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Cancer progression: a single cell perspective

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Abstract. – Tumor tissues are constituted by a dynamic diversity of malignant and non-malignant cells, which shape a puzzling biological ecosystem affecting cancer biology and response to treatments. Over the course of the tumoral disease, cancer cells acquire genotypic and phenotypic changes, allowing them to improve cellular fitness and overcome environmental and treatment constraints. This progression is depicted by an evolutionary process in which single cells expand as a result of an interaction between single-cell changes and the local microenvironment. Recent technological developments have made it possible to depict the development of cancer at the single-cell level, offering a novel method for understanding the biology of this complex disease. Here, we review those complex interactions from the perspective of single cells and introduce the concept of omics for single-cell studies. This review emphasizes the evolutionary dynamics that control cancer progression and the capacity of single cells to escape the local environment and colonize distant sites. We are assisting a rapid progression of studies carried out at the single-cell level, and we survey relevant single-cell technologies looking at multi-omics studies. These forefront approaches will address the combined contribution of both genetic and non-genetic factors to cancer progression and will pave the path for precision medicine in cancer.

Key Words:
Single cells, Cancer progression and evolution, Single cells omics.

Introduction

Cancer is a major cause of death throughout the world, and despite significant investments and advancements in research, cancer still exerts a massive burden on society with no immediate prospects for effective control[1,2]. Cancer is characterized as a sizable group of illnesses that result in unchecked cell proliferation and abnormal immune system recognition[3]. Cancer cells can flourish at unneeded levels within a tissue, spread beyond normal regulatory boundaries and invade neighboring tissues, even colonizing distant sites[4].

Over the years, a large range of cancer treatments, including surgery, chemotherapy, radiation therapy, and the most recent, immunotherapy, have been adopted to eradicate cancer cells or inhibit their proliferation. Although patients' survival times have been extended following clinical treatments, a large proportion of patients still experience recurrence and are unable to achieve long-term survival. We have a large understanding of cancer biology, but translating our knowledge into clinical practice is still a complex task due to the disease's demanding cellular complexity, dynamics, and evolutionary characteristics. All these components could provide both barriers to or opportunities for successful treatments[5]. The daunting complexity of cancer resides in the breadth and scope of its diversity, including genetics, cell phenotype, biological aspects, physiological conditions, and response to therapy. For example, an important aspect of cancer cells is the complexity of their genomes, which is characterized by a mutational burden, numerical chromosome changes, and local genome rearrangements[6]. All the above concepts and diverse aspects of cancer cells have a powerful resonance in the field of cancer therapeutics, and cancer treatments continue to be a major challenge for human medicine today.

A significant factor in the lethality of cancer is cancer evolution, a dynamic, complex, and adaptive clonal process that promotes cell robustness and is supported by genetic diversity and epigenetic plasticity[7]. Under an evolutionary framework, cancer cells adapt to their surroundings, suppress the host immune system's ability to recognize and attack tumors and deceive molecular and cellular

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protective mechanisms primed to prevent tumor formation[8]. Cancer progression begins when a normal cell transforms and expands into tumoral tissue, initiating a process that could potentially transform into a malignant state. During this process, cancer cells form a tumor microenvironment that protects them during tumorigenesis and makes it difficult for the host immune system to effectively eliminate harmful cells[9]. This evolutionary process can increase clonal fitness and empower cancer cells to grow more aggressively. As cancer cells evolve, their immunogenicity can fade and be lower than that of pathogenic microorganisms, which consequently induces very weak immune responses. Today's scientific research is improving our understanding of cancer by examining cancer progression at the single-cell level and using evolutionary theory to explain single-cell diversity, drug resistance, and cancer causation.

Here we review, under an evolutionary framework, the role of single cells in cancer development and their ability to spread through the body. We also attempt to succinctly summarise the most recent tools and techniques used to study cancer at the single-cell level, which is sure to revolutionize cancer medicine, all within a multi-omics context.

Tumor Heterogeneity

Cancers, during the progress of the disease, generally become heterogeneous, a condition characterized by the existence of many distinct cellular populations rather than a homogenous cluster of identical cells. The heterogeneity of cancer cells within a singular tumor, named intratumor heterogeneity (ITH), encompasses several angles that render tumors unique and has significant clinical consequences as it often provides fuel for resistance[10].

A single tumor bulk includes a diverse set of cells harboring distinct molecular signatures with differential levels of sensitivity to treatment[11]. The mechanisms that cause intratumoral heterogeneity are only partially understood and under constant debate. Arguably, the most widely recognized mechanism of tumor heterogeneity relies on genetics and involves single-cell mutations and chromosomal aberrations, which, when limited to a subset of cancer cells, establish distinct genetic populations or clones. Clones, on the other hand, may differ not only in terms of somatic genetic makeup but also in cell morphology, gene expression, and metastatic potential[12].

Genomics as a Source of Intratumoral Heterogeneity

In recent years, large-scale genomic studies[13] and advances in next-generation sequencing (NGS) have revealed details about tumor heterogeneity and the extent of genetic diversity within tumors. More recently, the adoption of long reads/third-generation sequencing has allowed for unprecedented characterization of structural variation as well as direct detection of epigenetic markers in native DNA and RNA. These long-read technologies are rapidly advancing and have improved power to resolve complex regions of the cancer genomes[14-16]. Large-scale genomic studies[14,17] that sequenced multiple cancer regions in space and time demonstrated the large repertoires of genetic alterations that exist in cancers, including single nucleotide variants, small insertions and deletions (indels), structural variants, and somatic copy number alterations. All those genomic alterations contribute to intratumor heterogeneity and occur in almost all cancer types, albeit at varying degrees[17].

Genomic instability is the most studied aspect of intratumor heterogeneity, although our understanding is still far from being sufficiently complete[14]. Genomic instability increases the probability and rate of mutations and thus drives the generation of multiple tumor subclones. When a single cell divides, stochastic somatic mutagenesis might happen in the form of nucleotide substitutions and/or small indels, even in the absence of internal and external mutagens[18]. Considering the constant turnover and the large size of the tumor cell populations, some stochastic mutations will inescapably affect genes with cancer relevance. The extent to which those stochastic mutations account for carcinogenesis still remains a subject of debate[19,20]. Many spontaneous human cancers show aneuploidy, a condition linked with chromosomal instability. Aneuploidy commonly rises as a consequence of whole genome doubling due to mitotic failure leading to tetraploidization[21]. Chromosomal instability involves loss, gains, and translocations of large fragments of genomic DNA and follows an increased rate of genomic mutation errors[22]. Certain tumors show a high mutational burden as a result of exogenous mutagens. An increased mutation rate and specific mutational signatures can rise because of exposure to exogenous mutagens such as UV-related mutagenesis in skin cancers and tobacco-related mutagenesis in oral, lung, and bladder cancers[23].

Genetically diverse clonal populations can bear distinct morphological patterns and distinct responses to treatment or environmental stimuli[24], but emerging evidence indicates that genetic alterations alone might be insufficient to fully explain the range of phenotypic diversity in solid malignancies. Intratumoral heterogeneity thus seems to arise at other levels beyond genetics, such as epigenetics, transcriptomics, and proteomics[25].

Multi-Omics Levels as a Source of Intratumoral Heterogeneity

Epigenomics

Intratumor heterogeneity can result from epigenetic variability, a reversible and heritable phenomenon involving changes that affect gene expression with no alteration in the underlying genetic background. Changes in gene expression caused by epigenetics allow cells to adapt to microenvironmental stimuli (acidity, oxygen, nutrient deprivation) and develop resistance mechanisms against clinical therapies[26]. Different environmental-sensitive mechanisms are responsible for epigenetic changes, such as DNA methylation, histone modification, and non-coding RNA acting on gene expression[3].

Epigenetics have shown to have an impact on tumor cell phenotypes[27], and alterations of the epigenetic system have been recognized as a hallmark of cancer[28]. In chronic lymphocytic leukemia, corrupted coordination of epigenetic modifications fuels cancer evolution and intratumoral heterogeneity. By exclusively activating and repressing histone modifications, leukemic cells can dysregulate gene expression and transcriptional output, thereby diversifying cell heterogeneity[29]. Various epigenetic studies[30-32] on different tumors, such as chronic lymphocytic leukaemia[30], prostate cancer[31], and glioma[32] demonstrated that DNA methylation patterns can complement genetic data to infer cancer evolution. A recent study[33] measured the co-evolution of the genome and epigenome in colorectal cancer. The study showed the existence of recurrent somatic alterations in regions of chromatin accessibility, including regulatory regions of cancer-driver genes that usually lack genetic mutations. Evidence of positive selection was found in chromatin modifier genes, and promoters/enhancers with mutations showed altered expression of associated genes. The interplay between epigenetic changes and the accumulation of somatic genetic alterations can thus also drive tumor evolution. Important tumor cell phenotypes, such as immune surveillance evasion, may thus be influenced by epigenetic alterations.

