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Review

Colorectal Cancer Subtypes: Developmental Origin and Microenvironmental Regulation

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Cancer is a heterogeneous disease and many cancer types do not represent a single entity, but are composed of biologically and clinically diverse subtypes. The subtype affiliation can influence prognosis and response to therapy. Recently, a multicenter colorectal cancer (CRC) subtyping consortium introduced a consensus molecular classification system for CRC. This will be of great benefit for future basic and clinical research since it enables uniform categorization of CRC specimens across different institutes and studies. The biological conformity observed within each consensus molecular subtype (CMS) holds promise for the design of subtype-specific treatment regimens. Herein, we review the CMSs of CRC with a focus on how multiple parameters, such as the origin, developmental route, and microenvironmental regulation shape distinct subtypes.

Trends

Colorectal cancer (CRC) is not a single disease; it can be classified into biologically and clinically distinct subgroups that can influence prognosis and response to therapy.

The biological conformity within each subtype suggests common underlying drivers and holds promise for the design of subtype-specific treatments.

Each CRC subtype presents a complex system of cancer and nontransformed stromal cells. Both the presence of microenvironmental components and their influence on epithelial tumor cells impact subtype affiliation.

CRC subtypes are being modeled *in vitro* using cell line panels for subtype-specific screening approaches, yet, more sophisticated model systems are needed to recapitulate the interplay with the tumor microenvironment.

Colorectal Cancer Is a Heterogeneous Disease

Colorectal cancer (CRC) patients show diversity with respect to prognosis and response to therapy and predicting these clinical parameters at the level of individual patients remains a holy grail in clinical practice. Accordingly, a large body of literature discusses the classification of CRC patients into clinically relevant groups. Single molecular markers – such as microsatellite instability (**MSI**; see Glossary) – can hold prognostic and predictive information; however, this only applies to a small group of patients [1,2]. The combination of multiple molecular markers allows more refined classification of patients and more accurate prognostication [3–5]. Yet, the molecular make up of a cancer does not always account for all its characteristics, highlighting the need for also assessing the activity of signaling pathways (Box 1) [6,7]. Conceivably, gene expression-based studies will aid to obtain a more comprehensive picture and allow drawing conclusions about underlying tumor biology. Recent advances in derivation and analysis of gene-expression profiles make it possible to stratify patients with a given cancer type into biologically homogeneous subgroups. Unbiased classification approaches [8] have been applied to multiple CRC data sets in several studies and indicate the existence of distinct subtypes. Because of differences in the generation of gene-expression profiles (e.g., RNA-extraction methods and gene-expression platforms) and data analysis (e.g., processing of data and algorithms to identify subgroups), the resulting classifications only overlap partially and consist of a number of subtypes ranging from 3 to 6 [9–16]. In an effort to generate a unified classification system for CRC, the International CRC Subtyping Consortium was formed in 2014 [17]. Despite these major efforts in identifying relevant cancer subgroups, the implementation of subtype information in the clinical management of patients is in its infancy: whereas sets of cancer patients can nowadays be easily classified, predicting subtype affiliation for individual patients remains challenging. Hence, the subtype classification system should not be viewed as a replacement for the current prognostic evaluation protocol; instead, its main value may derive from being used as a framework for detailed biological characterization of distinct CRC

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Box 1. Prognostic and Predictive Value of Molecular Markers

The MSI status of CRC specimens holds prognostic and predictive information: patients with MSI$^+$ tumors display better prognosis and appear to respond poorly to fluorouracil-based therapy [1,2]. In addition to genome-wide aberrations, single genetic alterations – such as mutations in the *KRAS* and *BRAF* oncogenes – present important clinical parameters. The *KRAS* mutation status, for instance, holds predictive value for patients with metastatic disease that would be eligible for anti-EGFR therapy [103,104]. CRCs responding to this targeted approach are characterized by high expression of the EGFR and its ligands, leading to physiological activation of the EGFR pathway in an autocrine manner [103,105–107]. The *KRAS*-mutant tumors do not respond to this treatment option due to activation of the MAPK pathway downstream of the EGFR. Yet, the use of the *KRAS* mutation status as predictive marker is complicated by the fact that only about 35% of non-*KRAS*-mutant tumors respond to this treatment, which could be explained by the presence of mutations in other downstream signaling components of the MAPK pathway – such as *BRAF* or *NRAS* – and possibly by mutations in *PIK3CA* activating a parallel pathway [108,109]. CRCs lacking alterations in the EGFR signaling pathway are characterized by constitutive activation of other key oncogenic signaling routes (e.g., by amplification of *ERBB2*, *FGFR1*, and *MET*), which represents another mode to evade EGFR signaling inhibition [11,110–112]. These data point to the apparent necessity of CRCs to activate the KRAS/BRAF pathway, but more importantly indicate a benefit from combining information about multiple molecular markers, which allows for more refined classification of patients and more accurate prognostication [3–5]. This approach also revealed that the prognostic value of individual mutations relies on the presence or absence of other molecular markers – the *BRAF*^{V600E} mutation, for example, is only associated with poor prognosis in tumors that are not microsatellite unstable [4]. Yet, defining the mutational spectrum of a cancer does not always reveal all its characteristics. Recently, it was, for instance, shown that there is a striking overlap in gene expression between tumors carrying a *BRAF*^{V600E} mutation and a subset of tumors – dubbed *BRAF* mutant-like – which lack this genetic event, but display the same gene-expression patterns and overlapping clinical features [6]. This analogy could result from mutational activation of the same signaling pathway and could predict similar response to targeted therapy [113], propelling whole pathways rather than single mutations into the spotlight [114]. In line with this is the observation that poor response to anti-EGFR therapy can be observed in tumors characterized by a gene-expression signature indicative of active MAPK or PI3K signaling pathways. Surprisingly, tumors lacking mutations in *KRAS*, *BRAF*, and *PIK3CA* can also fall into this category [7], indicating that assessing the mutation status of single genes or a combination of genes will be insufficient to predict treatment response.

subgroups. In this review, we summarize the recently developed consensus molecular classification system for CRC and discuss the parameters that can potentially impact subtype affiliation, such as the **cell-of-origin**, distinct developmental trajectories, or the tumor microenvironment. The identification and characterization of these parameters might reveal subtype-specific differences, which could be exploited to identify biomarkers, generate prediction signatures, and design drugs that target subtype-specific vulnerabilities. Furthermore, we describe model systems that recapitulate the steps of tumor development and progression of different CRC subtypes, which we believe will be crucial for in-depth understanding of underlying tumor biology and for designing new therapeutic strategies.

