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Review

Epigenetic diversity in hematopoietic neoplasms

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ABSTRACT

Tumor cell populations display a remarkable extent of variability in non-genetic characteristics such as DNA methylation, histone modification patterns, and differentiation levels of individual cells. It remains to be elucidated whether non-genetic heterogeneity is simply a byproduct of tumor evolution or instead a manifestation of a higher-order tissue organization that is maintained within the neoplasm to establish a differentiation hierarchy, a favorable microenvironment, or a buffer against changing selection pressures during tumorigenesis. Here, we review recent findings on epigenetic diversity, particularly heterogeneity in DNA methylation patterns in hematologic malignancies. We also address the implications of epigenetic heterogeneity for the clonal evolution of tumors and discuss its effects on gene expression and other genome functions in cancer.

Contents

1. Introduction

A series of recent studies have suggested that tumors may contain a large number of distinct clones, each defined by different landscapes of genetic and epigenetic alterations [1–5]. While most investigations have concentrated on the extent of genetic and genomic variability within tumor cells, non-genetic diversity has only recently come to the forefront of research interests. Such epigenetic heterogeneity among normal cells has long been noted as important since it drives tissue-specific differences among cells: as all cells in an individual share the same genotype (with the exception of somatic mutations, chromosomal losses and gains, and immunoglobulin rearrangements in immune system cells), the phenotypic identities and differentiation stages of normal cells are defined by non-genetic mechanisms. As such, non-genetic diversity in cells has been classified into two qualitatively different states [6]: deterministic variability and stochastic variability (Fig. 1). Deterministic variability refers to cell type-specific differences that are readily recapitulated across individuals and which lead to tissue-specific differentiation hierarchies. Stochastic variability, in contrast, leads to cell-to-

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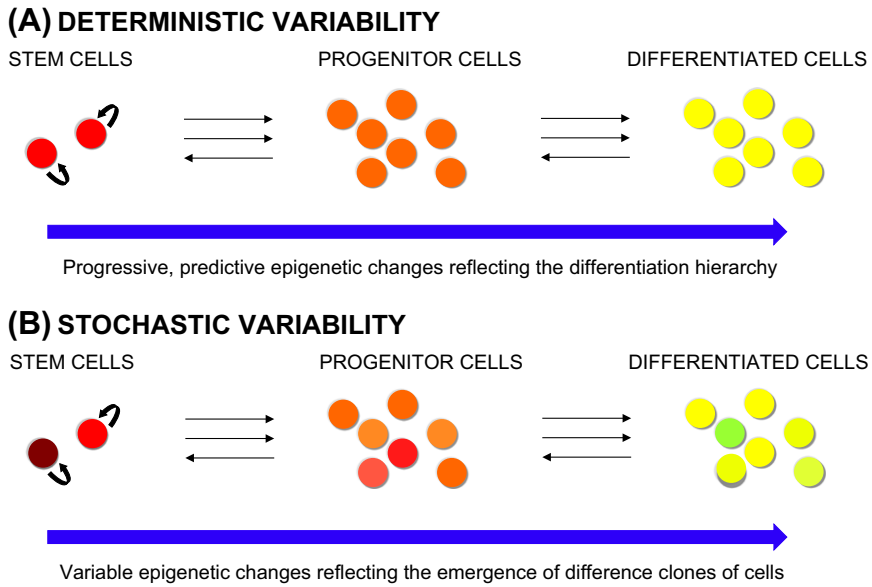

Fig. 1. Deterministic versus stochastic epigenetic heterogeneity. Deterministic epigenetic heterogeneity (a) arises during differentiation of cells, in which progressive and predictive changes are accumulated. In contrast, stochastic epigenetic heterogeneity (b) emerges due to unpredictable changes that differ between patients and clones.

cell variability within differentiation hierarchies such that even individual cells of the same differentiation stage display marked heterogeneity.

DNA methylation, histone modifications and non-coding RNAs are critical for establishing the epigenome of a cell. Epigenetic variability can thus arise via cell-to-cell differences in the patterning of DNA methylation, histone modifications, expression of protein coding genes and noncoding RNAs. For instance, DNA methylation patterns in cancer cells differ considerably from those in normal cells [7], and even within cancer cell populations, there is a large extent of diversity in DNA methylation patterns. The epigenetic patterning of individual cancer samples can even be used to cluster them into different subtypes. Figueroa et al. recently showed that classification of AML patients based on their DNA methylation profiles can help identify novel biologically distinct subtypes in acute myeloid leukemia and predict patient survival [8].

