CHAPTER 1

Cancer Heterogeneity
– a Multifaceted View

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SUMMARY

Cancers of various organs have been categorized into distinct subtypes following increasingly sophisticated taxonomies. Additionally, within a seemingly homogeneous subclass, individual cancers contain diverse tumor cell populations that display variability in important cancer-specific traits such as clonogenicity and invasive potential. Differences that exist between and within a given tumor type have both significantly hampered the proper selection of patients that might benefit from therapy, as well as the development of novel targeted agents. In this review we discuss the differences associated with organ-specific cancer subtypes and the factors that contribute to intra-tumor heterogeneity. It is of utmost importance to understand the biological causes that distinguish tumors as well as distinct tumor cell populations within malignancies, as these will ultimately point the way to more rational anti-cancer treatments.
**Introduction**

The first pathological reports made it clear that a single neoplasm presents with tremendous variability among individual tumor cells. This so called *intra*-tumor heterogeneity poses an important challenge for predicting tumor behavior and clinical outcome. By all means, differences not only exist within a single tumor, but also across individual patients presenting with cancers originating in the same organ. Increasing knowledge of this *inter*-tumor heterogeneity has led to an exhaustive categorization of tumor subsets according to staging, differentiation grade, cellular morphology and marker expression.

*Intra- and inter-tumor* heterogeneity and how they relate to each other are the main focus of this review. The emergence of advanced molecular and biochemical technologies have led to a better understanding of the numerous mechanisms that drive both faces of tumor heterogeneity. Indeed, gene expression arrays and next generation sequencing have paved the way to more comprehensive indexing of a broad variety of cancer types, such as breast and brain tumors [1-3], allowing their separation in robust subtypes with markedly different molecular and clinical qualities. In addition, scrutiny within individual tumors has reached tremendous sensitivity and many genetic and epigenetic modifications, as well as micro-environmental interactions, can now be dissected at the single cell level [4]. Despite all these efforts, we are only starting to appreciate the complexity of tumors and our limited understanding of the molecular mechanisms that drive individual cancers has precluded the development of curative targeted therapies. In more advanced disease for instance, tumors relapse in the vast majority of cases even after a seemingly successful therapy. This distressing fact has often been attributed to the presence of heterogeneity within tumors, and two main conceptual frameworks have been elaborated to conceptualize the link between *intra*-tumor heterogeneity and therapy resistance. The first, and most well established one, rests on classical evolutionary concepts and postulates that selective pressure acting on tumor cells ultimately leads to (epi-)genetically distinct, resistant clones [5, 6]. The second, and more recent theory, is based on the realization that subsets of tumor cells inherently have a dissimilar ability to drive tumor growth and metastasis [7, 8]. In this scenario, tumors are perceived as hierarchically organized tissues and differences between tumor cells are mainly explained by a gradient of differentiation that exists between cancer stem cells (CSCs), residing at the top of the hierarchy, and their more differentiated progeny. These two theories perfectly co-exist and are complementary rather than mutually exclusive [9]. In addition, tumor heterogeneity is not only defined by tumor cells alone, but also involves the presence of non-neoplastic cells that establish a tumor microenvironment.

Here, we provide an overview of several aspects that critically contribute to tumor heterogeneity (Figure 1) and discuss their implications in a clinical context. Moreover, we highlight the connections that exist between *intra-* and *inter-*tumoral heterogeneity in an effort to draw parallels that might contribute to our basic understanding of these two sides of the same coin.
Colon Cancer Heterogeneity: Stem Cells, Signals and Subtypes

Figure 1. Schematic representation of the various factors (diagonal, red boxes) that affect intra (x-axis, light blue) and inter-tumor (y-axis, light grey) heterogeneity. Several examples are depicted for each factor and for both forms of tumor heterogeneity.