Epigenetic modifications are exposed to a high error rate in replicating DNA and potentially are less faithfully propagated through cell division than genetic information, suggesting more epigenetic variability as tumors evolve[30]. Epigenetic changes in cancer cells are also responsible for cellular plasticity, a phenomenon where cell differentiation and reprogramming happen without alterations of the underlying DNA sequence[34]. Intratumoral epigenetic heterogeneity analysis has been mainly studied by profiling the DNA methylation status, as the employed methodology has limited challenges even at the single cell[35].

One key driver in epigenetics is histone modification, which modulates the structure of the chromatin and regulates the accessibility of DNA. Single-cell histone modification assays of breast cancer cells have recently shown the presence of cellular phenotypes with varying histone modification patterns[36]. Furthermore, histone acetylation plays a role in the development of breast cancer, and targeting histone enzymes such as histone deacetylases could be a novel approach for breast cancer therapy[37], as well as reducing host inflammation caused by silicone breast implants[38]. The inclusion of other, more challenging methods, such as the above histone modifications and open chromatin assays, in the present and near future can potentially revolutionize our understanding of the epigenomic role in tumor heterogeneity.

Transcriptomics

Transcriptomic profiling at the single-cell level is a very promising approach for studying intratumoral heterogeneity and cancer progression, as well as the tumor microenvironment. Changes in transcriptional activity and regulation generally underlie cellular phenotypic diversity, and single-cell transcriptomics provides a quantitative measurement of the molecular activity that underlies the phenotypic diversity. Furthermore, single-cell transcriptomics can characterize the various cell types dwelling in the tumoral microenvironment, such as immune cell subtypes, in a high-throughput and mostly unbiased manner[39].

Single-cell transcriptomic profiling of ductal carcinoma revealed a different gene expression driving heterogeneity and the invasive phenotype[40]. Single-cell transcriptomes of acute lymphoblastic leukemia across multiple mice models demonstrat-

ed that leukemic cells have a unique gene expression profile to respond to different chemotherapies. Leukemia expansion can also be spatially confined in single anatomical sites of the bone marrow and be driven by cells with distinct gene expression[41]. Genomics and transcriptomics analyses of lung squamous cell carcinoma with a moderate level of intratumor genetic heterogeneity showed that transcriptomic heterogeneity impacts cancer pathways and drives phenotypic heterogeneity. Cancer cells belonging to the same subclonal cell population differ in the transcriptomic makeup and proliferative potential, contingent on their localization in the tumor margin or resource-limited interior[42].

In colorectal cancer, intratumoral heterogeneity inferred *via* gene expression was found to be plastic, that is, to adjust in response to environmental changes rather than being heritable. This transcriptional plasticity is, therefore, widespread within a tumor[43].

Proteomics

Proteomic approaches to investigating intratumoral heterogeneity have long lagged behind genomics and transcriptomics, mainly owing to technical and biological limitations. A single gene can produce high amounts of proteins with different isoforms and modification states, which are challenging to detect at the single-cell level. There may also be complex regulation of protein expression at both translational and posttranslational levels, which adds to the technical difficulties. These factors make intratumor heterogeneity difficult to unravel using proteomics, especially at the single-cell level[44]. The proteome still remains a daunting aspect of tumor biology to comprehend, and more research will be required to supplement the already extensive studies of tumor transcriptomes and genomes.

Tumor Microenvironment as a Source of Heterogeneity

The tumor microenvironment (TME) is a complex and dynamic entity composed of cells (cellular component), secreted factors, and extracellular matrix (both non-cellular components) capable of inducing intratumoral heterogeneity and clonal progression, increasing multidrug resistance, and stimulating metastasis[45]. The composition of the tumor microenvironment is different among various tumor types, but hallmark components include immune cells, stromal cells, blood vessels, and extracellular matrix. It is believed that the TME can trigger a large variety of pro-tumorigenic signals, and consequently, it is an active promoter of cancer progression[46]. Cancer cells are able to adapt to their surrounding microenvironment and to shape it through the secretion of oncogenic signals and modification of local environmental conditions[47]. As a result, the phenotypic characteristics of cancer cells and the heterogeneity of the tumor microenvironment vary greatly depending on the tumor context. For example, in pancreatic cancer, a high collagen content of the extracellular matrix is associated with a poor prognosis and chemoresistance[48], whereas in breast cancer, tumor-associated macrophages and neutrophils living in the TME drive tumor cell plasticity through the secretion of specific cytokines[49].

The presence of tumor-infiltrating lymphocytes is considered a prognostic marker for lung cancer[50]. The tumor microenvironment can promote the formation of tumor niches, anatomically distinct regions within the tumor microenvironment, that are able to drive cancer progression, metastasis, and drug resistance[51,52].

TME can also have negative tumorigenesis effects, such as cancer growth inhibition, which can be induced by cancer-associated fibroblasts with tumor-suppressor activity. Cancer-associated fibroblasts, on the other hand, can also promote cancer angiogenesis, metastasis, and drug resistance, emphasizing the dual roles of TME in tumorigenesis being beneficial or harmful[53]. The tumor microenvironment represents an emerging target for clinical treatments.

Phenotypic Plasticity as a Source of Heterogeneity

Phenotypic plasticity is the ability of cancer cells to undergo dynamic, nongenetic cell state changes that amplify cancer heterogeneity and can promote metastasis and therapeutic evasion. In contrast to the heterogeneity caused by genetic changes usually considered permanent, phenotypic plasticity is dynamic, reversible, and receptive to regulation[54,55]. In this context, distinct cancer cell phenotypes can be found as a consequence of dynamic and reversible epigenetic and transcriptional mechanisms[56]. Phenotypic plasticity is now recognized as a hallmark of cancer[28].

Epigenetic alterations are usually considered drivers of phenotypic plasticity and cell state dynamics[57]. Epigenetic changes can simultaneously

affect many loci, leading to rapid shifts in regulatory programs and cell states. Acquired epigenetic alterations and cell states tend to be heritable and can persist for several generations before spontaneously reverting or changing in response to local signals. Different microenvironmental aspects such as hypoxia, tissue constraints, and chronic inflammation can both directly and indirectly affect cancer cell epigenetics and, thus, phenotypic plasticity[58]. According to mathematical models, reversible epigenetic mutations and fluctuations in gene expression patterns can confer transient drug resistance due to cell phenotypic switching between a drug-sensitive and drug-resistant state[59]. Intratumor epigenetic and transcriptomic heterogeneity, at least partly, can contribute to cancer cell phenotypes, dormancy, and metastasis[60].

Although non-genetic determinants are primarily responsible for phenotypic plasticity, genetic changes can also dynamically modulate cellular properties and result in phenotypic plasticity. In some cases, reversal genetic mutations can compensate for oncogenic genetic alterations, such as those reversion mutations in *BRCA* genes which are able to generate a cellular phenotype resistant poly ADP ribose polymerase to (PARP) inhibitors and chemotherapy[61]. In other cases, genetic alterations may impact the cellular phenotype *via* a dose-dependent mechanism. Extrachromosomal DNA amplification is an important driver of alterations in cancer. Unequal segregation of extrachromosomal DNA from a parental tumor cell to offspring cells rapidly increases tumor heterogeneity, granting cancer cells an additional layer of response to treatment and perhaps providing them with an evolutionary advantage[62]. Cancer cells with a higher copy number of oncogenic loci may have a proliferative advantage but may be sensitive to a targeted clinical treatment. Cells with lower copy numbers, on the other hand, may be transiently less sensitive to the same treatment, and under favorable conditions, within a few cell divisions may still gain a high-copy number state, feeding phenotypic plasticity.

Intrapatient and Interpatient Heterogeneity

Multiple tumors of the same type found in the same patient can have significant differences. The heterogeneous status of similarity or differences between anatomically distinct tumor sites is referred to as intrapatient (or intratumor) heterogeneity[63]. This kind of heterogeneity could result from intratumor heterogeneity, in which subpopulations of cells proliferate, differentiate, and move from one anatomical site to another, even at a distance, to form metastatic lesions. Heterogeneity between different malignant sites is the most common clinical observation in patients with advanced metastatic disease.

