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Clonal cooperativity in heterogenous cancers

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Abstract

Tumor heterogeneity is a major obstacle to the development of effective therapies and is thus an important focus of cancer research. Genetic and epigenetic alterations, as well as altered tumor microenvironments, result in tumors made up of diverse subclones with different genetic and phenotypic characteristics. Intratumor heterogeneity enables competition, but also supports clonal cooperation via cell-cell contact or secretion of factors, resulting in enhanced tumor progression. Here, we summarize recent findings related to interclonal interactions within a tumor and the therapeutic implications of such interactions, with an emphasis on how different subclones collaborate with each other to promote proliferation, metastasis and therapy-resistance. Furthermore, we propose that disruption of clonal cooperation by targeting key factors (such as Wnt and Hedgehog, amongst others) can be an alternative approach to improving clinical outcomes.

Introduction

The fact that tumors are non-uniform has been well accepted for decades, as cells with distinct morphologies and/or aneuploidy within a tumor have long been described by pathologists¹. More recently, advances in next-generation sequencing and digital pathology have provided abundant evidence for intratumor heterogeneity, which is observed not only by distinct cellular morphologies, but also by diverse mutation and expression profiles, metabolic profiles, and invasive potentials^{2,3}. Such heterogeneity is thought to play a crucial role in tumor growth, resistance, recurrence and metastasis^{3–7}.

Two well accepted models that explain the rise of tumor heterogeneity are the clonal evolution model^{8,9} and the cancer stem cell model^{10–12}. These two models are not mutually exclusive. Moreover, both models appreciate the impact of the tumor microenvironment

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(TME) on tumor cell fate^{13–15}. After all, only cells that have a competitive advantage (high cellular fitness) are selected within a given environment.

The presence of diverse genetic and phenotypic subclones within a tumor provides a substrate for Darwinian-like evolution, potentiating clonal cooperation and clonal competition. As a result, “unsuccessful” subclones can be outcompeted, whereas subclones possessing the greatest fitness will survive, accelerating the process of tumor progression^{9,16,17}. Despite the important role of clonal competition in shaping tumor composition, clonal cooperation can also substantially impact the cancer evolutionary trajectory^{18,19}, particularly in the context of metastasis and therapy resistance^{20–25}. Therefore, disruption of clonal cooperation may be critical to improve clinical outcomes.

In this review we discuss recent discoveries that have led to a more comprehensive landscape of the tumor environment, encompassing heterogeneous subclones and microenvironmental niches, with a focus on how clonal cooperation promotes tumor progression. Such knowledge should provide insight into developing therapies that target reciprocal interactions that occur amongst different tumor subpopulations.

The rise of intratumor heterogeneity

Genetic heterogeneity

Tumor cells often possess high genomic instability²⁶. Such instability results from dysfunction of pathways such as base and nucleotide excision repair, mismatch repair, double-strand break repair, DNA replication, telomere maintenance and chromosome segregation; allowing for rapid acquisition of genetic alterations^{5,27}.

The classic view of tumor evolution is that tumor initiating cells acquire driver mutations which endow them with a fitness advantage in their given microenvironment^{28–30}, after which various passenger mutations are sequentially accumulated as the tumor progresses^{5,8,9,27}. One theory as to why tumors accumulate passenger mutations proposes that in expanding tumor populations, the effects of genetic drift (random loss or fixation of genotypes) may be magnified, thus neutral and even deleterious mutants can be preserved during expansion, resulting in genetic diversity¹⁶. Although individual passenger mutations can be neutral or even slightly deleterious, combinatorial effects of multiple passenger mutations could confer a selective fitness advantage (through epistatic interactions)¹⁸. Eventually, different subclones likely undergo branched evolution rather than linear evolution^{2,9,16,27}, allowing tumor cells to follow diverse evolutionary paths. Branched evolution is a more likely path because selective sweep (a series of clonal expansions that dominate the whole neoplasm^{31,32}) can only occur if one subclone can sweep through the neoplasm before the next driver mutation emerges, which is unlikely given the high mutation rate of tumor cells⁹. Indeed, acute leukemias³³, breast carcinomas³⁴, colon carcinomas and adenomas³⁵, clear-cell renal carcinomas², pancreatic carcinomas³⁶ and prostate cancers³⁷ have all been reported to follow branched evolutionary trajectories.

The aforementioned “gradual” model of genetic alteration acquisition is challenged by catastrophic events such as chromosome rearrangements³⁸. Multiple studies suggest that

genomic alterations can be achieved not only by incremental steps but also in a single leap. For example, chromothripsis is a process in which hundreds of chromosomal rearrangements can take place across only a few chromosomes³⁹. Similarly, transient telomere dysfunction itself can result in drastic chromosomal instability allowing catastrophic genomic alterations to occur⁴⁰. Moreover, genome doubling, a potential intermediate phase to aneuploidy⁴¹, is yet another type of dramatic genomic change⁴². Intriguingly, although many cancer cells will not survive these types of catastrophic events, such events can rapidly lead to high genetic diversity, supplying large amounts of substrates for evolution to act upon.

