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Untangling the web of intratumour heterogeneity

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Intratumour heterogeneity (ITH) is a hallmark of cancer that drives tumour evolution and disease progression. Technological and computational advances have enabled us to assess ITH at unprecedented depths, yet this accumulating knowledge has not had a substantial clinical impact. This is in part due to a limited understanding of the functional relevance of ITH and the inadequacy of preclinical experimental models to reproduce it. Here, we discuss progress made in these areas and illuminate future directions.

Cancer is a global health and socioeconomic problem that is affecting an increasing number of people worldwide. Despite intensive research and new treatment strategies, most cancers are still incurable largely due to the high degree of tumour heterogeneity. Every tumour has a specific composition of cancer cell subclones, stromal cells and microenvironmental conditions that together define tumour growth, progression and treatment response. To tackle this complexity and to develop more effective therapies, we need to better understand the drivers of ITH and how different tumour traits influence each other to create phenotypes specific to heterogeneous tumours.

Recent technical and computational advances have allowed us to dissect tumours at extraordinary depths. We can analyse tumours at single-cell resolution and follow temporal and spatial patterns. Experimental models have also evolved, enabling us to mimic tumour heterogeneity and tumour–stroma interactions more faithfully. Moreover, innovations in mathematical modelling and artificial intelligence (AI) are helping to deconvolute the growing amount of multidimensional data in both clinical and experimental tumours. In this Review, we summarize our current knowledge of the drivers of ITH and highlight technological advances to characterize ITH. We further elaborate on the role of ITH in tumour evolution, including metastatic progression and immune escape.

Cell-autonomous sources of ITH

Tumours follow a Darwinian evolution mechanism in which dynamic microenvironments act on diverse cell populations to select for the fittest subclones. There are many sources of ITH, including genetic, epigenetic and microenvironmental[1,2], which we can broadly classify as cell-autonomous and non-cell-autonomous. Cell-autonomous factors drive mutant phenotypes only in mutant cells, whereas non-cell-autonomous factors cause other cells (regardless of their genotype) to exhibit a mutant phenotype. Cell-autonomous sources of ITH include the cell of origin of the cancer and cancer-cell-specific genetic and epigenetic alterations. Non-cell-autonomous factors can be derived from cancer cells or stromal cells and they include physical variables such as hypoxia and shear stress (Fig. 1). However, this classification is arbitrary as there are extensive interactions between cell-autonomous and non-cell-autonomous factors, as discussed below.

Tumours are thought to originate from normal tissue-specific progenitors or stem cells with proliferative capacity[3]. The tissue of origin and the differentiation capacity of the tumour-initiating cell (that is, the cell of origin) are generally preserved in the resulting tumour and can be a source of ITH, generating tumours composed of cancer cells that represent different lineages. Single-cell transcriptomic profiling of gastric adenocarcinoma peritoneal metastases[4] revealed colorectal-like, duodenal-like, gastric and entero-goblet cell subtypes. These potentially reflected differences in the normal cell-of-origin of cancer that correlated with clinical outcomes independent of histopathological features[4]. Patients with gastric-dominant metastases had shorter survival times compared with patients with mixed lesions; however, the small cohort size and poor overall survival limit these conclusions. Similarly, in a transgenic mouse model of *KEAP1*-deficient $KRAS^{G12D}$-mutant lung adenocarcinoma, double-mutant tumours arose from bronchiolar cells that lacked the pro-tumorigenic macrophage expansion characteristic of alveolar-origin tumours[5]. At the same time, metabolic reprogramming in the double-mutant tumours resulted in a dependency on the pentose phosphate pathway, thereby revealing a new therapeutic vulnerability for *KEAP1*-deficient $KRAS^{G12D}$-mutant lung cancer. These examples demonstrate that the normal cell-of-origin of cancer can be a source of ITH that can also affect the tumour microenvironment (TME).

In human tumours, the cell of origin is not always feasible to define in part because of epigenetic plasticity during disease progression that can lead to the acquisition of new cell states. In some cases, epigenetic plasticity and ITH can be caused by mutant or aberrantly expressed epigenetic regulators. Cell-of-origin and cell-type-specific epigenetic programmes have a major impact on tumorigenesis because the expression of the same oncogene in different cell types can have highly different outcomes. For example, in zebrafish and in human induced pluripotent stem cell models, the expression of $BRAF^{V600E}$ in neural crest and melanoblasts resulted in large transcriptional changes and tumour formation, whereas melanocytes showed limited response and did not efficiently transform[6]. Many of the genes induced by $BRAF^{V600E}$ in the transformation-permissive cell types encode epigenetic regulators including *ATAD2*, which by itself can switch cells to a transformation-permissive state through SOX10, a key neural crest transcription factor (TF). These findings could potentially explain why melanoma rarely develops despite the presence of the $BRAF^{V600E}$ mutation in nearly all nevi and why some tumour types might be less susceptible to lineage changes and are therefore less heterogenous.

