compared with PDXs, organoids are expected to speed up the bench-to-bedside pipeline due to their higher manageability. However, we are still missing metrics that adequately capture how and to what extent organoids deliver a clear prediction of the outcome in patients. There is no consensus on the adoption of common readouts of drug sensitivity (reduction of cell proliferation *versus* induction of apoptosis) and shared methodologies for data acquisition (digital imaging *versus* cell counts). Moreover, a direct comparison of the concordance between patient-matched PDXs and organoids in categorizing response or resistance to therapy has not been attempted so far on a systematic scale.

PDX studies could also provide potentially useful real-time information about drug toxicity, but gathering generalizable data on this aspect will likely prove daunting. A meta-analysis of adverse events in mice treated with various therapies has revealed large deviations among different studies, with a variable extent of animal weight loss or death toll that was apparently independent of mouse strain and dosage and rather attributable to facility- and operator-related factors (147). This inconsistency is compounded by idiosyncratic liabilities of defined mouse strains; for instance, SCID mice harbor a loss-of-function mutation in the catalytic subunit of DNA-dependent protein kinase, an enzyme required for efficient DNA double-strand break repair (148). Consequently, mouse strains that carry this mutation show increased total-body sensitivity to chemical or physical agents that damage DNA, such as irradiation, chemotherapy, and inhibitors of the DNA repair machinery. As always when dealing with resource platforms, standardized guidelines built on cumulative experience will be a prerequisite for direct transfer of preclinical results to patients.

Integration of the tumor immune microenvironment: humanized mice and co-cultures

The necessity of using immunocompromised mice to prevent xenograft rejection hampers the use of conventional PDX models to assess the efficacy of immunotherapies. Humanized mice are immunodeficient animals in which the human immune system is partially reconstituted by introducing $CD34^+$ hematopoietic stem cells (HSCs), peripheral blood mononuclear cells (PBMCs), or tumor-infiltrating lymphocytes (TILs) (6,149) (Figure 4). Attempts to generate humanized CRC models have been scant. Cell-line xenografts in mice engrafted with allogeneic or autologous human PBMCs showed delayed growth kinetics and increased infiltration of cytotoxic T cells after treatment with a combination of nivolumab and urelumab, a CD137 agonist monoclonal antibody that enhances T-cell and natural killer-cell antitumor activity (150). Similar results were observed in a dMMR/MSI-H PDX model, but not in a microsatellite stable model, after humanization with cord blood-derived $CD34^+$ cells and treatment with nivolumab (151).

Although humanized mice appear to recapitulate some of the effects of immunotherapy observed in patients, the procedure of mouse humanization is afflicted with several drawbacks. PBMC and TIL infusion typically causes severe graft-versus-host disease starting 2-5 weeks after injection (152,153), which restricts the investigative window to temporal limits that are hardy compatible with experimental needs. Transplantation of HSCs results in a more complete hematopoietic reconstitution, but their maturation as well as the effector functions of their differentiated progeny are compromised by the lack of cytokines, phagocytes, and HLA molecules of human origin in the mouse host. The application of genome editing technologies for mouse genetic engineering is expected to increase the extent of humanized cells and molecules in future murine models.

Another emerging asset to reconstruct the functional interactions between cancer cells and the immune microenvironment relies on hybrid organ-on-a-chip platforms, which allow the build-up of more complex multicellular systems (154). Reductionist methodologies involve the initial establishment of separate cultures of epithelial organoids and immune cells, followed by artificial reconstitution in co-

mingling experiments. This approach has been used to set up co-cultures of cancer cells from primary or metastatic CRC with high mutational burden and autologous PBMCs, wherein cancer cell organoids triggered antigen-specific stimulation of tumor-reactive cytotoxic T cells in the PBMC fraction (155) (Figure 4). More sophisticated air-liquid interface (ALI) methods have also been deployed that enable the *en bloc* preservation of the tumor epithelium and its endogenous immune stroma, including fibroblasts, tumor-associated macrophages, T and B lymphocytes, and natural killer cells (156). ALI cohesive units propagated from CRC biopsies retained the T cell receptor heterogeneity of the T cells present in original tumors and modeled the effects of nivolumab by recapitulating cytotoxic T cell expansion and antibody-dependent tumor cytotoxicity (156) (Figure 4). Further complexity could be achieved by integrating on-chip tumor immune microenvironments with biomimetic vascular-like structures for reconstitution of physiological functions of the microvascular tissue. This methodology has been used to develop a 3D chip-based model comprising a human CRC core and a surrounding vascularized network (157) and, in principle, might be upscaled to include microfluidic co-cultures of immune cells. Assessing the functional consequences of immune checkpoint blockade using advanced organoid technology is poised to complement existing descriptive biomarkers, such as neoantigen load, in the identification of patient-specific determinants of response to immunotherapy.

Concluding remarks

The clinical and experimental observations discussed above illustrate the power of population-level studies – both in patients and in the preclinical setting – to credential candidate predictive biomarkers and identify novel determinants of therapeutic response as well as novel targets. Recent evidence also highlights the value of patient-derived xenografts and organoids as tools to investigate subclonal dynamics during tumor evolution and functional heterogeneity under drug pressure. The credibility of patient-derived models in preserving the molecular architecture of the corresponding pre-derivation tumors is now supported by large-scale analytical efforts and the use of accurate genomic approaches. These merits notwithstanding, several issues remain, which are mostly related to the inability of PDXs and organoids to recapitulate heterotypic interactions between cancer cells, stromal cells, and immune cells. Mouse humanization procedures and co-culture assays are expected to aid the development of more holistic models that incorporate immune components. However, the impact of bone marrow reconstitution (let alone that of PBMC or TIL infusion) on the quality, quantity and topographical localization of immune infiltrates in transplanted tumors is difficult to assess, as is the influence of the host on the differentiation trajectories and functionality of transplanted human HSCs. Likewise, cocultures of cancer cell organoids with endogenous, syngeneic immune cells fail to mimic the subtleties of the tumor microenvironment in terms of complexity, representation, and reciprocal distribution of immune components. Another dimension of complexity is the difficulty – if not the impossibility – of replacing stromal elements such as endothelial cells and fibroblasts with their human counterparts; hence, the limitation remains that mouse-derived cytokines and growth factors in some cases do not crossreact with receptors that are expressed by human cancer cells.

A careful appraisal of the (vast) extent of information that can be reliably garnered by the use of patient-derived models, but also a clear understanding of their shortcomings, will be key to deliver robust, predictive and translationally relevant knowledge. This critical attitude will help triage and move to the clinic only those findings that emerge from conclusive and generalizable preclinical research and are motivated by responsible and limitation-aware methodological considerations.

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LEGENDS TO FIGURES

Figure 1: Application of colorectal cancer PDXs in translational research. PDX trials are conducted in parallel with or after clinical trials to "reverse validate" response biomarkers and genotype-response associations identified in patients (left panel). Cells dissociated from PDXs can be genetically manipulated and used in lineage tracing experiments to assess the temporal and spatial dynamics of functionally heterogeneous clones under drug pressure (middle panel). Genomic analysis of large-scale PDX collections enables the discovery of molecularly defined CRC subpopulations, which can be tested for the presence of potential therapeutic targets through pharmacologic experiments in vivo (right panel).

Figure 2: Application of genetically modified animal models of colorectal cancer in translational research. Genetically modified mice carrying targeted gene recombination of common mutations (*Apc*, *Kras*, *Tgfbr2* and *Trp53*) in intestinal stem cells develop immune-cold CRC tumors with high levels of stromal TGF-β; blockade of TGF-β signals prompts the recruitment of immune effector cells into the tumor microenvironment and sensitizes tumors to immunotherapy (left panel). Another mouse model develops metastatic CRC featuring a pronounced stromal reaction due to targeted expression of active Kras and Notch and loss of Trp53 in villin-positive intestinal cells; Notch-dependent production of TGF-β by cancer cells promotes tumor infiltration by neutrophils and metastatic dissemination, which can be blunted by inhibition of neutrophil recruitment or TGF-β signaling (right panel).

Figure 3: Application of CRC organoids in translation research. Patient-derived organoids from normal colon can be engineered to express drivers of colorectal tumorigenesis, alone and in combination; this approach allows to explore the contribution of each driver to tumor onset and progression and helps understand how and to what extent engineered organoid models recapitulate the biological characteristics of spontaneous tumors from patients (left panel). Mutational profiles, methylomics and/or RNA sequencing analysis of clonal organoids derived from individual cells of patients' tumors can be used to reconstruct phylogenetic trees and investigate CRC tumor evolution (middle panel). Organoids can be exploited in mid- to high-throughput drug screens, and results from pharmacologic analyses can be coupled with molecular profiles to extract associations between drug sensitivity and specific molecular traits (right panels).