Importantly, genomic alterations conferred by either gradual or catastrophic events are heritable. Thus, tumors become increasingly genetically heterogeneous as they progress.

Non-genetic heterogeneity

There is a high degree of phenotypic plasticity in tumor cells sharing the same genotype; governed by non-genetic mechanisms. Two types of non-genetic heterogeneity have been described: deterministic and stochastic⁶. In deterministic heterogeneity, cues from the microenvironment impinge on the tumor cell expression profile, thereby defining phenotypes. According to the cancer stem cell model, which is deterministic, progeny cells that originate from cancer stem cells can adjust their differentiation status based on the given tumor microenvironment^{10,43}. For instance, clonally related CD24+ and CD44+ breast cancer cells can exhibit distinct properties, where CD44+ cells with active TGF- β signaling express high levels of stem-cell markers and are associated with decreased patient survival, while CD24+ cells are much less aggressive⁴⁴. Nonetheless, even in the deterministic model, cellular phenotypes are dynamic.

In contrast, stochastic heterogeneity is a result of random fluctuation within the cells⁴⁵. Moreover, random “noise” within the cell, when surpassing a certain threshold, can change genetic circuits and epigenetic landscapes, and can even facilitate evolutionary transitions^{6,46,47}. For example, biochemical processes within cells often involve low-frequency events. Therefore, stochastic fluctuations can occur in the cell. Importantly, these fluctuations affect cellular processes such as signal transduction, gene expression and cell fate decision. For instance, in the early mouse embryo, both epiblast and primitive endoderm cells are derived from the inner cell mass. This lineage segregation is determined by random “salt and pepper” (mutually exclusive) expression patterns of Nanog and Gata6 in the inner cell mass. The epiblast cells are derived from the Nanog positive inner cell mass, whereas the primitive endoderm cells originate from the Gata6 positive inner cell mass^{48,49}. Another intriguing example is that sister cells exhibit different responsiveness to TRAIL-mediated apoptosis, and this cell-to-cell variability is caused by endogenous variation in the levels of apoptotic regulators (BID, BAX, BCL2, XIAP and caspase 3)⁵⁰. Thus, noise can result in phenotypic spectrums of tumor cells with given genotypes, thereby facilitating tumor evolution.

Although non-genetic heterogeneity does not involve genetic alterations, it can bestow upon cells a transient and/or stable advantageous phenotype. If such cells can maintain their phenotypes over several cell divisions, it is likely that new genetic mutations will

emerge^{11,51}. Thus, non-genetic heterogeneity can be temporarily heritable and provide substrates for selection as well.

Microenvironment and intratumor heterogeneity

The microenvironment, regarded as dynamic and heterogeneous, can serve as a double edged sword during tumor development, as it can shift from anti to tumor promotional as tumors progress^{15,52}. Three major sources of microenvironmental heterogeneity include the extracellular matrix (ECM), the vascular network and the immune cell infiltrate⁵³. Varying microhabitats confer diverse commanding signals and selective forces on tumor cells, contributing to intratumor heterogeneity⁵⁴. For example, high ECM stiffness releases Twist1 from its cytoplasmic binding partner G3BP2 [GTPase activating protein (SH3 domain) binding protein 2]^{55,56}, leading to Twist1 nuclear translocation, which results in epithelial-mesenchymal transition (EMT). Such an induced EMT then drives tumor invasion and metastasis⁵⁷. In addition, aberrant ECM remodeling can stimulate tumor growth, CSCs phenotypes, and has been experimentally shown to play a significant role in the progression of tumors (review⁵⁵). Insufficient vasculature can lead to hypoxia in tumors, which is associated with alteration of multiple signaling pathways and the induction of aggressive phenotypes⁵⁸, such as abnormal angiogenesis (e.g. VEGF and Ang2), inflammation (e.g. IL-6), CSC phenotypes (e.g. β -catenin and Oct4), EMT (e.g. Snail), secretion of growth factors (e.g. IGF1 and TGF- α) and resistance to radiotherapy, chemotherapy and immunotherapy (e.g. entering quiescence) (review⁵⁷). Finally, infiltration of immune cells into tumors, which was described by Rudolf Virchow in the 19th century, is now known to lead to considerable crosstalk between these cells and tumor cells. Ironically, immune cells can be altered by tumor cells as tumors progress, changing them from suppressive to promotional, and thus tumor infiltrating immune cells play crucial roles in eliminating or fostering tumor cells at different stages of tumor development^{59,60}. For example, macrophage phenotypes largely depend on microenvironmental cues, and thus, distinct macrophages can be educated by tumor cells to serve different roles in tumor progression. In early stage tumors, macrophages can stimulate tumor growth by generating an inflammatory niche and by inducing angiogenesis to supply nutrients for tumor cells. Then, as tumors progress, subpopulations of macrophages may facilitate primary tumor cell dissemination by enhancing their migration and invasion, while different subpopulations of macrophages can prepare the pre-metastatic niche in secondary sites to allow tumor cells to colonize (review^{58,59}).

