Review

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Epigenetics reloaded: the single-cell revolution

Poonam Bheda and Robert Schneider

Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC), CNRS UMR 7104/Inserm U964/Université de Strasbourg, 67400 Illkirch, France

Mechanistically, how epigenetic states are inherited through cellular divisions remains an important open question in the chromatin field and beyond. Defining the heritability of epigenetic states and the underlying chromatin-based mechanisms within a population of cells is complicated due to cell heterogeneity combined with varying levels of stability of these states; thus, efforts must be focused toward single-cell analyses. The approaches presented here constitute the forefront of epigenetics research at the single-cell level using classic and innovative methods to dissect epigenetics mechanisms from the limited material available in a single cell. This review further outlines exciting future avenues of research to address the significance of epigenetic heterogeneity and the contributions of microfluidics technologies to single-cell isolation and analysis.

A single-cell look at epigenetic inheritance mechanisms

In a completely gene-deterministic world, all genetically identical cells should have the same phenotype. However, we know that this is not the case; in multicellular organisms, cells undergo differentiation to give rise to various lineages and even when considering single-cell organisms, isogenic cells do not behave the same. While some of the differences between isogenic cells can be attributed to stochastic heterogeneity, others can arise from diversity in their epigenome (see Glossary).

Epigenetic mechanisms underlie the transmission of changes in phenotypic traits to progeny independent of alterations in the DNA sequence. Epigenetic phenomena include memory and/or maintenance of distinct transcriptional states that by themselves can arise from 'predetermined' programs or environmental signals retained in the absence of the original stimuli. Such processes include gene silencing, position-effect variegation, X-chromosome inactivation, cellular differentiation, and transcriptional memory [1–3]. Key to this regulation are noncoding RNAs (ncRNAs) [4], chromatin modifications [5,6], chromatin-modifying and -binding factors, and chromosome architecture [7]. Thus, chromatin-based mechanisms are the foundation of epigenetic processes and therefore analysis of chromatin states forms a large part of epigenetic studies.

Epigenetic mechanisms can be inherited through cellular divisions in single-cell organisms or to cellular and generational progeny in multicellular organisms. Unlike the highly stable genome, epigenetic signatures are metastable, with different epigenetic phenomena having different degrees of stability and variability [8–10]. However, the extent to which the establishment or erasure of specific epigenetic signatures is predetermined, heritable, and/or stochastic is unclear. Some epigenetic phenomena are stably inherited across many cell divisions and/or generations, while others are maintained through only one or a few generations. There is also considerable variability among individual cells in the level of specific epigenetic processes. Position-effect variegation is a classic example of this cell-to-cell variability [1]. This cell heterogeneity, combined with the varying levels of stability, makes it difficult to define heritability in a population of cells. While population experiments provide some mechanistic information for persistent epigenetic processes present in a significant percentage of cells, they miss the intricacies of individual-cell responses. Therefore, it would be of interest to track the maintenance, inheritance, and variability of epigenetic processes in single cells and their progeny (Figure 1).

The recent emphasis on single-cell rather than population-based data has pushed the limits of resolution for every type of analysis and has been particularly informative in deciphering epigenetic mechanisms. Variability in transcriptional responses in single cells and the role of chromatin modifications, chromatin-modifying enzymes, binding proteins, and ncRNAs has become a major avenue of research. This, along with the push for high-throughput analyses, has moved the epigenetics frontier to include single-cell 'omics' approaches. These studies will be vital to identify carriers of epigenetic information and understand the extent to which an epigenetic process is heritable. In this review, we present techniques geared toward single-cell epigenetics research (Figure 2) and discuss recent insights achieved in our understanding of epigenetic mechanisms that have come from their use.

Genomic sequencing-based epigenomic methods

The role of chromatin in epigenetic processes has focused research in epigenetics toward multiple techniques involving lysis of populations of cells to analyze gene expression (transcriptomics) and correlations with chromatin

Corresponding author: Schneider, R. (schneidr@igbmc.fr).
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Glossary

Bisulfite sequencing: a method to differentiate between methylated/hydroxymethylated DNA and unmethylated DNA. Bisulfite converts unmethylated cytosines to uracil while methylated and hydroxymethylated cytosines are unaffected. Conversion is usually followed by PCR amplification and NGS to identify the methylation status [28,29].

Bromodomain: a protein domain found in some modification-reader proteins that recognizes acetylated lysine residues such as those on histones. Although conserved, bromodomains can be selective toward acetylation of specific lysine residues.

Cas9: RNA-guided nuclease found in Archaea and some eubacteria for immunity against foreign DNA. These proteins have been exploited for their ability to be directed to a specific genomic locus by synthetic sgRNAs, which hybridize with their complementary DNA for targeted recruitment [55]. See also TALE.

Chromatin: histone–DNA complex that packages DNA in the nucleus of a eukaryotic cell.

Chromatin immunoprecipitation (ChIP): a technique to identify genomic DNA sequences that are associated with chromatin-associated factors or histone modifications. Antibodies against proteins or histone modifications are used to purify the DNA associated with the protein or modification of interest, followed by locus-specific PCR or NGS methods.

Chromatin-modifying enzymes: proteins that catalyze modifications of DNA or histones.

DNA adenine methyltransferase identification (DamID): DamID functions by expressing a Dam fused to the protein of interest. Dam is not present in higher eukaryotes but can be expressed to methylate adenines that come in close contact with the enzyme. These methylated adenines can then be mapped, allowing the identification of binding sites in an antibody-independent approach.