Similarly, the expression of mutant SMARCA4 (the gene encodes a SNF/SWI chromatin remodelling complex subunit) in

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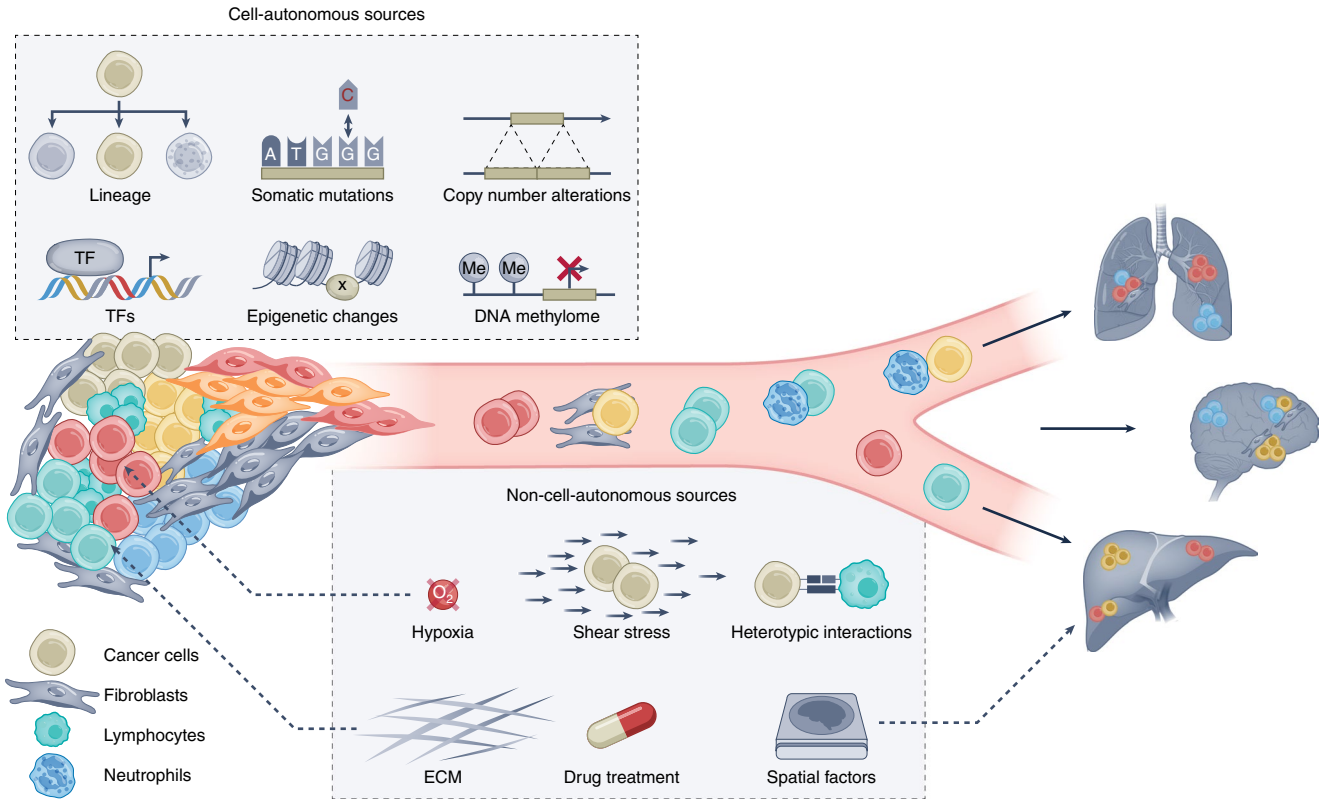

Fig. 1 | Cell-autonomous and non-cell-autonomous sources of ITH. ITH can already be present at tumour initiation and is continuously reprogrammed during progression. Cell-autonomous sources of ITH include the normal cell-of-origin of cancer, genetic alterations (point mutations and copy number changes), epigenetic plasticity (variability in histone and DNA methylation patterns) and transcription factors (TFs). Non-cell-autonomous sources include cues from the TME such as hypoxia, shear stress, ECM stiffness and heterotypical interactions with non-malignant cells (for example, neutrophils, lymphocytes and fibroblasts) as well as selection by cancer therapies. Both sources of ITH interact with each other to promote tumour initiation and progression, such as distant metastases in different organs (for example, lung, brain and liver).

the *Kras*$^{LSL\text{-}G12D/+}$;*Trp53*$^{fl/fl}$ mouse model of lung cancer disrupted lung-specific epigenetic programmes in lung club cells[7]. Moreover, it increased the susceptibility to oncogenic transformation to enable the outgrowth of epigenetically heterogenous metastatic tumours[7]. SMARC4 can be a component of all three SWI/SNF complexes (that is, cBAF, ncBAF and PBAF) that regulate chromatin landscapes. Therefore, the loss of normal SMARCA4 function leads to global chromatin changes and decreases the accessibility of lung-lineage-specific loci. Mutations in *SMARC4* are common in a wide variety of cancer types and are associated with undifferentiated features and poor clinical outcomes[8]. Cells with highly plastic epigenetic states have high tumorigenic and metastatic potential and therapeutic resistance[9,10], which provides motivation for the therapeutic targeting of these cells and the underlying mutations.

Genetic heterogeneity is a main source of functionally relevant ITH. Cancer cells often harbour defects in DNA repair pathways that increase mutational burden and chromosomal instability[11,12]. Thus, somatic mutations or copy number alterations (SCNAs) commonly arise and their phenotypic consequences trigger clonal selection in cancer progression. Small-scale focal SCNAs are thought to be indicative of selection for individual cancer driver genes. *MYC* is a commonly amplified cancer driver gene and MYC-driven tumours are sensitive to BET bromodomain inhibitors[13]. However, *MYC* amplification can be heterogeneous within tumours, which in turn causes heterogeneity in therapeutic sensitivity. In B cell lymphoma, ex vivo drug responses of cells from CD48^{high}CD62L^{+} subclones exhibited exclusive sensitivity towards BET inhibitors owing to the specific copy number gain of *MYC* at chromosome 8q24 (ref. [14]). Similarly, in a pancreatic ductal adenocarcinoma mouse model (KPCXY model) and utilizing multifluorescence lineage-labelling, clones with focal *MYC* amplification possessed higher metastatic potential by recruiting premetastatic macrophages through CXCL3 and MIF[15]. Although small-scale focal SCNAs at specific well-characterized genetic loci are relatively easy to be interpreted, they only represent the tip of the iceberg. Large-scale genomic imbalance on the order of megabases occurs as subchromosome copy number variants or whole-genome aneuploidy. In patients with relapsed–refractory multiple myeloma, single-cell omics profiling revealed a rare subpopulation of cells (~2%) with a wide-range chromosome 1q gain that frequently expanded during different treatments, such as carfilzomib and dexamethasone, and was associated with inferior outcomes[16]. Conversely, aneuploidy is associated with either reduced or enhanced clonal fitness in a context-dependent manner, which is in part due to the need for an optimal oncogene-to-tumour suppressor gene balance to fully support tumour growth[17]. Last, global chromosomal copy number heterogeneity is a common prognostic feature across all cancer types, which was demonstrated using a scalable quantification method and single-sample copy number profiles[18]. The impact of other genetic structural genetic variants, such as gene fusions and non-coding alterations on ITH, is still poorly defined.