Notably, in addition to effects of the microenvironment on tumor cells, spatial constraints restrict competition to the immediately neighboring subpopulations. Thus, selective sweep may be unachievable even by highly fit subpopulations, contributing to the generation and maintenance of intratumor heterogeneity⁶¹. Therefore, creating a relatively homogeneous and steady tumor microenvironment in early stage tumors may be an alternative approach to constrain tumor heterogeneity and decelerate tumor evolution.

Clonal cooperation and tumor malignancy

Given high intratumor heterogeneity, the tumor mass is thought to function in a similar manner as an evolving ecosystem involving intense interactions among various cellular components^{62–64}. Even though most interactions among subclones are neutral, detectable interactions should be positive or negative, resulting in a phenotypic switch⁶⁵. Recently, a multi-agent computational model postulated that cooperative cells tend to be spatially segregated from exploitive cells, and that the cooperators can increase their abundance faster than defectors, potentiating the maintenance of this collaborative interaction^{18,66}. Below we outline how positive subclonal interactions promote tumor growth, metastasis and therapeutic resistance via cell-cell physical communication, paracrine effects or remodeling of the tumor microenvironment.

Clonal cooperation induces tumor growth

Cell proliferation is regulated by intrinsic signaling, nutrient availability, growth factors, ECM composition and many other factors. Cooperation between tumor subpopulations can significantly contribute to tumor growth via increasing growth factor abundance and activating pro-proliferative signaling pathways. For example, in *Drosophila*, Ras activation, in cooperation with mitochondrial defects in imaginal epithelium, cooperatively induced the production of reactive oxygen species (ROS), leading to the activation of JNK signaling and cellular senescence. The cellular senescence that arose exhibited a senescence-associated secretory phenotype, which includes secretion of interleukins, chemokines, growth factors, proteases and ECM components⁶⁷). Activated JNK coupled with oncogenic Ras could then inactivate the hippo pathway in senescent cells, stimulating secretion of Unpaired (an Interleukin-6 homologue) and Wingless (a Wnt homologue). Eventually, these secreted factors could cooperate with Ras signaling in neighboring cells (which have normal mitochondrial function), resulting in overgrowth of neighboring tissue^{68,69}.

Interclonal cooperation has also been extensively described in mouse models. For instance, in glioblastoma multiforme (GBM), amplification of wildtype (wt) EGFR and EGFR mutations (not amplified) are frequently observed in a heterogeneous manner. Using human glioma cells (U87) and immortalized mouse astrocytes (mAstr-*Ink4a/Arf*^{-/-}), it was demonstrated that glioma cells with mutant EGFR express IL-6 and/or LIF cytokines, thus activating amplified wt EGFR in neighboring cells and resulting in heightened proliferation⁷⁰. Similarly, in a breast cancer context, aberrant expression of Wnt1 can generate mammary tumors consisting of both basal Hras^{mut} Wnt1^{low} and luminal Hras^{wt} Wnt1^{high} cell subtypes. Importantly, both subclones were shown to be necessary for full tumor expansion, as the basal cells relied on Wnt1 secreted by luminal subclones for growth²⁵.

Intriguingly, apoptosis can also induce proliferation in neighboring cells. “Cell competition” has been intensively studied in *Drosophila*: a process in which apoptosis of cells with low fitness (loser) is induced by neighboring cells with high fitness (winner). This mechanism is thought to be a surveillance mechanism to maintain tissue homeostasis by removing mutated cells with reduced fitness⁷¹. Moreover, cell competition is not limited to flies, as it has been observed in the early mouse embryo. During normal development, Myc is heterogeneously

expressed in epiblasts, and epiblast cells with high Myc levels eliminate the ones with lower Myc levels to refine the epiblast cell population⁷². However, increasing evidence has shown that cell competition can also be utilized by tumor cells to promote tumor progression^{23,73,74}. For instance, in *Drosophila*, growth of Rab5 mutant tumors is dependent on the cell-competition-induced apoptosis inside the tumor. In this case, competition between cells acts in a tumor promotional manner by up-regulating JNK and Wg (a Wnt homologue) signaling in the viable tumor cells, thereby enhancing their proliferation⁷⁴. Similarly, dying “oncogenic niche cells” (ONCs, cells which promote tumor progression non-cell autonomously via interaction with surrounding cells) have been shown to stimulate growth in neighboring tissues²³. Cell death is often induced (potentially by cell competition) in ONCs, however, because ONCs express high levels of caspase inhibitors, cell death can’t be executed in these cells. Eventually, ONCs go on to exhibit SASP, which stimulates neighboring cell growth via Dpp (a BMP/TGF β homologue) and Wg signaling^{23,75,76}. This phenomena has also been observed in human patients, where apoptotic tumor cells can activate a “phoenix rising” pathway to stimulate wound healing and tissue regeneration⁷⁷. Specifically, activated caspase 3/7 in apoptotic tumor cells caused downstream prostaglandin (PGE2) secretion, promoting growth of neighboring tumor cells^{77,78}.