Consensus Molecular Subtypes of CRC

Gene Expression-Based Identification of CRC Subtypes

Unifying independent gene expression-based CRC classification approaches, the CRC Subtyping Consortium was able to classify a large number of CRC samples into one of four main consensus molecular subtypes (CMSs): (i) the CMS1 MSI immune group, in which tumors display a diffuse immune infiltrate and are frequently associated with MSI, the **CpG island methylator phenotype (CIMP)**, and the *BRAF*^{V600E} mutation; (ii) the CMS2 canonical subtype, which is characterized by an epithelial gene-expression signature. This group was recognized as the classical type of CRC due to high levels of **chromosomal instability (CIN)** and activated WNT signaling, suggesting it to follow the canonical path of CRC development [18]; (iii) the CMS3 metabolic subgroup, based on gene expression, CMS3 tumors also possess a strong epithelial component and additionally show deregulation of metabolic processes. On the molecular level, these tumors frequently display mutations in *KRAS*, in line with an increase in metabolic pathways, and are generally CIMP low; and (iv) the CMS4 mesenchymal type, which presents with a large fraction of stromal cells and the activation of genes associated with **epithelial–mesenchymal transition (EMT)**, angiogenesis, transforming growth factor-β (TGFβ) signaling, and matrix remodeling. Each of these subtypes associates with specific biological programs and distinct activated signaling pathways [e.g., Janus kinase/signal transducers and activators of

Glossary

Aneuploidy: a cell is aneuploid if it carries an abnormal number of chromosomes (i.e., more or less than 46 chromosomes in a human cell).
Cell-of-origin: cell that is the target of the first mutational hit(s) that may lead to malignant transformation.
Cetuximab: monoclonal antibody that binds to the epidermal growth factor receptor and inhibits its activation and thus downstream signaling. Cetuximab has been approved for the treatment of metastatic CRC without *KRAS* mutation.
CIMP: the CpG island methylator phenotype is defined as hypermethylation of CpG islands, which are frequently located in promoter regions of genes. Therefore, CIMP can lead to epigenetic silencing of tumor suppressor genes such as *CDKN2A*.
CIN: chromosomal instability is present in approximately two-thirds of CRC cases and refers to tumors with aneuploid chromosome sets carrying multiple structural and numerical aberrations. The causes of CIN are not well defined.
CRISPR-Cas: the clustered regularly interspaced short palindromic repeat-CRISPR-associated nuclease system is a prokaryotic immune system that protects bacteria from invading foreign genetic sequences. The components of this system can be used for genome editing, that is, for knocking out specific genes or for replacing a given genetic sequence with the sequence of interest.
EMT: cancer cells undergoing the epithelial–mesenchymal transition program lose epithelial characteristics, such as polarity and cell–cell adhesion, and gain mesenchymal features that allow them to leave the primary site and disseminate to distant organs.
HNPCC: hereditary nonpolyposis colorectal cancer syndrome, also known as Lynch syndrome, is a cancer syndrome associated with the increased risk of developing colorectal cancer, among others, due to the inheritance of mutations inactivating components of the DNA mismatch repair system.
MSI: microsatellite instability; MSI$^+$ tumors are characterized by a near-diploid genome and instability in the form of insertions and deletions in microsatellite regions. MSI is caused

transcription (JAK-STAT) in CMS1; WNT and SRC in CMS2; and integrin-β3, TGFβ, and vascular endothelial growth factor/vascular endothelial growth factor receptor in CMS4 (Figure 1, Key Figure)]. This reinforces the potential of developing subtype-specific targeting regimens [17]. Yet, approximately 13% of all CRC specimens cannot be assigned to one of the subtypes and represent either mixed or intermediate samples [17]. In this respect, it is important to note that the sampling procedure might impact subtype affiliation. Since different regions, as well as single cells within a given tumor, can be classified into distinct subtypes [19–21], a larger area of sampling could include tumor regions with different subtype affiliations. The influence of sampling techniques and the extent of intratumor heterogeneity with respect to affiliation to the CMSs of CRC should therefore be investigated in follow-up studies [22]. Furthermore, with the foreseeable development of more sophisticated classification algorithms, the CMSs of CRC may evolve in the coming years and should therefore not be considered as a final classification. We believe that it will be important to combine this gene expression-based classification with information about genetic mutations, epigenetic wiring, and activation of signaling pathways by microenvironmental cues to draw a comprehensive picture of CRC subtypes.

Intriguingly, an overlap of subtypes is observed in distinct solid cancers, suggesting that deregulation of similar signaling pathways with comparable phenotypic outcomes is a common trait across cancers in different organs. Therefore, lessons can be learnt from analogies between tumors of similar subtypes arising in different organs and from extrapolating biological characteristics and treatment responses. Parallels can, for instance, be drawn between CMS3 of CRC and a metabolic, genomically stable group of gastric cancers [23,24]. In fact, Hoadley *et al.* [25] recently described the identification of 11 'integrated subtypes' across 12 cancer types, revealing a striking conformity between gene expression in cancers derived from different organs. Incorporating this subtype information into the current prognostic protocols improves the prediction of survival, highlighting the clinical significance of this approach.

Modeling CRC Subtypes

The biological conformity observed within cancer subtypes holds promise for the design of subtype-specific treatment strategies. Cell line panels can be classified into the same subtypes as primary CRCs, and thus facilitate subtype-specific screenings [9]. This approach revealed, for instance, that cell lines belonging to the mesenchymal colon cancer subtype are more resistant to the anti-epidermal growth factor receptor (EGFR) antibody **cetuximab**. Importantly, this finding is mirrored in the clinic: patients with mesenchymal colon cancers do not seem to benefit from cetuximab treatment irrespective of their *KRAS* mutation status [12]. Although cell line panels are an invaluable tool for high-throughput screening approaches due to easy propagation and extensive annotation [26–28], they have numerous drawbacks, such as the adaptation to culture conditions during continuous propagation. Recent advances in *ex vivo* culture systems allowed the generation of so-called organoid cultures from patient-derived specimens. Organoids are defined as self-organizing systems that contain both stem cells and their progeny, and thus constitute *in vitro* models of healthy organs (in case of normal tissue used for isolation) or patient tumors [29]. CRC organoids display the same mutations and copy number variations as primary tumors and at similar frequencies, implying that collections of organoid cultures will faithfully reflect the heterogeneity observed at the population level [30]. The generation of organoid biobanks may soon permit *ex vivo* therapeutic screenings of cancer subtypes with the goal of designing personalized treatments [30]. Moreover, tissue specimens obtained from patient tumors can also be transplanted into immune-deficient mice to form so-called patient-derived xenografts (PDXs). This strategy has been used successfully to determine sensitivity of patient-derived tumor tissue to specific drugs [31–33].