Epigenetic ITH is a major source of phenotypic heterogeneity owing to stochastic and microenvironmental inputs, and often arises with aberrations of histone modifiers and DNA methylomes[19]. In contrast to genetic ITH, these highly dynamic epigenetic programmes are likely to account for acquired traits triggered by external stimuli with a reversible nature, such as tolerance to

therapeutic agents[2]. Examples of epigenetic regulators that modulate ITH and treatment resistance are the histone H3 lysine 4 (H3K4) demethylase 5A (KDM5A) and 5B (KDM5B). Higher levels of these enzymes increase cellular transcriptomic heterogeneity and resistance to endocrine therapy in oestrogen-receptor-positive (ER^+) breast cancer[20] and to EGFR inhibitors in non-small cell lung cancer[21]. As these enzymes are therapeutically targetable, their inhibition could decrease ITH and therefore increase the efficacy of targeted treatments and chemotherapies. This idea is well supported by the increased sensitivity of ER^+ breast cancer lines to anti-oestrogen therapy following genetic deletion of *KDM5B* or pharmacological inhibition of KDM5 activity[20]. *KDM5B* and *KDM5A* are commonly overexpressed and amplified in a subset of tumours, which demonstrates that genetic and epigenetic sources of ITH are intertwined in heterogeneous tumours.

Another example of interactions between genetic and epigenetic sources of ITH is the link between mutations in *KMT2C* (which encodes a H3K4 methyltransferase) and *APOBEC* mutagenesis in multiple cancer types[22]. Tumours with *KMT2C* mutations have an excess *APOBEC* mutational signature and altered patterns of open chromatin regions. Loss of KMT2C induces DNA replication stress, which increases APOBEC expression and deaminase activity. Analyses of three-dimensional chromatin structures using the Hi-C technique revealed *APOBEC* mutation clusters in interacting regions, which potentially reflect replication factories. Replication stress also perturbed DNA repair pathways, which further increased genetic instability and ITH.

DNA methylation is the best-understood stable epigenetic mark, but its role in the regulation of clonal and cellular ITH is still poorly defined. Investigations of clonal and transient DNA methylation and transcriptional states have been performed. In one study[23], the Luria–Delbruck experimental design (that is, repeated sampling over time and comparison of profiles of single cells in a bulk founder population to that of single cell subclones derived from the same founder population) was applied. Longitudinal single-cell DNA methylation and transcriptional profiling of clonal colon cancer populations revealed dynamic DNA methylation signatures tightly associated with a slow drifting spectrum of epithelial-to-mesenchymal transition (EMT) states, which was apparently independent of genetic variants. Although DNA methylation remained relatively stable in most of the genome, high-degree clonal and cellular variability was observed in CpG islands, which reflect epigenetic instability.

A recent study[24] utilized single-cell profiling and an expressed barcoding strategy for lineage tracing in a mouse model of MLL–AF9 fusion-driven acute myeloid leukaemia to characterize ITH. The authors found that non-genetic determinants of ITH dictate malignant clonal dominance and that the transcriptional features of cancer-initiating cells are heritable[24]. Higher transcriptional heterogeneity was associated with higher clonal fitness, and leukaemia stem cells had variable clonal outputs that were also heritable. Notably, clonal dominance was associated with the high expression of *Slpi* (which encodes a secretory leukocyte protease inhibitor) and downregulation of genes involved in antigen processing and presentation, which implicated immune escape as a key determinant of clonal fitness.

Apart from epigenetic regulators, TFs can also drive ITH. This was exemplified by the recognition of new intrinsic neuroendocrine prostate cancer subtypes based on the expression of the neuronal TFs ASCL1 and NEUROD1. These caused divergent transcriptomic, epigenetic and histopathological features towards two subpopulations in the same tumour[25]. ASCL1- and NEUROD1-driven subpopulations were transcriptionally distinct, characterized by cytokine response and enrichment in brain developmental pathways, respectively. Notably, NEUROD1-driven cells also exhibited a specific copy number amplification at 14p and 7p, which suggested that ITH governed by TFs and SCNAs could be tightly linked and occur together in the same subpopulation.

ITH is often shaped by the coordinated action of multiple TFs instead of one master regulator. Cancer cells are commonly subjected to environmental stress, which leads to a specific stress-like cell state conserved across species and governed by TF networks including Fosb, Jun and Fosab[26]. Cancer cells in a stress-like state are enriched for heat-shock-response genes and exhibit higher tumour seeding capacity as well as MEK and BRAF inhibitor resistance in melanoma. Other studies have demonstrated a crucial role for heat shock signalling in enabling morphological evolution in lower eukaryotes such as *Drosophila*[27] and more recently in human tumours through the TF HSF1 (ref. [28]). Thus, the association of a stress-like cancer cell state with increased tumour initiation and therapeutic resistance may reflect increased phenotypic heterogeneity in these cell populations.