Using mathematical models, it has been proposed that non-cell-autonomous-induced tumor growth (such as that described above), combined with clonal interference, can stabilize intratumor heterogeneity thereby maintaining interclonal interactions¹⁹. This stabilization occurs because a positive growth effect on subclones can confer a fitness advantage to them, and because multiple subclones with high fitness will then interfere with each other, inhibiting expansion of individual subclones. Despite the advantages conferred by cooperation between subpopulations, clonal cooperation can also lead to tumor collapse if the driver subclone, that stimulates other subclones to grow non-cell autonomously, gets outcompeted by fast-growing exploitive subclones^{19,79}. This may lead to tumor collapse because it leads to homogeneity within the tumor, and thus may result in the tumor being incapable of responding to adverse conditions such as hypoxia if “incompetent communicators” become dominant⁸⁰.

Clonal cooperation induces metastasis

It has long been acknowledged that subpopulations that originate from the same tumor can possess different metastatic potentials^{81,82}. Moreover, a positive correlation between tumor heterogeneity and metastasis has been established^{53,83}. Despite the clear importance of diverse intrinsic characteristics of different subpopulations, collaborative interactions present among subpopulations can additionally facilitate the metastatic cascade. Subpopulations may gain a selective advantage during the metastatic process, if inter-clonal cooperation is present⁸⁴.

Decades ago, several studies demonstrated that metastatic subclones can enhance the metastatic potential of non-metastatic subclones^{82,85–87}. O’Grady and colleagues found that the rat mammary carcinoma cell line, B1, consists of two stable subtypes which were characterized as epithelioid cells (E-cells) and myoepithelioid (M-cells) cells. Interestingly, collagenase could only be sufficiently secreted when both cellular types were present. In

brief, a soluble factor released by M-cells was able to induce collagenase secretion by E-cells, indicating that inter-clonal cooperation may promote at least local invasiveness⁸⁷. Another study conducted by Miller and colleagues directly demonstrated that, in a syngeneic mouse mammary tumor model, presence of a metastatic subpopulation (410.4 cells) in the circulation was able to enhance the metastatic potential of non-metastatic subpopulations located in either subcutaneous sites (168 cells) or in circulation (67 cells)⁸². Thus, both studies demonstrated clear cooperativity between metastatic and non-metastatic cells, although neither elucidated the molecular mechanisms behind the clonal-cooperation-induced metastasis.

Several recent studies have revisited how clonal cooperation mechanistically influences metastasis. Clonal cooperation may facilitate metastasis in different ways; either by causing a phenotypic switch (active escape) or through microenvironment remodeling (passive escape). Importantly, these two mechanisms are not mutually exclusive. As an example of phenotypic switch, poorly metastatic F1 melanoma cells could uptake Met 72 tumor antigen containing exosomes, released by the BL6–10 highly metastatic melanoma cells, thereby increasing their own expression of Met 72 tumor antigen and their metastatic ability⁸⁸. Similarly, Lieberman and colleagues presented that, in the context of breast cancer, murine (4T1E) and human (BPLER) breast cancer cell lines could transfer their metastatic potential via secretion of miR-200-containing extracellular vesicles, leading to mesenchymal-to-epithelial transition in non-metastatic cells (4TO7 cells and MB-231 cells), which increases their ability to colonize secondary sites. Strikingly, these extracellular vesicles were not only found in the primary site, but they could penetrate basement membrane and enter the circulation, thus influencing tumor cells at a distance⁸⁹. Phenotypic switch can also be simply induced by paracrine factors. For example, using the PC-3 prostate cancer cell model it was demonstrated that non-CSC subpopulations (PC-3S) could secrete the matricellular protein SPARC, which then induced the invasiveness of a CSC-enriched subpopulation (PC-3M), leading to heightened metastasis in lungs⁹⁰. (Fig. 1 active/passive models)

As outlined above, a second means through which clonal cooperativity may mediate metastasis is via microenvironment remodeling. The process of epithelial to mesenchymal transition (EMT) is a well-described mediator of metastasis, and has been shown to increase invasiveness of tumor cells that also have enhanced ECM remodeling ability. Thus, studies focusing on how EMT cells and non-EMT cells interact during metastasis may provide intriguing mechanistic insights. For example, Hu and colleagues conducted an elegant study using a hamster cheek pouch carcinoma model in which the authors demonstrated that mice inoculated subcutaneously with EMT (expressing high p12^{CDK2-AP1}) cells had these cells in the bloodstream, but metastases did not form. In contrast, when they subcutaneously injected the non-EMT cells, these cells could not be found in the bloodstream, nor were they able to form metastases at secondary sites. However, the non-EMT cells could form lung metastasis by intravenous inoculation, whereas the EMT cells could not. Importantly, subcutaneous co-injection of EMT and non-EMT cells led to the presence of both cell types in the circulation, and to the presence of overt metastases that were composed entirely of the non-EMT cells. These data suggest that EMT cells are likely responsible for degrading the ECM and breaching the basement membrane, allowing the non-EMT cells to intravasate⁹¹. Once these

cells have made it into the bloodstream, they are then capable of extravasating and colonizing the secondary site.