Figure 4: Incorporating the immune system into patient-derived models. The immune system of immunocompromised mice can be partially reconstituted with different approaches of variable efficacy, from infusions of PBMCs or TILs to transplantation of HSCs derived from the bone marrow or umbilical cord blood; once humanized, mice can be xenografted with patient-derived tumors and treated with immunotherapy to assess tumor growth kinetics and intratumor representation of immune cells before and after treatment (left panel). Cocultures of immune cells and cancer cells can be performed by co-mingling tumor organoids and autologous PBMCs or by implementing ALI methods that allow the preservation of the tumor epithelium and the associated immune stroma in cohesive units; both approaches are permissive for expansion of tumor-specific cytotoxic T cells (right panel).

Rational treatment of colorectal cancer: A reverse tale of men, mice, and culture dishes

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Abstract

Stratification of colorectal cancer (CRC) into subgroups that differ in their response to therapy was initially informed by correlative studies in patients, which were based on statistical associations between the presence of specific biomarkers and treatment outcome. Recently, preclinical model systems based on propagatable patient-derived tumor samples (xenografts and organoids) have yielded an improved understanding of disease biology, which has translated into the functional validation of descriptive correlations and the discovery of novel response determinants, therapeutic targets, and mechanisms of tumor adaptation and drug resistance. We critically review the contribution of patient-derived tumor models to advancing CRC molecular characterization, discuss their influence on clinical decision-making, and highlight emerging challenges in the interpretation and clinical transferability of results obtainable with such approaches.

Significance

Association studies in patients with CRC have led to the identification of response biomarkers, some of which have been implemented as companion diagnostics for therapeutic decisions. By enabling biological investigation in a clinically relevant experimental context, patient-derived CRC models have proved useful to model the causal role of such biomarkers in dictating drug sensitivity and are providing fresh knowledge on new actionable targets, dynamics of tumor evolution and adaptation, and mechanisms of drug resistance.

Introduction

Colorectal cancer (CRC) is the third most frequent cause of cancer-related deaths in both the United States and Europe [1, 2]. Around 25% of individuals harbor metastatic disease at the time of diagnosis, while approximately 50% of patients will develop metastases later [3] . Although 5-year survival rates experienced by patients with metastatic CRC (mCRC) remain low (14%) [1], therapeutic options developed in the past two decades have prolonged the median overall survival (OS) from 12 to 30 months [4]. This survival advantage can be attributed to improved surgical techniques and the use of more effective systemic therapies. At least partially, more informed treatment decisions based on molecular response predictors have also helped increase life expectancy, but biomarker recognition has been slow and often inconclusive due to the difficulty of substantiating correlative observations in patients with functional investigation in clinically relevant model systems. Similarly, while genomic datasets have offered an instructive compendium of the genes that are frequently altered in CRC [5], the question whether the aberrant protein products of such genes represent effective therapeutic targets remains hard to address in the absence of adequate translational tools.

The availability of large collections of patient-derived tumor samples that can be propagated in mice (xenografts) and in three-dimensional cultures (organoids) has spearheaded attempts to afford biomarker-response associations with mechanistic annotation and has facilitated studies aimed to model cancer progression and acquisition of drug resistance [6, 7]. Herein, we provide an overview of current therapies and related biomarkers, as implemented in patients with mCRC, and discuss how patient-derived xenografts and organoids have been deployed to go beyond correlative descriptions and to illuminate fundamental biological and clinical aspects of CRC, including drug repurposing efforts that have rapidly moved to the clinical space. Further, we consider the practical implications and the limitations of using such models in terms of clinical applicability and predictivity.

Empirical and biomarker-driven treatments: Correlative response predictors in patients

The standard-of-care treatment for mCRC patients includes cytotoxic agents and biological targeted compounds, which are administered cumulatively based on the empirical observation that multi-agent therapeutic cocktails are more effective than monotherapies [8, 9]. Although some patients receive important clinical benefit from these regimens, responses are typically limited to a fraction of individuals. The polarized distribution of responsive and non-responsive patients likely derives from the genomic and functional heterogeneity of mCRC tumors, which display patient-to-patient molecular differences that influence treatment outcome. While the application of ‘omics’ technologies has been instrumental to enrich for potential responders to targeted therapies, this has been unsuccessful for chemotherapy, in part due to its often incompletely understood and diverse mechanisms of action.

Chemotherapy

The fluoropyrimidine antimetabolite 5-fluorouracil (5-FU) is injected intravenously together with leucovorin (LV), a biomodulator of 5-FU that has been shown to enhance its activity. Capecitabine, another fluoropyrimidine, is given orally. 5-FU/LV is most often administered in combination with oxaliplatin, a platinum compound endowed with inter- and intra-strand DNA cross-linking activity (FOLFOX) [10], or irinotecan, a topoisomerase I inhibitor (FOLFIRI) [11]. Either combination is more effective than 5-FU/LV alone in increasing response rates and prolonging progression-free survival (PFS), but at the cost of more pronounced toxicity [10-12]. The triplet association of 5-FU/LV, oxaliplatin and irinotecan (FOLFOXIRI) is also being increasingly used in mCRC patients with adequate performance status [13-15]. Other therapeutic options include capecitabine plus oxaliplatin (CAPOX/XELOX) and capecitabine plus irinotecan (XELIRI). CAPOX/XELOX has shown analogous efficacy and safety compared to FOLFOX and it is now proposed as first- or second-line therapy in patients refractory to irinotecan-based chemotherapy [16]. In the case of XELIRI, the phase 3 trials BICC-C and EORTC-415 have documented severe gastrointestinal side-effects compared to FOLFIRI [17, 18]. However, a modified regimen with reduced doses of both capecitabine and irinotecan (mXELIRI) was well tolerated, proved to be non-inferior to FOLFIRI in terms of OS, and is now proposed as an alternative second-line backbone treatment [19]. In patients who have

progressed after all standard therapies, a statistically significant (but modest) improvement in OS can be obtained with TAS-102, an agent that combines trifluridine (a nucleoside analog) and tipiracil hydrochloride (an inhibitor of thymidine phosphorylase) [20].

Potential determinants of response to chemotherapy have been brought to the fore based on the mechanism of action and metabolism of the various agents. However, the application of such predictors in clinical practice has been hampered by inconsistent results among different case series and poor diagnostic sensitivity and specificity. In some instances, in consonance with data from targeted therapies, drug target overexpression may be a positive determinant of sensitivity. For example, high expression of thymidylate synthase (TS), a direct target of 5-FU, has been associated with longer survival in CRC patients treated with adjuvant 5-FU-based therapy in some studies [21, 22]; however, other reports have not confirmed the positive predictive value of TS overexpression [23, 24] (Table 1). Likewise, elevated levels of topoisomerase 1 appear to predict better response to irinotecan [25] (Table 1). The activity of drug metabolic pathways is also likely to affect chemosensitivity. Dihydropyrimidine dehydrogenase (DPD) is a rate-limiting enzyme in fluoropyrimidine catabolism. High expression of DPD has been documented in tumors from patients with reduced sensitivity to capecitabine [26], with or without irinotecan [27], whereas inactivating polymorphisms of the *DYPD* gene (encoding DPD) have been associated with acute toxicity over the course of fluoropyrimidines-based therapy [28-30] (Table 1). In the same vein, deleterious polymorphisms of the *UGT1A1* gene (encoding glucuronosyltransferase, a key enzyme of irinotecan metabolism) are more frequent in patients who experience severe toxicity during treatment with irinotecan-based regimens [31, 32] (Table 1).

Responsiveness to chemotherapy may also be related to defects in DNA repair mechanisms after chemotherapy-induced DNA damage, leading to abnormalities in DNA replication and/or chromosome segregation that culminate in cancer cell death. Excision repair cross-complementation group 1 (ERCC1) is a key effector of DNA repair mechanisms and influences the tumor DNA-targeting effect of oxaliplatin. Some studies have shown that low transcript expression of the *ERCC1* gene correlates with longer survival of patients treated with FOLFOX [33] (Table 1). Similar findings were reported for an *ERCC1* polymorphism at codon 118, which is expected to result in decreased *ERCC1* gene expression [34] (Table 1). These correlations, however, have not been confirmed in other datasets, especially when ERCC1 protein amounts rather than transcript expression were analyzed [35, 36]. Functional studies are needed to deepen mechanistic investigation of the relationship between DNA repair deficiency and chemosensitization. More in general, we advocate a revamping of biologically oriented research as a means to disentangle the intricacies behind chemotherapy efficacy (or lack of), including the evaluation of how genetic and epigenetic modifications in components of DNA repair pathways shape response to cytotoxic agents. A clearer understanding of the cellular and molecular underpinnings of chemotherapy activity in clinically relevant experimental models is a necessary prelude to the nomination of response biomarkers above and beyond descriptive variables in patients.