One major restriction of these approaches is the fact that cell lines and patient-derived organoids and xenografts represent the end point of carcinogenesis. Yet, the specification into a particular

by a defective mismatch repair system in the sporadic form most frequently due to inactivation of one of its main components, *MLH1*, by promoter hypermethylation.

Multipotency: a cell is multipotent if it possesses the capability to differentiate into multiple, yet limited, cell types.

Virtual microdissection: mathematical decomposition of gene-expression data of tumor samples based on predefined signatures. This approach aims to digitally separate gene expression from normal, malignant, and stromal components within one tumor to identify cell type-specific gene expression and pathway activation.

Key Figure

The Consensus Molecular Subtypes of Colorectal Cancer Represent Biologically and Clinically Distinct Subgroups

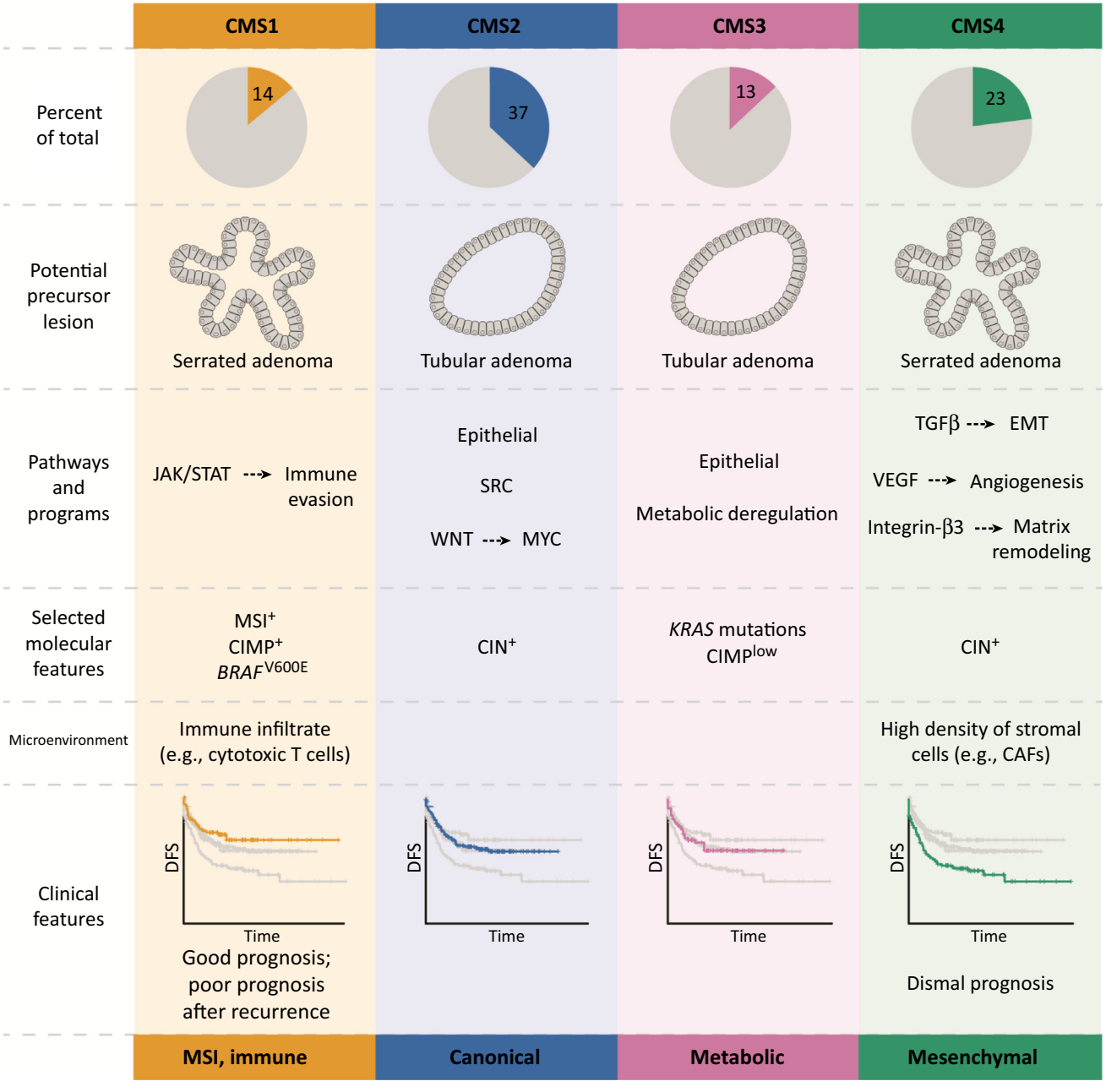


Figure 1. Each consensus molecular subtype (CMS) constitutes a distinct entity judged by molecular and clinical features (Guinney *et al.* [17]). Precursor lesions are assigned to the CMSs based on gene expression (Fessler *et al.* [65]). Active pathways and programs are linked with arrows based on their potential connection. CAFs, cancer-associated fibroblasts; CIMP, CpG island methylator phenotype; CIN, chromosomal instability; EMT, epithelial–mesenchymal transition; JAK/STAT, Janus kinase/signal transducers and activators of transcription; MSI, microsatellite instability; TGF, transforming growth factor; VEGF, vascular endothelial growth factor.

cancer subtype may occur early in tumor development and be dictated by multiple parameters during progression to malignancy. Understanding these cues may be necessary for detailed biological characterization of distinct cancer subtypes.