Part I, The Actors of Tumor Heterogeneity

The genetic contribution. Genetic variation is undoubtedly the most established foundation of both intra- and inter-tumor heterogeneity. The stepwise acquisition of mutations during cancer evolution was first advocated by Nowell in 1976 [10], and later on substantiated by a large body of experimental work. As tumors progress, multiple, and changing, environmental pressures operate on the cancer cell population and select for clones that are best endowed to survive and respond to these changes (Figure 2A). Adaptation can be enabled by genetic mutations that confer a selective advantage to a particular clone [6]. This process is reiterated as long as the tumor ecosystem evolves, leading to the formation of complex clonal patterns. In their landmark paper, Fearon and Vogelstein crystallized this concept by looking at different mutations in a series of patients comparing normal epithelium with cancer precursor lesions and cancer tissue. They reported increasing amount of mutations with more advanced disease, but importantly, also proposed a preferential sequence of events by inferring the prevalence of certain mutations at each stage [11]. Today the picture has expanded considerably and a full genomic landscape has been drawn for a large variety of malignancies [12-14], only to strengthen the notion that clonal evolution is crucial to tumorigenesis. Individual cancers can harbor a handful [15, 16], or more than hundreds of mutations [17]. This startling diversity has urged efforts to define patterns and
sets of mutations that actively contribute to disease initiation and progression, also dubbed “driver mutations” as compared to bystander or “passenger mutations” that are not causally implicated in oncogenesis but are carried along and accumulate as cancers develop. Identifying the biological nature of driver mutations in tumors, such as those conferring a growth advantage, is relatively straightforward, but the actual benefit they provide on tumor cells remains poorly investigated. Indeed, few studies have attempted to quantify the evolutionary advantage mutations confer to a clone [18]. Models where clone size can be easily measured longitudinally [19] are needed for this purpose such as the normal colonic crypt, which provides a unique architecture to study stem cell homeostasis. Colonic crypts become monoclonal during time following a pattern of neutral drift dynamics in which functionally equivalent stem cells either expand or disappear stochastically until they either take over the crypt or are lost [20, 21]. Therefore, a mutation specifically initiated in stem cells can affect neutral drift and the speed at which an affected crypt reaches monoclonality. This can subsequently be used to infer the evolutionary benefit of that particular mutation [L.V., unpublished work]. In contrast to the lack of quantitative measurements on genetic hits, it is more apparent that distinct mutations have different impact on tumor heterogeneity. For instance, inactivation of the tumor suppressor MLH1 in colorectal cancer (CRC) impairs the DNA damage response and leads to the formation of tumors with a high mutation rate and a so-called hypermutator phenotype [22]. Finally, assessing clonal heterogeneity with current techniques requires the evaluation of topologically distinct regions. This is beautifully illustrated in renal carcinoma where whole exome sequencing of multiple distinct tumor regions has revealed considerable genetic disparities [23].

Genomic differences do not solely exist within an individual cancer but certainly, and arguably most prominently, among cancers. It is quite evident that the range of mutations between seemingly identical cancers can vary substantially between patients. Even the type of driver mutation(s) found in each cancer can highly differ. This has often resulted in the identification of molecular subsets of cancers that are made up of diverse mutational spectra (Figure 2D). In the case of CRC, many previous reports have attempted to classify this disease in different categories according to its respective genetic defects [24, 25]. For instance, activating mutations of the BRAF oncogene are associated with a particular type of CRC that displays instability in microsatellite (MSI) DNA repeats. Another category shows chromosomal instability (CIN) and associates with mutations in the TPS3 and KRAS genes. Although certain mutation patterns associate with a subset of cancers, the link between the genetic make-up of a tumor and its phenotype is fairly weak. For example, on the most abstract level, similar mutations can result in a vastly different phenotype, while the same phenotype can result from different mutations. CRC patients with BRAF mutations illustrate the former, and have markedly different biology and clinical outcome whether they present with MSI or not [24]. Conversely, gastrointestinal stromal tumors (GISTs) that are induced by either c-KIT or PDGFR-α mutations, present with remarkably similar histology and therapy response [26]. Furthermore, our group has recently shown that unbiased determination of the most biologically distinct CRCs using gene expression arrays results in the identification of robust subsets that show key differences in prognosis despite a significant overlap in their mutations spectra [27], which further
demonstrate that determining mutations *per se* is insufficient to fully appreciate biological and, as a consequence, clinical behavior.