EGFR monoclonal antibodies

The EGFR monoclonal antibodies cetuximab and panitumumab are currently used in association with FOLXOX or FOLFIRI in the first- or second-line treatment of patients with *KRAS* or *NRAS* wild-type tumors [37]. The restricted use of anti-EGFR antibodies to patients with RAS wild-type mCRC is the result of a population-level biomarker-development strategy motivated by the plausible rationale that constitutive activation of signaling pathways downstream from EGFR – such as those triggered by RAS mutations – should bypass EGFR inhibition and therefore obviate sensitivity to EGFR targeted agents. Evidence of the correlation between RAS genetic alterations and lack of response to EGFR blockade was initially limited to tumors with exon 2 *KRAS* mutations [38] and was later extended to *KRAS* exons 3 and 4 and *NRAS* exons 2, 3 and 4 [39] (Table 1). The predictive value of the association was so strong that retrospective studies in patients were deemed sufficiently powered to guide the development of companion diagnostics for the routine assessment of "RAS extended" mutations in mCRC patients, even in the absence of prospective validation.

While the use of RAS mutation panels for the exclusion of mCRC patients who will not benefit from anti-EGFR therapy is now commonplace, the quest for positive response predictors has lagged behind. EGFR is hardly ever mutated or amplified in CRC, indicating that tumor dependency on the EGFR pathway does not have an evident genetic basis. Retrospective correlative studies have documented higher tumor expression of the EGFR ligands amphiregulin and epiregulin in patients who respond to EGFR antibodies [40, 41] (Table 1). Hence, mCRC tumors that are sensitive to EGFR neutralization appear to rely on EGFR signals owing to ligand-mediated autocrine or paracrine receptor activation. This knowledge has not translated into clinical-grade methods for selection of potential responders due to the difficulty of dichotomizing continuous variables, such as transcript or protein expression, into a digital cut off for univocal allocation of patients to treatment.

Anti-angiogenic therapy

The first-line treatment for patients with mCRCs harboring *KRAS* or *NRAS* mutations comprises either FOLFIRI or FOLFOX plus the anti-VEGF antibody bevacizumab [42, 43]. Whether the addition of VEGF-targeting agents to chemotherapy provides comparable or superior benefit to anti-EGFR antibodies in the context of *KRAS*/*NRAS* wild-type tumors is still a matter of debate [44]. Beyond bevacizumab, two other anti-angiogenic drugs have been proved to positively impact on PFS and response rates when combined with chemotherapeutic agents in late lines of treatment: aflibercept (an anti-VEGF-A, VEGF-B, and placental growth factor) [45] and ramucirumab (an anti-VEGF receptor 2) [46]. Finally, heavily treated chemorefractory patients can experience slightly longer OS when treated with the multikinase inhibitor regorafenib, which also targets pro-angiogenic receptors [47].

At present, there are no validated predictors of response to anti-angiogenic agents [48]. Clinical reports have shown a correlation between more marked responsiveness to bevacizumab and low baseline levels of VEGF-A splice isoforms [49], VEGF-D [50], HGF [51], interleukin-8 [52] or the VEGF-A coreceptor neuropilin-1 [53], either in plasma or tumors (Table 1). Germline polymorphisms of VEGF-A [54, 55], VEGF receptor-1 [56] and inflammation- and endoplasmic reticulum-associated genes [57] also show a significant interaction with bevacizumab effectiveness (Table 1). Other potential biomarkers predicting bevacizumab therapeutic efficacy include loss of chromosome 18q11.2-q12.1 [58] and, more in general, a high degree of chromosomal instability (CIN) [59] (Table 1). In the case of regorafenib, initial studies suggest that low expression of vascular cell adhesion protein 1 (VCAM-1) may be associated with better response [60] (Table 1). Polymorphisms of genes related to the C-C motif chemokine ligand 5/C-C motif chemokine receptor 5 pathway (which regulates VEGF-A expression) also predict efficacy in mCRC patients treated with regorafenib [61] (Table 1).

The fact that the expression levels of angiogenic targets positively associate with sensitivity to anti-angiogenic agents has a pharmacokinetic basis ascribable to the relative stoichiometry of drug-target interactions. The biological significance of other candidate biomarkers, such as copy number alterations, is of difficult interpretation in the absence of functional modeling in adequate experimental systems. Mechanistic investigation of stromal-directed therapies is complicated by a dearth of preclinical resources, which typically consist of syngeneic transplants, genetically modified mice, or xenografts. All approaches have limitations: on the one hand, "mouse-only" models hardly recapitulate the inter-patient heterogeneity and population diversity of human tumors – a severe drawback for biomarker discovery research; on the other hand, xenografts are by definition a chimeric source of angiogenic factors, which can be concomitantly released by human cells of the tumor and murine stromal and inflammatory cells of the host. Therefore, due to species specificity, the cross-talk between heterologous cellular compartments is biased, and therapeutic antibodies cannot have full capacity at the organismal level because they selectively target either murine or human antigens.

Other targeted therapies

All the above therapies are administered in the absence of positive molecular selection; the only criterion is patient exclusion based on the presence of negative response biomarkers, as epitomized by the established association between *KRAS*/*NRAS* mutations and lack of response to EGFR blockade. Recently, a number of low-frequency aberrations in kinase-encoding genes have been identified in mCRC that result in constitutive activation of the corresponding protein products. Alterations in the

ERRB2 gene (mostly gene amplification) are detected in around 5% of *KRAS*, *NRAS* or *BRAF* wild-type mCRCs and lead to overexpression and hyperactivation of the encoded HER2 tyrosine kinase receptor. Phase 2 studies in patients selected for having HER2-positive mCRC tumors have shown that dual inhibition of HER2 using the anti-HER2 antibody trastuzumab and the EGFR/HER2 small-molecule inhibitor lapatinib (HERACLES trial) or the combination of trastuzumab and the anti-HER2 antibody pertuzumab (MyPathway trial) have considerable clinical efficacy, with around 30% response rates in heavily pretreated patients [62, 63] (Table 1). Notably, in both studies responsive patients had tumors with higher *ERBB2* gene copy number than resistant patients, consistent with the assumption that higher *ERBB2* gene dosage translates into stronger kinase activation, hence in more profound tumor dependency on HER2 signaling.

Kinase fusions originating from chromosomal translocations and resulting in constitutive activation of neurotrophic receptor tyrosine kinase 1 (NTRK1), NTRK2, NTRK3, anaplastic lymphoma kinase (ALK), and RET account for approximately 1-2% of *KRAS*, *NRAS* or *BRAF* wild-type mCRCs. These rearrangements are enriched in right-sided RAS wild-type tumors and, while typically portending a dismal prognosis [64], they predict therapeutic benefit of inhibitors such as entrectinib (targeting NTRK, ROS1 and ALK) [65, 66] and ponatinib (targeting various tyrosine kinases including RET) [67, 68] (Table 1).

BRAF gene alterations (with a dominant prevalence of V600E activating mutations) are found in 7-10% of mCRCs [69, 70] and are generally mutually exclusive with *KRAS* and *NRAS* mutations, indicating that a single oncogenic hit on the ERK MAPK pathway is sufficient to sustain tumorigenicity. The most recent guidelines recommend an upfront intensified regimen with triplet chemotherapy (FOLFOXIRI) plus bevacizumab, a schedule that – however – is not limited to *BRAF* mutant tumors. Selective BRAF targeting with specific inhibitors has proven ineffective in patients with *BRAF*-mutant mCRC due to feedback reactivation of EGFR signaling, which substitutes for BRAF blockade in stimulating the MAPK pathway [71, 72]. This observation has prompted the design of clinical trials aimed at evaluating the efficacy of combined BRAF and EGFR inhibition in patients with *BRAF* mutant mCRC. In a recent phase 3 study testing cetuximab and the BRAF inhibitor encorafenib versus cetuximab and irinotecan (or FOLFIRI), combined EGFR and BRAF blockade significantly improved response rates and OS compared with standard therapy. This superior activity was further enhanced by concomitant MEK inhibition [73] (Table 1). These results have led to the FDA approval of encorafenib plus cetuximab in previously treated patients with *BRAF* mutant mCRC.

Despite their high prevalence (approximately 50% of all CRCs), *KRAS* and *NRAS* mutant tumors are still treated with conventional chemotherapy and anti-angiogenic agents. Hopes are now placed on the use of KRAS G12C covalent inhibitors, which are currently tested in patients with *KRAS* G12C solid tumors. Initial results seem to indicate that response rates to these drugs are relatively high in patients with non-small cell lung cancer but limited in mCRC patients, likely due to retained sensitivity of CRC tumors to upstream EGFR signaling [74] (Table 1). These observations echo findings in *BRAF* mutant tumors [71, 72] and strengthen the notion that EGFR signaling needs to be concomitantly neutralized to achieve better responses to drugs targeting the RAS-MAPK pathway in mCRC.