Parameters Influencing CRC Subtype Affiliation

Each CRC subtype represents a complex system in which cell intrinsic parameters (e.g., hierarchical and anatomical location, and the type and sequence of genetic lesions) and extrinsic cues (e.g., the tumor microenvironment and the microbiome) shape the phenotype of tumors and influence subtype affiliation. As we discuss in the following section, the microenvironment not only accounts for a substantial proportion of subtype-specific gene-expression profiles, but also determines biological and clinical behavior of cancer cells. Furthermore, the acquisition of specific biological traits and/or activity of signaling pathways may become wired into a cell during malignant transformation. Therefore, detailed insight into subtype biology can be obtained not just from the tissue at hand, but also from tracing the development and origin of specific cancer subtypes.

Cell-of-Origin

In an attempt to explain the existence of biologically heterogeneous groups lacking unifying mutations within one cancer type, the cell-of-origin was propelled into the spotlight (reviewed in [34]). Since specific biological programs may be active in distinct cells-of-origin, identifying this cell for different cancer subtypes could yield information about activation of signaling pathways and requirements for external stimuli during the early steps of tumor development. Several potential cells-of-origin have been described for CRC. In mouse models, aberrant activation of the WNT pathway in intestinal stem cells results in rapid development of adenomatous lesions [35,36]. By contrast, directing activation of the WNT pathway to more differentiated cells only rarely induces tumor formation [35]. Intestinal stem cells can be identified using various markers, pointing to the existence of multiple stem-like cell populations. It has therefore been proposed that the intestine has a 'stem cell compartment' rather than individual stem cells (reviewed in [37]). Moreover, the hierarchical organization of the colon is not unidirectional and more-differentiated cells can gain tumor-initiating potential if receiving the proper stimuli. Exposure to a favorable environment – for example, inflammation that induces nuclear factor-κB (NF-κB) signaling – instigates bidirectionality, inflicting stem cell properties and tumor-initiating capacity on non-stem cells of the intestine [38,39]. Similar results have been obtained for other organs, where not only proliferative progenitor cells, but also terminally differentiated cells can be targeted for transformation (e.g., neurons in the murine brain can dedifferentiate and gain tumor-initiating capacity upon loss of tumor suppressors and activation of oncogenic signaling [40]).

These concepts expand the pool of candidate cells-of-origin, allowing the speculation that different cancer subtypes originate from distinct hierarchical cells-of-origin (Figure 2A.1). Indeed, the CRC classification system proposed by Sadanandam *et al.* [13] draws parallels between gene-expression programs active in distinct subsets of healthy intestinal cells and specific CRC entities. Nonetheless, the hypothesis that the cell-of-origin can be inferred from the differentiation state of the tumor has been challenged by studies in breast cancer [41,42]. The conclusion that the tumor phenotype does not always relate to the cell population targeted for transformation was confirmed by two recent studies. Making use of mouse models, the most frequent mutation observed in human breast cancer – *PIK3CA*^{H1047R} – was introduced into lineage-restricted cells of the mammary gland [43,44]. This oncogenic hit results in reversion of unipotent lineage-committed cells to stem-like cells. The so-inferred **multipotency** permits subsequent multilineage differentiation of transformed cells, resulting in heterogeneous tumors. This indicates that histology and expression of differentiation markers of specific breast cancer subtypes do not directly imply a relation to the cell-of-origin. However, these studies reported different degrees of tumor aggressiveness depending on which cell was targeted with the *PIK3CA*^{H1047R} mutation, indicating that the cell-of-origin is indeed responsible for specific features of a distinct tumor type