Attempts are under way to assess the extent of epigenetic heterogeneity at different levels. Capturing heterogeneity in histone modifications and non-coding RNAs requires single cell profiling technologies, which remain technically difficult. However, due to recent advances in sequencing technologies, it has become feasible to interrogate DNA methylation patterns and heterogeneity therein at a genome-wide scale. Hence, in this review, we focus primarily on DNA methylation patterns in hematopoietic and other cancer types. We first discuss deterministic DNA methylation patterning in cancer, with a focus on B-cell ontogeny and lymphomagenesis. We then review stochastic DNA methylation patterning in lymphomas. We conclude by discussing functional implications of DNA methylation heterogeneity, and by comparing the patterns observed in leukemias and lymphomas to those identified in other cancer types.

1.1. Deterministic DNA methylation patterning in cancer

While all somatic cells in the body have a similar genomic content, the epigenetic makeup of cells and their tissue microenvironment may have a crucial role to play in tumor initiation, as suggested by the different cells of origin of individual cancer types. According to the cancer stem cell model, epigenetic heterogeneity during neoplastic transformation persists as a reflection of the developmental hierarchy within the tumor (Fig. 2). Indeed, gene expression profiles of relatively differentiated and stem cell-like subpopulations in cancers cluster more closely to their counterparts in normal tissues than they do to each other [9,10]. Baylin and colleagues recently identified a DNA hypermethylation module that marks a stem cell signature and promotes self-renewal and persistence of tumor cells [11]. These and other observations have led to the formulation of the epigenetic progenitor model of cancer [12]. This model postulates that epigenetic aberrations of progenitor cells are a key determinant not only of the predisposition to cancer, but also of the dynamics of tumor progression and the extent of heterogeneity within the tumor that arises from these progenitor cells.

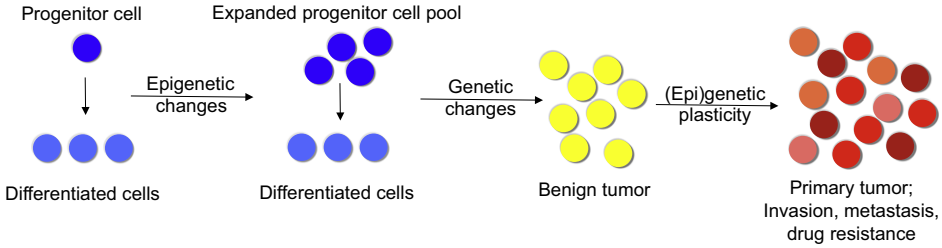

Fig. 2. The epigenetic progenitor model. According to the epigenetic progenitor model, cancer arises in three steps. First is an epigenetic alteration of stem/progenitor cells within a given tissue, which is mediated by aberrant regulation of tumor-progenitor genes. Second is a gatekeeper mutation (of tumor-suppressor genes or oncogenes). Although these GKMs are themselves monoclonal, the expanded or altered progenitor compartment increases the risk of cancer when such a mutation occurs and the frequency of subsequent primary tumors (shown as separately arising tumors). Third is genetic and epigenetic instability, which leads to increased tumor evolution. Note that many of the properties of advanced tumors (invasion, metastasis and drug resistance) are inherent properties of the progenitor cells that give rise to the primary tumor and do not require other mutations (highlighting the importance of epigenetic factors in tumor progression).

The first step, according to the epigenetic progenitor model, is an epigenetic disruption of a progenitor cell population, which leads to a polyclonal population of cells within a particular tissue. This disruption might perturb the normal balance of cell division within this tissue, for instance by changing the ratio of symmetric to asymmetric cell division or the relative frequency of certain progeny cell types. These cells then accumulate a number of genetic and/or epigenetic alterations, which allow them to undergo cycles of clonal expansion and selection. Finally, the model posits that cancer involves genetic and epigenetic plasticity — an enhanced ability to evolve the phenotypes of cells through both genetic and epigenetic mechanisms. This plasticity then explains the widespread heterogeneity observed within and across human tumors. This model has attractive implications for understanding the interactions between tumors and the (micro)environment, the mechanisms for generating heterogeneity, as well as other biological and etiological characteristics of human tumors [12].