Interestingly, a similar phenomenon was uncovered in a zebrafish melanoma xenograft model, where inherently invasive cells exhibiting high protease activity were able to deposit ECM leading to co-invasion of poorly invasive cells. The poorly invasive cells did not undergo a phenotype switch, but instead, the inherently invasive cells switched their invasion pattern from protease-independent to MT1-MMP-dependent, mediating ECM degradation and aiding the invasion of poorly invasive cells. Thus, the subpopulation with low invasiveness passively benefited from the microenvironmental remodeling ability of the highly invasive subpopulation⁹².

One can imagine that much of the time clonal-cooperation-induced metastasis is a combination of both active and passive mechanisms. Indeed, using a PC-3 prostate cancer model, Thomson and colleagues demonstrated that microenvironmental cues can induce some tumor initiating cells (TICs) to undergo a permanent EMT, thus losing their CSCs properties. These first generation “mesenchymalized” cells can then non-cell autonomously induce other TICs to undergo permanent or transient EMT, resulting in increased invasiveness of the tumor entity on a more global scale, ultimately leading to the breach of local barriers. Such a breach of local barriers then allows not only the EMT cells (active escape) but also the non-EMT cells (passive escape) to leave the primary site. Eventually, non-EMT and transient EMT cells will then colonize secondary sites, since they retain CSCs properties. In short, some metastatic cells actively participate in enhancing tumor invasiveness under the stimulation of “mesenchymalized” cells by undergoing transient EMT, while others can passively follow the paved path to disseminate⁹³.

Collectively, tumor subpopulations are able to initiate their cooperation within the primary site, leading to enhanced invasiveness of the whole tumor mass via active and/or passive mechanisms. Hence, disruption of clonal cooperation present in primary tumors may be an effective approach to suppress metastasis.

Clonal cooperation induces therapeutic resistance

In addition to influencing metastasis, an association between intratumor heterogeneity and therapeutic resistance, relapse and poor prognosis has been established across cancers, as a result of both genome plasticity and cooperation among subclones^{94–97}.

Recently, several studies have demonstrated that cancer cells promote therapy resistance and relapse at least in part through clonal cooperation^{20,78,98}. For instance, it was demonstrated that cetuximab-resistant, KRAS^{mut} colorectal cancer cells were not only resistant to EGFR blockade, but were also able to increase resistance of surrounding KRAS^{wt} sensitive cells in a paracrine manner, via their ability to secrete TGF α and amphiregulin⁹⁸. In addition to influencing neighboring cells via secretion of key factors, tumor cells can boost their resistance through direct cell-cell interactions. A recent study by Winkler and colleagues demonstrated that astrocytoma cells could interconnect by extending ultra-long protrusions, referred to as microtubes. Importantly, Connexin 43 was critical for the formation and function of these microtubes. These microtubes allowed for multicellular communication

through gap junctions and were critical for invasion and proliferation in the brain. Furthermore, these microtubes could be used for repair once this multicellular network was damaged. After exposing astrocytoma cells to a fatal laser dose, new microtubes were extended to the dead cells if the ablated cell was a member of the interconnected network, and a new-formed nucleus migrated to the original location of the ablated cell through the microtubes. Therefore, interconnected astrocytoma cells exhibit much higher radiotherapy resistance than unconnected ones²⁰. In addition, many other cooperative mechanisms may contribute to therapy resistance. For example, one can imagine that subclones expressing high levels of immune-inhibitory molecules (e.g. PD-L1) may facilitate other subclones to escape from immune therapies such as chimeric antigen receptor (CAR) T-cell therapy, by creating an immune-suppressive TME^{99–102}.

Altogether, cooperation occurring among subpopulations can profoundly influence sensitivity of tumor cells to therapeutics via secreted factors or direct cell-cell interactions. Thus, reducing intratumor heterogeneity or disrupting existing clonal cooperation may be critical to overcoming resistance and postponing relapse.

Therapeutic implications of intratumor heterogeneity

As outlined above, intratumor heterogeneity positively correlates with shorter time to relapse and increased multidrug resistance in different types of cancers. Decades ago, Dexter and colleagues had already identified a correlation between intraneoplastic diversity and therapy failure¹⁰³, which has since been supported in numerous studies^{104–106}. For example, it was reported that mutant-allele tumor heterogeneity (MATH), which measures clonal diversity by quantifying mutant-allele fractions in tumors, is associated with adverse outcomes in head and neck squamous cell carcinoma (HNSCC)¹⁰⁶. Similarly, in chronic lymphocytic leukemia, Landau and colleagues showed that the presence of subclonal driver mutations was not only an independent risk factor for cancer progression but also an indicator of relapse¹⁰⁴. However, mechanistic underpinnings of how heterogeneity drives therapy resistance have only more recently been uncovered.