Another example of TF-driven ITH and tumour-promoting subclonal cooperation is the regulation of proliferative and invasive melanoma subpopulations by differing levels of the neural crest TF TFAP2 (ref. [29]). These subclones form heterotypic structures in which proliferative cells surround invasive cells, and the resulting circulating tumour cell (CTC) clusters facilitate collective dissemination and metastatic outgrowth. An invasive (mesenchymal) phenotype is usually associated with the secretion of cytokines that promote angiogenesis and suppress antitumour immune responses (for example, TGFβ). This might explain why invasive subclones are maintained within primary tumours despite their lower proliferation rate.

Non-cell-autonomous sources of ITH

Tissue architecture and normal cellular interactions are some of the main barriers to tumour initiation, and alterations in the microenvironment due to physiological (for example, ageing) or pathological (for example, inflammation) conditions favour tumorigenesis[30]. TME heterogeneity is greatly affected by ageing[31] and probably by many other host factors, such as gender and germline polymorphisms, which highlights the need to also subclassify cancer-associated non-malignant cells in the host. The TME in primary tumours is highly heterogeneous and composed of various tissue-resident cells altered by the tumour and recruited cells from distant sites[32], and this heterogeneity further increases in metastatic disease. Cancer-associated fibroblasts (CAFs) are highly heterogeneous and can be classified into four distinct subtypes (CAF-S1 to CAF-S4) distinguished by the expression of FAP, CD29, αSMA, PDPN and PDGFRβ in both primary breast tumours[33] and in lymph node (LN) metastases of breast cancer[34]. In primary tumours, CAF-S1 constitute an immunosuppressive environment by secreting CXCL12 and recruiting $CD4^+CD25^+$ T lymphocytes and promote their differentiation to $CD25^+FOXP3^+$ regulatory T cells. In contrast, in LN metastases, CAF-S1 induce EMT and cancer cell invasion through CXCL12 and TGFβ. CAF-S4 are similar to CAF-S1 as they also show enrichment in primary triple-negative breast cancers and promote metastasis in LNs, but they are functionally different and act through the NOTCH signalling pathway[34].

The TME is a major source of non-cell-autonomous factors that affect cancer epigenetic and transcriptional landscapes and drive the selection of cancer cells with the highest fitness during disease progression[35]. Phenotypic diversifications also entangle with each other as a combinatory consequence of sensing a multitude of environmental inputs from distinct spatial elements. An example of this is the spatial heterogeneities of hepatocellular carcinoma CTCs enumerated from different vascular sites, including the hepatic vein, the peripheral artery and the peripheral and portal vein[36]. Each site is linked to a specific transcriptomic pattern and different degrees of heterogeneity. Pseudotemporal kinetic analysis depicted the

striking evolutionary route of these four sites when following the anatomical blood flow pathways of CTC dissemination.

Physical cues of the TME, such as hypoxia and mechanical pressure, also serve as selection pressures during progression and extensively rewire ITH[37,38]. A hypoxic gradient is present either within a single solid tumour (for example, spatial distance from vessels)[39] or among different metastatic niches[40]. Mechanisms of adaptation to hypoxia are highly variable among different cancer cell subpopulations. In glioblastoma, long-term hypoxia exposure of patient-derived cancer cell cultures diversified 16 subpopulations with distinct adaptation kinetics towards hypoxia, which could be discriminated by the heterogeneous expression of the stem cell markers CD133, CD44, CD15 and A2B5 (ref. [41]). Cells with a more plastic adaptive state accelerate tumorigenesis; therefore, hypoxia in primary tumours is associated with higher risk of progression. Hypoxia can also dictate dormant cell states within tumours. For example, hypoxia in breast cancer induced a subset of disseminated tumour cells (DTCs) displaying high NRF2, DEC2, p27 and TGFβ expression to evade chemotherapy through an increased dormant capacity[42].

Stiffness of the extracellular matrix (ECM) has been characterized as a pivotal modulator of subclonal selection, in part by serving as a physical barrier and creating spatially distinct niches within tumours[37]. Vitronectin, a glycoprotein related to a stiff matrix, is highly abundant in high-risk neuroblastoma, a paediatric solid tumour associated with poor outcomes. Neuroblastoma growth in hydrogel co-cultures with Schwann cells revealed a positive selection for cancer cell subclones with chromosome 9 aberration in stiffer matrix as well as in xenografts implanted in vitronectin-knockout mice[43]. Importantly, 40% of high-risk neuroblastoma clinical samples harbour chromosome 9 aberrations with an imbalance of *DOCK8* and *KANK1*, genes that encode proteins that regulate cell shape and motility. This finding also supports the idea that cell-autonomous and non-cell-autonomous sources of ITH contextually intersect, and genetic aberrations can confer a fitness advantage to environmental stress.

Heterotypic interactions between cancer and non-malignant cells at different anatomical locations also increase ITH through paracrine loops. In pancreatic cancer, CAFs reshape ITH by prompting the diversification of distinct subpopulations towards EMT and proliferative phenotypes through STAT3 and MAPK signalling, which are spatially correlated with the architecture of tumour gland types[44]. In the context of blood circulation, neutrophil-educated CTCs exclusively retained cell cycle progression that is mediated by VCAM1 compared with single CTCs[45] in both patients with breast cancer and in mouse models of breast cancer.

Non-cell-autonomous and cell-autonomous factors are highly intertwined. Reshaping of the TME by a genetically defined dominant clone can facilitate the outgrowth of less fit minor subclones. In a mouse model of colorectal cancer, invasive metastatic clones with $Apc^{\Delta 716}$, $Kras^{G12D}$, $Tgfbr2^{-/-}$ or $Trp53^{R270H}$ mutations created a fibrotic niche by activating hepatic stellate cells through TGFβ signalling to facilitate metastatic colonization of other non-metastatic clones[46]. Likewise, external stimuli can promote new genomic mutagenesis in cancer cell subclones that accelerates therapeutic resistance. In a longitudinal genomic study of patients with acute lymphoblastic leukaemia, early relapse highly relied on bona fide resistance mutations induced by chemotherapy[47]. A new thiopurine-treatment-induced mutagenesis signature was found to be responsible for 46% of acquired resistance mutations in *NT5C2*, *PRPS1*, *NR3C1* and *TP53*.