1.2. Genetic alterations driving DNA methylation patterning in cancer

Epigenetic abnormalities in cancer genomes are often caused by genetic alterations. For instance, several studies have found recurrent mutations in epigenetic modifier enzymes and pathways involved in DNA methylation, demethylation as well as chromatin packaging, histone modification and chromatin remodeling (see [13] for a comprehensive review). Genetically driven DNA methylation abnormalities in the genome usually follow deterministic patterns and can thus be distinguished from other methylation changes due to other causes [13].

Mutations in DNA methyltransferases (e.g. DNMT1, DNMT3A, DNMT3B), a family of enzymes involved in incorporating methyl groups to CpG sites in DNA, are found in diverse types of cancer. DNMT3A mutations have been reported in different subgroups of acute myeloid leukemia [14] and also in solid tumors [15]. Usually, these mutations either affect the enzymatic activity of the protein or change its binding affinity to histones. Recent studies indicate that another DNA methyltransferase, DNMT3B, acts as a haploinsufficient tumor suppressor gene in MYC-induced lymphomagenesis [16]. In a murine model, DNMT1 haploinsufficiency affects leukemia stem cell function via derepression of bivalent chromatin domains [17]. Perhaps not surprisingly, mutations in the DNMTs are associated with changes in methylation patterns.

Like DNMTs, the TET family of DNA demethylating enzymes are also frequent targets of alterations in hematopoietic and solid tumors. The TET1-MLL translocation t(10,11) has been found in AML [18] and more recently in lymphoblastic lymphoma [19]. Functionally deleterious mutations in TET2 occur at a high frequency (~7.5%) in AML patients [20], and those tend to be mutually exclusive with IDH1 and IDH2 mutations [21]. Mutant IDH enzymes produce 2-hydroxyglutarate (2HG), which competes with the TET substrate α-ketoglutarate, leading to global methylation abnormalities in tumor genomes. IDH1/2-mutant AMLs usually display specific genome-wide DNA methylation signatures. Another major factor is AID, which is an APOBEC family cytidine deaminase enzyme that plays a role in DNA demethylation [22] and has also been implicated in tumorigenesis.

A third type of DNA methylation regulators includes CTCF elements, which help mark the boundaries of chromatin domains and are important for the maintenance of methylation signatures. Chr16q22.1, which harbors the CTCF gene locus, is frequently deleted in many cancer types [23]. It has also recently been shown that CTCF haploinsufficiency destabilizes global DNA methylation patterns and increases cancer predisposition [24].

In addition, alterations in genes involved in other types of epigenetic deregulation such as chromatin packaging (e.g. H3F3A), histone modification (e.g. EZH2), and chromatin remodeling (e.g. ARIDIA) are common in different types of cancer [13]. Given the crosstalk among different epigenetic modifications and chromatin organization, it is conceivable that these changes also indirectly affect methylation patterns in tumor genomes at a local or global scale.

1.3. Deterministic variability and plasticity of DNA methylation during B-cell ontogeny and lymphomagenesis

B-cell lymphomas represent a heterogeneous group of diseases, which arise from mature B-cells that have left the bone marrow compartment. Many B-cell lymphomas arise from a germinal center B-cell due to the high proliferative rate and unique hypermutability of centroblasts undergoing somatic hypermutation (SHM) and class switch recombination (CSR) [25–27]; expression of the Bcl6 transcription factor that suppresses sensing and the response to genotoxic stress [28,29]; and a unique functional activity of AID, which is physiologically involved in creating genetic diversification of Ig-variable loci, while also targeting many somatic transcription factors [27,30,31]. While genomic diversification is well described as part of the germinal center reaction [32,33] its epigenetic consequences are currently being unraveled. Based on the epigenetic progenitor model described above, germinal center B-cells represent a “progenitor cell population” that give rise to germinal center-derived lymphomas. Differentiation of B-cells is associated with deterministic changes in the epigenome that have been delineated in many studies. Ji et al. [34], for instance, studied epigenetic modifications that accompany the differentiation of multipotent progenitors (MPPs) into various hematopoietic lineages, including lymphoid, myeloid, and erythroid lineages, using Comprehensive High-throughput Arrays for Relative Methylation (CHARM) that examine 4.6 million CpG sites. These studies demonstrated that lymphoid commitment involved a larger extent of methylation than other lineages, supported by the fact that treatment with DNA methyltransferase inhibitors resulted in myeloid predominance. Methylation of CpG shores displayed a greater correlation with gene expression, supporting a previous finding by Irizzary et al. [35] in colon cancer.