Immunotherapy

About 15% of CRCs display defective functionality of mismatch repair (MMR) proteins, which participate in the correction of base-pair mismatches occurring during DNA replication (especially at the level of repetitive DNA sequences called microsatellites). Deficient-MRR (dMMR) tumors with mutations in 30% or more microsatellites (defined as dMMR/MSI-H, *i.e*. with high microsatellite instability) tend to accumulate nonsynonymous mutations; this increased mutational burden can translate into a higher neoantigen load, which makes some dMMR/MSI-H tumors immunogenic and sensitive to immune checkpoint blockade [75]. Accordingly, single-agent therapy with the anti-PD-1 antibodies pembrolizumab or nivolumab and combination therapy with nivolumab and the anti-CTLA-4 antibody ipilimumab have been approved for second-line treatment of patients with chemorefractory dMMR/MSI-H mCRC [76-78] (Table 1). Based on recent data from the phase 3 KEYNOTE-177 trial, pembrolizumab appears to be superior to standard-of-care chemotherapy in improving PFS in the first-

line setting; this information has led to the FDA approval of pembrolizumab as first-line therapy for patients with unresectable or metastatic dMMR/MSI-H colorectal cancer [79].

The assessment of MSI status is now routinely performed for selecting patients likely to respond to immunotherapy, but only a subgroup of individuals with MSI mCRC receive clinical benefit from this treatment. Not always are the protein products of somatic DNA variants efficiently presented by MHC molecules, which means that tumor mutational burden only partially contributes to neoantigen load. Likewise, although an association between high neoantigen load and pronounced immune cell infiltration has been repeatedly documented, the presence of an active immune microenvironment has not predictive value for immunotherapy sensitivity [80]. HLA binding prediction tools and artificial intelligence algorithms for multiparametric imaging of immune cell representation and topography in tumors are expected to yield more reliable molecular biomarkers for effective patient stratification in the immuno-oncology space.

Preclinical models for understanding and predicting therapeutic response in CRC

Biomarkers that predict patient response to treatment are usually identified using population-based association studies, in which clinical outcome is correlated with a statistically significant enrichment for a specific molecular trait (typically, a genetic alteration) in subjects who do or do not respond to a given therapy. Albeit useful for clinical decision making, this approach fails to inform whether therapeutically relevant response predictors causally influence drug sensitivity and does not provide insight into the mechanistic underpinnings of the observed correlations. In a complementary perspective, studies using cancer cell lines enable extracting functional annotations and modeling cause-effect relationships; however, cell lines are by definition limited in number; thus, they do not recapitulate the spectrum of genetic heterogeneity spanned by patient tumors. Recently, patient-derived platforms that reflect the diversity of cancers, while retaining experimental manipulability and clinical fidelity, have been developed with the aim to characterize response biomarkers, investigate tumor adaptation under drug pressure, and understand the evolutionary principles of tumor progression. CRC has been – and still is - a testing arena for such efforts.

Patient-derived xenografts (PDXs) for validation of targeted therapy biomarkers

Surgically derived tumor samples that are implanted in mice (known as patient-derived xenografts, PDXs) retain the inherent features of different tumors from different patients [6, 81]. Vast PDX collections are therefore expected to capture inter-patient tumor heterogeneity at the population level in a clinically relevant *in vivo* setting [82]. CRC is a paradigmatic example of the importance of PDX-based research for large-scale genotype-response associations, predictive biomarker identification, and therapeutic studies [83, 84] (Figure 1). In 2011 a systematic survey of *KRAS* and *NRAS* mutations in more than 100 mCRC PDXs, coupled with annotation of sensitivity to cetuximab, produced a dataset with both confirmatory and discovery aspects [85]. On the one hand, the association between *KRAS* mutations in exon 2 and *de novo* resistance to EGFR blockade – which had emerged from clinical studies some years earlier [86] – was 'reverse validated' in PDXs and found to be coherent with patient data [85] (Table 1). On the other hand, results in PDXs were among the first to illustrate that *KRAS* mutations in exons 3 and 4 and *NRAS* mutations predict lack of response to EGFR antibodies [85, 87]. This finding would receive ultimate clinical recognition only two years later, when a retrospective-prospective analysis concluded that patients with tumors harboring 'RAS extended' mutations treated with anti-EGFR antibodies had inferior PFS and OS compared with patients with *KRAS*/*NRAS* wild-type tumors [39].

While PDXs appear to have adequate predictive power for cancer cell-directed treatments, they lose value when dealing with therapies against stromal components – such as cancer-associated fibroblasts, endothelial cells, and inflammatory cells – and cells of the adaptive immune system. Indeed, the host must be immunocompromised to tolerate the graft, and human stromal cells are substituted with murine counterparts over serial passaging [6]. But this drawback bears some advantages: the chimeric nature of PDXs has been leveraged to decompose – from bulk tumors – cancer cell-specific and stromal signals using analytical methods that distinguish human *versus* mouse transcripts. This

exercise has increased the granularity and informative merit of gene expression classifications. For example, the clinical aggressiveness of a poor-prognosis transcriptional subtype named CMS4 had been initially ascribed to the ability of cancer cells to undergo epithelial-mesenchymal transition (EMT), a phenotypic switch that instigates cell motility and invasion [8, 88]. With the possibility to discriminate between human and mouse transcripts, it became clear that – together with displaying some cancer cell-autonomous EMT traits – the vast majority of mesenchymal CMS4 tumors are in fact characterized by a heavy content of stromal cells, which likely foster the malignant characteristics of this subtype by conveying mitogenic, pro-invasive and anti-apoptotic cues [89]. In the same vein, CRIS, a new CRC classification based only on PDX human transcripts, identified subtypes endowed with prognostic and predictive significance and showing limited overlap with transcriptional classes obtained from whole bulk CRCs [90]. Moreover, by focusing on cancer-cell intrinsic gene expression features that are not influenced by stromal abundance in isolated, randomly taken tumor samples, CRIS demonstrated higher accuracy in clustering CRCs by patient-of-origin rather than tumor region-of-origin [91].

PDXs to study the clonal dynamics of CRC tumors under chemotherapy pressure

Tumors are composed of heterogeneous cell subsets that display different proliferation kinetics, susceptibility to apoptosis, and sensitivity to drug insults [92]. Some works have used PDX models to investigate the clonal propagation dynamics of CRC subpopulations, both during spontaneous tumor growth and under drug pressure (Figure 1). DNA copy number alteration profiling and deep sequencing of mutational hotspots were combined with lentiviral lineage tracking to follow the progeny of single CRC cells over serial xenografts and to investigate the relative contribution of genetic and nongenetic mechanisms to the functional heterogeneity of the individual cancer cells [93]. While genetically identical clones remained stable upon serial transplantation, lentivirally marked lineages were variable within each clone, with pronounced differences in proliferation rates, ability to persist, and susceptibility to exhaust through passages [93]. Likewise, treatment of xenografts with irinotecan did not result in a detectable bottleneck or selection for novel genetic clones; rather, chemotherapy shaped a new dominance of previously dormant lineages and culled actively proliferating progeny [93]. Together, these results indicate that cancer cells subpopulations can be genetically homogeneous (and stable) but functionally heterogeneous (and plastic) in CRC.

The finding that CRC cancer cells oscillate between periods of dormancy and activity appears to have a positional determination. Using a tamoxifen-inducible labeling system to stochastically mark cancer cells in mouse xenografts of patient-derived spheroids, coupled with computational modeling, Lenos and colleagues documented that CRC grows through surface expansion [94]. This peripheral accretion is driven by the local availability of mitogenic gradients secreted by cancer-associated fibroblasts, which are sensed only by cancer cells located in the outermost zone of the tumor [94] (Figure 1). Chemotherapy with 5-FU and oxaliplatin reduced tumor growth rates but did not affect the residual dynamics of surface growth, indicating that microenvironmental influences dictating spatially confined cell proliferation are not interfered by cytotoxic treatment [94]. Other studies with multicolor lineage tracing approaches in xenografts of CRC primary cultures and cell lines confirmed that tumor outgrowth is geometrically orchestrated by large proliferating clones confined at the leading tumor edge, whilst small quiescent clones reside in the center [95, 96].

PDXs and genetically modified animal models for target discovery

Besides providing preclinical hints for response biomarker validation, PDXs have also been deployed for testing therapeutic options in newly identified, molecularly circumscribed mCRC subsets (Figure 1). Amplification of the *ERBB2* oncogene was detected in some *KRAS*/*NRAS* wild-type, cetuximab-resistant PDX models, and was found to predict response to HER2 targeted therapies in PDX-based preclinical trials [85, 97] (Table 1). Other clinically actionable alterations were shown to be enriched in *KRAS*/*NRAS* wild-type, cetuximab-refractory PDXs and patients, including activating mutations of *ERBB2* and *MAP2K1* (encoding the RAS downstream effector MEK1), amplification of the tyrosine kinase receptors *MET* and *FGFR1*, and outlier overexpression of the survival factor *IGF2* [98-101]. In general, the sole inhibition of the hyperactive oncoproteins proved to be ineffective in PDXs, but treated tumors were invariably sensitized to concomitant EGFR blockade. Subsequent clinical studies confirmed that patients with HER2-positive mCRC tend to respond poorly to EGFR antibodies and

can benefit from dual treatments against HER2 and EGFR [62, 63]. Similarly, MEK1 mutations were found to predict resistance to EGFR inhibition and response to a combination of trametinib (a MEK inhibitor) and panitumumab in patients [102].