Besides indirect interactions through the TME, cancer cell subclones can directly interact with each other through juxtracrine and paracrine interactions, many involving known oncogenic pathways such as WNT and NOTCH signalling[48]. WNT plays important roles in tissue stem cell maintenance, and dependence on WNT signalling is a common feature of cancer cells with stem-cell-like features. However, WNT-secreting and WNT-dependent cell populations are distinct and cooperative as observed in human breast and lung carcinomas and in experimental models[49,50]. Such cooperative interactions between more differentiated and $LGR5^{+}$ stem-cell-like cancer cells potentially explain the preservation of differentiation hierarchies observed in some tumours such as colorectal cancer[51]. Cooperation between cells of two different lineages is also observed in small-cell lung cancer, a highly aggressive neuroendocrine lung tumour, through the heterogeneous activation of NOTCH[52]. Endogenous activation of NOTCH in a subset of cancer cells prevented neuroendocrine differentiation and resulted in a slow-growing phenotype resistant to chemotherapies. These non-neuroendocrine small-cell lung cancer cells also supported the growth of the neuroendocrine cancer cells favouring the selection for cooperative subpopulations within tumours.

Finally, the degree and sources of ITH are variable during different stages of tumour evolution. Thus, inference of ITH determinants also needs to consider temporal trajectories.

Experimental approaches to characterize ITH

Single-cell omics technologies facilitate detailed high-dimensional heterogeneity measurements within tumours[53,54] (representative recent single-cell multiplexed technologies are summarized in Table 1). However, our understanding of the functional relevance of ITH is limited in part due to the paucity of suitable experimental models. Profiling patient specimens only gives a glimpse of the generally already advanced tumour, as detecting initiating steps and repeated sampling during progression are rarely feasible, especially in solid tumours. Although such snapshots can identify dominant and minor subclones, they are not sufficient to deconvolute clonal interactions, including elimination and competition. Mechanistic investigation of these questions requires robust experimental models that faithfully reproduce clinical scenarios and record evolutionary trajectories. We list recent examples of such models in Table 2 and highlight a few below.

Molecular barcoding[55,56] and optical approaches[57] were developed years ago to decipher cellular heterogeneity and to track subclones, and recent technological advances have enabled their combination with multi-omics profiling at single-cell resolution. The recently described ClonMapper barcoding system[58] allows single-cell transcriptomics mapped to clonality and enables the retrospective study of specific clones of interest with a clonal retrievable function. Application of this technology revealed subpopulation composition was associated with survivorship trajectory in a chronic lymphocytic leukaemia cell line under chemotherapy. It also reported clone-specific durable evolutionary transcriptomes by enabling the recovery of the same clone before, during and after treatment.

The fast-evolving CRISPR technology has expedited the development of multiple lineage tracking methodologies and conferred advantages in resolving temporal relationships among different passages to identify ancestors and offspring. A molecular recording technology[59] based on Cas9-enabeled inherited allele insertions and deletions (INDEL) paired with single-cell RNA sequencing (scRNA-seq) was applied to reconstruct the phylogenetic trees and molecular drivers of lung cancer metastases in xenograft models. Single-cell tracking of cancer cells over time revealed a high degree of heterogeneity for metastatic capacity and identified candidate genes driving these features. A related macsGESTALT system[60] based on an inducible CRISPR–Cas9 lineage recorder pointed out a role for rare, late-hybrid EMT states in promoting pancreatic cancer metastasis in vivo. Meanwhile, the cell lineage access driven by an edition sequence (CLADES) technology[61] also exploits CRISPR–Cas9 to build genetic switches to activate and inactivate fluorescent reporter genes in a predetermined order. CLADES enabled the temporal resolution of lineage development under perturbations grounded on the sequential cascades of reporters in the progeny[61].

Table 1 | Representative multiplexed single-cell omics technologies to assess ITH

Technology	Targeted omics	Application	Refs.
ASTAR-seq	Transcriptome, accessible chromatin	Mouse embryonic stem cells under naive, primed and pluripotent states	95
scCAT-seq		Human pre-implantation embryos	96
sciCAR-seq		Lung cancer cell lines treated with dexamethasone, adult mouse kidney	96
SNARE-seq		Neonatal and adult mouse cerebral cortices	97
SHARE-seq		Mouse skin cells	86
G&T-seq	Genomic DNA, transcriptome	Human breast cancer cell lines, mouse pluripotent stem cells	98
CITE-seq	Transcriptome, epitomes	Cord blood mononuclear cells	99
Paired-Tag	Transcriptome, histone modification	Adult mouse frontal cortex and hippocampus	100
ASAP-seq	Accessible chromatin, epitomes	Human peripheral blood mononuclear cells	101
epi-gSCAR	Genomic DNA, DNA methylome	Cells derived from acute myeloid leukaemia	102
scNOMe-seq	Accessible chromatin, DNA methylome	Human lymphoblastoid cell line and immortalized leukaemia cell line	103
scMT-seq	Transcriptome, DNA methylome	Adult mouse sensory neurons	104
inCITE-seq	Transcriptome, intranuclear protein	Mouse brain neural activity with pharmacological perturbation	105
scRNA-seq+ST	Spatial resolved transcriptome	Human pancreatic tumour, breast tumour	82,106
XYZseq		Syngeneic human colon adenocarcinoma murine model	107
scNMT-seq	Transcriptome, accessible genome, DNA methylome	Differentiating mouse embryonic stem cells	108
TEA-seq	Transcriptome, accessible chromatin, epitomes	Human peripheral blood mononuclear cells	109
DOGMA-seq			101

Last, the Watermelon system[62] simultaneously recorded the origin of each lung cancer cell clone and proliferative status under EGFR inhibitor selection. This method revealed sharply divergent lineage and metabolic programmes in cycling and non-cycling persisters[62].