During early lymphoid development, the epigenetic landscape shows a marked plasticity: differentially methylated regions (DMRs) between MPPs and common lymphocyte progenitors (CLPs) showed a loss of methylation in the latter, while later during transition to the DN1 stage (double negative stage 1), 15-fold more DMRs showed a gain of methylation. Interestingly, *DNMT1* hypomorphic mice have normal myeloid, but diminished lymphoid development [36], suggestive of the crucial role that DNMT1 plays in DNA methylation during lineage commitment. Deaton et al. recently confirmed prior findings that not only methylation of CpGs close to TSS has biological significance, but also that CpGs in intergenic and intragenic CpG islands confirm cell-type specificity of DNA methylation [37,38]. This study indicated that DNA methylation changes mirrored the developmental distance of cell types much better than gene expression, suggesting a greater stability of DNA methylation marks. Emerging data suggests that the patterning of DMRs between normal B-cell and lymphoma subtypes can point to the evolutionary distance between subtypes as well as predict patient survival [39,40].

B-cells undergoing the germinal center transit continue to modify their epigenetic landscape with many stage-specific DMRs: we recently demonstrated in primary human tonsillar naïve B-cells and germinal center B-cells that entry into the germinal center is associated with a predominant loss of methylation in nearly all of the 235 differentially methylated genes that are enriched for NFkB and MAP kinase pathways [31]. Interestingly, many tumors undergo a dramatic loss of DNA methylation with as much as 20–60% less 5 methyl cytosine (5mC) in tumor cells [41,42]. Such DNA hypomethylation predominantly affects gene bodies and repetitive sequences [43]. Our data also points to the loss of DNA methylation in lymphomas [39,40] and may reflect their origin from the germinal center cells. Thus, germinal center B-cells may function as a progenitor cell population for many subtypes of lymphomas and may equip those lymphomas with critical, but subsequently mutated or aberrantly expressed epigenetic factors [37,38]. Loss of

methylation may then result in chromosomal instability leading to numerous chromosomal aberrations, not unlike those seen in DLBCLs [44]. It can also lead to reactivation of transposable elements [45,46] and loss of imprinting.

Somatic mutations in DNA methyltransferases may contribute to chromosomal instability, as supported by the observation of increased mutation frequencies in patients with germline mutations in *DNMT3b* [47]. DNMT1 is the main methyltransferase expressed in germinal center B-cells, along with smaller quantities of DNMT3b. Both are likely responsible for resetting the DNA methylation profile during commitment to plasma cells and memory B-cells, and for aberrant methylation during the malignant transformation of those precursor cells [48]. Overexpression of DNMT1 and DNMT3b has furthermore been linked to advanced clinical stages of DLBCLs [49].

Germinal center B-cells have some qualities of stem cells: the capacity to differentiate into multiple cell lineages, albeit in a more restricted way in the case of germinal center B-cell, and the ability to tolerate genotoxic stress [28,29]. Thus, the deterministic DNA methylation patterning described above must be the consequence of tissue–specific transcription factors and also epigenetic factors that possess stage-specific expression patterns [50].

Indeed, key transcription factors have been shown to maintain the differentiated cellular identity [51–53]. Loss of expression of those transcription factors may result in the induction of pluripotent states with the ability to give rise to other lineages. B-cell lineage commitment, for instance, requires PAX5 [54,55] and its loss results in a decrease in mature characteristics and the generation of B-cells that are similar to uncommitted progenitors [56]. These cells can repopulate the entire hematopoietic system including T-cells, myeloid cells and macrophages. Propagation of the cell fate program with a progressive silencing of genes driving alternative fates even in the presence of key transcription factors requires epigenetic modifications like in PcG proteins [57], which create repressive histone marks such as H3K27Me3 that, together with DNA methylation marks, lead to a repressive chromatin environment and a compaction of chromatin [58,59]. The fact that the differentiation fate depends on epigenetic plasticity is underscored by an elegant experiment by Bhutani et al. [22] in which mouse embryonic stem (ES) cells were fused with human fibroblasts. This resulted in a large proportion of fused cells, which initiated reprogramming into pluripotent cells as early as on day 1 after the fusion. These cells displayed increased expression of human NANOG and OCT4 that depended on progressive demethylation of their promoters [22]. These experiments led to similar results as reprogramming of iPS cells [60].