Another type of tumor heterogeneity is interpatient heterogeneity, which refers to differences in the same tumor type in different patients. It refers to differences in tumor genotypes and phenotypes between individual patients. Interpatient heterogeneity is often observed in clinics and is largely associated with different somatic mutations acquired in different genes or in different domains within the same gene[64]. As a result, patients with the same type of cancer may have very different clinical outcomes. Investigations on the causes of interpatient heterogeneity are still far from exhaustive, but this heterogeneity should be considered for designing more efficient and safer tumor drug therapies[65].

Cancer Progression: Darwinian Evolution

The fundamental principle of a Darwinian evolutionary system is the phenotypic variation of reproductive individuals linked by common descent that undergoes natural selection for the fittest variants. Under a Darwinian framework, individuals with traits best fitting the environment will favorably survive and produce offspring bearing those advantageously inherited traits. Over time increasing accumulation of traits or, at the genomic level, genetic mutations, can lead to the formation of a new species, particularly in isolated and reproductive populations[66]. Modern cancer biology and genomics have corroborated cancer progression as a complex Darwinian, adaptive system, with cancer cells being the equivalent of asexually reproducing, unicellular quasi-species. Tumors are frequently identified as a large population of genetically diverse groups of cells (clones or subclones) competing with one another for a limited set of nutrients and metabolites under the selective pressures of endogenous (e.g., microenvironmental pressures and tissue barriers) and exogenous (e.g., therapy) factors. The outcome of this competition is the survival of clones best fitted to flourish under

very specific conditions. Because Darwinian selection is highly contingent and blind to the future, many dominant clones at one point in time may reach evolutionary dead ends and die out, while others, often a minority, may be able to persist[5].

Over the last two decades, numerous cancer genomics studies[67,68] based on next-generation sequencing have characterized various aspects of cancer progression and supported the concept that cancer development is a process that adheres to Darwinian evolution. One of the earliest genomics studies[68] sequenced bulk tissues from primary and metastatic sites to show the somatic evolution of a lobular breast tumor. According to the study, the somatic genomic landscape of the metastasis at the time of diagnosis was significantly more heterogeneous than that of the primary tumor, indicating that somatic evolution had occurred between the primary and metastasis sites. Intratumor heterogeneity across different regions of the same tumor was reported as the result of an evolutionary process[69] by genetically profiling thirty tumor samples from four patients with renal cell carcinoma. Furthermore, the same study found evidence of parallel evolution for multiple tumor suppressor genes (*SETD2*, *PTEN*, and *KDM5C*), implying that selective pressures drive the inactivation of the same gene multiple times within a single tumor. Bulk tumor profiling of twenty-one breast tumors revealed an extensive genetic variation within individual breast tumors, demonstrating the role of selection in cancer progression[70]. The genomic characterization of multiple metastases from prostate tumors in ten patients identified, as a common event, metastasis to metastasis seeding consistent with monoclonal and polyclonal dispersal[71]. Within an evolutionary framework, phylogenetic methods can be used to reconstruct the clonal composition and progression of cancer, as well as the time and onset of metastasis. A primary and metastatic bulk genome sequencing of a colorectal cancer patient combined with phylogenomic and serial sampling from different stages of the disease allowed us to infer the timing of tumor evolution from initiation[72].

Although multiple sampling can provide high resolution for inferring clonal evolution, only single-cell analysis can give a complete picture of clonal dynamics and tumor evolution history. Darwinian evolution can provide a distinct evolutionary framework for understanding single-cell progression in cancer through the application of three concepts: genetic variation, inheritance at the single-cell level, and selection. Early evidence of Darwinian evolution at single cell level was presented by Nick Navin and colleagues for a breast cancer patient[73] and by the Mel Greaves group for hematological malignancy, therefore providing detailed subclonal genetic architectures and phylogenies[74,75].

Targeted single-cell DNA sequencing of acute myeloid leukemia (AML) samples uncovered complex clonal evolution within AML that bulk sequencing did not reveal[76]. Single-cell DNA sequencing from non-small-cell lung cancer patients depicted the clonal evolution and adaptation of cancer cells during targeted therapy. Distinct driver alterations conferring drug treatment resistance coexist within individual cancer cells[77].

Single-cell transcriptomics, due to its high resolution, allows for the confident detection of genes expressed at the single-cell level. Single-cell RNA sequencing has recently emerged as a valuable tool for studying tumor evolutionary dynamics[78]. Single-cell transcriptomics was used to depict stromal evolution in animal models affected by pancreatic ductal adenocarcinoma. During cancer evolution, a population of carcinoma-associated fibroblasts, a TGFβ-driven cell lineage, was found to be prevalent in the tumor microenvironment. These fibroblasts can express LRRC15 (leucine-rich repeat containing 15) proteins, which are absent in normal tissue but abundant in cancerous tissues where they surround cancer cells. The presence of these carcinoma-associated fibroblasts is associated with poor outcomes in immunotherapy trial[79]. A combined transcriptomics and whole genome single-cell sequencing strategy was used to dissect the clonal evolution of hepatocellular carcinoma and investigate the relationship between genetic and phenotypic heterogeneity. Key genetic events were observed to occur early in cancer evolution when primary sites and metastases shared a common origin but then evolved independently. Genetic diversity was found to be related to single-cell transcriptomic phenotype[80]. Another study[81] of hepatocellular carcinoma that combined single-cell DNA and RNA sequencing found an accumulation of copy number mutations consistent with a two-phase evolution pattern. A first phase of non-Darwinian punctuated evolution was followed by a second phase of gradual Darwinian evolution in which single cells continuously accumulated genetic aberrations and adapted to the selection pressure.

Clonal Evolution in a Darwinian Framework: Linear, Branched, Convergent, and Parallel Evolution

Evolutionary clonal lineages and the chronology of somatic mutations occurring over time can be inferred through a phylogenetic inference[82] for which general modes of tumor evolution have been suggested. Despite still being debated, two main evolutionary modes have been proposed within a Darwinian framework: linear evolution and branching evolution. Other major proposed modes, such as neutral evolution and punctuated evolution, do not reflect a Darwinian scheme.

The linear evolution mode provides evidence for selective sweeps occurring during tumor evolution. The occurrence of new driver mutations with a strong selective survival advantage raises the fitness of the cells carrying those mutations, which outcompete all clones. Cells that do not carry those mutations will succumb to evolutionary pressure and die. The resulting phylogenetic tree is expected to have a major dominant clone, with only rare intermediate clones persisting from the previous selective sweeps (Figure 1). Some breast cancer cells appear to spread linearly, supporting a unidirectional, linear cancer evolution in some breast cancer patients[83,84].

Branching evolution results from the instability of the cancer genome. Clones diverge from a common ancestor, evolve in parallel, and separate from the previous generation; this process results in multiple clonal lineages. Unlike linear evolution, selective sweeps are uncommon, and multiple clones with the same increased fitness expand simultaneously. The resulting phylogenetic tree is expected to include intermediate clones as well as those clones expanded as a result of positive selection acting on subclonal lineages (Figure 1). Branching evolution has been reported in many human cancers, including, among others, acute