Beyond tracking cancer cells, unravelling cancer cell and niche interactions, both cell-to-cell connections and cellular responses to various TME stresses such as hypoxia, is another underexplored area that requires robust in vivo models for preclinical investigation. Cancer cell subpopulations respond differently to external stressors, and this is linked to disease recurrence mechanisms, especially therapeutic responses[63]. To visualize this heterogeneity in stress responses, several reporter systems have been developed that enable the monitoring of cells both in vitro and in vivo. A hypoxia fate-mapping system built on a synthetic HIF1 response element driving a fluorescent reporter was used to identify pre-hypoxic and post-hypoxic breast cancer cells in three-dimensional spheroids and in xenografts[64]. The use of this reporter enabled the identification of a phenotype that was resistant to reactive oxygen species and enriched in post-hypoxic cells that promoted metastasis. A similar approach was used to design an in vivo pH ratiometric bioluminescent sensor, pHLuc, which produces a pH-responsive green fluorescence[65]. This marker captured the heterogeneous tumour response to acidosis in human synovial sarcoma xenograft models[65]. The use of these reporters in experimental models is ideal for investigating how non-cell-autonomous environmental factors shape ITH and for characterizing the functional relevance of heterogeneity in response.

Several recently developed models have enabled the decoding of cellular interactomes during tumour progression to evaluate the impact of non-malignant niche cells on ITH. The sLP-mCherry niche-labelling system[66] is based on a cancer-cell-released cell-penetrating fluorescent protein (mCherry), which is taken up by neighbouring cells and sustained intracellularly for up to 120 h. By applying this strategy in the 4T1 mouse mammary tumour model, specific stem-cell-like features in lung metastatic niches were uncovered. The related GFP-based Touching Nexus (G-bToN) tool[67] is built on nanobody-directed fluorescent protein transfer that allows high-resolution detection and quantification of distinct physical cell–cell interactions in cancer models, including cancer cell attachment with endothelial cells, T cells and neurons.

Undoubtedly, the next major ambition of ITH model development is to probe sources of cell-autonomous and non-cell-autonomous ITH simultaneously at single-cell resolution in a combined manner in vivo, preferentially using noninvasive approaches such as molecular imaging.

The impact of ITH on tumour progression

Quantitative measures of ITH in clinical samples have demonstrated that higher ITH is associated with higher risk of recurrence, regardless of cancer type and treatment[68]. High levels of ITH enable tumour adaptation to changes of the TME in many ways, which drives the selection for more aggressive phenotypes[69]. Several models have been proposed to explain tumour evolution, including linear, branched, punctuated and neutral evolution (discussed in detail in a recent review[1] and summarized in Table 3 and Fig. 2a). Although each of these models acknowledge the presence of ITH, not all recognize its functional relevance in tumour evolution. Most tumour evolutionary traits are defined by 'driver mutations' as an origin[70] even though evolution is driven by phenotypic traits. Parallel evolution either in primary and metastatic lesions or in different TMEs of the same tumour can result in higher ITH over time[71]. Evidence for a parallel evolution of intratumour subclones has been found by comparing copy number variant properties of different biopsies from a single tumour[72]. Independent of the evolutionary traits, consecutive mutations or alterations act synergistically to support tumour growth[73]. Such synergies are not restricted to specific cell clones but also occur among different lineages within the tumour through clonal or subclonal cooperation[48,74] (Fig. 2b). This has been demonstrated in a xenograft model of ITH generated by deriving different subclones expressing various secreted proteins from the same breast cancer cell line[75]. Here, certain subclones such as IL11-expressing cells highly supported the outgrowth

Table 2 | Experimental models to investigate ITH

Model type	Model name	Principle and advantages	Application	Refs.
Clone tracking	ClonMapper	Expression DNA barcoding and specific clone retrievable	Chronic lymphocytic leukaemia cell line chemoresistance	58
	BSVTK labelling	Five fluorophore combination for clonal tracking	Effects of different metastatic niches to triple-negative breast cancer subclones	110
Lineage tracking	Polylox	Endogenous Cre-*loxP* barcodes recombination	Haematopoietic stem cell specification during mouse embryonic development	111
	Rainbow-seq	Fluorescent marker-based lineage separation	Embryo division from two-cell stage	112
	Cas9-INDEL tracking system	Cas9-triggered inheritable allelic INDEL+ scRNA-seq	Phylogenetic rates, routes and molecular drivers of lung cancer metastasis	59
	macsGESTALT	Cas9-triggered inheritable allelic INDEL + scRNA-seq	EMT status of pancreatic cancer metastasis	60
	CLADES	Cas9-triggered fluorescent reporter activation and inactivation	Cascade progression across fly generations	61
	Watermelon	Fluorescence-based simultaneous clone and proliferation status tracking	Lung cancer cycling persister cells under EGFR inhibition	62
TME probing	Hypoxia fate-mapping system	Transgenic mouse with HIF-1 response element construct	Post-hypoxic cell adaption in mouse mammary tumour development	64
	pHLuc	pH-responsive fluorescent construct	Acidosis heterogeneity in sarcoma xenograft model	65
	sLP-mCherry	Secreted mCherry tag to label niche cells	Breast-cancer-associated parenchymal cells in lung metastatic niche	66
	G-bToN	Nanobody-directed fluorescent protein transfer	Heterotypic interactions between cancer cells and endothelial, immune cells and neuron	67
Patient-derived	Organoid	Ex vivo cultures and biobanks from patient specimens	Drug screen and functional studies in multiple cancer types	113–121
	Patient-derived xenograft (PDX), circulating-tumour-cell-derived explant (CDX) model	In vivo cultures and biobanks from patient specimens		122–124