Thus the epigenetic plasticity during B-cell development depends on the maintenance of a complex pattern of histone modifications and DNA methylation. The breakdown of normal patterning in lymphomas is evident in the example of histone methyltransferase enhancer of zeste-2 (EZH2) [57]. Both normal germinal center B-cells and ES cells express high levels of EZH2 — a member of the PRC2 polycomb complex that mediates transcriptional repression through methylation of H3K27 [57]. The 1800 promoter targets of EZH2 in germinal center B-cells are mostly overlapping with those of ES cells and are predominantly hypomethylated in normal B-cells. The targets include cell cycle-related tumor suppressor genes; it has also been shown that in lymphomas, many targets of PRC2 are hypermethylated and may also carry H3K27Me3 marks, which represent the breakdown of regulatory epigenetic mechanisms [40]. These events may be due to mutations in the SET domain of EZH2 that have been detected in up to 12% of follicular lymphomas and 21.7% of GCB-like DLBCLs [61,62] (Fig. 3).

1.4. Stochastic DNA methylation patterning in lymphomagenesis

Stochastic variability, in contrast, leads to cell-to-cell variability within differentiation hierarchies such that even individual cells of the same differentiation stage display marked heterogeneity. It remains unclear whether the stochastic cell-to-cell variation in DNA methylation patterns arises due to poor fidelity of the DNA-methylating and -demethylating enzymes, or whether it represents a mechanism for introducing an epigenetic buffer state into a cell population. Recent work on methylomes of aging peripheral blood cells suggested that cells undergo age-related epigenetic drift [63] that is accelerated in cancer cells. Either way, the results of this stochastic variability are

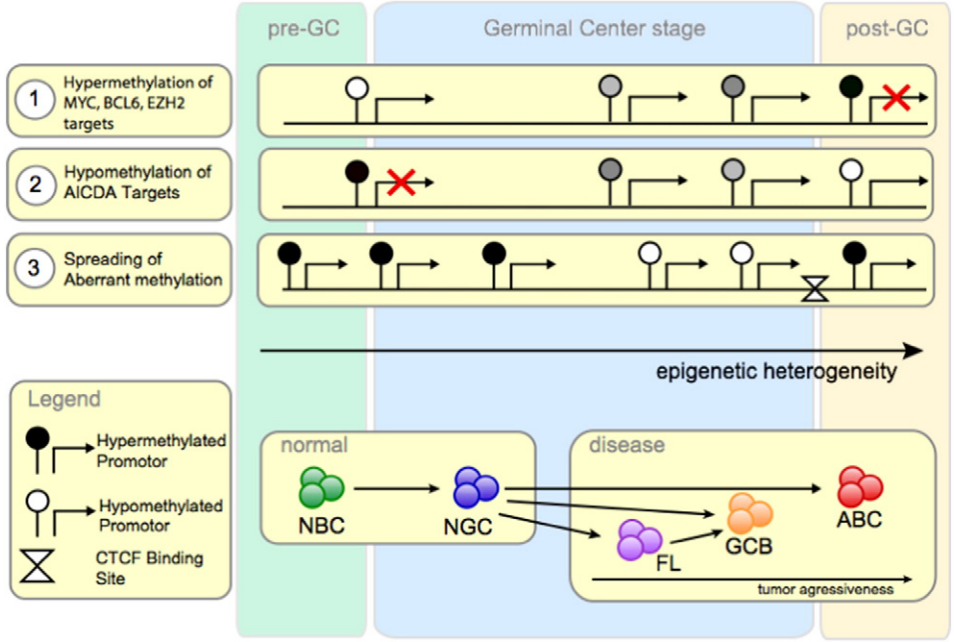

Fig. 3. Changes in DNA methylation during B cell development and lymphomagenesis. Graphical representation of the aberrant changes in DNA methylation at preGC (pre germinal center stage), germinal center stage and postGC (post germinal center stage) as cells progress from naïve B-cells (NBC) to germinal center B-cells (NGC) and during neoplastic transformation into Follicular Lymphoma (FL), GCB-like (GCB) and ABC-like (ABC) diffuse large B-cell lymphoma. Main characteristic aberrant changes in DNA methylation are: 1. Hypermethylation of EZH2 target sites 2. Hypomethylation of AICDA target sites 3. Spreading of aberrant methylation limited by CTCF binding. Epigenetic diversity is also increasing as cells progress through the germinal center and transform into lymphoma.

that cell-to-cell epigenetic variability cannot be explained by cellular differentiation states, gene expression patterns, or any other characteristics usually related to the deterministic patterns outlined above. Thus, such heterogeneity represents an unpredictable addition of variability into a cell population of otherwise similar characteristics.