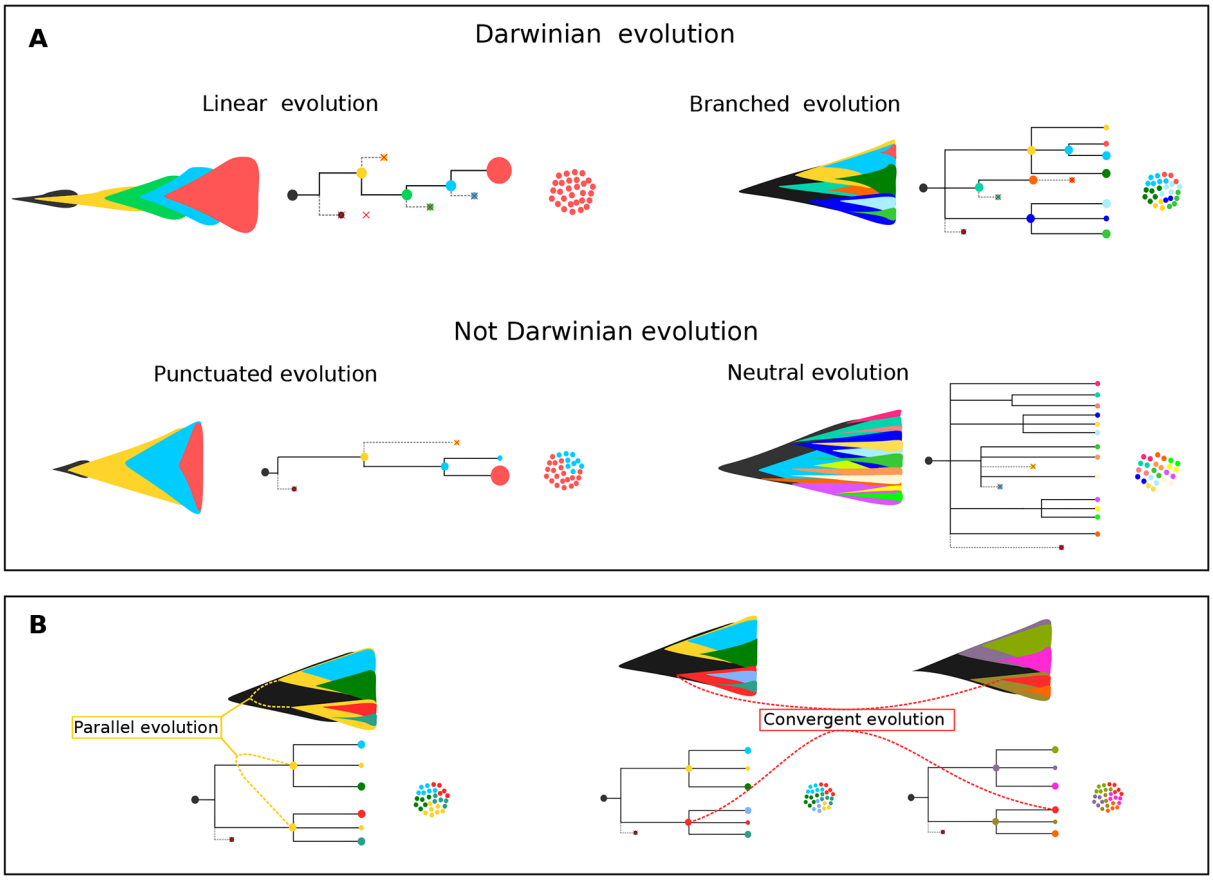

Figure 1. Modes of cancer evolution. **A**, The four typical cancer evolutionary processes within a Darwinian (top) and non-Darwinian evolutionary framework (bottom). Each evolutionary model is described by Muller plots representing dynamic changes in clonal size over time (left), phylogenetic trees (center), and a schematic representation of generated intratumoral heterogeneity (right). Colors indicate different clones and subclones. **B**, Two different aspects of cancer evolution: parallel and convergent evolution, illustrated by Muller plots, phylogenetic trees, and intratumoral heterogeneity.

lymphoblastic and myeloid leukaemia[85,86], colorectal cancer[87], melanoma[88], brain cancer[89,90], and also breast cancer[70,91,92].

Regardless of linear or branching architecture, clonal expansion can result in convergent or parallel evolution (Figure 1). The independent evolution of similar traits in unrelated species or clades dwelling in different habitats is referred to as convergent evolution. Convergent evolution occurs in cancer when different tumor sites in the same patient bear a similar mutation pattern, accounting for similar external environments. Convergent evolution also occurs in different affected patients where cancer clones follow a similar eco-evolutionary path and exhibit the same mutational patterns independently. Convergent evolution was observed in metastatic colorectal cancer drug-treated patients, who developed multiple convergent somatic mutations regardless of their pre-existing mutations. Under convergent evolution mode, cancer cells can generate drug-resistant mechanisms[93]. Parallel evolution occurs when independent species or clades acquire similar traits while coexisting in the same eco-space. Parallel evolution in cancer refers to different clonal populations within the same cancer side that evolve or retain a specific gene mutation at the same time. Cancer cells enduring parallel evolution tend to be consistent in their evolutionary direction as they might undergo the same or similar external pressure. Parallel evolution has been observed in many early human cancers, resulting in genetic heterogeneity as well as multilineage progression. Parallel evolution has been reported, among others inmyeloma[94], lung cancer[95], colorectal cancer[96], and within *in vivo* models able to resemble the genesis of human breast cancer[97]. Convergent and parallel evolution are not mutually exclusive. This can be seen in clear-cell renal cell carcinoma, where some patients independently had mutations converging on the *VHL* pathway, while others had parallel evolution for mutations in the same genes or pathways within distinct tumor subclones[98]. Patterns of linear and parallel evolutionary patterns were suggested to coexist in osteosarcoma, a common primary bone malignancy, for which the lung is the most frequent site of metastasis. A significant intertumor heterogeneity between tumors and metastatic sites was also suggested, with metastatic mutant genes being enriched in the *MAPK* pathway[99]. *MAPK* regulates cell fate by transducing a myriad of growth-factor signals, some of which might be considered potential therapeutic targets in osteosarcoma[100].

Cancer Progression: non-Darwinian Evolution

The Darwinian model alone seems to be insufficient to fully explain the entire spectrum of evolutionary behaviors in cancer. Distinct mutation accumulation rates in somatic cells, unsustainable genetic loads, and high levels of genetic variation challenged Darwinian evolution[101,102]. A Darwinian model would typically have orders of magnitude less genetic diversity than a non-Darwinian model that best fits the broad genetic diversity found in some tumors[103]. Furthermore, somatic mutations are common in healthy tissues, even in cancer-driver genes, suggesting that genetic mechanisms alone may not be sufficient to drive malignant transformation[104,105]. A growing body of evidence suggests that non-Darwinian mechanisms play an important role in tumor evolution. Evolutionary processes such as neutral evolution, macroevolutionary changes, and the impact of non-genetic determinants are emerging as important drivers of cancer progression.

Neutral Evolution

The neutral model of cancer evolution is based on Kimura's work on population genetics, who first supported a neutral process for molecular evolution. The neutral model advocates that selection is weak and most evolutionary changes are not caused by natural selection but by stochastic genetic drift. Most genetic variants will be neutral, with no selection or fitness change, especially at small population sizes. The few and rare variants with an impact on fitness will be predominantly deleterious and will be eliminated by purifying selection. However, neutral evolution does not exclude the possibility of occasional strong positive selection, especially after the purifying selection has removed harmful mutations[106].

Under a neutral model, cancer-driving mutations accumulate in a clonal fashion prior to tumor initiation as a consequence of aging and carcinogenic action. The genetic heterogeneity observed in tumors may often arise as a result of the random fixation of neutral alterations that play no functional role in promoting tumor growth and evolution (Figure 1). The proposed Big Bang model of cancer initiation[107] is grounded in neutral evolution, in which cancer develops as a result of a single expansion of many intermixed clones. The emergence of novel variants

is entirely governed by genetic drift, which quietly drives the accumulation of mutations over time. Consequently, at the onset of cancer, all the mutations responsible for cancer initiation are already present, and their presence is sufficient for tumor formation and development. However, the model does not rule out the possibility of selective forces, such as therapeutic pressure, acting on previously arisen neutral alterations to drive clonal expansion[108,109]. The Big Bang model explains why most mutations in tumors are detected in the early stages of cancer. The power of the model is still controversial, being either criticised[110] or supported[111], and the role of neutral evolution in cancer is still hotly debated, despite the fact that neutral evolution has been observed in various cancers, including colon, stomach, and lung cancers[109].

Macroevolution: Punctuated Evolution

A model of punctuated evolution, also known as "discontinuous equilibrium", has been recently applied to the field of cancer evolution. The punctuated evolution model borrowed from evolutionary biology explains an apparently abrupt change in a species' phenotype. Evolution happens in short and intense bursts of time interspersed over long periods of time, during which apparently no 'important evolution' happened. This pattern of events is described as punctuated equilibrium[112].

Increasing evidence points to the possibility that, in some circumstances, cancer progression may follow a punctuated evolution (Figure 1). Cancer cells may remain stagnant for a long time before a large number of genomic aberrations occur suddenly and in short bursts of time, driving cancerogenesis[113,114]. Those cancer cells could acquire multiple driver alterations at the same time and potentially achieve greater fitness than would be possible through a gradual accumulation of alterations, as in a Darwinian model[115].

Chromosomal instability and significant genomic rearrangements occur as a result of punctuated evolution. Examples of these catastrophic events in cancer genomes include chromoplexy, chromothripsis, breakage-fusion-bridge cycles, and other similar events. The phenotypic impact of the genetic changes achieved through punctuated evolution is frequently deleterious, but in rare cases, will result in a significant increase in fitness and the generation of highly adapted and efficient cancer cells[116]. Punctuated evolution has been described in triple-negative breast cancer, where the majority of copy number aberrations are acquired at the earliest stages of tumor evolution[117] and prostate cancer genomes[114].