Table 3 | Models of tumour evolution

Evolutionary model	Key features	Role of ITH	Refs.
Linear	Step-wise accumulation of mutations in the dominant clone	No functional relevance, only the dominant clone is relevant	125,126
Branched	Coexisting subclones with both shared (truncal) and specific mutations, recurrence can occur due to selection for the minor subclone	Multiple co-evolving subclones create specific phenotypes	127
Punctuated	Large number of genetic aberrations acquired at the same time at early stages followed by selection for a few dominant clones	Bursts of ITH followed by clonal dominance	128
Neutral	Many subclones but no evidence for selection, changes in clonal frequencies are due to random drift	There is ITH, but it has no functional relevance	129

of the whole tumour even as a minor subpopulation, which demonstrates that tumour drivers do not have to become dominant clones. Additional mechanistic analysis and mathematical modelling demonstrated that ITH is maintained by non-cell-autonomous drivers. Further evidence for subclonal cooperation comes from a transgenic MMTV–Wnt-driven breast cancer mouse model, in which *Hras*-mutated basal cells were fuelled by Wnt1-secreting luminal cells to enhance mutual tumour growth[49].

Functional cooperation also occurs between tumour and stromal cells. In a mouse model of pancreatic cancer[76], *Kras*-driven neoplastic cells had high expression of cytokine receptors for cytokines secreted by recruited T helper 2 cells that were found in close proximity. This affected metabolic states and supporting tumorigenesis[76]. Similarly, in a mouse model of liver cancer, necroptotic cells in the microenvironment of pre-tumorigenic cells induced a switch to the tumorigenic subtype showing heterogenic plasticity[77].

Intercellular communication is particularly relevant during metastatic colonization, as different metastatic niches favour the survival and outgrowth of different disseminated cancer cell populations[78]. Using a mouse model of experimental metastases based on the MDA-MB-231 breast cancer cell line, two different studies

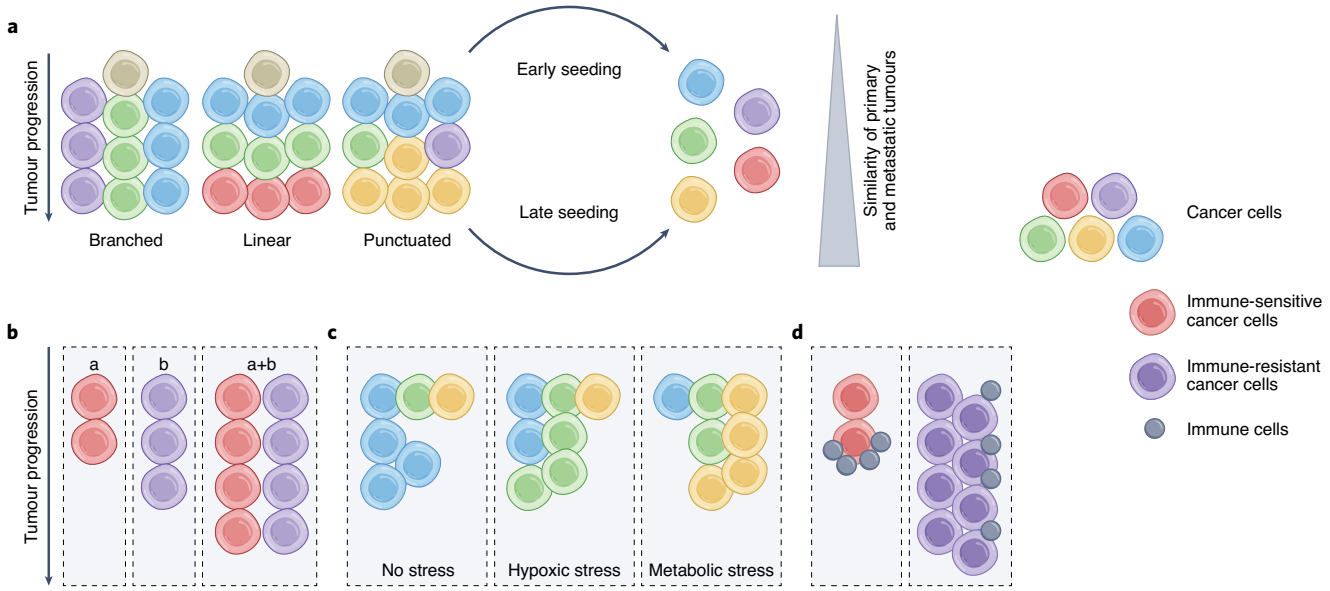

Fig. 2 | Clonality, adaption and progression of heterogeneous tumours. a, Subclonal heterogeneity can be derived in different ways. In the branched evolution, subclones cumulatively diverge (branch) at different time points from a parental clone into different directions, whereas clones in the linear model do not diverge and there is always a single dominant clone. Subclones in the punctuated model are less cumulative as they are defined early during tumour evolution. Regardless of the underlying model, similarities between primary and metastatic tumours are generally lower when cells seed early from the tumour, whereas similarities are higher when cell seeding occurs at later time points during tumour evolution. **b**, Distinct tumour subclones have different progression and growth rates that can be greatly enhanced when subclones cooperate. **c**, Different subclones have different responses to specific microenvironmental challenges, which result in the outgrowth of different subclones in different conditions. For example, in a resource-rich environment (no stress), subclones with higher intrinsic proliferative capacity will expand. By contrast, in hypoxic conditions, subclones that are more able to adapt to oxygen deprivation will become the prevalent population. Similarly, metabolic stress such as acidosis favours the outgrowth of another cancer cell subpopulation. **d**, Certain subclones can efficiently be cleared by immune cells, whereas other subclones can evade them through different mechanisms. The immune system can detect and eliminate immune-sensitive cancer cells, for example, via direct killing through cytotoxic T cells. However, some cancer cells evade immune-mediated elimination (immune-resistant cancer cells).

identified cell populations specifically enriched in lung and bone metastases, each harbouring specific tropism-specific gene signatures[79,80]. This implies that certain subclones in the parental cells have a pre-existing or acquired fitness advantage to grow in different metastatic niches of various organs.