**The impact of epigenetics to tumor heterogeneity.** Besides genetic diversity, there is increasing recognition that epigenetic changes, such as DNA methylation and histone de-acetylation, can occur throughout tumorigenesis [28]. These changes, and the subsequent biological processes they affect, are inherited during cell division without altering the DNA sequence. Aberrant DNA methylation is a hallmark detected in many cancer types that often present with global loss of DNA methylation [29]. More specific alterations are also frequently seen, such as those that target *p16* [30], which is a prime example of methylation-mediated inactivation of tumor suppressor genes. In addition to contributing to tumorigenesis, epigenetic alterations are also linked to tumor heterogeneity in numerous ways. Within an individual tumor, DNA methylation patterns are particularly polymorphic, even at single DNA promoter regions that are frequently methylated, which suggests the presence of heterogeneous cell populations with regards to DNA methylation [31]. In breast cancers, subsets of tumor cells differentially methylate the *TGFBR2* promoter, which results in differential TGF pathway activity [32]. Furthermore, methylation-mediated silencing of key gatekeeper genes, such as *MLH1* might fuel clonal evolution as it promotes a hypermutation phenotype [22]. Interestingly, this particular case is directly related to inter-tumor heterogeneity since *MLH1* methylation is a defining feature of the MSI CRC subtype [25]. Moreover, methylation of a subset of Wnt target genes has been shown to identify colon cancers with poor prognosis [33]. Finally, certain subtypes of CRCs and gliomas have been even defined by a high level of methylation at the CpG rich promoter regions of a subset of genes [34, 35]. The reversible nature of epigenetic modifications has attracted much research efforts to develop agents that specifically target the enzymes responsible of these changes and are discussed more broadly elsewhere [36].

**The role of cancer stem cells in tumor heterogeneity.** Normal tissue homeostasis is orchestrated by complex interactions between cells and their microenvironment that maintain a critical balance between proliferation and differentiation. Importantly, all these processes occur in an ecosystem that is genetically identical and relies on a gradient of differentiation between stem cells and their differentiated progeny. An equivalent paradigm is not novel in cancer [37], but only recent advances in technologies have made it possible to critically investigate the role of cancer cells endowed with stem cell properties. Initially described in leukemia, the concept of cancer stem cells (CSCs) has been rapidly embraced by the scientific community and led to their identification in virtually all tumor types [7-9]. The principle generally relies on the use of markers that enrich for cells able to propagate the malignancy in serial xenotransplantation assays. The exact identity of CSCs in many tumors is still disputed [38], especially due to the imperfect nature of the current methodologies available to assess cancer stemness. Although more recent reports lend support for the existence of CSCs in endogenously growing neoplasia [39-41], these also have their own caveats. For instance, both intestinal adenoma and skin papillomas used in these studies are only benign precursor lesions that progress infrequently to carcinomas. The CSC concept nevertheless provides an additional source of heterogeneity
in cancer; heterogeneous cell populations are defined by their differentiation state, non-differentiated (or CSCs) as compared to differentiated cells, as well as the various differentiation lineages that can be adopted (Figure 2B). Importantly, the genetic and CSC models are not mutually exclusive but rather complementary in fuelling tumor heterogeneity [9]. For example, CSCs are -by definition- the only cells with self-renewal capacity and as such would serve as repository for clonal evolution to take place. Reciprocally, genetic mutations such as inactivation of TP53 can influence the CSC content [42]. The molecular attributes that maintain the CSC state remain largely elusive but we have gained much more insights into the molecular determinants of cancer stemness. For example, colon CSCs were shown to possess relatively higher levels of Wnt pathway activity as compared to their differentiated progeny despite the presence of Wnt activating mutations [43]. While deregulated in all colon carcinomas, Wnt does not necessarily define colon CSCs in every tumor and there might be tumors in which CSCs rely on alternative signal transduction cascades or that do not even follow a hierarchical organization [38, 44], as illustrated in CRC or breast cancer cell lines [45, 46].

