Journal of Genetics and Genomics 43 (2016) 503–511

Contents lists available at ScienceDirect

Journal of Genetics and Genomics

Journal homepage: www.journals.elsevier.com/journal-of-genetics-and-genomics/

Original research

Epigenetic modulations rendering cell-to-cell variability and phenotypic metastability

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ARTICLE INFO

Article history:
Received 8 January 2016
Received in revised form
12 May 2016
Accepted 25 May 2016
Available online 27 May 2016

Keywords:
Intraclonal heterogeneity
Epigenetic regulation
DNA methylation
Differential histone marks
Metastable expression

ABSTRACT

Tumor cells display phenotypic plasticity and heterogeneity due to genetic and epigenetic variations which limit the predictability of therapeutic interventions. Chromatin modifications can arise stochastically but can also be a consequence of environmental influences such as the microenvironment of cancer cells. A better understanding of the impact and dynamics of epigenetic modulation at defined chromosomal sites is required to get access to the underlying mechanisms. We investigated the epigenetic modulations leading to cell-to-cell heterogeneity in a tumor cell line model. To this end, we analyzed expression variance in 80 genetically uniform cell populations having a single-copy reporter randomly integrated in the genome. Single-cell analysis showed high intraclonal heterogeneity. Epigenetic characterization revealed that expression heterogeneity was accompanied by differential histone marks whereas contribution of DNA methylation could be excluded. Strikingly, some clones revealed a highly dynamic, stochastically altered chromatin state of the transgene cassette which was accompanied with a metastable expression pattern. In contrast, other clones represented a robust chromatin state of the transgene cassette with a stable expression pattern. Together, these results elucidate locus-specific epigenetic modulation in gene expression that contributes to phenotypic heterogeneity of cells and might account for cellular plasticity.

1. Introduction

The heterogeneity and cellular plasticity observed in cancer cell populations represent a major hurdle in treating cancer patients. The development of resistance in the metastatic cells limits the utility of the therapeutic remedies. While most of this heterogeneity was previously thought to be due to genetic alterations and inherent genetic instability of cancer cells (Marusyk et al., 2012), there is increasing evidence showing that genetic mutations cannot be held as a sole cause of this heterogeneity (Marjanovic et al., 2013). Studies have shown that the disruption in the epigenetic marks can also be an important intrinsic factor that might result in cellular heterogeneity and plasticity (Huang, 2013; Marjanovic et al., 2013).

The phenotypic reversibility and metastability frequently observed in tumor populations are considered to be significantly contributed by dynamic chromatin markings (Huang, 2013; Marjanovic et al., 2013). These structures might act as sensors and effectors (mediators) to adjust the selection pressure exerted by the cellular microenvironment.

Epigenetic modifications are known to critically affect the chromatin state. This includes changes in the methylation pattern of DNA as well as specific histone modifications such as methylation and acetylation on the specific amino acid residues of histones (Ghavifekr Fakhr et al., 2013). Thereby, the differential accessibility and/or binding of DNA sequences by a set of proteins are realized. Together, this modulates the efficiency of transcription and as a consequence the cellular phenotype (Cui et al., 2013; Li, 2013; Buck et al., 2014). DNA methylation is one of the best characterized epigenetic modifications. It predominately involves addition of a methyl group to the position 5 of cytosine residues that are coupled to guanine (CpG motifs) (Crider et al., 2012). Histone modifications comprise a set of modifications like methylation

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http://dx.doi.org/10.1016/j.jgg.2016.05.008

and acetylation that can occur on specific residues (e.g., lysine) present on the histone tails. These modifications form a histone code that modulates gene expression by allowing or preventing access to chromatin, thereby acting as a guide for the transcription factors and other regulatory proteins. In recent times, a large number of studies have permitted a partial unraveling of this code (Misri et al., 2008; Gacek and Strauss, 2012). Generally, DNA hypermethylation and certain histone markings like trimethylation of lysine 27 on histone H3 have been considered to suppress gene expression, whereas the DNA hypomethylation and histone H3 acetylation were shown to be associated with active gene expression (Kirmizis et al., 2004; Yu et al., 2007; Pauler et al., 2009; Connolly et al., 2013).

Most of our understanding of the role of epigenetics in cancer is based on studies of differential expression of cellular oncogenes and tumor suppressor genes in their natural chromosomal context. However, tumor progression is frequently accompanied with an inherent genomic instability. As a result of genomic rearrangements, deletions and translocations can occur. As a consequence, genes are subjected to influences arising from new genetic environments.

We aimed at a better understanding of the phenotypic variation of gene expression that may occur if genes are exposed to novel chromosomal environments. To simulate this situation, we investigated the epigenetic mechanism(s) underlying the alterations in expression of single-copy transgenes randomly integrated into chromosomal sites of a tumor cell line without selection pressure. Interestingly, we could correlate the expression phenotype with defined histone modifications. Depending on the particular chromosomal site, these chromatin modifications were either stable or dynamically changed upon prolonged cultivation. Together, the results highlight the plasticity of chromatin modulation upon rearrangement and resulting phenotypic variations in cancer cells.