PDXs have been shown to recapitulate clinical reality also in terms of depth of response. Similar to metastases in patients, mCRC PDXs that respond to EGFR antibodies can experience massive shrinkage but are hardly ever eradicated. The residual cancer cells that withstand upfront drug treatments act as a reservoir for the stochastic acquisition of resistance-conferring mutations, with the ensuing expansion of subclones responsible for tumor relapse [103]. Recent evidence indicates that residual mCRC PDXs (and residual tumors in patients) at maximal response to prolonged anti-EGFR therapy relax their dependency on EGFR signals by reducing the expression of genes encoding EGFR-activating ligands and increasing alternate HER2/HER3 pathway activity, while becoming similar to slowly-cycling secretory precursors of the normal intestine [104]. The finding that cetuximab-tolerant residual tumors exhibit decreased abundance of EGFR cognate ligands is consistent with the clinical observation that patients with mCRC tumors expressing low levels of amphiregulin and epiregulin tend to respond less to EGFR antibodies [40, 41] (Table 1). Pseudodifferentiation into tissue-specific lineages has been documented as a mechanism of therapy resistance in other tumors; for example, the manifestation of neuroendocrine traits is a hallmark of emerging resistance to EGFR inhibitors and anti-androgen treatment in lung and prostate cancer, respectively [105, 106]. In the context of CRC, cetuximab-induced phenotypic reprogramming towards a secretory fate with high HER2/HER3 signaling makes cancer cells vulnerable to concomitant targeting of EGFR, HER2 and HER3, as shown by reduction of residual disease burden and prolonged time to relapse after treatment discontinuation in PDX trials with a Pan-HER antibody [104].

As noted above, PDXs are inadequate tools for predicting response to therapies against stromal and immune cells. Genetically modified mouse models develop autochthonous CRC tumors in an immune-competent background, but the artificial introduction of founder oncogenic mutations may result in evolutionary trajectories different from those occurring in spontaneous tumors [107]. This limitation has been addressed by engineering the ordered expression of salient mutant oncoproteins along the linear progression sequence that typifies human CRC, with the aim to more faithfully recapitulate the natural history of human tumors (Figure 2). Animals with targeted gene recombination of common mutations (Apc, Kras, Tgfbr2, and Trp53, known as AKTP) to intestinal stem cells developed invasive CRC adenocarcinomas with hallmarks of human microsatellite-stable tumors, including low mutational burden and scant T-cell infiltration [108]. Moreover, AKTP tumors had an abundant representation of carcinoma-associated fibroblasts engaged in massive deposition of extracellular matrix and profuse secretion of TGF-β. Importantly, increased TGFβ in the tumor microenvironment was found to be a major determinant of T-cell exclusion, and blockade of TGFβ signaling rendered tumors more T cell-inflamed and susceptible to immunotherapy [108] (Figure 2). Another mouse model harboring Kras and Trp53 mutations along with hyperactive Notch signaling in intestinal cells developed metastatic tumors with serrated morphology, extensive stromal content, and gene expression profiles similar to those of poor-prognosis tumors in patients [109]. In these mouse tumors, hyperactivation of the Notch pathway resulted in secretion of TGF-β by cancer cells, which prompted neutrophil accumulation in the tumor stroma and neutrophil-dependent metastatic dissemination. Accordingly, targeting neutrophil recruitment or TGF-β signaling reduced metastatic burden [109] (Figure 2). Collectively, these results underscore the value of transgenic mice as investigational models to explore the interplay between genetic alterations and the immune-competent tumor microenvironment and to integrate PDX-based research on cancer cell-intrinsic vulnerabilities.

Patient-derived organoids for mechanistic investigation and pharmacologic studies

PDXs represent more authentic working models than conventional cell-line xenografts to study how cancer cells evolve and react to therapies in a clinically relevant scenario that reflects organismal complexity. However, PDXs are not endowed with sufficient experimental tractability to distill causality from description, nor do they show sufficient scalability to enable high-throughput pharmacogenomic screens. Short-term culture of tumor sections allows for *in vitro* screening at a reasonably large scale [110], but it is constrained by the fact that the proliferative capacity of the

cultures dissipates over time. To overcome these limitations, three-dimensional organotypic or 'organoid' long-term culture methods have been developed that combine the experimental flexibility of "immortalized" *in vitro* systems with the tissue context of animal studies [111]. For colon, normal organoids containing only epithelial cells can be derived by culturing primary nontransformed intestinal tissue in Matrigel – a gelatinous mixture made of laminin-rich extracellular matrix and growth factors – supplemented with additional growth factors that mimic the intestinal niche [112, 113] (Figure 3). CRC organoids require less stringent combinations of niche factors than normal intestinal organoids [114, 115].

Patient-derived normal and CRC organoids have been leveraged to advance cancer modeling and decompose mechanisms of CRC tumorigenesis. Using CRISPR/Cas9-based genome editing, Matano et al. sequentially introduced loss-of-function mutations of *APC*, *SMAD4* and *TP53* and gain-of-function mutations of *KRAS* and/or *PIK3CA* in normal human colon organoids, followed by growth selection under customized cell culture conditions [116]. Organoids engineered to express all five mutations formed small, highly differentiated tumors with limited local infiltration after implantation under the kidney subcapsule in mice, and were unable to metastasize to the liver after injection into the spleen. Conversely, organoids from patients' advanced tumors that had accumulated spontaneous oncogenic mutations during their evolutionary history displayed robust renal subcapsular growth and produced prominent spleen-to-liver dissemination [116] (Figure 3). These results suggest that the ectopic introduction of canonical driver mutations in normal human intestinal cells results in incipient tumor formation but is not sufficient for a CRC tumor to manifest an invasive and metastatic phenotype. Additional lesions that drive full-blown CRC malignancy may be fueled by epigenetic modifications and CIN; indeed, the engineered organoids largely lacked karyotypic or DNA methylation aberrations, which were instead abundantly present in patient-derived CRC organoids [116]. The acquisition of gene copy number alterations after genetic manipulation of normal intestinal organoids appears to be influenced by experimental variables; for example, different from Matano et al., Drost et al. found that combined loss of *APC* and *TP53* in normal human colon organoids was sufficient for the appearance of CIN and massive aneuploidy [117]. In patient-derived CRC organoids, a combination of genetic lineage tracing and ablation systems revealed robust functional plasticity. $LGR5^+$ cells were shown to act as cancer stem cells that constantly fueled tumor growth through self-renewal and at the same time were able to morph into differentiated post-mitotic cells. Selective ablation of $LGR5^+$ cells transiently regressed tumors; however, this shrinkage was followed by tumor regrowth due to the replenishment of the $LGR5^+$ pool by differentiated cells that had reacquired stem-like features [118].

Clonal organoids derived from isolated cells can be considered as proxies for the single cells from which they originate (Figure 3). Phylogenetic trees constructed through deep genomic analysis of CRC clonal organoids revealed that driver mutations commonly found in CRC (such as those in *APC*, *KRAS*, and *TP53*) were present in all organoids, that is, they were trunk mutations common to all cells of the original tumor. However, many 'private' mutations could be detected in the distal branches of the phylogenetic trees, indicating that they had arisen later during tumor progression and had contributed to tumor genetic diversification [119]. These results are in line with the 'big bang' model of CRC tumorigenesis, according to which genetic variants that confer selective advantages occur early in a cancer's evolution and are followed by the neutral expansion of genetically different but equally fit subclones [120, 121]. Stable alterations of DNA methylation and transcriptome states were also observed in cloncal organoids, with phylogenetic topologies similar to the mutation-based trees (Figure 3). Conversely, response to drugs commonly used in CRC was variable – especially with chemotherapeutic agents – and not linked to the geographical location of the organoid-initiating cells in the original tumor or the genetic distance between clones [119]. Similar to that observed in PDX-based lineage tracking experiments [93], these results suggest that diversification in biological behavior has no evident correlation with the extent of mutational diversification.

The application of organoid technology in systematic high-throughput drug screens to validate clinically relevant response biomarkers and nominate new ones is rapidly expanding (Figure 3). A seminal study with a library of 83 compounds tested in 19 CRC organoids confirmed the association between *KRAS* mutations and lack of response to EGFR blockade as well as general refractoriness of

BRAF-mutant tumors to BRAF inhibitors, as observed in the clinic [114]. This effort also identified loss-of-function mutations in *RNF43*, resulting in cell hypersensitivity to secreted WNT factors, as predictive biomarkers of CRC susceptibility to neutralization of autocrine/paracrine activation of the WNT pathway [114]. CRC organoids have also been shown to recapitulate the clinical response of the donor patient to cetuximab, regorafenib, and TAS-102 [122]. Interestingly, organoids derived from a patient with regorafenib-sensitive liver metastases proved to be resistant to the drug when cultured *ex vivo* as isolated cancer cells; however, liver orthotopic xenografts developed from the same organoids coopted the host's blood vessels and displayed reduced vascularity after regorafenib administration, in keeping with the assumption that response to regorafenib is mainly driven by its antiangiogenic activity [122]. A concordance between organoid viability and patient response was also found in the case of irinotecan monotherapy and FOLFIRI, but not when FOLFOX was used [123]. Possibly, stromal and immune components absent in organoid cultures tune sensitivity to oxaliplatin more than they do with other drugs, or reliable response to oxaliplatin requires tailored culture conditions that are less stringent for other chemotherapeutics. Finally, organoids derived from rectal cancer have been demonstrated to predict clinical and histopathologic responses to neoadjuvant chemoradiation, as observed in matched donor patients [124, 125].