Poor penetrance of therapeutic agents can be the first obstacle to achieving a high therapeutic index^{53,107}. As a result of heterogeneous distribution of drugs, as well as leakiness of abnormal vascular networks¹⁰⁸, tumor cells will be exposed to differing concentrations of therapeutic agents¹⁰⁹. Therefore, individual tumor cells will adapt to different drug concentrations by utilizing diverse mechanisms, in part dependent on the level of drug to which they are exposed. Such mechanisms include increasing drug efflux¹¹⁰, suppressing apoptotic signaling¹¹¹, entering quiescence¹¹², activating alternative signaling pathways¹¹³ and collaborating with the tumor microenvironment¹¹⁴. Nonetheless, many tumor cells are unable to survive a given therapeutic insult. Thus, it is of interest to explore whether tumor resistance is predominantly driven by expansion of pre-existing resistant subclones, or by de novo alterations of surviving cells.

Vogelstein and colleagues recently demonstrated that after EGFR blockade treatment (panitumumab), circulating KRAS^{mut} DNA was detectable in the sera of 38% patients bearing KRAS^{wt} colorectal tumors. Given their mathematical modeling, the authors suggest

that those mutations must have been present prior to the panitumumab treatment, indicating that acquired resistance can arise from undetectable pre-existing subclones¹¹⁵. Similarly, it has been demonstrated that MET amplification, which contributes to resistance to EGFR inhibition, is present in non-small cell lung cancer (NSCLC) subpopulations before EGFR inhibitor treatment¹¹⁶. Recently, an impressive study by Stegmeier and colleagues, in which a high-complexity barcode library was used to perform high-resolution tracking of tumor cells [both in NSCLC and chronic myeloid leukemia (CML)], clearly demonstrated that the majority of resistant subclones originated from rare pre-existing subpopulations, which gained a fitness advantage upon treatment challenge (EGFR inhibitor and c-Met inhibitor for NSCLC; ABL inhibitor for CML). This study not only validated that acquired resistance can be derived from rare pre-existing resistant subclones, but also suggested that expansion of pre-existing resistant subclones outweighs de novo alterations in mediating tumor resistance¹¹⁷. Moreover, Engelman and colleagues further dissected which evolutionary path(s) NSCLC cells can follow to generate their resistance to EGFR inhibition, and suggested that two routes can lead to a 2nd generation EGFR inhibitor gatekeeper mutation (EGFR^{T790M}) in the tumor mass. One route is through the pre-existence of a small number of EGFR^{T790M} positive cells, which can drive the growth of early-resistant subclones by selection. The second route is through rare apoptosis-suppressive EGFR^{T790M} negative cells which can also tolerate EGFR inhibition, and then slowly acquire the EGFR^{T790M} mutation through evolution, giving rise to late-resistant subclones¹¹⁸. Interestingly, the late-resistant subclones are also resistant to 3rd generation EGFR inhibitors, which target the EGFR^{T790M} mutation, due to their ability to suppress apoptosis. This study provides temporal clues of how different resistant subclones arise during resistance development, and further confirms the probability that pre-existing resistant subclones will dominate resistant tumors after treatments. Taken together, a simplified tumor resistance development model (after initial treatment) can be proposed: upon initial treatment challenge, both intrinsic resistant subclones and death-tolerant subclones will survive and get released from clonal interference from drug-sensitive subclones. Then, intrinsic resistant subclones will expand rapidly and form early-resistant populations, taking up a large portion of the tumor, while death-tolerant subclones can gain resistance through de novo alterations and form the late-resistant populations, taking up a small proportion of the tumor. (Fig. 2)

Most therapies induce massive cytotoxic effects, which function as double-edged swords. Even though cytotoxic effects can contribute to eliminating tumor cells, they can also cause a series of adverse responses such as local genomic instability^{119,120}, SASP in surviving cells^{67,121,122}, chronic inflammation^{123,124}, heightened regional hypoxia¹²⁵, wound healing responses¹²⁶, accelerated tumor evolution¹²⁷ and destruction of tissue homeostasis. Noteworthy, SASP, inflammation, hypoxia and wound healing responses can all facilitate the emergence of cells with CSC properties^{128–132}, which often possess high therapy resistance. Furthermore, inflammation, combined with mutagens released by dead cells, can further promote genomic instability of surviving cells^{119,120,133,134}, contributing to intratumor heterogeneity. Additionally, cytotoxic effects induce intense selection pressures which rapidly drive tumor evolution, thus potentiating the appearance of resistant subclones¹²⁷. Inflammation plays a critical role in these intertwined adverse responses, rendering anti-inflammatory agents a tempting adjuvant treatment to diminish tumor resistance and

intratumor heterogeneity. Alternatively, due to aforementioned disadvantages of cytotoxic effects, cytostatic agents may provide better clinical outcome for certain patients¹³⁵, based on the idea of trying to contain cancer as a chronic disease.