Heterogenous primary tumours are more likely to progress to metastatic disease and to produce polyclonal metastases. In an experimental model of heterogeneous breast cancer, subclonal cooperation in primary tumours drove metastatic progression through tumour-induced systemic alterations[81]. Specifically, polyclonal tumours composed of *FIGF* and *IL11*-expressing subclones activated mesenchymal stromal cells in the lungs, which then recruited neutrophils forming the metastatic niche and enabling the outgrowth of metastases. Notably, most metastatic lesions were also polyclonal, composed of both cancer driver (for example, IL-11-expressing) and neutral (for example, parental non-metastatic) subclones. All these examples highlight various ways of selection of the fittest clones either through tumour cell intrinsic or TME-driven mechanisms (Fig. 2c).

The role of ITH in immune escape

Cancer and immune cell interactions shape tumour evolution in multiple ways[82]. Molecular alterations in cancer cells generate antigens that are recognized by the immune cells as 'foreign' to trigger an antitumour immune response[83]. This process of detection, elimination and eventually escape of tumour subpopulations from immune attacks is called immunoediting[84]. Massively parallel sequencing of highly immunogenic sarcomas identified tumour-specific antigens that are recognized by T cells and cleared in immunocompetent mice[85]. However, subclones lacking this antigen escape immune elimination and eventually grow out.

Mechanisms involved in immune escape also play a role in resistance to immunotherapies (for example, immune checkpoint inhibitors) that have shown success in a subset of cancer types[86]. Although these therapies do not directly target cancer cells, they were initially thought to be agnostic to ITH or even be more effective in tumours with high genetic ITH, including cancers deficient in mismatch repair[87]. However, a study in a mouse model of UVB-driven melanoma[88] highlighted the importance of both subclonal and genetic ITH for effective antitumour immune responses. A high tumour mutational burden resulted in more tumour neoantigens to facilitate immune recognition and therefore improved tumour clearance by the immune system. However, a high degree of subclonal ITH facilitated immune escape, presumably due to less effective immune responses against minor subclones. Thus, successful tumour growth requires an optimal balance of different types of ITH.

Immune surveillance also selects for cancer cells with immunosuppressive properties, which can be direct as cancer-cell-specific traits or indirect through modification of the TME (Fig. 2d). For example, loss of the tumour suppressor *PTEN* in melanoma cells led to the secretion of immunosuppressive cytokines[89]. In patients, loss of *PTEN* is correlated with a lower T cell infiltration[89]. A comprehensive study demonstrated that stemness (mesenchymal) features of cancers highly correlated with ITH and immunosuppressive pathways among various cancer types[90]. An example of indirect immune escape mechanisms is the increased recruitment of tumour-associated macrophages in patients with liver cancer, which then suppress T cell infiltration and activity[91]. Immune cells

also display a high degree of ITH, as demonstrated in a group of patients with non-small cell lung cancer, from whom the same tumour had highly infiltrated ('hot') regions and low infiltrated ('cold') regions[92]. This suggests that a single biopsy might not always reflect the immune environment of a tumour; therefore, there is a strong need to better evaluate microenvironmental and spatial ITH in patients with cancer.

Summary and outlook

Despite recent achievements in developing sophisticated technologies to assess ITH, the clinical utility of ITH is still limited. One hurdle is the limited feasibility to assess ITH in standard clinical samples due to cost and the need for special experimental or computational tools. However, for some cancer types, such as HER2$^+$ breast cancer in which *ERBB2* fluorescence in situ hybridization is routinely performed, ITH for *ERBB2* copy number is now part of the pathology report and it predicts the success of HER2-targeted therapies[93]. The increasing use of AI is likely to overcome the feasibility problem, as AI can recognize ITH even from slides stained with haematoxylin and eosin used for cancer diagnosis[94]. However, measuring ITH by itself will not improve treatment outcomes unless we have effective therapeutic strategies for heterogeneous tumours, which requires an improved mechanistic understanding of ITH, the discovery of suitable therapeutic agents and the rational design of combination therapies. Progress in these areas will require improved preclinical models that faithfully reproduce the heterogeneity of the human disease, including both interindividual and intratumour variation. Outbred rodent strains and tumour induction in a cell agnostic and stochastic manner (for example, use of mutagens) are promising approaches that could be exploited. Because epigenetic ITH is more extensive than genetic ITH and modifiable by targeting epigenetic enzymes or TFs, combining epigenetic agents with targeted or chemotherapies holds promise for improving the treatment of heterogeneous tumours. AI and mathematical modelling will also help with predicting optimal combination therapies in a specific patient at a specific stage of the disease, leading to truly individualized precision medicine. Based on the intense investigations in these areas, we anticipate important progress in the near future.

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Competing interests

K.P. serves on the Scientific Advisory Board of Acrivon Therapeutics, Vividion Therapeutics, Scorpion Therapeutics and the Novartis Institute for BioMedical Research, holds equity options in Scorpion Therapeutics, is a consultant to Aria Pharmaceuticals, received honorarium from AstraZeneca and New Equilibrium Biosciences, and has an institutional research agreement with Novartis. The remaining authors declare no competing interests.

Additional information

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