Challenges and emerging opportunities

The utilization of living biobanks of tumor samples holds considerable promise for *in vivo* and *in vitro* interrogation of clinically actionable pathways and for the study of tumor evolution. But the use of patient-derived models should be accompanied by careful appreciation of their real potential not only as platforms for biomarker validation and target discovery but also as reliable proxies of the biological and molecular fingerprints of matched tumors in donor patients. A critical knowledge of the accuracy of patient-derived models in retaining the characteristics of original tumors is crucial for assessing their ability to predict drug activity in the clinic.

Preservation of genomic architecture in propagated tumor-derived models

An ongoing debate revolves around the question whether serially passaged PDXs and long-cultured organoids preserve the genomic makeup, in terms of copy number alterations (CNAs), of their pre-derivation counterparts. Using gene expression microarray data to infer large-scale CNA profiles, Ben-David et al. reported extensive copy number divergence between the pre-implantation tumor of origin and the corresponding xenograft at the first *in vivo* passage, which was exacerbated along serial propagations [126]. This raised concerns that mouse-specific selective pressures may "artificially" influence PDX tumor evolution, with implications for the ability of PDXs to faithfully model patient treatment response. However, expression-based CNA calling only enables assessment of aberrations at the gross scale of chromosomal arms. Recently, a joint effort of the National Cancer Institute PDXNet consortium and the EurOPDX consortium produced a DNA-based enumeration of copy number profiles at high segmental resolution in a large collection of PDX models [127; Woo et al., Nat.Genet., accepted in principle]. This analysis did not confirm systematic copy number deviation between patient tumors and PDXs; rather, it documented high CNA retention during PDX engraftment and passaging (both globally and at the level of cancer-related genes) for many tumor types including CRC. Notably, CNA variations between pre-implantation and xenografted tumors were comparable to differences in multi-region samples of tumors in patients, indicating that the impact of PDX-associated CNA drift is similar to the natural intratumoral evolution that occurs in patients.

Somatic mutations, typically assessed by whole exome sequencing, are largely concordant between original tumors and matched PDXs, even though evolutionary neutral subclonal alterations may arise at low allele frequency during PDX propagation [128]. In CRC, mutations in known oncogenic drivers are retained in PDXs when present in matched patient tumors and do not appear *de novo* in mouse-passaged tumors [84]. An overall preservation of CNA and mutational landscape, with the caveat that the number of samples analyzed so far is limited, has also been observed in CRC organoids as compared with the corresponding patient tumors [84, 114, 129]. However, CIN CRC organoids tend to tolerate mitotic errors, which results in the accrual of chromosome mis-segregations over time [130].

Similarly, an accumulation of synonymous and nonsynonymous mutations has been noted during prolonged culturing of MSI CRC organoids [115].

The hurdles of co-clinical trials

If patient-derived models are high-fidelity "avatars" of pre-derivation tumor samples, they could be used – in principle – for real-time assessment of drug sensitivity, which may be reverse-exploited to guide treatment decisions in donor patients. Co-clinical trials have been proposed in which PDX mice are treated with panels of drugs – either agents with broad-brush anticancer activity or targeted compounds based on molecular predictors; then, when a positive signal for a specific therapy emerges, the information is transferred back to the donor patient for clinical evaluation [81, 131]. While intriguing, an approach of this kind requires that therapeutic outcomes be univocally deciphered and rigorously interpreted. For example, spurious positive signals may arise for treatments that delay tumor growth, resulting in tumors that are smaller than untreated controls at end point, but larger than they were at treatment initiation. This information may be indicative of biological sensitivity (*i.e.*, the drug reduces cancer cell proliferation) but has little clinical relevance; indeed, in patients, a lesion that enlarges during treatment (even to a relatively small extent) denotes tumor progression, and the therapy is usually discontinued due to lack of efficacy.

Another issue with the execution of PDX-based co-clinical trials is the need to cope with quick turnarounds. Results in mice must be promptly returned to donor patients to inform treatment decisions. However, research with PDXs notoriously implies long-term and time-consuming experiments. "Cutting corners" in the name of rapidity, for example by reducing the number of animals tested in each treatment cohort, would lead to insufficiently powered studies and scientifically unreliable conclusions. Compared with PDXs, organoids are expected to speed up the bench-to-bedside pipeline due to their higher manageability. However, we are still missing metrics that adequately capture how and to what extent organoids deliver a clear prediction of the outcome in patients. There is no consensus on the adoption of common readouts of drug sensitivity (reduction of cell proliferation *versus* induction of apoptosis) and shared methodologies for data acquisition (digital imaging *versus* cell counts). Moreover, a direct comparison of the concordance between patient-matched PDXs and organoids in categorizing response or resistance to therapy has not been attempted so far on a systematic scale. As always when dealing with resource platforms, standardized guidelines built on cumulative experience will be a prerequisite for direct transfer of preclinical results to patients.

Integration of the tumor immune microenvironment: humanized mice and co-cultures

The necessity of using immunocompromised mice to prevent xenograft rejection hampers the use of conventional PDX models to assess the efficacy of immunotherapies. Humanized mice are immunodeficient animals in which the human immune system is partially reconstituted by introducing $CD34^+$ hematopoietic stem cells (HSCs), peripheral blood mononuclear cells (PBMCs), or tumor-infiltrating lymphocytes (TILs) [6, 132] (Figure 4). Attempts to generate humanized CRC models have been scant. Cell-line xenografts in mice engrafted with allogeneic or autologous human PBMCs showed delayed growth kinetics and increased infiltration of cytotoxic T cells after treatment with a combination of nivolumab and urelumab, a CD137 agonist monoclonal antibody that enhances T-cell and natural killer-cell antitumor activity [133]. Similar results were observed in a dMMR/MSI-H PDX model, but not in a microsatellite stable model, after humanization with cord blood-derived $CD34^+$ cells and treatment with nivolumab [134].

Although humanized mice appear to recapitulate some of the effects of immunotherapy observed in patients, the procedure of mouse humanization is afflicted with several drawbacks. PBMC and TIL infusion typically causes severe graft-versus-host disease starting 2-5 weeks after injection [135, 136], which restricts the investigative window to temporal limits that are hardy compatible with experimental needs. Transplantation of HSCs results in a more complete hematopoietic reconstitution, but their maturation as well as the effector functions of their differentiated progeny are compromised by the lack of cytokines, phagocytes, and HLA molecules of human origin in the mouse host. The application of genome editing technologies for mouse genetic engineering is expected to increase the extent of humanized cells and molecules in future murine models.

Another emerging asset to reconstruct the functional interactions between cancer cells and the immune microenvironment relies on hybrid organ-on-a-chip platforms, which allow the build-up of more complex multicellular systems [137]. Reductionist methodologies involve the initial establishment of separate cultures of epithelial organoids and immune cells, followed by artificial reconstitution in co-mingling experiments. This approach has been used to set up co-cultures of cancer cells from primary or metastatic CRC with high mutational burden and autologous PBMCs, wherein cancer cell organoids triggered antigen-specific stimulation of tumor-reactive cytotoxic T cells in the PBMC fraction [138] (Figure 4). More sophisticated air-liquid interface (ALI) methods have also been deployed that enable the *en bloc* preservation of the tumor epithelium and its endogenous immune stroma, including fibroblasts, tumor-associated macrophages, T and B lymphocytes, and natural killer cells [139]. ALI cohesive units propagated from CRC biopsies retained the T cell receptor heterogeneity of the T cells present in original tumors and modeled the effects of nivolumab by recapitulating cytotoxic T cell expansion and antibody-dependent tumor cytotoxicity [139] (Figure 4). Assessing the functional consequences of immune checkpoint blockade using organoid technology is poised to complement existing descriptive biomarkers, such as neoantigen load, in the identification of patient-specific determinants of response to immunotherapy.