The role of CSCs in regulating inter-tumor heterogeneity is more elusive, although subtype specific regulation of CSC traits has also been noted. Firstly, experimental mouse models of lung cancer with distinct genetic backgrounds were shown to critically impact on the identity of the CSC population [47]. Secondly, an elegant study by Caldas et al., reported that TGFβ potently induces a stem cell phenotype in basal (and claudin low) subtypes, but has a dramatically different and even an opposing role in other breast subtypes such as the luminal one (Figure 2E) [48]. Finally, besides xenotransplantation assays, analyses of phylogenic trees of tumor populations have also resolved that CSC fractions vary among cancers [49]. These subtype-specific features of CSCs might explain some long-lasting inconsistencies in the field such as the discrepancies associated with markers commonly used to identify CSC populations [38]. Regardless, these results point towards the CSC phenotype as an important contributor to heterogeneity across cancer subtypes. The implications of the CSC model for tumor growth are further illustrated by mathematical modeling of tumors that follow this model, which has revealed that hierarchical organization promotes the acquisition of novel genetic and epigenetic traits and thereafter, increased heterogeneity [50]. It should be noted however that differentiation hierarchy is not the only non-genetic explanation of tumor heterogeneity. In some instances, more stochastic processes seem to dominate the scene [45, 51].

The influence of the tumor microenvironment on heterogeneity. Environmental cues play a major role in tumor development and progression, but also determine the heterogeneity observed within and across tumors. The tumor microenvironment (TME) comprises a wide variety of non-neoplastic cells co-opted by tumor cells that influence the various steps of cancer development. The TME includes cancer-associated fibroblasts, endothelial cells and infiltrating immune cells with further subdivisions and promotes tumor heterogeneity in various ways. The mere presence of TME components is by itself a source of heterogeneity and these cell types can be distributed in different tumor microenvironments, such as the invasive versus non-invasive edge [52]. In addition, they can be a source of inter-tumor heterogeneity, being differentially represented among distinct tumors from a similar pathological type. Extrinsic factors produced
by stromal cells also affect tumor heterogeneity by mediating changes in clonal evolution rate or stem cell content. For example, chronic inflammation can lead to oxidative stress and increased reactive oxygen species production that are potent inducers of DNA damage [53]. Consequently, this may influence intra-tumor heterogeneity in tumor regions with localized inflammation, but also affect cancer subtypes that strongly associate with chronic inflammation.