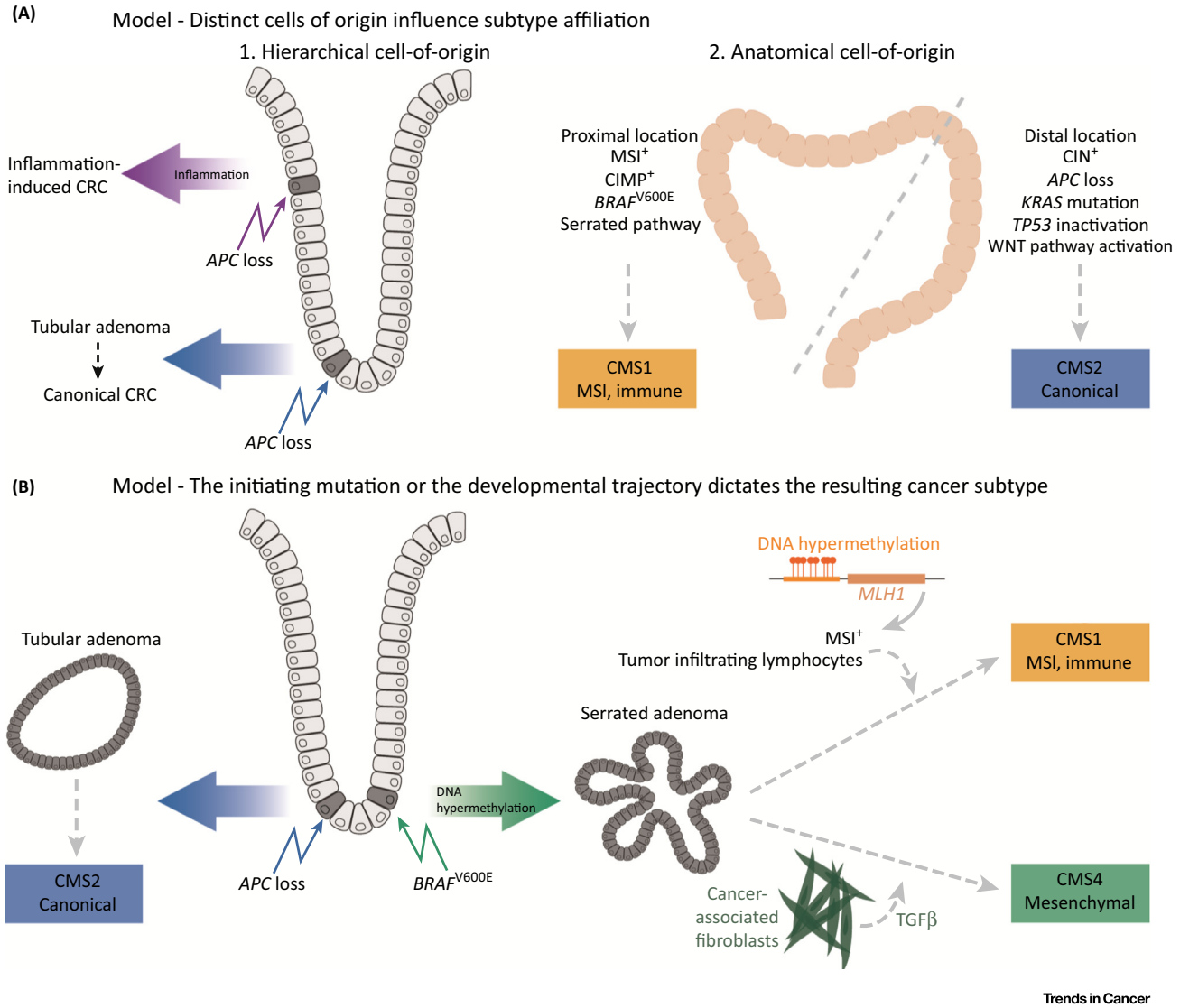


Figure 2. Multiple Parameters Shape Subtype Affiliation During Tumor Development and Progression. Models for how the cell-of-origin, the transformation-initiating mutation, or distinct developmental paths could dictate subtype affiliation. (A) 1. Loss of *APC* in two distinct cell types within the crypt hierarchy could lead to two different tumor forms: in case of a cell located in the crypt base, the resulting lesion could be a tubular adenoma further progressing to the canonical type of colorectal cancer (CRC). If the cell-of-origin is located further up the crypt, additional stimuli might be necessary for tumor development, such as inflammation, which could lead to the development of inflammation-induced CRC. 2. The anatomical location of the cell-of-origin might influence subtype affiliation. In the case of transformation of a cell located in the proximal colon, the resulting tumor frequently displays MSI, CIMP, and the $BRAF^{V600E}$ mutation, mirroring the features associated with the CMS1 of CRC. By contrast, tumors initiated by transformation of a cell in the distal colon display high percentages of *APC*, *KRAS*, and *TP53* mutations, as well as CIN, resembling the molecular make up frequently observed in the canonical CMS2 of CRC. (B) *APC* loss in a cell located in the crypt base can lead to the formation of a tubular adenoma, whereas an activating mutation in the *BRAF* oncogene afflicting the same cell type could spawn serrated adenomas. In both cases, adenoma formation may require additional aberrations, such as DNA hypermethylation in the serrated neoplasia pathway. Moreover, adenomas initiated by the same mutation could diverge upon additional stimuli such as a distinct microenvironmental composition or additional genetic lesions. CIMP, CpG island methylator phenotype; CIN, chromosomal instability; MSI, microsatellite instability; TGF, transforming growth factor.

[43,44]. Whereas similar results have been obtained for T-cell acute lymphoblastic leukemia [45] and glioblastoma [46], it is still a matter of debate whether the hierarchical localization of the cell-of-origin dictates subtype affiliation in CRC. Such a role may be less likely in CRC, since the identity of cells within the colonic crypt hierarchy is enforced by signals derived from the microenvironment and distinct cell types do not seem to differ in epigenetic marks, such as DNA methylation as well as histone methylation and acetylation [47,48]. In line with this notion is

the high rate of convertibility between specific cell types within the colonic crypt [49]. Several studies reported the interconversion of distinct pools of stem cells [50–52] and the reversion of precursor cells to stem-like cells upon the induction of stress [53]. Thus, mutational activation of an oncogenic signaling pathway could overcome microenvironmental stimuli leading to differentiation and allow transformation, resulting in highly similar tumors, despite distinct hierarchical cells-of-origin. In contrast to the uncertainty about distinct hierarchical cells-of-origin, it is now well established that the transformation of cells located in distinct anatomical regions of the colon results in specific cancer entities (Figure 2A.2). Based on different embryonal origins, the colon is divided into the proximal right-sided and the distal left-sided parts [54]. Tumors arising in these two locations are characterized by distinct molecular make ups: opposed to right-sided colon cancers that most frequently display MSI, the CIMP, and the $BRAF^{V600E}$ mutation, the majority of left-sided tumors are chromosomally unstable and are characterized by genetic insults occurring in classical CRCs (*APC* loss, *KRAS* mutations, *TP53* inactivation; Figure 2A.2) [54,55]. Tumor location also allows a more refined assessment of certain tumor types (e.g., microsatellite-stable tumors). Microsatellite-stable cancers in the proximal colon associate with worse clinical outcome than distal microsatellite-stable tumors [54]. A possible explanation is cell intrinsic differences, as discordant gene-expression profiles have been described for cells located in the proximal colon compared with the distal colon [56]. Additionally, cell extrinsic factors – such as the microbiome – differ between these two locations [54]. Yet, features of the proximal colon may not convert abruptly into properties of the distal colon when passing the splenic flexure, but this may instead represent a continuous transition [57]. Therefore, it remains to be investigated whether a strict separation into right- and left-sided colon cancer will be of similar use in clinical management as the separation into colon and rectal cancer. Nevertheless, from a biological perspective, this separation holds great value as a combination of location-specific variables might be responsible for distinct cancer phenotypes. In this mélange, the cell-of-origin could dictate the activity of gene-expression programs and wiring of signaling pathways in early transformed cells. Therefore, identifying its characteristics for distinct cancer subtypes might allow subtype-specific tailoring of treatment regimens with a focus on early intervention strategies.