Concluding remarks

The clinical and experimental observations discussed above illustrate the power of population-level studies – both in patients and in the preclinical setting – to credential candidate predictive biomarkers and identify novel determinants of therapeutic response as well as novel targets. Recent evidence also highlights the value of patient-derived xenografts and organoids as tools to investigate subclonal dynamics during tumor evolution and functional heterogeneity under drug pressure. The credibility of patient-derived models in preserving the molecular architecture of the corresponding pre-derivation tumors is now supported by large-scale analytical efforts and the use of accurate genomic approaches. These merits notwithstanding, several issues remain, which are mostly related to the inability of PDXs and organoids to recapitulate heterotypic interactions between cancer cells, stromal cells, and immune cells. Mouse humanization procedures and co-culture assays are expected to aid the development of more holistic models that incorporate immune components. However, the impact of bone marrow reconstitution (let alone that of PBMC or TIL infusion) on the quality, quantity and topographical localization of immune infiltrates in transplanted tumors is difficult to assess, as is the influence of the host on the differentiation trajectories and functionality of transplanted human HSCs. Likewise, cocultures of cancer cell organoids with endogenous, syngeneic immune cells fail to mimic the subtleties of the tumor microenvironment in terms of complexity, representation, and reciprocal distribution of immune components. Another dimension of complexity is the difficulty – if not the impossibility – of replacing stromal elements such as endothelial cells and fibroblasts with their human counterparts; hence, the limitation remains that mouse-derived cytokines and growth factors in some cases do not crossreact with receptors that are expressed by human cancer cells.

A careful appraisal of the (vast) extent of information that can be reliably garnered by the use of patient-derived models, but also a clear understanding of their shortcomings, will be key to deliver robust, predictive and translationally relevant knowledge. This critical attitude will help triage and move to the clinic only those findings that emerge from conclusive and generalizable preclinical research and are motivated by responsible and limitation-aware methodological considerations.

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LEGENDS TO FIGURES

Figure 1: Application of colorectal cancer PDXs in translational research. PDX trials are conducted in parallel with or after clinical trials to "reverse validate" response biomarkers and genotype-response associations identified in patients (left panel). Cells dissociated from PDXs can be genetically manipulated and used in lineage tracing experiments to assess the temporal and spatial dynamics of functionally heterogeneous clones under drug pressure (middle panel). Genomic analysis of large-scale PDX collections enables the discovery of molecularly defined CRC subpopulations, which can be tested for the presence of potential therapeutic targets through pharmacologic experiments in vivo (right panel).

Figure 2: Application of genetically modified animal models of colorectal cancer in translational research. Genetically modified mice carrying targeted gene recombination of common mutations (Apc, Kras, Tgfbr2 and Trp53) in intestinal stem cells develop immune-cold CRC tumors with high levels of stromal TGF-β; blockade of TGF-β signals prompts the recruitment of immune effector cells into the tumor microenvironment and sensitizes tumors to immunotherapy (left panel). Another mouse model develops metastatic CRC featuring a pronounced stromal reaction due to targeted expression of active Kras and Notch and loss of Trp53 in villin-positive intestinal cells; Notch-dependent production of TGF-β by cancer cells promotes tumor infiltration by neutrophils and metastatic dissemination, which can be blunted by inhibition of neutrophil recruitment or TGF-β signaling (right panel).

Figure 3: Application of CRC organoids in translation research. Patient-derived organoids from normal colon can be engineered to express drivers of colorectal tumorigenesis, alone and in combination; this approach allows to explore the contribution of each driver to tumor onset and progression and helps understand how and to what extent engineered organoid models recapitulate the biological characteristics of spontaneous tumors from patients (left panel). Mutational profiles, methylomics and/or RNA sequencing analysis of clonal organoids derived from individual cells of patients' tumors can be used to reconstruct phylogenetic trees and investigate CRC tumor evolution (middle panel). Organoids can be exploited in mid- to high-throughput drug screens, and results from pharmacologic analyses can be coupled with molecular profiles to extract associations between drug sensitivity and specific molecular traits (right panels).

Figure 4: Incorporating the immune system into patient-derived models. The immune system of immunocompromised mice can be partially reconstituted with different approaches of variable efficacy, from infusions of PBMCs or TILs to transplantation of HSCs derived from the bone marrow or umbilical cord blood; once humanized, mice can be xenografted with patient-derived tumors and treated with immunotherapy to assess tumor growth kinetics and intratumor representation of immune cells before and after treatment (left panel). Cocultures of immune cells and cancer cells can be performed by co-mingling tumor organoids and autologous PBMCs or by implementing ALI methods that allow the preservation of the tumor epithelium and the associated immune stroma in cohesive units; both approaches are permissive for expansion of tumor-specific cytotoxic T cells (right panel).

Table 1. Validated and proposed biomarkers of response to existing therapies in colorectal cancer

Therapeutic agent	Biomarker	Analyzed in patients	Analyzed in preclinical models	Ref.
Chemotherapy				
Fluoropyrimidine (5-FU – Capecitabine)	▲ Thymidylate synthase (Resp)	YES	NO	21, 22
	▲ Dihydropyrimidine Dehydrogenase (Resist)	YES	NO	26, 27
Irinotecan	▲ Topoisomerase I (Resp)	YES	NO	25
	● *UGT1A1* (Tox)	YES	NO	31, 32
Oxaliplatin	▼ ERCC1 (Resp)	YES	NO	33, 34
EGFR monoclonal antibodies				
Cetuximab/panitumumab	★ *RAS (KRAS/NRAS)* (Resist)	YES	YES	38, 39, 85
	▲ Amphiregulin and epiregulin (Resp)	YES	YES	40, 41, 104
Anti-angiogenic therapy				
Bevacizumab	▼ VEGF-A, VEGF-D, HGF, IL-8, and neuropilin-1 (Resp)	YES	NO	49-53
	● VEGF-A, VEGF-R1 and inflammation and ER-associated genes (Resp)	YES	NO	54-57
	18q11.2-q12.1 loss and CIN (Resp)	YES	NO	58, 59
Regorafenib	▼ VCAM-1 (Resp)	YES	NO	60
	● CCL5/CCR5 pathway genes (Resp)	YES	NO	61
Other targeted therapies				
Trastuzumab + pertuzumab/lapatinib	▲ *HER2* (Resp)	YES	YES	62, 63, 85, 97
Entrectinib, ponatinib	▲ *NTRK, ROS1, ALK, RET* (Resp)	YES	NO	65-68
Encorafenib + cetuximab with or without binimetinib	★ *BRAF* (Resp)	YES	YES	73
AMG510	★ $KRAS^{G12C}$(Resp)	YES	YES	74
Pembrolizumab/nivolumab nivolumab + ipilimumab	dMMR/MSI-H (Resp)	YES	YES	75-79