In the context of CSCs, the TME regulates tumor hierarchy and stem cell traits. Normal stem cell homeostasis is ensured by the integration of signals that fine-tune the balance between self-renewal and lineage commitment. Part of these signals emanate from the microenvironment, commonly referred to as the stem cell niche [54]. An equivalent entity has been shown to possess similar albeit aberrant functions in cancer. For instance, tumor-associated myofibroblasts produce HGF that binds to c-MET and activates Wnt activity to support colon CSCs [43]. The Notch ligands DLL4 and more recently Jagged1 were shown to have similar effects [55, 56]. In glioma, endothelial cells co-localize to and are required for glioma stem cell maintenance [57]. Altogether, these and other studies [55] demonstrate the impact of the TME on the heterogeneity of tumors. Perhaps even more importantly, factors provided by tumor-associated cells not only maintain stem cell attributes but can also induce the phenotype in more-differentiated cells (Figure 2C). For instance, HGF was shown to induce differentiated colon cancer cells to revert to a CSC state [43] and similarly TGF-β triggers an EMT–CSC program in mammary epithelial cells [58]. This extrinsic control of tumor cell flexibility poses an obvious challenge in the design of therapies, especially those aimed at targeting the clonogenic core of tumors [59]. The composition of the TME not only plays an important role in regulating tumor cell properties, but can also be used as a defining peculiarity among different cancer subtypes. For example, activation of CD8+ T cells in specific tumor regions predicts tumor recurrence in colon cancer [52]. Furthermore, stromal-derived profiles from breast cancer have also been associated with cancer subtypes endorsed with worse outcome (Figure 2F) [60]. Interestingly, the impact of the TME might also be specific to certain tumor subtypes. Mammary tumor metastasis in the MMTV-PyMT breast cancer model crucially depends on the recruitment of CD4+ T cells [61] whereas MMTV-NeuT-derived tumors do not [62]. The recognition of the TME as a defining feature of cancer subtypes is crucial especially when differences in mutations or stem cell content cannot explain the apparent diversity sufficiently. For example, similar mutations might accumulate in a different sequential order, leading to a distinct requirement towards the environment. Alternatively, analogous mutations following the same sequence of events might give rise to divergent molecular subtypes depending on the inflammatory or environmental response.

The effect of the cell of origin on tumor heterogeneity. Cancer develops from a single founding cell, the identity of which is still highly debated [63, 64]. As such, the cell of origin will seldom be considered as a source of intra-tumor heterogeneity. In stark contrast, it can be a source for inter-tumor heterogeneity and mapping the cell of origin of cancer subtypes is intensely investigated. A striking example was recently reported in medulloblastomas, one of the most frequent pediatric brain tumors originally thought to arise predominantly in the cerebellum. At least two major subtypes have been described, one driven by aberrant Sonic Hedgehog signaling (SHH) while the other seems to depend on Wnt activation, and respectively have
Cancer Heterogeneity: a Multifaceted View

a dismal and good prognosis. By comparing the transcriptomes of these subtypes to those of distinct precursor cells, Gibson et al. revealed that tumors from the SHH subtype were closely associated with committed cerebellar granule neuron precursor cells and develop from the cerebellum [65]. In contrast, the Wnt-driven subtype associated with a gene expression profile of neural precursor cells located in the embryonic dorsal brainstem. Several additional examples have been described [66] and it is expected that many molecular distinct subtypes from various tumors will be quickly trailed back to their origin.

In summary, we have reviewed here two forms of tumor heterogeneity, namely intra- and inter-tumor heterogeneity, and discussed various sources that promote them (Figure 1). We have first described the genetic model that defines heterogeneity as a result of clonal selection and accumulation of genetic mutations in tumor cells and subsequently discussed an alternative model, the cancer stem cell concept that posits that heterogeneity is a consequence of differentiation gradients between the CSC and their differentiated progeny. Finally we have concluded this first part by presenting the role of the tumor microenvironment and the cell of origin.

Figure 2. Intra-tumor heterogeneity (A,B,C): A) From a common ancestor (blue founder cell), different subclones (represented by different colors) are emerging due to selection and distinct mutations. B) In the CSC model, clonal evolution still takes place but only acts on the CSCs. These cells can self-renew and give rise to all the various cell lineages present in a tumor (differentiated cells in respective colors). C) The TME affects intra-tumor heterogeneity by its composition (myofibroblasts in grey and immune cells in white) as well as their derived factors that can induce the reversion of differentiated cell to CSCs. Inter-tumor heterogeneity (D,E,F): D) The MSI or CIN colon cancer subtypes are distinct subtypes that associate with BRAF or APC and KRAS mutations, respectively. E) TGF-β pathway activation results in an increase or decrease of CSC phenotype when triggered in the basal or luminal breast cancer subtype respectively. F) SDPP (stroma derived prognostic profiles) can be used to identify breast cancer patients that differ in their survival probability.
PART II, THE IMPLICATIONS AND INTERCONNECTIONS OF BOTH FORMS OF CANCER HETEROGENEITY