2. Results

2.1. Expression heterogeneity in HEK293T clones with a single-copy GFP expression cassette

To establish an *in vitro* system to study the mechanism(s) that cause epigenetically mediated variation in gene expression, we used genetically stable single-copy transgenes as sentinels. To simulate the influence of epigenetic variations in different chromosomal sites, we analyzed randomly chosen integration sites of a sentinel transgene. We employed SV40 T antigen-transfected human HEK293T cells which represent a model for cancer stem cells (Debeb et al., 2010). To set up a strategy to identify chromosomal sites that support transgene expression, we employed a transgene screening cassette comprising the human cytomegalovirus (CMV) promoter that drives a reporter gene encoding a stable GFP protein (half-life >20 h (Corish and Tyler-Smith, 1999)). This promoter was shown to be susceptible to epigenetic modifications (Grassi et al., 2003; Mehta et al., 2009; Hsu et al., 2010). As a reliable method for achieving single-copy integrations, lentiviral transduction was used. A self-inactivating (SIN) lentiviral vector with a deletion of the viral promoter in the 3′ long terminal repeat (LTR) was employed to avoid interference of the viral regulatory elements with the CMV promoter upon infection (Fig. S1). To ensure single-copy integration of the screening cassette, infection was performed at a multiplicity of infection (MOI) of 0.01 using a standardized protocol. Thereby, statistically, 99% of expressing cells carry a single-copy integration of the expression cassette; in previous studies, we confirmed this protocol with respect to the efficient generation of single copy integrations (see Materials and methods for further details) (Schucht et al., 2006; Gama-Norton et al., 2011). Ten days after lentiviral infection, single cells with high (>10^3 arbitrary units (a.u.)) and low (10^1–10^3 a.u.) GFP fluorescence were sorted by FACS and clonally expanded. This state was defined as passage 0. At passage 2 after sorting, flow cytometry analysis was performed for 55 and 25 clones that had been established from the high and low GFP expressing population, respectively. At this time point, the clonal cells were expanded about 100,000 folds corresponding to about 17 generations. Such cell clones represent sentinel genes whose expression is dominated by the respective chromosomal neighborhoods.

The FACS analysis revealed large differences in GFP expression in the individual clonal cell populations. None of the 25 cell clones established from the cells sorted for low-level expression showed GFP expression at this time point (less than 0.4% expressing cells, data not shown). Cell clones established from the 55 high GFP expressing cells showed variable expression with high clone-to-clone variation (Fig. S2 for overview and Fig. 1 for details of representative clones). One of the clones (clone 42^T) even showed a dramatically decreased expression. We observed variable mean expression levels and a high intraclonal variation of expression in individual clones. Some HEK293T clones (e.g., clone 12^T and 35^T) showed a more homogeneous expression phenotype while others (e.g., clones 31^T and 42^T) displayed a pronounced variation of expression.

For further in-depth characterization, we selected five HEK293T clones with different levels of heterogeneity (12^T, 17^T, 31^T, 42^T and 54^T). In all of the clones, a distinct population of low/non-expressing cells was detected (Fig. 1). To separate GFP positive expressing (PS) and GFP non-expressing cells (NS) from these five clonal populations, cells were sorted at passage 3 after infection which corresponds about 20 cell generations (Fig. 2A for overall scheme and Fig. S3 for sorting details). To exclude that non-expressing cells were a result of contamination by non-transgenic cells, genomic DNA was isolated from the five NS populations. PCR was used to confirm transgene integration for all populations (data not shown).

To evaluate the stability of the expression phenotype of the sorted populations, the selected cell clones were expanded for further 25 passages (corresponding to a total of about 110 cell generations) and re-analyzed for respective expression. The subpopulations of clones 12^T, 17^T and 54^T showed a stable phenotype upon extended cultivation: the PS populations remained positive and the NS populations also remained negative for GFP (Fig. 2B). In contrast, the subpopulations of clones 31^T and 42^T changed their phenotype: the NS populations of these clones shifted towards higher expression levels, while the PS populations showed a partial loss of GFP expression. As a result, the respective populations partially merged. Thus, these cell clones undergo a continuous modulation of the phenotype from the non-expressing state to the expressing state and *vice versa*, thereby exhibiting a highly dynamic, metastable phenotypic state.

2.2. Intraclonal heterogeneity is not correlated to differential CpG methylation

Phenotypic loss of expression has been frequently associated with a high degree of DNA methylation in CpG islands (Esteller, 2002; Cohen et al., 2008; Kaise et al., 2008; Liu et al., 2010; Tahara et al., 2010). Thus, we hypothesized that the heterogeneity in transgene expression might be modulated by epigenetic modification of the promoter sequence. We analyzed the DNA methylation status in the NS and PS populations immediately after second sorting (passage 3). In particular, we focused our analysis on a 283-bp fragment of the CMV promoter encompassing the TATA box and essential transcription factor binding