▲ High expression ▼ Low expression ● Polymorphisms ★ Mutations

Resp, response; Resist, resistance; Tox, toxicity

Figure 1, Avolio & Trusolino

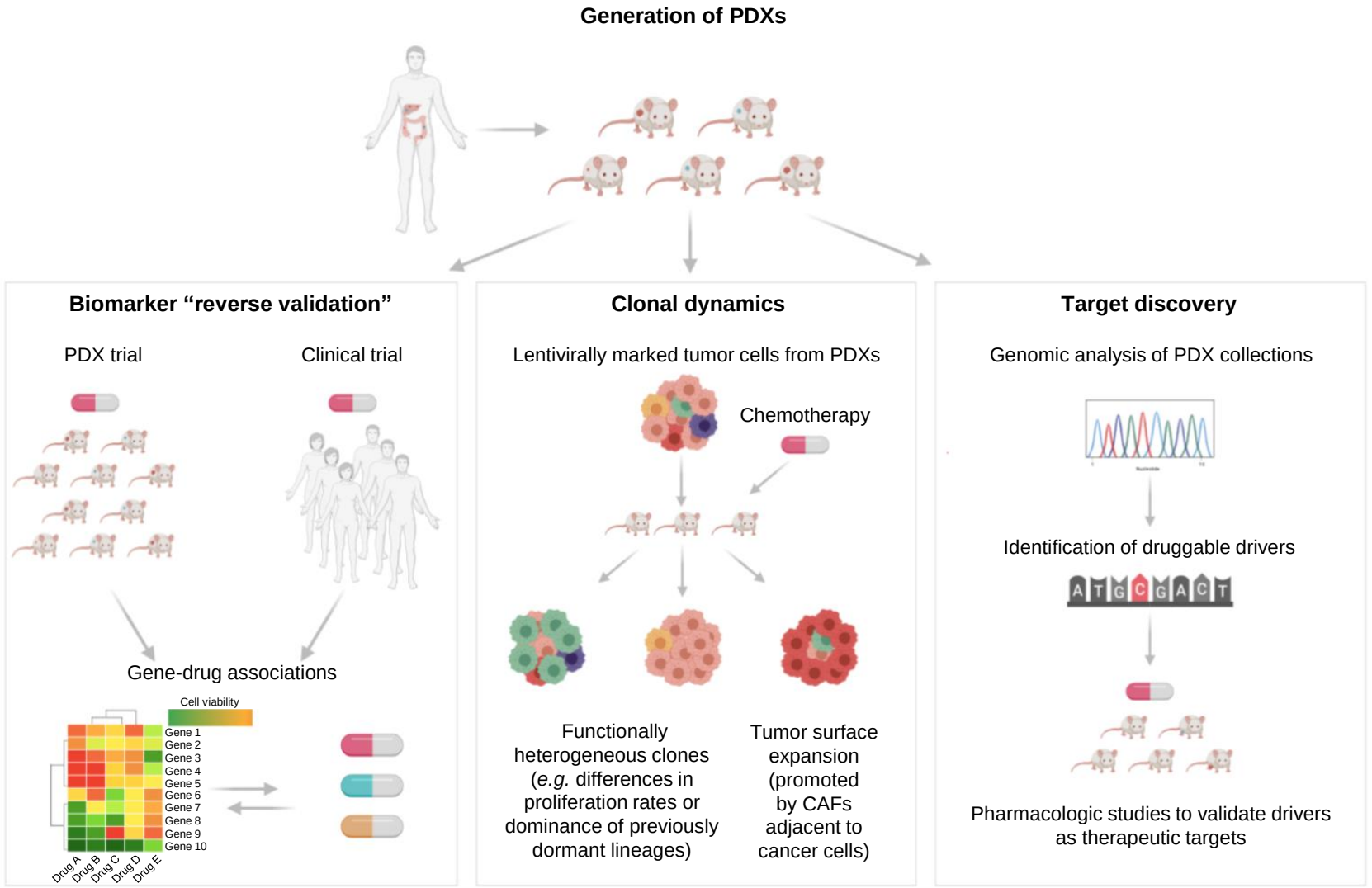

Figure 2, Avolio & Trusolino

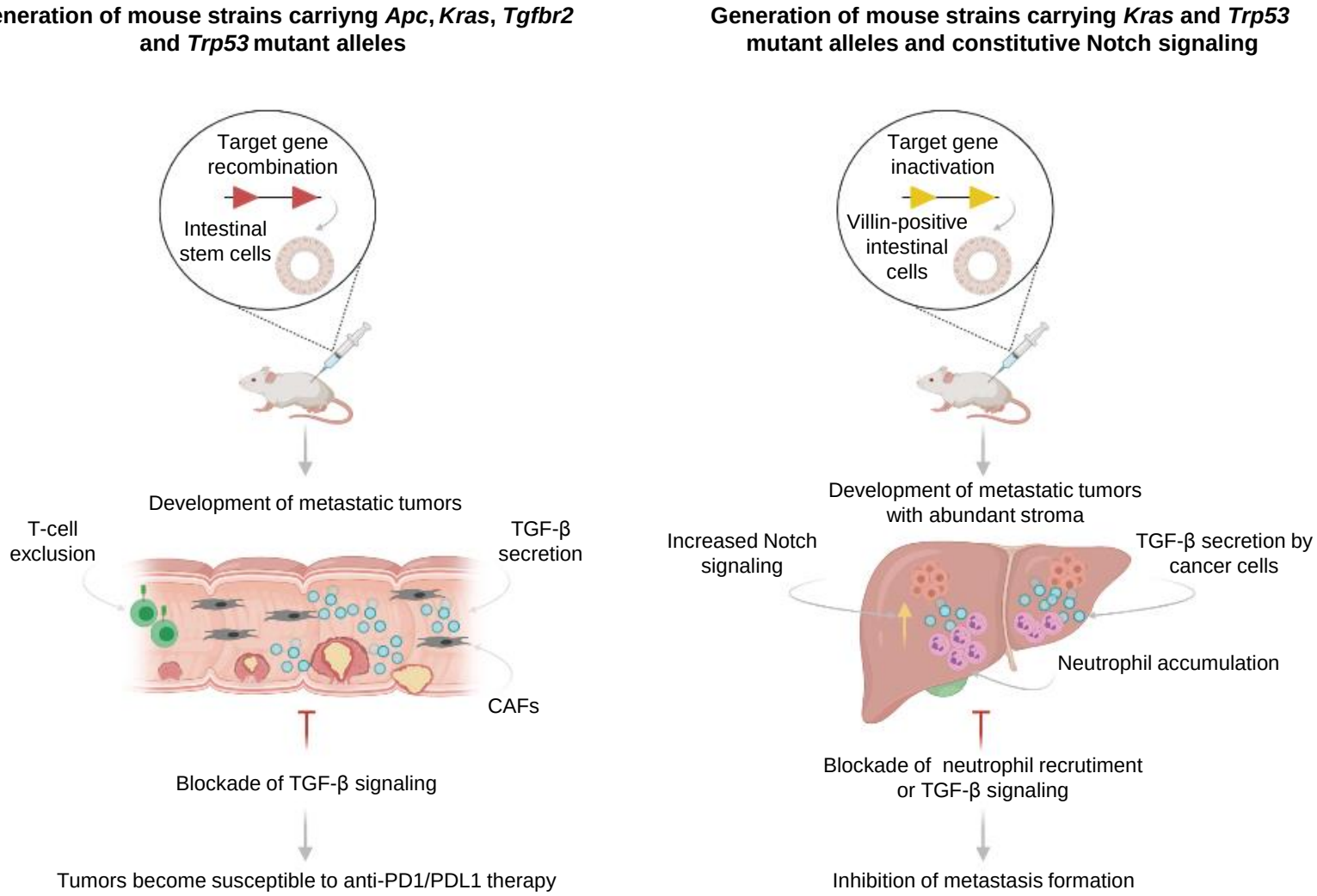

Figure 3, Avolio & Trusolino

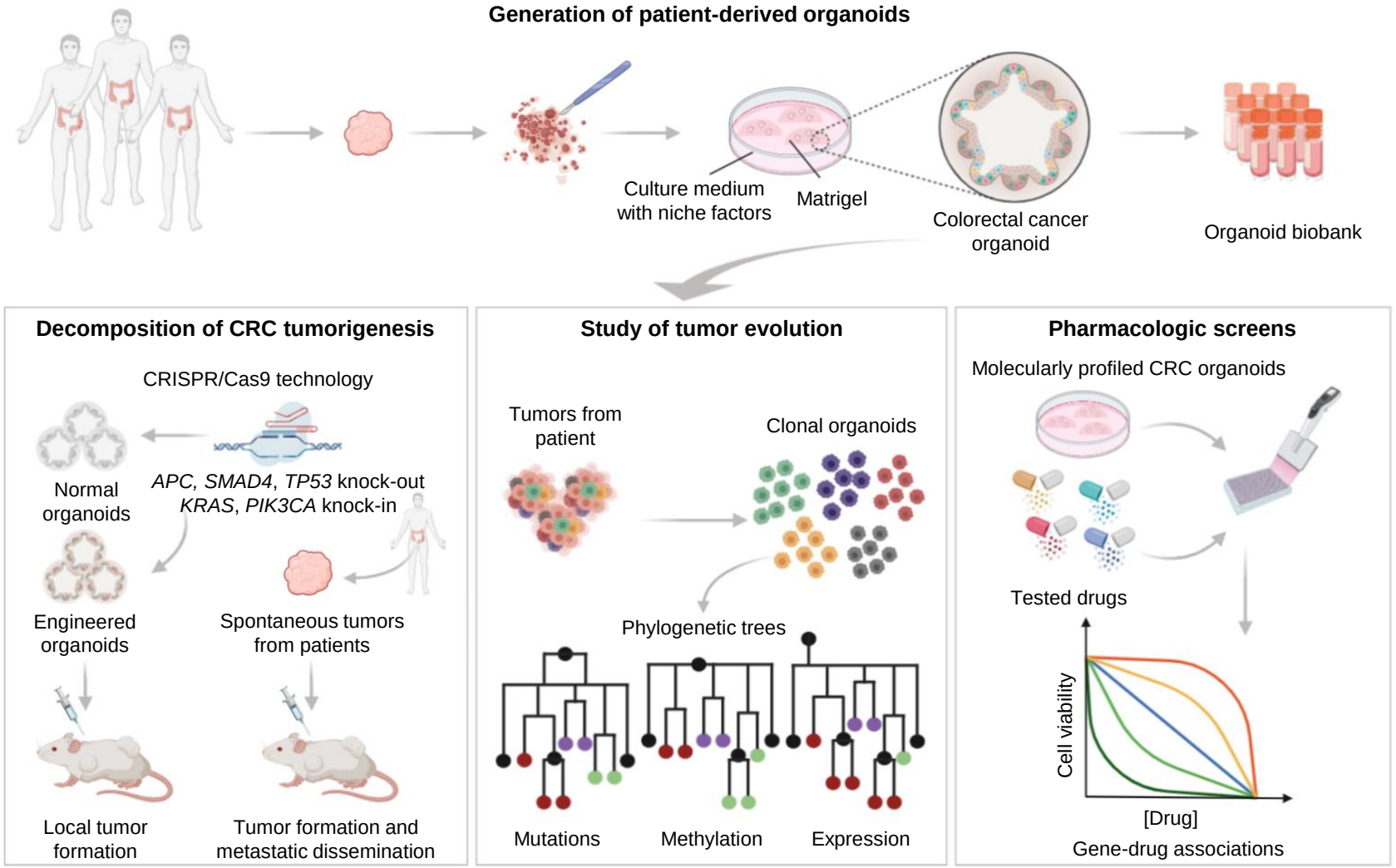

Figure 4, Avolio & Trusolino