Developmental Pathways

The developmental trajectory of benign tumorous lesions into fully metastatic carcinomas is well characterized at the molecular level in CRC, mainly through the analysis of transformed material obtained from individuals with inherited syndromes predisposing to CRC [58]. Different pathways of CRC development have been described, and intriguingly, an overlap with distinct CRC subtypes can be observed. For instance – due to high levels of WNT pathway activity and high frequency of chromosomally unstable tumors – the CMS2 group is thought to develop via the classical adenoma–carcinoma sequence. Tubular adenomas – precursor lesions associated with this path of CRC development – are frequently initiated by inactivation of the *APC* gene [18,59]. Subsequent genetic alterations include activation of the *KRAS* oncogene, development of CIN, and *TP53* inactivation [58]. The influence of distinct developmental trajectories on subtype affiliation becomes apparent when comparing this mutational sequence to the one observed in individuals with hereditary nonpolyposis CRC (**HNPCC**) syndrome. Early genetic lesions giving rise to adenomas overlap with those observed in the classical path of CRC development, namely, loss of *APC* and activation of *KRAS*. Yet, HNPCC patients carry germ-line mutations in genes of the mismatch repair pathway and are therefore predisposed to develop MSI^{+} CRC. Because of mismatch repair pathway deficiency, genes afflicted during tumor progression, such as *BAX*, differ from the classical sequence [58]. Hence, even though initiating mutations are identical, additional genetic lesions and most likely environmental stimuli determine the molecular make up and subtype affiliation of the resulting tumor. Thus, the subtype affiliation of a given lesion is not necessarily installed from the beginning but can be shaped throughout tumor development. Accordingly, a large proportion of CMS1 and a subset of CMS4

tumors carry an activating mutation in *BRAF*, which has been described as an initiating mutation in the serrated neoplasia pathway to CRC [60–62]. Based on molecular markers and gene expression, the serrated neoplasia pathway has been suggested to give rise to good and poor prognosis groups of CRC [63–65]. The developmental trajectory of serrated adenomas carrying the same molecular features (*BRAF*^{V600E} and DNA hypermethylation) into either CMS1 or CMS4 tumors remains to be examined. Molecular and microenvironmental features of the resulting tumors allow speculation about critical cues: a large proportion of CMS1 tumors are MSI^{+}, a feature that is frequently installed by *MLH1* promoter hypermethylation in sporadic CRCs [66] and leads to attraction of tumor-infiltrating lymphocytes [67], a cellular subset that is abundantly present in CMS1 tumors [17]. CMS4 tumors, by contrast, are characterized by a large stromal component and a mesenchymal appearance [17]. Furthermore, based on gene-expression profiling, the TGFβ pathway is active in CMS4 tumors and serrated adenomas are predicted to progress to this cancer subtype [12]. Therefore, we speculate that attraction of cancer-associated fibroblasts (CAFs) that secrete TGFβ could induce EMT in transformed cells and direct serrated adenomas to the mesenchymal CMS4 (Figure 2B). Accordingly, both murine and human organoid cultures that carry an activating mutation in the mitogen-activated protein kinase (MAPK) pathway show an attenuation of the apoptotic phenotype induced by TGFβ [65,68], and an EMT-like response prevails in a human *BRAF*^{V600E}-mutant culture upon stimulation with TGFβ [65]. Whether these factors can determine the development of subtypes remains to be investigated. It is important to note that not all CMS1 and CMS4 cancers display *BRAF*^{V600E} mutations, while this oncogenic hit is detected in the majority of serrated lesions [60]. In line with this notion is the observation that the TGFβ response of *BRAF*- and *KRAS*-mutant organoids overlaps [65,68], suggesting the existence of other – potentially *KRAS*-mutant – precursor lesions developing to CMS1 and CMS4 malignancies.

Detailed biological and functional understanding regarding cancer subtype development via distinct routes can be achieved by deriving organoid cultures of precursor lesions from individuals with inherited CRC syndromes. Genetic manipulation can lead to the delineation of the sequence of mutations directing the development to specific CRC subtypes. Furthermore, coculture experiments with specific nontransformed cell subsets, such as fibroblasts, or stimulation with defined growth factors, can shed light on the role of microenvironmental components in determining subtype affiliation. This could result in therapeutic strategies preventing or significantly decelerating progression of still-benign lesions to CRC in patients with inherited CRC syndromes. Moreover, biological programs associated with distinct CRC subtypes can already be active at the precursor stage [12] and these cultures may allow the identification of signals triggering these programs.

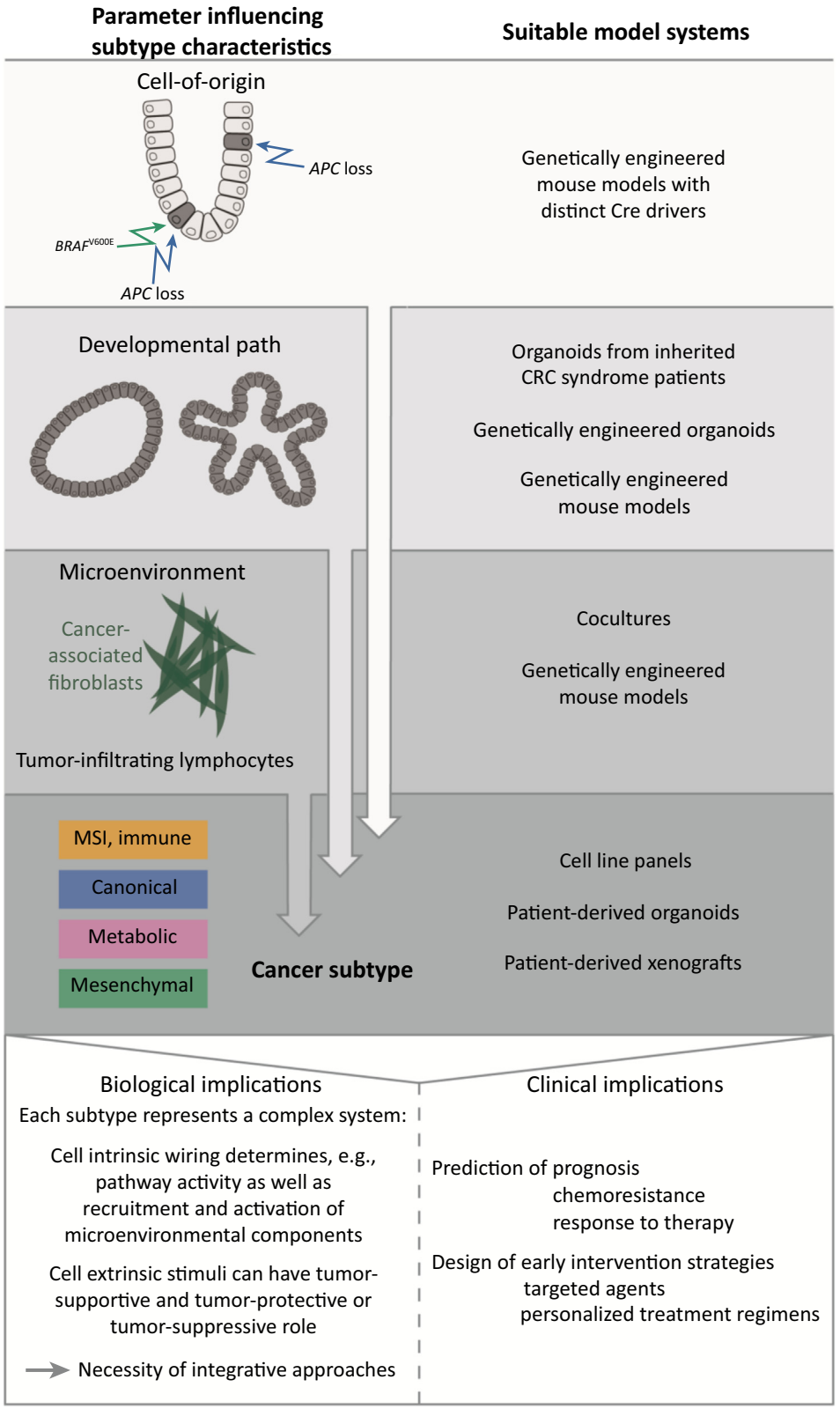
In addition to the sequence of mutations, studying polyps from familial CRC patients also provides information about the role of the initiating mutation. Whereas *APC* loss results in tubular adenomas most likely developing into CMS2 tumors with relatively good prognosis, activation of the *BRAF* oncogene initiates formation of serrated adenomas that have the potential to develop into poor-prognosis CMS4 tumors (Figure 2B). Because of major advances in genome engineering, studies of preneoplastic material from familial CRC syndrome patients can nowadays be complemented with organoid cultures carrying single genetic hits, modeling the earliest stage of cancer development. In contrast to adenomas obtained from patients, which in addition to the initiating event likely carry genetic and/or epigenetic insults with inter-patient variation, genetic manipulation of organoid cultures from the healthy intestine results in genetically well-defined model systems. Typical CRC-associated mutations were introduced into human intestinal organoids by the clustered regularly interspaced short palindromic repeat-CRISPR-associated nuclease system **(CRISPR/Cas9)** [69–74]. This approach allows the examination of specific effects inferred by individual mutations and has, for instance, revealed that inactivating mutations in the tumor suppressors *APC* and *TP53* together are sufficient to induce CIN in the form of