Non-Genetic Determinants of Cancer Evolution

A growing body of evidence suggests that, during cancer evolution, phenotypic changes or adaptations to the microenvironment are driven not only by genetic alterations but also by non-genetic and often non-heritable determinants such as cell plasticity and the status of the tumor microenvironment[24].

Cell Plasticity as Mediator of Cancer Evolution

Cell plasticity is a property of both healthy and cancer cells that refers to the cell's ability to acquire a new identity or to adopt an alternative state in response to intrinsic and extrinsic signals. In normal physiology, cellular plasticity drives many important biological functions, such as cellular differentiation, development, and wound healing[118]. In cancer, cellular plasticity is a means by which tumors adapt to their microenvironments[109]. This property enables cancer cells to quickly react to dynamic changes in the tumor microenvironment and to precisely tune their response to stressors like inflammation and therapy[119]. Additionally, it has been proposed that under a neutral framework, cellular plasticity, rather than clonal selection, may be responsible for phenotypic changes and adaptation to various microenvironments[109]. In contrast to genetic alterations, which have binary and largely irreversible effects, cell plasticity is reversible because it reflects dynamic and reversible epigenetic and transcriptional changes[56]. The epithelial-mesenchymal transition (EMT) is the most widely studied example of phenotypic plasticity, in which cells with an epithelial phenotype transform into a mesenchymal phenotype while maintaining the ability to revert to their epithelial state. EMT typically occurs during embryogenesis and wound healing, when epithelial cells drop their polarity, lose cell-cell adhesions, and invade the stroma to generate tissue. The EMT mechanism is often hi-

jacked by carcinoma cells for tumors and metastatic progression[120].

Cellular plasticity has been demonstrated as an adaptive mechanism to escape therapeutic pressure. For instance, mounting evidence suggests that cell plasticity occurs in glioblastoma (GBM), the most common malignant brain tumor in adults with high levels of therapeutic resistance and a very high rate of tumor recurrence. GBM shows limited genetic evolution at recurrence, implying that resistance mechanisms largely operate at the phenotypic level, where cancer cells have different gradients of transcriptomic states with multiple axes of variation[121]. Similarly, in melanoma patient-derived xenografts models, multiple therapeutic-resistant cell populations evolve in response to drug treatment by diversifying transcriptional states[122]. Although cell plasticity is crucial, malignant melanoma cells can still show treatment resistance brought on by genetic changes. This shows that different resistance mechanisms are possible in tumor evolution and that frequently, one determinant does not preclude the other[123].

Tumor Microenvironment: Evolution in Process

The tumor microenvironment is a complex and ever-changing entity that includes immune cells, stromal cells, blood vessels, and the extracellular matrix that surrounds and feeds a cancer cell. The tumor microenvironment plays a key role in the progression of cancer[46] and the therapeutic response[124]. The cancer microenvironment may act as a selective force to tune the evolution of cancer cells into clones able to breach lymphatic or vascular channels and spread to regional lymph nodes and distant sites[125]. Changes in the tumor microenvironment were found to be associated with progression from pre-invasive to invasive human lung adenocarcinoma[126]. The fact that cancer incidence rapidly increases with age may be linked to the microenvironment. The presence of malignant cells could reflect an age-related physiological decline of the soma, which, when combined with a weakened immune system, may lower the threshold for pre-malignant cell clonal expansions[127]. Interestingly, the mutational burden induced by environmental agents varies by tissue type, suggesting tissue-specific differences in toxicokinetic, DNA repair activity, and also tumor microenvironment[128].

When Cancer Comes Back: the Metastatic Recurrence

Metastasis is a process arising across multiple organs and on different timescales that involves the dissemination of cancer cells from a primary tumor to a distant organ in the body. Metastases, rather than primary tumors, are mainly responsible for most cancer deaths; in lung cancer, the low survival rate reflects the proportion of patients diagnosed with metastatic cancer. Despite remarkable advances in understanding and treating primary tumors, the five-year survival rate related to metastatic cancer remains very poor[129].

Because of its continuous and dynamic nature, the metastatic process has been difficult to investigate and is still not fully understood. The basic form of the metastatic process involves a living cancer cell entering, surviving, and exiting the bloodstream and colonizing a remote tissue with a potentially hostile microenvironment. Metastasis is a very inefficient process because most cancer cells that leave the primary site either die stranded in capillaries or undergo apoptosis within 24 hours of exiting the bloodstream[130]. Dispersion of cancer cells from a primary site can start early during tumor progression[131,132], but only a subset of cancer cells develops into metastatic tumors[133].

The metastatic course of cancer cells can vary significantly depending on the cancer type. In some cancer types, malignant cells primarily spread to a single organ (e.g., prostate cancer cells to bone, pancreatic cancer cells to the liver), whereas in others, cancer cells can metastasize to different organs either sequentially (e.g., colorectal cancer cells, often first to the liver, then to the lungs and brain) or simultaneously (e.g., lung cancer cells, frequently to the liver and brain)[134]. In brain metastases (the majority of which arise from lung/breast cancer and malignant melanoma), cancer cells must cross the blood-brain barrier to initiate metastatic progression. Cancer cells can weaken the barrier's integrity by changing its permeability, structural integrity, and active efflux of molecules (this condition is known as the blood-tumor barrier)[135]. Once penetrated, cancer cells initiate brain metastasis, which can result in significant neurological impairments, intracranial hemorrhage, and seizures, all of which have potentially devastating consequences[136,137].

The mechanisms underlying metastatic spread to multiple organs remain unknown, and their biological details are still challenging to disentangle.

Genetics Determinants of Metastasis

Cancer cells are not under positive selection to metastasize, but a set of key adaptations may increase their likelihood of spreading to distant organs. In order to earn the ability to become metastatic, a cancer cell must acquire potentially metastatic traits such as immune evasion, mobility, and the capacity to survive and proliferate at distant sites[130]. When traits are acquired, the cell is exposed to particular conditions, such as a variety of somatic changes, a particular microenvironment, and selection pressure, all of which have an impact on the strength of the cell's fitness[63,138]. Metastatic cells may also need to evade the actions of metastasis suppressor genes, a class of genes able to inhibit the metastasis process without preventing primary tumor formation, such as*nm23-H1* in melanoma or *KISS1* in breast cancer[139]. Metastatic cells are frequently characterized by a high burden of somatic alterations, primarily passengers with only a subset, driver mutations, conferring fitness advantage to metastatic clones[140-142]. Patterns of metastatic dissemination have been associated with genomic alterations, and genomic instability is strongly correlated with metastatic burden[143]. High levels of somatic copy-number aberrations are often described in metastatic cancer cells[144]. Somatic mutations found in a metastatic cell are not simply a reflection of metastatic mutations but also represent an archaeological record of the mutational burden. Some somatic alterations are likely to have been acquired by ancestral cancer cells at the primary site, while others will be gained following metastatic dissemination. A large number of genes with somatic mutations are known to promote cancer cell dissemination. These genes promote cell invasion, circulation, and extravasation, as well as cell capacity to induce metastatic niches, co-opt organ-specific stromal components, and perform other pro-metastatic functions[130,133]. When disseminated cancer cells adapt to a specific host tissue environment, metastatic-promoting genes will be preferentially expressed[145]. Breast cancer cells selected for preferential brain metastasis express higher levels of genes known to facilitate blood-brain barrier passage[146]. Analysis of a large number of metastatic breast cancers disclosed nine cancer genes (*AKT1*, *ESR1*, *GATA3*, *KMT2C*, *NCOR1*, *NF1*, *RIC8A*, *RB1,* and *TP53*) more frequently mutated in a metastatic context and potentially driving metastasis[142]. In addition, metastasis could be triggered by aberrant DNA methylation patterns (epigenetic alterations) capable of driving colonisation[147].