Clinical implications. The mere presence of heterogeneity, regardless of the underlying foundation that promotes it, has a profound clinical impact. In the case of inter-tumor heterogeneity, the presence of oncogenic mutations is often used to guide treatment decisions. Two main situations can be described: On the one hand, the presence of a mutation could be directly predictive for a lack of response to a particular treatment. This is the case in metastatic CRC, where anti-EGFR therapy is relatively effective in KRAS wildtype cancer subtypes, but is not effective in KRAS mutant tumors [67]. On the other hand, certain tumors are “addicted” to oncogenic aberrations and targeting these mutations has proven clinically useful in those tumors. For instance, melanomas with a BRAF mutation are eligible and sensitive to treatment with the BRAF inhibitor vemurafenib as opposed to BRAF wildtype tumors [68]. As mentioned previously, mutation-based categorization incompletely recapitulates the complexity and diversity of cancer subtypes and sub-classification of tumors based on gene expression profiles potentially improves clinical decisions. In this respect, unbiased identification of CRCs based on gene expression recently revealed the presence of a resistant subset to anti-EGFR treatment, independent of KRAS mutation status [27]. Similarly, different pancreatic adenocarcinoma subtypes show differential response to anti-EGFR treatment despite the presence of KRAS mutations [69]. The use of genomic technologies has resulted in an extensive characterization of breast cancers into distinct molecular subtypes and has yielded a major clinical benefit for some of these subsets. For example, the use of tamoxifen [70] (an estrogen receptor antagonist) and trastuzumab [71] (an anti-HER2 monoclonal antibody) has critically improved the survival of patients belonging to the estrogen receptor or HER2 subtype, respectively.

Intra-tumor heterogeneity also impacts clinical outcome. In diagnostic terms, the choice of a therapeutic intervention is constrained by topological heterogeneity, as current sampling procedures do not yield a fully representative picture of a tumor [23]. Furthermore, diagnosis is generally derived from the primary tumor whereas treatment often aims at eradicating metastatic disease that presents with phenotypic attributes that progressed beyond that of the primary tumor. Finally, much of our knowledge on tumor heterogeneity is derived from ensemble measurements, which reflect changes present only in the majority of the cells. Although emerging, the genetic make-up of single cells within a tumor [4] remains a formidable challenge, but will result in a better interpretation of clonal genotypes. Question remains whether these diverse issues are rate-limiting steps that need to be resolved to improve clinical outcome for patients. In other words, is it essential to obtain multi-regional sampling in order to have a correct diagnosis and successful therapy? For instance, some approaches might be available to filter through tumor complexity. One of them relies on the use of xenotransplantation since xenografts of human breast primary tumors display an enrichment of mutations present in the metastatic lesion counterpart. This suggests that this method could be used as an extra step to enrich for the most aggressive clones [72], from which diagnosis and therapy design should be made. Furthermore, the clinical benefit of obtaining genetic information on single tumor cells
remains uncertain as it will only provide a complex architectural view of the clonal genotype without further revealing the dependence of tumor cells on specific growth factors or signaling pathways. Finally, the relevance of single cell-derived diagnosis is unclear, as data derived from these cells will not provide any direct information on the remnant tumor cell population and other more accessible methods can be used to infer clonal relations [5, 73].