aneuploidy [73]. The mutations studied so far recapitulate the classical path of CRC development and it would be of importance to study the effect of different initiating mutations with the aim to compare the resulting structures with the CMSs of CRC. It is important to note, however, that tumor cell intrinsic changes occurring in patients not only determine malignant cell behavior, but also have profound effects on normal nontransformed cells, thus shaping the tumor microenvironment. In turn, distinct cellular subsets within the microenvironment influence malignant cells in various ways. The lack of microenvironmental components in organoid culture systems could therefore distort gene-expression profiles and might be an explanation for the fact that not all CMSs of CRC are represented in a CRC organoid biobank [75]. Hence, for a detailed understanding of subtype biology it is of utmost importance to consider the aforementioned modifications in cancer cells in an integrative approach that acknowledges the complex interactions with microenvironmental components.

Microenvironmental Regulation

In the last years it became increasingly clear that tumors are not only clonal outgrowths of deregulated cancer cells, but that they are – just as normal organs – composed of many different cell types, and that interactions between these diverse components are pivotal for tumor development and progression [76]. Tumor cell extrinsic stimuli derived from the microenvironment may play crucial roles in the development of cancer heterogeneity. MSI^+ CMS1 CRCs, for instance, are characterized by a large number of tumor-infiltrating lymphocytes [67], and the presence of cytotoxic T cells may dictate the better prognosis of this patient subgroup [77]. Although counterintuitive at first, inflammatory responses can also be detrimental for cancer patients, as they can aid malignant tumor progression under certain circumstances. In the case of chronic inflammatory conditions in the intestine, summarized as inflammatory bowel disease, inflammation precedes tumor formation and has been linked to increased risk of CRC development [78]. Interestingly, it was suggested that inflammatory bowel disease-associated CRC follows an inflammatory carcinogenesis route giving rise to a distinct form of CRC [79]. This could be explained by an alternative cell-of-origin, as inflammation-induced NF-κB signaling primes non-stem cells in the murine intestine for transformation [38].

As in normal organs, the stroma constitutes a major part of the tumor mass. CMS4 tumors are characterized by a high density of stromal cells, such as lymphoid, myeloid, and endothelial cells, as well as CAFs [80]. CAFs are a mesenchymal cell subset that is susceptible to manipulation by malignant cells and *vice versa* CAFs can influence a multitude of processes in transformed cells, such as growth and invasion [81]. It is a matter of debate whether the mesenchymal appearance of the CMS4 of CRC is caused by epithelial cells that undergo EMT and thus gain a mesenchymal phenotype, or solely by the presence of more stroma or a higher activation state of stromal components [22,82–84]. Both epithelial cells undergoing EMT and high amounts of stroma are linked to dismal outcome in various cancer types [85–91]. The cause of increased stromal cell recruitment or activation remains obscure. Laser capture microdissection experiments separating stroma from epithelium prior to gene-expression analyses may yield detailed insight into the activation state of the stroma and the epithelial phenotype in distinct cancer subtypes. Intriguingly, stroma from breast cancer patients acquired using laser capture microdissection predicts prognosis independent of cancer subtype [92]. Similarly, **virtual microdissection** revealed that not only malignant cells but also stromal components of pancreatic adenocarcinomas exist in distinct states that associate with prognosis and that can help refine cancer classification [93]. These observations suggest that the tumor stroma strongly affects tumor cell behavior and that it is an important determinant of gene-expression profiles derived from whole tumor samples. Conversely, Calon *et al.* [94] have shown that only malignant cells that induce stromal activation in distant organs are able to colonize the distant site, allowing the speculation that malignant transformation of epithelial cells shapes the stromal compartment. The so-influenced stromal niche might in turn affect