Lymphomagenesis provides a useful example for the extent of stochastic variability on both the genomic and the epigenomic level. Many mature B-cell lymphomas arise from germinal and post-germinal center B-cells and carry the burden of Ig mutations as well as somatic mutations caused by AID, which is expressed in the germinal center [64–66]. AID not only initiates SHM and CSR targeting Ig genes, but also regulates non-immunoglobulin genes like *CD79A*, *MYC*, *PAX5*, *BCL6*, and *MIR142*, among others [67–69], leading to potentially deleterious mutations and also chromosomal abnormalities [70]. The role of AICDA in genomic diversification, whether physiologic or pathologic, is well-established [30,71]. Recently, AID has been implicated in having a novel function as a DNA demethylase [72,73]. In zebrafish, *AICDA* and Apobec family genes are necessary to demethylate exogenous DNA [74], and in mouse primordial germ cells, *AICDA* knock-out results in genome-wide hypermethylation [75] (Fig. 3).

AID can deaminate cytidine residues in single stranded DNA through its interaction with stalled POL II RNA polymerase resulting in U:G mismatches that can be repaired by base-repair excision or mismatch repair pathways [76]. This AID-induced cytidine deamination and repair results in a removal of methylated cytidine and its replacement with unmethylated cytidine, thus causing effective demethylation [77]. Interestingly, this activity of AID does not depend on DNA replication, as shown by the heterokaryon experiments by Bhutani et al. [22]. This activity puts AID into the forefront of research interests as both epigenetic mutator in actively dividing tumor cells and also in "cancer stem cells" that are quiescent in the G0/G1 phase of the cell cycle. Interestingly, naïve B-cells predominantly loose methylation after entry into the germinal center reaction, with 223 of 225 DMRs being hypomethylated in germinal center B-cells [48]. Hypomethylated loci were found to have a statistically significant enrichment for the putative AICDA binding RGYW motif [78] and for the experimentally proven targets of AICDA using CHIP seq [77]. This observation is highly suggestive of a demethylase function of AICDA in germinal center B-cells. AID chip-seq data in in vitro activated B lymphocytes [77] revealed a broad binding pattern of AID at more than 12,000 loci outside of Ig genes, and particularly at actively transcribed genes. Presumably, binding of AID to POL II through a multiprotein complex allows a certain extent of promiscuity with which AID associates with single stranded DNA and transcribed genes. This scenario can lead to stochastic removal of methylation at heterogeneous sites in the genome. Indeed, centroblasts exhibit a larger extent of inter- and intra-sample heterogeneity in DNA methylation with an attenuation of the normal bimodal distribution of methylation and the emergence of many sites with a variable level of methylation between individual cells [48]. Nevertheless, recent work by Fritz et al. [79] did not confirm a demethylating function of AID in mouse splenic B-cells that were activated ex vivo. Further studies will consolidate our understanding of the role AID plays in epigenetic modifications within B-cells. Finally, epigenetic diversification is not unlike genetic diversification and may contribute to the clonal evolution of normal B-cells, but also to the emergence of "novel" malignant clones in lymphomas.

Many factors like DNA methyltransferases may also contribute to a less programmatic deposition of cytosine methylation marks, possibly due to somatic mutations like in the case of DNMT3A and leukemias [80,81], or due to the breakdown of chromatin domain packaging as a result of aberrant expression of insulator proteins like CTCF [82,83].

1.5. The functional implications of epigenetic heterogeneity

We are just at the beginning of our understanding of the implications of epigenetic heterogeneity in normal development and tumorigenesis. Recent findings suggest that apparently normal pluripotent stem cells are epigenetically heterogeneous, and that such heterogeneity contributes to functional variability that could change the signaling response of developmental pathways leading to lineage bias, or 'lock' the pluripotency network leading to residual pluripotent cells [84,85]. Epigenetic heterogeneity is generally considerably higher in tumors. While the study of epigenetic heterogeneity in hematopoietic malignancies has recently led to much enthusiasm, most of the evidence regarding functional implications of epigenetic heterogeneity stems from solid tumors. Indeed, it has been observed that the risk of most cancer types increases with age, as does the extent of epigenetic abnormalities [12]. Increased epigenetic heterogeneity and loss of DNA methylation in aging but otherwise normal tissues expose genomic DNA to DNA damage and mutations. In vitro and in vivo studies have shown that global DNA hypomethylation can lead to chromosomal instability and an increased incidence of tumor formation [86,87], thus providing a functional link between epigenetic modifications and cancer incidence. For instance, epigenetic silencing of DNA repair genes, such as *MGMT*, *BRCA1*, and *MLH1*, prevents their repair activities and thus contributes to an early onset of tumor types such as breast and colon cancer [88–90].