Clinical strategies to overcome intratumor heterogeneity

Characterization of intratumor heterogeneity

Accurate characterization of tumor heterogeneity will be required if we are to optimize therapy regimes. With the substantial progress that has been made in non-invasive imaging and high throughput sequencing, which can be integrated with an increasingly sophisticated computational workflow, it is becoming more and more feasible to characterize genetic and phenotypic heterogeneity of specific tumors, even before treatment begins^{136–141}. For example, Lambin and colleagues have shown that, by performing radiomic analysis on computed tomography (CT) data, one can quantify tumor shape, as well as intensity and texture, and these radiomics features can be compared with clinical data and gene-expression patterns to allow for prognostic radiomic signatures to be obtained¹⁴⁰. Intriguingly, Rosenfeld and colleagues successfully tracked genomic evolution of advanced breast, ovarian and lung cancers in response to therapy using serial sequencing of plasma DNA. The authors detected increased mutant alleles in plasma with the emergence of therapy resistance, indicating genomic alteration in solid tumors can be measured by sequencing circulating DNA released from tumor cells¹³⁷. Thus, advanced non-invasive analyses may enable researchers to overcome biased sampling issues that were previously an issue with conventional sequencing approaches, thus gaining a more quantitative view of intratumor heterogeneity. Moreover, accurate measures of intratumor heterogeneity can help estimate the risk of mortality across cancers¹⁴².

Targeting clonal cooperation

Although ablation of dominant tumor subclones using targeted therapy has improved clinical outcome substantially¹⁴³, taking clonal cooperation into consideration may be an effective therapeutic avenue, particularly when targets identified may affect multiple aggressive phenotypes. Wnt1, matrix metalloproteinases (MMPs), miR-200, TGF α/β , amphiregulin, PGE2 and deregulated Hedgehog (Hh) signaling^{144–146} could all promote tumor progression non-cell autonomously, making them potential candidates for disruption of clonal cooperation. In addition, inhibition of secreted factors may suppress multiple exploitive subclones, reducing evolved drug resistance. Such effects have been reported in modeling of drugs targeting pathogens¹⁴⁷. Below we outline recent progress in Wnt and Hh signaling inhibition, and some obstacles to targeting factors such as MMPs and miR-200.

Wnt inhibition—Wnt signaling may be an attractive pathway to target to inhibit clonal cooperativity. The canonical pathway is activated in response to Wnt secretion, which occurs following extensive post-translational modification of the Wnt proteins. Secreted Wnts then bind to Frizzled receptors on recipient cells, resulting in a signaling cascade that ultimately releases β catenin from degradation and enhances nuclear entry of β catenin, leading to TCF-LEF/ β -catenin mediated transcription¹⁴⁸. In addition to the critical non-cell autonomous role of Wnt1 in supporting multiple subpopulations in tumors, Wnt signaling has been reported

to be associated with tumor growth¹⁴⁹, migration, invasion¹⁵⁰ and CSC phenotypes¹⁵¹ in a cell-autonomous manner. Therefore, inhibition of Wnt signaling may limit tumor progression from multiple aspects. However, targeting essential developmental signaling pathways is challenging, because somatic stem cells often utilize the above pathways for tissue repair and homeostasis of the stem cell niche¹⁴⁸. In particular, targeting Wnt signaling would be anticipated to have significant adverse effects in the gut, where this pathway is critical for stem cell maintenance^{148,152,153}. Because a precise balance has to be achieved between the healing and harmful effects of Wnt pathway inhibition, to date, no inhibitors targeting the Wnt pathway have been approved^{148,152,154}. However, exciting new research from Virshup and colleagues has shown that pharmacological inhibition of Porcupine, which is a membrane-bound O-acyltransferase in charge of palmitoylation of all Wnts to enable their normal secretion and activity, has promising efficacy in MMTV-WNT1 mouse mammary tumors, without apparent toxicities^{155,156}. Indeed, these inhibitors are currently in clinical trials, and are a promising approach to inhibiting Wnt signaling perhaps without associated side effects¹⁴⁸.

Hedgehog inhibition—Like Wnt signaling, Hedgehog (Hh) signaling is another intriguing pathway to target because of its cell-autonomous and non cell-autonomous role in tumor progression. In brief, the Hh pathway is activated when Hh ligands (Sonic Hedgehog, Indian Hedgehog and Desert Hedgehog) bind to their receptors, Patched 1 or 2 (Ptch1/2), releasing Smoothened (Smo) from Ptch suppression, and allowing Smo to activate Gli transcription factors. Heightened Hh signaling is known to stimulate growth in a variety of cancers including basal cell carcinomas (BCCs), medulloblastomas, pancreatic, breast and prostate cancers¹⁴⁶. In addition, cells carrying deregulated Hh activity were demonstrated to induce overgrowth and resistance to apoptosis in wildtype neighboring cells^{144,145}. Interestingly, Tuveson and colleagues have demonstrated that inhibition of Hh signaling using the Smo inhibitor, IPI-926, led to an increase in gemcitabine delivery (as the inhibitor increased angiogenesis) in pancreatic ductal adenocarcinoma (PDA) using a KPC (K-Ras^{+/LSLG12D}; p53^{R172H/+}) mouse model¹⁵⁷.