DNA cytosine methylation: cytosines in DNA can be methylated/hydroxymethylated, which is generally associated with transcriptional repression.

Fluorescence *in situ* hybridization (FISH): can detect DNA regions and nascent transcription of specific RNAs [39].

Förster resonance energy transfer (FRET): occurs between two chromophores when the donor chromophore excites the second acceptor chromophore when in close proximity [56].

Hi-C: a genome-wide, high-throughput method to detect chromosomal interactions, based on the 3C method, in which DNA interactions are detected by crosslinking of interacting regions followed by restriction digestion of the DNA and ligation of the crosslinked pieces, which are then processed in various ways. Hi-C uses biotinylation before ligation to mark ligation sites, which is subsequently used to purify ligation products, which are then sequenced by NGS [21,22,62].

Histones: small basic proteins important for organization of DNA. The core histones H2A, H2B, H3, and H4 form octameric complexes around which DNA is wrapped, while histone H1 is bound to DNA between these octamers. Histones are modified by post-translational modifications, of which the most well known are acetylation, methylation, phosphorylation, and ubiquitination, which can, for example, affect the affinity of the histones for DNA or recruit specific binding proteins.

Immunofluorescence (IF): fluorescently labeled antibodies with specificity for the modification or protein of interest are used for visualization in fixed cells by microscopy.

^{m6}A-Tracer: a methylated adenine (^{m6}A)-binding domain fused to GFP that allows the visualization of adenines in DNA that have been methylated by the Dam methyltransferase [52].

Multiphoton fluorescence-lifetime imaging microscopy–Förster resonance energy transfer (FLIM–FRET): a highly quantitative type of FRET (see above) in which the fluorescence lifetime of the donor fluorophore provides the readout for FRET such that the lifetime fluorescence of the donor decreases when FRET occurs when the donor and acceptor are in close proximity [53].

Next-generation sequencing (NGS): massively parallelized DNA-sequencing methods for high-throughput analyses [63,64].

Nucleosome: the basic unit of chromatin, a histone–DNA complex comprising approximately 146 bp of DNA wrapped around an octamer of two of each of four histone proteins (H2A, H2B, H3, and H4).

Quantitative reverse-transcription PCR (qRT-PCR): an RNA detection and relative quantification method. Conventional qRT-PCR uses a reverse transcriptase to make cDNA from RNA and then applies locus-specific primers to amplify the cDNA of interest.

RNA sequencing (RNA-seq): a method to detect RNAs globally using NGS methods [13,14].

Transcription activator-like effectors (TALEs): originally involved in bacterial infection of plants, these proteins recognize DNA sequences by specific amino acids in a variable region of a repeat domain that has been exploited for redirecting TALE fusions (e.g., with GFP) to specific DNA sequences of interest [65]. See also Cas9.

states including chromosome architecture, chromatin modifications, and/or nucleosome occupancy. Assays to study these chromatin states on a genome-wide scale all rely on PCR or high-throughput DNA-sequencing methods collectively called 'next-generation sequencing' (NGS).

With the advent of single-cell sequencing, it is now possible to resolve the transcriptome, genome organization, and some modifications of a single cell on a genome-wide scale. Due to the limited sample, single-cell sequencing experiments require the isolation of individual cells (Box 1) and amplification steps in very small volumes. Like all omics methods involving NGS, the techniques described below require significant bioinformatic analysis, which is currently the major bottleneck for these studies. While their drawback in epigenetic inheritance studies is the lack of time-resolved analyses of the same cells, insights gained from single-cell epigenomics is vast and invaluable.

RNA analysis for open reading frame (ORF) and ncRNA expression: quantitative reverse-transcription PCR (qRT-PCR) and RNA sequencing (RNA-seq)

Transcriptomics methods determine locus-specific or whole-genome expression profiles. Since epigenetic changes are associated with heritable effects in ORF and ncRNA expression [11–14], it is vital to compare RNA expression. Single-cell transcriptomics studies differences in transcription profiles between individual cells, for example, to compare different cells of the same embryo to identify cell type-specific changes during development or variability among fully differentiated cells to observe differences between cells of the same cell type. These studies have relied on RNA expression analysis in single cells by either qRT-PCR or RNA-seq, often employing microfluidics platforms (Box 1) [11–14]. qRT-PCR has been used to examine the transcriptional profile of individual mouse cells from the oocyte to early blastocyst stage of embryogenesis, consequently identifying key epigenetic regulators for each developmental stage [15]. RNA expression analyses have also implicated ncRNAs as regulators of gene expression. For example, nuclear long ncRNAs can function either as antisense RNAs or as scaffolds for targeting chromatin-modifying enzymes to specific locations [16]. Single-cell transcript data can afford a deeper understanding of the variability of ncRNA expression, with simultaneous information on downstream effects influencing ORF expression. In the future, single-cell transcriptomics might also be important for understanding disease processes and could be used to reveal differences among cancerous cells and normal cells from the same tissue within a single patient to better understand the initiation of cancer.

Chromosome-conformation analysis: Hi-C

Chromosomal looping, such as enhancer interactions with promoters or targeting of nuclear lamin-associated domains (LADs) to the nuclear periphery, has been implicated in the regulation of transcription and epigenetic memory. Chromosome-conformation capture (3C) techniques decipher these interactions to study spatial genome organization. These techniques include the original 3C and variations such as 4C, 5C, and Hi-C. Here, DNA interactions are detected by a series of steps including crosslinking of the

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