Changes in DNA methylation and histone modification patterning can have profound effects on the regulation of gene transcription [91]. For instance, epigenetic silencing of tumor suppressor gene expression [92] may lead to dramatic differences in cellular behavior, thus providing a substrate for natural selection to work on in such clones, so that these epigenetic changes increase in frequency within the population. At a larger scale, epigenetic alterations of specific intracellular signaling pathways also modulate the phenotype of cells. Methylation of the estrogen receptor CpG island, for example, has been observed to increase the risk of colorectal tumorigenesis [93], while epigenetic silencing of SFRP leads to Wnt pathway activation, thereby promoting cell proliferation in colon crypt cells [94]. Similarly, competing non-coding RNAs, whose expression is epigenetically regulated, alter the expression of tumor suppressor genes such as *PTEN* and thereby modify the phenotype of cells [95], again providing a substrate for clonal selection within tumor cell populations. Finally, it was recently proposed that the long non-coding RNA *HOTAIR* reprograms the chromatin state of cells, thereby promoting metastasis [96].

Similarly, epigenetic alterations can have important implications for the spatial architecture of chromatin [91] as well as for replication timing of DNA. These characteristics are, at least partly, determined by epigenetic states of genomic regions. Recent work has demonstrated that replication timing as well as the higher-order nuclear organization of genomic material predetermines the risk of certain genomic areas to be altered during tumorigenesis, thus providing an opportunity for a predictive model for the size and length distribution of somatic copy number alterations in cancer genomes [97].

We recently proposed a model that abnormal and stochastic hypomethylation in genomic regions enriched for potential G-quadruplex motifs may act as a mutagenic factor driving tissue-specific mutational landscapes in cancer [98]. According to this model, the stability of G-quadruplex secondary structures in the genome is regulated by chemical modifications such as methylation of CpGs that reside within the G-quadruplex consensus, such that in hypomethylated areas of the genome, these secondary structures are more stable [99]. Stable G-quadruplex structures then have the capacity of stalling the movement of DNA polymerase and introducing DNA breaks as well as loss or gain of genomic material [100,101].

Similarly, epigenetic regulation of repeat elements may also contribute to changing phenotypes of cells on the road to cancer. For instance, changes in the methylation patterns of Long and Short Interspersed Elements (LINEs and SINEs) are frequent in cancer cells. Altered expression of satellite repeats in pancreatic and other cancers is associated with overexpression of LINE-1 elements, which in turn leads to a drastic remodeling of the cancer genome [102]. A recent study discovered 7743 somatic LINE-1 insertions in healthy brain samples [103], suggesting

that these alterations may play a more common role in human tissues than hitherto realized. Furthermore, hypomethylation of intragenic LINE-1 can repress gene expression [104] of particular genes, thereby providing a further mechanism for the regulation of cellular behavior through epigenetic means.

During tumorigenesis and therapy, epigenetic heterogeneity (together with genetic changes) allows tumor cells to explore new niches of epigenetic and fitness landscapes, ultimately driving them towards cancer attractor phenotypes [105] — stable cellular states that support the malignant phenotype and offer a growth advantage against the selective pressure imposed by the microenvironment [106,107]. Genetic and epigenetic diversity present within a tumor cell population also increases the risk of drug resistance and is often a marker for poor survival [108]. Indeed, in DLBCL, a large extent of epigenetic heterogeneity is correlated with poor survival [39]. Even when the extent of genetic diversity is minimal, epigenetic heterogeneity allows distinct clones to explore different evolutionary niches in a reversible manner [108], and epigenetic plasticity can potentially lead a cell population to regain clonal diversity once the selection pressure (e.g. a particular chemotherapeutic drug) is removed [109]. Recent studies indicate that 5-aza-2′-deoxcytidine (5-AZA-CdR) and 3-deazaneplanocin-A (DZNep) together can synergistically reactivate genes that are selectively silenced by EZH2 and PRC2 (polycomb group repressive complex 2) mediated mechanisms in leukemic cells, and lead to a loss of proliferative potential [110].