Therapy response is also affected by heterogeneity as targeted drugs often suffer from their highly selective nature towards specific gene alterations. When a selective pressure, such as therapy, is applied, the fittest, i.e. the most resistant, subclones are invariably selected to survive and expand to eventually clinically manifest as a tumor relapse [5, 6]. Mutations can directly affect the target itself, as in the case of chronic myelogenous leukemia (CML) when treated with imatinib [74]. Alternatively, mutations can act synergistically to reactivate signaling pathways that are targeted. As mentioned above, relapses of metastatic CRC treated with anti-EGFR are frequently accompanied with mutations in KRAS, a downstream effector of EGFR signaling [67]. Alternatively, feedback loop mechanisms are often activated to confer resistance. One such example has been recently described in CRC where resistance to BRAF inhibition is bypassed by EGFR over-expression [75]. It is still debated whether these examples, or others reported [76], are due to the acquisition of novel mutations or affect the selection of pre-existing genetic variants. Furthermore, mutation-independent mechanisms [51] have also been reported. In CML, a variant of the ABL kinase, a crucial target of the drug imatinib, can be detected before the treatment in patients that will develop resistance to that drug [77]. Similarly, the emergence of KRAS mutant clones can be noticed months before radiographic progression in metastatic CRC patients treated with anti-EGFR therapy [78]. More recently, longitudinal assessment of genomic alterations before and after treatment in a panel of chronic lymphocytic leukemias has revealed that an adverse clinical outcome was related to the expansion of subclones that were already present prior to treatment [73].

Importantly, in the context of CSCs, therapy seems to select for already pre-existing more resistant tumor cells. It is generally assumed that these cells are relatively more resistant to a variety of treatments [59, 79]. Evidently this has a major clinical impact, as eradication of this subpopulation should be the priority when designing novel therapeutic interventions. For example, leukemic stem cells identified in CML are reportedly more resistant to the drug imatinib than their differentiated progeny. Similar experimental evidence comes from glioma where CSC marker expressing cells more efficiently repair DNA damage [80] or the ability of breast CSCs to maintain low levels of reactive oxygen species, which protects them against radiation [81]. More clinical examples have also been reported, as for instance in GISTs, where patients that showed complete disease remission as long as they were maintained under imatinib treatment, quickly recurred after imatinib withdrawal [82]. This suggests that a fraction of cancer cells remained untouched during the treatment and caused the relapse.

Estimates of CSC numbers might relate to metastatic potential and it is therefore not too surprising that many efforts have been made to better identify tumors that are at a higher risk of relapse, with the assumption that they would present with a higher CSC content. The identification of poor prognosis patients in breast [83], colon cancers [33, 84] and leukemia
Colon Cancer Heterogeneity: Stem cells, Signals and Subtypes

[85] based on their association with CSC signatures of various experimental design has indeed supported the relevance of CSCs in a clinical setting. Frequently, the underlying biological association of the tumor transcriptome to a CSC signature was insinuated to reflect the fraction of CSCs present in a tumor. Although these associations might exist in the case where malignancies have a high CSC content, it is unlikely to be a general feature, since most cancers are believed to harbor only a minute amount of CSCs. Consequently, several important conclusions can be drawn from the prognostic power of CSC signatures. First, CSC-associated expression profiles are clinically relevant since elements of stemness influence the clinical outcome of various cancers. Second, the association of CSC-derived profiles with prognosis should be interpreted with caution. This is well-illustrated in CRC where a mouse intestinal stem cell (ISC) derived signature was shown to strongly associate with patients at a high risk of relapses [84] suggesting that stem cell content could be of importance. However our data shows that the presence of ISC related genes in both pure ISC or colon CSC-derived gene signatures is not a key determinant of the prognostic power of these signatures [33]. Importantly, in that particular case ISC/CSC signatures point to a distinct, more immature and poorly differentiated subset, and thereby reflect a clonal trait of the malignant tissue, rather than CSC numbers.