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tumor behavior and direct cancers to a more malignant state. Do malignant cells remain dependent on microenvironmental stimuli throughout development and progression, and is continuous crosstalk between normal and transformed cells necessary for maintaining subtype affiliation? If so, it would not be surprising that culture systems as well as PDX models do not recapitulate all cancer subtypes [75,83]. The gene-expression signals derived from microenvironmental components and also their role in shaping the cancer cell phenotype are most likely major determinants in cancer subtype affiliation. Indeed, immune cells and fibroblasts themselves as well as their interaction with malignant cells have been shown to critically influence not only biological characteristics but also clinical behavior (reviewed in [95]). To date, we lack model systems that fully recapitulate the complex ecosystem of cancers in patients. Whereas cell intrinsic effects can be easily recapitulated in cell culture systems, modeling the interplay with the microenvironment is more challenging. In the case of PDX models, the tumor piece interacts with the murine organism and nontransformed cells are incorporated into the tumor. However, studying cancer subtypes in this system is a cost- and labor-intensive approach and might be further restrained by difficulties in detecting molecular cancer subtypes based on gene expression. As detailed earlier, nonepithelial cells constitute a major proportion of the cancer mass and a stroma-derived signal might influence the classification of tumors into distinct subtypes. Therefore, the replacement of human stroma with stroma of murine origin in PDXs complicates the comparison of gene-expression data. Indeed, the mesenchymal subtype, which contains a large stromal component, could in initial studies not be detected in a panel of PDXs [83]. This is due to the fact that the original classifiers were built on whole tumor samples and comprises epithelial and stromal genes, of which the latter are mouse-derived in PDX models, and therefore not detected properly by the gene-expression analyses employed. However, this does not mean that the mesenchymal subtype does not exist in PDX models and also does not warrant the conclusion that the mesenchymal subtype is solely defined by stromal components. Several groups are currently trying to generate classifiers that work independent of stroma to determine the underlying cancer biology. We hypothesize that malignant tumor cells recruit and activate stromal components and that the ability to do so differs depending on subtype affiliation. Next to the problem of identifying distinct subtypes in PDX models, species specificity may preclude the effective communication between human tumor cells and murine stromal cells. For example, it was shown that murine hepatocyte growth factor only inadequately stimulates the human c-Met receptor [96]. In addition, immune components, which can influence biological and clinical parameters (for instance, in CMS1 tumors), will not be part of the equation due to the need of immune-compromised mice for xenotransplantation. Genetically engineered mouse models more faithfully recapitulate this interaction and allow incorporation of multiple components that can shape the resulting cancer subtype. Nevertheless, the utility of this system is restricted by the long period of tumor development, limited amount of mutations that can be introduced, the short life span of a mouse that often prevents the development of fully malignant and metastatic carcinomas, and human–mouse differences (reviewed in [97]). Furthermore, it remains to be investigated how adenomas and carcinomas arising in genetically engineered mouse models relate to the cancer subtypes detected in patients. Results from our laboratory point to *BRAF*^{V600E}-mutant organoids as a valuable *in vitro* model for serrated adenomas [65]. Importantly, gastrointestinal tumors initiated by the *Braf*^{V600E} mutation in mice also closely resemble human serrated adenomas [98] and might thus provide a system to study the intricate crosstalk between *BRAF*^{V600E}-mutant cells and specific microenvironmental components.

Figure 3. Each Colorectal Cancer (CRC) Subtype Represents a Complex System Shaped by Multiple Parameters. The distinct steps of tumor development and the microenvironmental composition shape the resulting cancer subtype. Information can be obtained from model systems and the tissue at hand and this information impacts the biological as well as clinical understanding of subtypes within a given tumor. We propose that an integrated approach considering knowledge gained from each dimension is necessary for an in-depth biological characterization and the design of successful treatment strategies. MSI, microsatellite instability.

Concluding Remarks

It is becoming increasingly clear that subtypes of a given type of cancer are as heterogeneous as unrelated malignancies arising in different organs [99]. Each subtype should therefore be considered as distinct entity and characterized as such. Subtype-specific vulnerabilities identified this way could be exploited for the design of tailored treatment regimens. While this approach has been successfully employed in breast cancer [100], it remains to be investigated if treatments specific for CRC subtypes will be similarly beneficial. To understand the efficacy or failure of specific agents, we believe that it is paramount to consider not just the current state of the tumor by analyzing the end-stage carcinomatous tissue derived from patients, but to also complement this knowledge with information from early stage disease. As we discuss herein, distinct steps of tumor development and the composition of the tumor microenvironment influence the resulting cancer subtype (Figure 3). It is important to note, however, that not only intertumor, but also intratumor and microenvironmental heterogeneity could severely impair the efficacy of novel therapeutics and lead to resistance and relapse [95,101]. In addition, spatial and temporal differences need to be considered: specimens from different areas of the tumor might be classified into different subgroups in molecular analyses, potentially due to the presence of more or distinct types of tumor stroma [22]. Furthermore, tumors that recur might not belong to the same subtype as the primary malignancy [102]. In this respect, it is important to take into consideration that most, if not all, subtyping studies use primary cancer material, whereas disseminated and metastatic cells could be viewed as the main target of chemotherapeutic agents (see Outstanding Questions).

Future research should aim to devise reliable culture techniques and model systems that account for all forms of tumor heterogeneity. We suspect that integrating information from multiple model systems with data from primary specimens will be essential for delineating the origin and nature of different subtypes within a given malignancy and to shed light on the causes of subtype-specific characteristics. Furthermore, we advocate the use of integrative approaches, which acknowledge that each cancer subtype is in fact a complex mélange of nontransformed and malignant cells. Even though the extent of tumor heterogeneity can be overwhelming and presents a major challenge for the clinical management of individual patients, it is important to note that only due to heterogeneity it is possible to design tailored therapeutic approaches.

Outstanding Questions

What is the role of intratumor heterogeneity in subtype affiliation and what is the nature of mixed/intermediate samples?

Does the hierarchical location of the cell-of-origin within the colonic crypt determine subtype affiliation?

Why do colorectal cancers of the mesenchymal consensus molecular subtype have increased amounts of stroma and what are the signals responsible for stroma recruitment?

Can the signal that distinguishes distinct subtypes be also found in the epithelial tumor cell fraction?

Is a continuous crosstalk between epithelial and stromal cells necessary to maintain adherence to a specific subtype?

Can a model system that accurately reflects all forms of heterogeneity and the interplay with the tumor microenvironment be designed?

Is subtype affiliation maintained throughout metastatic dissemination, that is, do primary tumors and matching metastatic samples belong to the same subtype?

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