The detection of somatic mutations in epigenetic modifier genes like *TET2*, *DNMT3A*, *EZH2*, and *IDH1/2* in myeloproliferative neoplasms (MPN) and lymphomas has provided the basis for evaluating epigenetic therapy in the clinic [111–113]. Results from a phase 1 study of the histone deacetylase inhibitor (HDAC) Panobinostat (LBH589) revealed pronounced anti-MPN effects, including decreases in JAK/STAT signaling, *JAK2* V617F allele burden and inflammatory cytokine levels; however, only 1 patient (3%) experienced a clinical response [114]. Pilot studies with other HDACs are ongoing [115–117].

Aberrant hypermethylation of genes and the overall disruption of genome-wide methylation patterns has also served as the rationale for testing the efficacy of hypomethylating agents in different cancer types. Up to date there have been 13 phase I or II clinical trials (a total of 305 patients and 18 tumor types) treated with the hypomethylating agent DAC in solid tumors (for a review see [118]). In MDS and MPN, hypomethylating agents like 5-azacytidine have led to promising results: up to 52% of MDS patients and 24% of MPN patients were reported to experience a partial response to 5-aza [119–121]. Many studies documented a limited clinical response using demethylating agents as monotherapy, but improved responses were observed when epigenetic treatment was followed by chemotherapy, immunotherapy or targeted therapy. Clozel et al. revealed a mechanism by which the hypomethylating agent decitabine reverses chemoresistance to doxorubicin in DLBCLs [122]: prolonged exposure to low doses of decitabine reprogrammed chemoresistant cells and made them chemoresponsive. A phase I clinical study evaluating azacitidine priming followed by standard chemoimmunotherapy in high-risk patients newly diagnosed with DLBCL resulted in a high rate of complete remission. In this study, reversal of aberrant methylation in 9 genes was associated with chemosensitization, with SMAD1 being a critical one. In xenograft models, low doses of decitabine led to a demethylated genome with minimal DNA damage, allowing for derepression of some key genes that drive resistance.

In sum, the efficacy of hypomethylating agents across different types of tumors is variable, but the key anti-cancer effect of these drugs seems to stem from reducing stochastic heterogeneity of tumors via genome demethylation. In addition, these agents demethylate key tumor suppressor genes and genes that allow chemosensitization. Future work will help demonstrate the efficacy of these agents in combination with other drugs as well as mechanisms of sensitivity and resistance.

These observations, together, provide a model for how proliferating cells are able to broaden their set of phenotypic characteristics. These options then allow a population of cancer cells to induce genomic instability, optimally respond to changing environmental conditions such as the onset of therapeutic interventions, the invasion into new microhabitats, and the challenges of angiogenesis and immune system interactions. Phenotypic heterogeneity by means of epigenetic variability thus provides a key advantage to tumor cells: that of evolvability.

2. Conclusions

Here we have provided an overview of the extent of variability in non-genetic characteristics such as DNA methylation, histone modification patterns, and differentiation levels in individual cells within a tumor. We particularly focused on heterogeneity in DNA methylation patterns in hematologic malignancies. We also addressed the implications of epigenetic heterogeneity for the clonal evolution of tumors and discussed its effects on gene expression and other genome functions in cancer.

Much work has been done to elucidate the causes and consequences of epigenetic variability in cancer; however, many open problems still remain. For instance, a more comprehensive characterization of epigenetic heterogeneity in cell populations depends on the development of methodologies that allow for single cell profiling of genome-wide epigenetic patterning. Such methodology is starting to be developed, as exemplified by recent work developing single cell reduced representation bisulfite sequencing (scRRBS) protocols [123], but single cell methodology for other types of epigenetic changes, such as histone modifications etc., is currently not available. The development of such methods will be an important goal of the field. Hand in hand with the inception and testing of such assays comes the computational analysis of such data. Since gold standard methods are not yet available, it remains to be seen how new single cell methods can be validated and tested, and how noise can be distinguished from single cell variability of methylation patterns. Close collaboration between computational and experimental researchers will be necessary to push the frontier on single cell variability. Other areas of future investigation include broader testing of epigenetic therapeutics, the elucidation of causes and consequences of epigenetic diversity across cell types and in response to selection pressures such as drug exposure and dissemination, and the development of predictive models to aid in these processes.

Authorship

All authors contributed to conception of the manuscript as well as its writing. The authors have no conflicts to report.

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