**Connecting inter- and intra-tumor heterogeneity.** As described above, both forms of heterogeneity influence clinical outcome in many different ways. The mere presence of intra-tumoral heterogeneity impinges on adequate diagnosis and frequently results in therapy resistance. Moreover, the realization that distinct molecular subsets exist requires a shift in cancer drug development, from a ‘one-size fits all chemotherapy’ to a more personalized or group-based drug design or even directed towards specific tumor clones [86]. Unfortunately, our crude understanding of the molecular mechanism that drive intra-tumor heterogeneity coupled with uncertainty on the diversity of existing molecular subtypes may preclude the development of significant improved therapies, especially for more advanced, metastatic disease.

Can one find analogy between these two forms of heterogeneity that could be exploited in order to improve rational therapy design? In other words, could a better appreciation of the biological foundation of distinct subtypes be potentially used to infer the behavior of intra-tumor heterogeneity? A recent study of acquired resistance to EGFR inhibitors in lung cancer revealed that recurrent tumors in a fraction of patients undergo a subtype shift from non-small cell to small cell carcinoma [87]. The acquisition of the small cell lineage is marked by the acquisition of traits specific for this particular lineage, including sensitivity to conventional chemotherapy. Although the authors conclude that a lineage switch occurred by an unknown mechanism, it is equally feasible that robust subsets of lung cancer were pre-existing within these tumors and contracted or expanded during the therapy. Although this observation remains anecdotal, the presence of heterogeneous tumor cell population in a tumor could be a reflection of the diverse molecular subtypes that belong to a malignancy (Figure 3).
Conclusion and Outlook

The recognition that tumors are heterogeneous entities is a fact but the crucial question remains how to use that knowledge and turn it into a clinical benefit. For instance, heterogeneity in Barrett esophagus measured by several indexes initially developed for ecological studies, is an important predictor of progression to esophageal carcinoma and a similar study performed in breast tissue suggests that the degree of heterogeneity could be a general predictor for disease progression [88]. Whether the presence of more clones is crucial for progression or simply the reflection of genomic instable tumors in that case remains to be established. Ingeniously designed targeted therapeutic agents are tested in pre-clinical models at tremendous speed, but at a high cost as well. Tumor heterogeneity reflects the biggest challenge for drug development as single agents that target known aberrations, to which cancer cells may even be addicted (such as BCR-ABL in CML) may simply fail due to the presence of distinct non-targeted clones. Moreover, a better appreciation of inter-tumor heterogeneity and how this may affect tumor response is needed when drug responses are being evaluated. For example, drug x may be discarded because it does not produce clinical benefit in the overall population, although it might be very potent in a small subset of the population given that the latter is represented adequately. This is not straightforward...
since the identification of subtypes is generally achieved on primary tumor-derived material whereas phase II clinical trials are usually performed on late stage cancers that might not necessarily encompass the same diversity of subtypes than those present in early stages. Assuming the latter is true, how do we progress towards more effective treatments? One of the limiting steps there is the use the appropriate models to tackle these questions. Mouse models have been cleverly engineered to recapitulate crucial aspects of human tumor progression but, in particular, fail to reflect the heterogeneity that is present in human cancers both intra- and inter-tumorally. Human xenograft models are superior in that respect as they are directly derived from patients and can be maintained almost indefinitely in immune-compromised mice. The main criticism here rests on the lack of interaction with the microenvironment and the selection of tumor cells and clones that are more apt to survive in the mouse environment. Moreover, drug screening in this setting is cumbersome. A potential alternative is to fall back on established and well-characterized cancer cell lines. Studies have begun to elucidate drug response in large panels of cell lines [89], but these lack sufficient insight into the extent to which these represent the cancer population. Based on the unbiased identification of distinct subtypes of primary colon cancers, we have recently identified corresponding subtypes of colon cancer cell lines that share important biological attributes with primary cancers such as invasive properties and therapy resistance [27]. Research in this area will be facilitated by a wealth of data such as genotype, drug response and expression profiles that are available for a wide range of cell lines from various lineages. Although clearly imperfect, these simple in vitro models might be the first critical step to understand the different biological grounds that are hard-wired into the distinct cancer entities.

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