Phenotypic Plasticity as Determinants of Metastasis

The ability of a cancer cell to metastasize is caused not only by (epi)genetic and genomic instability but also by phenotypic plasticity or the ability of a malignant progenitor cell to undergo extensive phenotypic variation. Phenotypic plasticity critically increases the likelihood of a cancer cell metastasizing by allowing the cell to adapt to microenvironments, overcome metastasis barriers, and resist therapy[148]. Cancer cells gaining certain plasticity acquire the ability to invade the underlying mesenchyme, to intravasate and extravasate blood circulation, and, finally, to colonise distant organs[130]. In colorectal carcinoma (CRC) cells are able to acquire phenotypic plasticity through differential gene expression. Under certain environmental conditions, CRC cells that express the L1 cell adhesion molecule gene (*L1CAM*) acquire metastasis-initiating capacity. The *L1CAM* gene is usually expressed under non-cancerous conditions after an epithelium injury in order to be regenerated by intestinal progenitor cells. In a CRC environment, loss of epithelial integrity promotes *L1CAM* expression in malignant cells, driving cancer cells to a highly plastic regenerative phenotype capable of metastasis and skipping anoikis. The expression of *L1CAM* is not necessary for adenoma initiation, but it is required for orthotopic carcinoma propagation, liver metastatic colonisation, and chemoresistance[149]. Differential gene expression has also been observed in prostate cancer. Increased expression of brain-derived neurotrophic factor/tropomyosin receptor kinase B (*BDNF/TrkB*) has been shown to promote prostate cancer progression by inducing epithelial-mesenchymal transition and anoikis resistance[150-152].

The Metastatic Power of the Epithelial-Mesenchymal Transition

The capacity of a malignant cell to undergo an epithelial-mesenchymal transition can increase the probability of a cell metastasizing. EMT is characterized by specific patterns of gene expression changes, with epithelial cells losing their adherent tight junctions and acquiring a mesenchymal phenotype, potentially resulting in increased mobility to distant locations[153]. Cancer cells with EMT characteristics can migrate from

the primary site, invade, and colonize a distal site, and then reverse the process of mesenchymal-epithelial transition (MET) and initiate metastatic growth[154,155]. In carcinomas, EMT abilities are mainly driven by SNAIL, TWIST, and ZEB transcription factors, together with microRNAs that balance the regulatory network[156]. Furthermore, TGF-β, in cooperation with other pathways, particularly the Ras-MAPK signaling pathway, is a powerful inducer of EMT by promoting intratumoral fibrosis and supporting tumor growth[157].

For over a decade, the role of EMT and its reverse process, MET, in promoting metastasis has been accepted[156], but the concept of a hybrid epithelial-mesenchymal phenotype has gained increasing importance and challenged the importance of EMT[158,159]. Cancer cells during the transition from epithelial to completely mesenchymal states undergo several transition states, intermediate epithelial-mesenchymal hybrid states with tumor-propagating cell capacity. In contrast to the full mesenchymal state, hybrid states do not undergo reverse MET, and other mechanisms other than MET may contribute to the metastatic potential of those hybrid cell populations[54].

Circulating Tumor and Single Disseminated Tumor Cells

Cancer cells arising from primary tumors may, sooner or later, breach into the vascular system, resulting in the intravasation of circulating tumor cells (CTCs) capable of traveling to distant sites and possibly seeding new metastatic colonies[160]. CTCs can circulate through the vascular system as individual cells or multicellular clusters, but they usually only last a few seconds or minutes before becoming trapped in small-bore microvessels in distant tissues. Despite their large volume, which makes it difficult to penetrate micro-vessel, experimental models revealed that CTC clusters have a superior ability to seed metastasis[161]. CTC clusters can line up into a chain of single cells held together by adhesive interactions allowing them to successfully penetrate 5 μm to 10 μm blood vessels[162]. Notably, CTCs, whether in a cluster or single cells, frequently carry combinations of epithelial and mesenchymal traits, highlighting the importance of EMT in cancer dissemination[163]. Detection of CTC in the bloodstream has been proposed as a potential biomarker for early cancer prognosis. Despite a rich body of literature supporting the biomarker possibility, the sensitivity of current CTC detection assays still remains a limitation, and concerns have arisen[164].

Cancer cells that have left a primary site may also go into dormancy, an inactive, lazy state in which they persist over time as single disseminated tumor cells (DTCs) and are capable of reactivating proliferation after protracted latency periods (this phenomenon is known as clinical dormancy). For some cancer types, such as breast, prostate, and kidney cancers, malignant cells may persist dormant for many years, even decades, despite successful courses of clinical therapy. While establishing a direct link between a new metastatic colony and a previously dormant cancer cell is difficult, the interaction of DTCs with bone marrow may be crucial. DTCs have been found in the bone marrow after successful therapy in various cancer types (including breast, and colon cancers), suggesting both the bone marrow as a potential reservoir of DTCs and DTCs as a possible source of cancer relapse[165]. To explain recurrence from DTCs, two different models of dormancy have been proposed: tumor mass dormancy and cellular dormancy. In the first model, the tumor stops growing either at the primary site or at metastatic dissemination as a consequence of the equilibrium between cancer cell proliferation and death. This state can be induced by the tumor's angiogenic state (angiogenic dormancy) and/or by the surrounding immune system priming dormant cells to avoid immune surveillance (immune-mediated dormancy). In the cellular dormancy model, disseminated cancer cells enter a quiescent state characterized by minimal proliferation, minimal death, reversibility, and failure to form a colony. Different factors can contribute to this kind of dormancy, including extracellular matrix status, metastatic niche conditions, a hypoxic microenvironmental state, and endoplasmic reticulum stress[166]. The microenvironment can play a critical role in dormancy through dormancy-inducing signals. In head-and-neck squamous carcinoma, a high concentration of TGF-β2 in the bone marrow can induce dormancy in DTCs *via* TGF-β-RI and TGF-β-RIII signalling[167]. Dormancy can also be induced by BMP ligands. In prostate carcinoma, bone stromal cells expressing BM7 can induce dormancy on prostate cells[168], whereas BMP4 ligand expression in the lung maintains dormancy on disseminated breast cancer cells. In contrast, DAND5, a member of the DAN family proteins, by inhibiting BMP signaling, induces dormant metastasis, initiating cells to reactivate in the lung[169].

Establishing a Niche

In ecology, a niche generally refers to all those environmental factors and interspecies relationships that influence a species' growth and distribution. This concept can be applied to cancer progression by referring to the dynamic, anatomical, and environmental conditions, as well as cell-to-cell interactions, that drive cancer cell adaptation. Good examples include the hematopoietic stem cell niche and the perivascular niche. Dormant DTCs may dwell in specialized niches that support their survival, hamper their proliferation, and protect them from therapeutic agents[170]. In order to dwell and survive in a specialized niche, cancer cells must be prepared to respond to the extracellular matrix and metabolic signals, to the stromal architecture, and to cell-to-cell contacts. All these factors can be collectively referred to as 'metastasis niche[171,172]. In addition to adapting to the microenvironment in which they are colonizing, metastatic cancer cells must also compete with normally residing cells for environmental resources. Cytokines are increasingly being recognized as critical factors in creating a permissive microenvironment for metastatic growth by bridging communications between cancer and dweller cells. Prostate cancer cells metastasizing to the bone will most likely target a stem-cell niche and address the CXCL12/CXCR4 pathway, which is normally reserved for hematopoietic stem cell physiologic regulation[173]. Overexpression of the cytokine CXCL12 and its receptor CXCR4 in malignant prostate cells strongly promotes proliferation, migration, and invasion[174]. Primary tumors can also release tumor-derived factors such as chemokines, cytokines, and hormones, which prime distant organs to nurture the arriving cancer cells, resulting in the formation of a pre-metastatic niche[175]. This supportive metastatic microenvironment at distant sites is frequently characterized by increased vascular permeability, reorganization of the extracellular matrix, recruitment of bone marrow-derived cells, angiogenesis, and immunosuppression[176]. Angiogenesis is a hallmark of cancer and aids tumor progression in many ways[4]. Cancer therapies targeting the vascular endothelial growth factor (VEGF), a potent angiogenic factor, have been suggested[177].

Investigate Single Cells in the -omics Time

Two revolutionary methods for sequencing nucleic acids at the single-cell level were introduced around a decade ago. The first method allowed for the sequencing of single-cell DNA (scDNA-seq) and the inference of tumor evolution using individual cells[73]. The second method described the whole transcriptome at the scale of single cells through single-cell RNA-sequencing (scRNA-seq)[178]. Since the introduction of these two single-cell sequencing (SCS) methods, the field has advanced rapidly, and thousands of cells can now be sequenced in parallel, delivering unprecedented resolution of single-cell phenotypes and remarkable insights into cancer progression.