Currently, vismodegib, the first FDA-approved Smo inhibitor, has shown promising effects in tumors where Hh signaling is altered via mutations, such as basal cell carcinoma¹⁵⁸. In a BCC study, vismodegib reduced both the size of existing BCCs and per-patient rate of new surgically eligible BCCs. Moreover, all BCCs regressed in some patients¹⁵⁹. However, acquired resistance rapidly develops after Smo inhibitor treatment, through the development of secondary mutations which disrupt binding of the drug to Smo¹⁶⁰, or activation of Gli through compensatory pathways (e.g. mTOR) bypassing Smo¹⁶¹. Furthermore, it was reported that the inflammatory cytokine, osteopontin (OPN), activates Gli in a non-canonical manner (bypassing Smo), resulting in EMT and enhanced drug resistance¹⁶². Therefore, direct targeting of the downstream Gli transcription factors using small molecules such as GANT58 and GANT61 may be a better approach to suppress Hh signaling¹⁶³.

Limitations of MMP and miR-200 inhibition—It has been well established that, due to their ability to remodel ECM, MMPs can significantly contribute to tumor invasion and metastasis by increasing cellular invasiveness and liberating growth factors stored in the

ECM^{92,164,165}. Furthermore, as mentioned above, MMPs can non cell-autonomously induce invasion. These data suggest that MMP intervention should be a promising therapeutic strategy. Unfortunately, more than 50 MMP inhibitors have failed in clinical trials due to low specificity and resultant severe toxicities^{166,167}. Even though targeting broad-spectrum MMPs may be unwise, it may still be beneficial to selectively inhibit membrane-anchored MMPs using antibody-based agents^{168,169}. For example, it was recently shown that DX-2400, an antibody selectively inhibits MMP-14 (a membrane-bound zinc endopeptidase), suppresses angiogenesis and tumor progression in xenograft models¹⁷⁰

Although miR-200-containing vesicles can promote non-metastatic breast cancer cells to metastasize, even at a distance, by inducing MET⁸⁹, inhibition of the miR-200 family should be carefully evaluated. miR-200 family members suppress the E-cadherin repressors ZEB1 and ZEB2, thereby increasing E-cadherin levels and inhibiting EMT and stem cell like phenotypes^{171–175}. Because the EMT-MET axis plays a multifaceted role during the metastatic cascade, in which EMT is thought to play an important role in early metastatic dissemination^{176–178}, whereas MET is thought to be required for metastatic outgrowth at the secondary site^{176–182}, inhibition of a key regulator of MET may not be a wise choice. Of note, the plastic nature of tumor cells, allowing them to switch between epithelial and mesenchymal states, may be the key to successful metastasis^{178,183}, and novel approaches to inhibit this plasticity in the primary tumor may be a worthwhile approach.

The above examples are only a few of many pathways/molecules that could be targeted to limit tumor cell cooperatively and inhibit tumor progression. A comprehensive characterization of tumor heterogeneity will allow us to uncover many more avenues through which to inhibit clonal cooperation in addition to those mentioned above. As always, the balance between efficacy and toxicity will need to be considered.

Conclusions

In summary, due to genomic instability and the heterogeneous tumor microenvironment, various selection pressures cause tumor cells to undergo branched evolution forming a heterogeneous and dynamic tumor. Diverse subclones can establish their cooperation through paracrine, cell-cell contact and microenvironment remodeling, which allows them to exhibit a fitness advantage during tumor progression. Thus, disruption of these clonal cooperations may disturb the balance in the tumor ecosystem, leading to the repression of tumor growth, metastasis, therapy resistance, and hopefully, tumor collapse.

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Fig. 1. Mechanisms of metastasis of heterogeneous tumors

(A) Active Metastasis- Heterogeneous tumors contain a small percentage of innately aggressive subclones that secrete exosomes and/or release paracrine factors to enhance the aggressive properties of nearby cells. Aggressive and “transformed” subclones then actively perform the various steps of metastasis together. (B) Alternatively, innately aggressive subclones within the heterogeneous tumor remodel the tumor microenvironment, without acting directly on neighboring tumor cells. These microenvironmental alterations, such as degradation of the ECM, pave the path for innately “non-aggressive” tumor subclones to passively escape the primary tumor site and to enter the bloodstream (intravasate) and eventually metastasize. These two models for metastasis are not mutually exclusive and allow for overall increase in metastatic incidence of heterogeneous primary tumors.

Fig. 2. Therapy resistance in heterogeneous tumors

Treatment of heterogeneous tumors with targeted therapies leads to the elimination of therapy-sensitive subclones, leaving behind intrinsically drug-resistant as well as death-resistant subclones. Drug-resistant subclones, which represent the “early” resistant tumor cell subpopulations, will then repopulate the majority of the tumor. Continual treatment challenges or stochastic events can cause de novo mutations in the death-resistant subclones,

rendering them therapy-resistant and allowing them to occupy the tumor as “late” resistant subpopulations.

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