Despite significant progress in single cell methods over the last decade, cancer research continues to struggle to fully adopt single cell profiling, and traditional approaches of sequencing a mixture of cancer cells (termed bulk sequencing) are still widely used, particularly in genomics studies[179]. Single cell sequencing has some limitations at the moment when compared to bulk sequencing methods; SCS is time-consuming and requires a rigid experimental design, which is often impractical because it requires live single cell dissection. SCS, compared to traditional bulk sequencing, requires challenging experimental procedures, burdensome downstream data analysis, and might suffer higher technical variations. Despite some advantages over SCS, bulk sequencing provides an average signal from different cell types and only gives rough estimates of cancer clones present in a tumor sample. As a consequence, bulk methods cannot resolve cell-to-cell variations of heterogeneous cell populations and are unable to dissect the cellular components of a small number of cells[180]. Single-cell sequencing methods, on the other hand, can resolve the confounding effects of different cell types in heterogeneous samples that cannot be separated using traditional bulk methods. Single-cell sequencing has the capability to measure the -omics (genomic, epigenomic, and transcriptomic) heterogeneity of a cellular population, and the changes happening at these levels. A significant shift toward single-cell technologies is expected over the next few years, even if it is already occurring (Figure 2).

The recent commercialization of a plethora of sequencing methods provides stable platforms for single-cell sequencing, and an increasing number of single-cell methods have been developed to profile a specific omics layer. Although single-cell sequencing and omics studies have grown in popularity in cancer research, their use in clinical studies remains limited. Costs, throughput, the lack of a rigorously standardized methodology, and straightforward reproducibility remain critical issues.

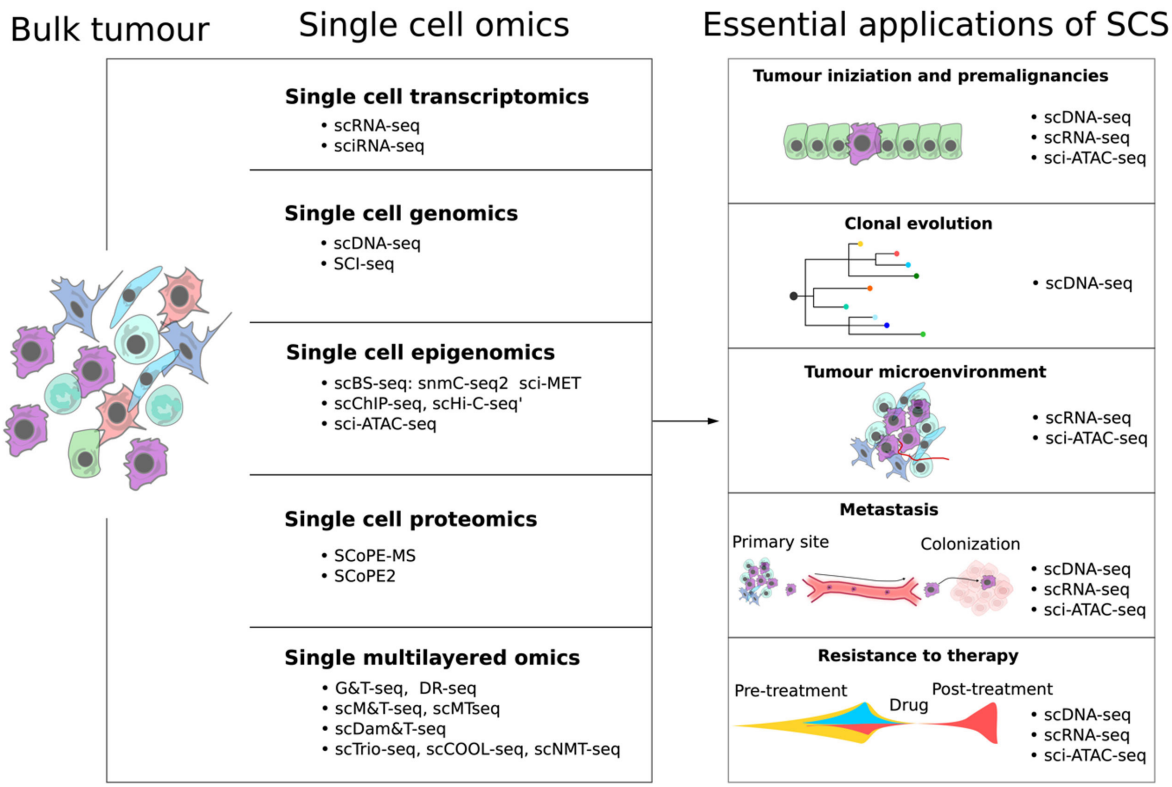

Figure 2. Single cells cancer research applications. Schematic of single cell omics fields and relative technologies. Essential single-cell sequencing (SCS) applications include investigating tumor initiation, clonal evolution, tumor microenvironment, metastatic dissemination, and therapeutic resistance.

Single Cell Transcriptomics

Single-cell RNA sequencing provides a precise understanding of the transcriptomic status of a heterogeneous cell population, which may include tumor cells, immune cells, fibroblasts, and endothelial cells, among others. It estimates the number of transcripts present in a single cell. Despite a variety of methodologies existing for single-cell RNA sequencing, nearly all involve poly-A selection and are thus suitable for mRNA. This limitation burdens the capability to investigate non-polyadenylated transcripts, such as small nucleolar RNAs, histone mRNAs, pre-mRNAs, and long noncoding RNAs, which may have diverse regulatory roles in cancer[181]. For the measurement of transcripts in each cell, reverse transcription of very small amounts of RNA and complementary DNA (cDNA) amplification must be performed, followed by the sequencing of the amplified molecules. This goal can then be met using two distinct procedures: full-length transcript sequencing and 3'/5'-tag capturing sequencing.

The first method generates full-length cDNA-amplified products and achieves full-length coverage across transcripts; an example of this approach is given by Smart-seq2, a method that allows the generation of full-length cDNA libraries from individual cells[182]. Despite the benefit of having complete coverage, full-length transcript sequencing requires processing hundreds to thousands of single cells with small amounts of liquid, which is currently difficult to accomplish. However, this approach is constantly evolving, and third-generation sequencing, such as Pacific BioSciences (PacBio) technology, now allows for the generation of high-confidence full-length transcripts as well as the detection of RNA isoforms of individual cells[183].

Different and widely used technologies use 3'/5'-tag capturing protocols where sequencing barcodes are located at the 3' or 5' end of the RNA. Microdroplet-based systems such as Chromium (10× Genomics) employ an oil droplet to carry out reverse transcriptase and generate cDNA for a single cell. Each oil droplet contains a cell/nucleus, reaction liquid, and a barcoded

bead. The resulting cDNA library can then be sequenced. Microwell-based systems, such as the BD Rhapsody, accommodate only one cell and one oligo-barcoded bead in each microwell, which captures mRNA molecules after single-cell lysis. After that, all of the beads are combined into a single tube for reverse transcription and cDNA amplification, library construction, and sequencing. Both microdroplet and microwell protocols allow for the easy handling of thousands of single cells and typically result in high cell throughput.

For even higher throughput and lower costs, a novel method known as single-cell combinatorial indexing (sciRNA-seq) has been proposed. This is a combinatorial indexing method with a complex procedure that uniquely barcodes the RNA contents of each cell[184]. Although sci-RNA has a high single-cell throughput, it generates sparse coverage resulting in a lower efficiency of the generated sequences[185].

Full-length transcript approaches typically have high sensitivity of gene detection but low cell throughput (e.g., 100-300 cells for Smart-seq2). Methods based on 3'/5' -end transcripts usually have high cell throughput (e.g., 1,000-10,000 cells for microdroplet/microwell-based systems, ≥ 50,000 cells for sciRNA-seq) but low detection accuracy[186].

As single-cell sequencing generates sparse, multidimensional data, suitable bioinformatics tools should be selected according to the research purposes and the data set. For example, Seurat[187] is an R package for scRNA-seq analysis that includes data filtering, normalization, scaling, dimensionality reduction, clustering, and visualization.

Single Cell Genomics

Single-cell genome sequencing allows for the profiling of genetic heterogeneity in single-cell populations and is widely used to study the somatic mutational landscape of cancer cells. Individual cells' genomes can be sequenced using either the low cell-throughput (10-100) method of single-nucleus sequencing[73] or the high cell throughput (10,000-20,000) approach based on combinatorial indexing and termed SCI-seq[188]. Both methods have sparse coverage and are primarily useful for identifying copy number variations.

The main limitation of single-cell DNA sequencing is the amount of DNA that can be extracted from a single cell, which is usually very small and requires an amplification step prior to sequencing. Various whole-genome amplification (WGA) methods, such as multiple displacement amplification[189], multiple annealing, and looping-based amplification cycles[190], have been developed to amplify the genomic DNA of individual cells. Because a human single cell contains only two copies of genomic DNA, WGA is extremely challenging and frequently results in nonuniform genome coverage, allelic dropout, and amplification artifacts after the sequencing step. All of these factors are currently the main limitations of scDNA-seq, and we have yet to see a significant technological advancement capable of overcoming those limitations. However, various bioinformatics tools such as SCcaller[191], LiRA[192], and Conbase[193] have been developed to address these limitations and are capable of detecting somatic variants despite the aforementioned constraints.

Single Cell Epigenomics

Individual cells' epigenetic status can be assessed using three main strategies: 1) profiling the whole-genome DNA methylation status; 2) determining the genome-wide binding sites of DNA-associated proteins; 3) estimating the chromatin accessibility level.

Single-cell DNA methylation profiling can be estimated through single-cell bisulfite sequencing (scBS-seq)[194], which converts unmethylated cytosines (C) to uracils (U) by bisulfite treatment and estimates the CpG methylation status across the entire genome. Two recent scBS-seq approaches, snmC-seq2[195] and sci-MET[196], can profile the single-cell DNA methylome at high cell throughput (1,000-5,000). However, estimating methylation levels in single cells remains difficult and can result in high levels of artefactual sequences such as adapter dimers, a low mapping rate, or a small insert size.

The following methods can be used to investigate the genome-wide binding sites of DNA-associated proteins: single-cell ChIP-seq (scChIP-seq)[197] (cell throughput: 1,000-10,000) and single-cell Hi-C (scHi-C-seq) (cell throughput: 1-10)[198]. ScChIP-seq can be performed in a highly parallel manner using a droplet microfluidics-based procedure called Drop-ChIP, unique molecular barcodes, and next-generation sequencing[197]. Chromatin immunoprecipitation of a single cell followed by scChIP-seq is usually used for mapping histone modifications, transcription factors, and other protein-DNA interactions genome-wide. ScHi-C-

seq is a chromosome conformation capture assay that quantifies interactions between closely spaced genomic loci in 3D space to examine the spatial, three-dimensional genome organization of a single cell. Despite the technology's promise for genome 3D mapping, it suffers from extreme data sparsity, making analysis difficult[199].

The sci-ATAC-seq (single-cell assay for transposase accessible chromatin sequencing; cell throughput: 10,000-20,000)[200] can be used to investigate chromatin accessibility across the entire genome at the single-cell level. Sci-ATAC-seq identifies open chromatin patterns and assesses how chromatin packaging influences gene expression. The technology uses the Tn5 transposase, which tags open chromatin regions with sequencing adaptors. Tagged regions are then PCR amplified and sequenced. Several single-cell platforms, including the Chromium systems (10x Genomics), enable single-cell ATAC-seq.

Single Cell Proteomics

Single-cell proteomics is a field in rapid development aiming at comprehensively measuring the expression patterns of proteins at a given point in time at the single-cell level. Single-cell proteomics adds another level of investigation beyond the above-described omics methods, as it can interrogate the post-translational states of a single cell. Most traditional methods for identifying and quantifying proteins rely on antibodies barcoded with DNA sequences, fluorophores, or transition metals, as well as mass spectrometry (MS). These methods have limited identification capabilities because they cannot simultaneously identify a large number of proteins and frequently rely on antibodies with low target specificity, resulting in nonspecific protein detections[201]. Furthermore, some technical issues concerning the amount of sample required and the extent of protein coverage complicate the application of traditional mass spectrometry to single cells. These challenges are now being addressed by emerging MS-based technologies that do not rely on antibodies, such as Single Cell ProtEomics by MS (SCoPE-MS)[202] and its second-generation approach, SCoPE2[203]. This methodology allows for high-throughput protein quantification in single cells using mass spectrometry. Single cells are isolated by FACS or CellenONE into multiwell plates, lysed, and then their peptides are tagged by isobaric labeling for multiplexed analysis and quantification by mass spectrometry.

A note of mention goes to the recent field of single-cell metabolomics, which, theoretically linking genomics, transcriptomics, and proteomics, brings connections between genotype and phenotype at the single-cell level and can be important to assess the efficacy of drug delivery to targeted cells.

A special mention should be made of the emerging field of single-cell metabolomics, which theoretically connects genomics, transcriptomics, and proteomics. Single-cell metabolomics establishes links between genotype and phenotype, which can be essential for evaluating how effective drugs are delivered to particular cells[204]. A single-cell metabolome can be profiled using different methodologies, such as nanospray desorption electrospray ionization (nano-DESI)[205] and laser desorption/ionization droplet delivery mass spectrometry (LDIDD-MS)[206].

Single Cell Multilayered Omics

Once a single cell is used for any single omics layer, the same cell cannot be used anymore to measure other layers of omics information. This single unimodal factor can be a big limitation in multi-omics studies, as multi-omics data inferred from different cells may suffer from both biological and technical variations. Each type of omics is, in fact, measured using unique protocols from different cells, which could impact the robustness of the analysis. However, currently, a number of techniques have been developed to analyze two or more omics layers from the same single cell[207]. For measuring two different omics modalities, G&T-seq[208] and DR-seq[209] allow an integrated and simultaneous genome and transcriptome analysis. Both methods achieve an accuracy in the copy number and expression profile comparable to that attained by the unimodal methods previously mentioned. For simultaneous profiling of the transcriptome and methylome from the same cell, scM&T-seq[210] and scMTseq[211] can be used. ScDam&T-seq[212] can be employed to profile the transcriptome and to measure protein-DNA interactions in the same cell at the same time. The ability to simultaneously study three different omics in a single cell is becoming more common. Examples include scTrio-seq[213] for sequencing the genome (copy number variations), methylome, and transcriptome; scCOOL-seq[214] for estimating chromatin state, DNA methylation, and the genome (copy number variation and ploidy); and

scNMT-seq[215] for investigating the transcriptome, DNA methylation, and nucleosome.

Conclusions

The importance of cancer progression fuelled by clonal evolution has grown in the last ten years. Tumor heterogeneity, the most common cause of antitumor drug resistance, is caused by the genetic, epigenetic, and microenvironmental selective pressures that cancer cells face as the disease progresses. Many evolutionary models, including parallel evolution and linear evolution based on Darwin's theory, have been adopted in an effort to understand the evolutionary causes of cancer. However, mounting evidence suggests that the dynamics of cancer evolution sometimes appear to defy Darwinian principles. Darwinian mechanisms have historically been grounded on a gene-centric understanding of evolution, but increasing evidence suggests that non-genetic factors, such as cell plasticity and tumor microenvironment, play a role in the evolution of cancer. Whether viewed from a Darwinian or non-Darwinian perspective, many of the various evolutionary patterns result in cancer drug resistance. The probability of therapy resistance and metastasis is likely explained by cell traits that promote adaptation and cancer evolution. Understanding the mechanisms underlying cancer evolution would allow for the development of therapies that target the cell's ability to evolve while also improving drug efficacy and preventing metastases. This is a win-win situation.

With the advent of single-cell sequencing technologies, cancer research is entering a new phase. These new sequencing technologies have enormous potential for use in cancer research, allowing researchers to answer a wide range of biological and clinical questions (Figure 2). SCS can be used to accurately compare the gene expression of different cancer cells and their (epi-)genetic profiles, including those from primary and metastatic tumors, and thereby to create omics maps of single cancer cells. It is now possible to identify clinically significant tumor subpopulations, such as those with drug resistance or metastatic progression, thanks to the widespread adoption and increased throughput of scRNA-seq platforms. Furthermore, scDNA-seq methods can be used to determine whether resistant clones acquired resistance mutations as a result of treatment (acquired resistance) or if they existed in the tumor mass prior to therapy and were selected after (adaptive resistance). With the advancement of technology, mass spectrometry is also becoming able to characterize thousands of proteins at the level of the individual cell. Experiments that evaluate multiple omics layers in the same single cell provide new insights into the mechanisms that govern the state, diversity, and evolution of cancer cells.

In closing, the studies we examined herald the beginning of a new era in single-cell research, an omics-oriented era that is rapidly expanding in capacity, scale, and resolution. SCS and multi-omics have already revolutionized many areas of cancer research and are poised to have an even greater impact in the clinic. Overall, we anticipate that the adoption of SCS in oncology over the next ten years will significantly improve cancer detection and treatment, as well as significantly enhance cancer patient diagnosis and care.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

Conceptualization L.E. and M.A.; writing, collection/researching of articles, original draft preparation, and illustrations, L.E.; writing, review and editing, S.T., A.G., and M.A.; supervision, M.A. All authors have read and agreed to the published version of the manuscript.

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