Opportunities for single-cell approaches in cancer epigenomics

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Layman's summary

Cells become cancerous via the accumulation of alterations that either change the sequence of DNA (genetic mutations) or that affect the regulation of gene expression without changing the underlying DNA sequence (epigenetic mutations). These epigenetic alterations can consist of the addition or removal of methyl groups to the DNA/RNA sequence (DNA/RNA methylation), the addition or removal of chemical groups to the proteins around which DNA is wrapped (histone modifications), or interactions with RNA molecules that are not translated into proteins but have a regulatory effect (non-coding RNAs).

As epigenetic modifications do not modify the DNA sequence, we rely on experimental procedures (chemical conversions, antibody binding, or enzymatic cleaving) to alter the sequenced DNA to indicate the presence or absence of the epigenetic modification of interest. Methods that study epigenetic modifications at a single-cell level allow us to appreciate the diversity in the epigenetic landscapes of a cell population. Single-cell epigenomic methods do not only directly target epigenetic modifications, but also study their effects on how accessible to the transcription machinery DNA sequences are, and how the DNA is organized within the cell. On top of that, some methods allow us to simultaneously study epigenetic modifications and their consequences on gene expression (at the RNA or protein level).

Single-cell epigenomic methods can provide vital information on the role of epigenetics in cancer development that would be missed by traditional bulk methods, as they can be used to identify subpopulations of cells with distinct epigenetic landscapes within the same tumor. This is essential to study the role of epigenetics across different cancer (sub)types in carcinogenesis, drug resistance, and progression. On top of that, single-cell methods can be used to capture the epigenomic landscape of circulating tumor cells found in the blood of cancer patients. Studies focused on circulating tumor cells could clarify the epigenetic mechanisms behind cancer metastasis, as well as help to develop new minimally invasive diagnostic methods.

Overall, the newly developed single-cell epigenomics provides new opportunities for innovative research into the epigenetic mechanism behind cancer. This can lead to further innovations in cancer diagnosis, prognosis, and treatment.

Abstract

Epigenetic alterations are reversible modifications that alter the way gene expression is regulated without changing the underlying DNA sequence. They play a key role in both healthy development, as well as in diseases like cancer. During the last decade, we have seen the development of novel methods to study the different layers of the epigenome (DNA methylation, histone modifications, non-coding RNAs, chromatin accessibility, and chromatin architecture) at a single-cell level. These methods provide a unique opportunity to study the impact of epigenetics in the development of intra-tumor heterogeneity and its effects on cancer development, drug resistance, and progression towards metastasis. In this review, we provide an overview of how epigenetic modifications regulate gene expression and the currently existing single-cell epigenomic methods, as well as an explanation on how these methods can be used to expand our current knowledge on the role of epigenetics in cancer.

Abbreviations

TME	–	Tumor micro-environment
m5C	–	5-methylcytosine
5hmC	–	5-hydroxymethylcytosine
5fC	–	5-formylcytosine
5acC	–	5-carboxylcytosine
m6A	–	N6-methyladenosine
MBD	–	Methyl-binding domain protein
DNMT	–	DNA methyltransferase
TET	–	Ten-eleven translocation methyl-cytosine dioxygenases
PTM	–	Post-translational modification
HAT	–	Histone acetyltransferases
HDAC	–	Histone deacetylases
HMT	–	Histone methyltransferase
HMD	–	Histone demethylase
ncRNAs	–	Non-coding RNAs
sncRNAs	–	Small non-coding RNAs (<200 nucleotides)
lncRNAs	–	Long non-coding RNAs (>200 nucleotides)
miRNAs	–	Micro non-coding RNAs (<20 nucleotides)
circRNAs	–	Circular non-coding RNAs
FACS	–	Fluorescence-activated cell sorting
BS	–	Bisulfite conversion
Dam	–	Deoxyadenosine methylase
CTC	–	Circulating tumor cell
PC	–	Prostate cancer
NSCLC	–	Non-small cell lung cancer
ALL	–	Acute lymphoid leukemia
MRT	–	Malignant rhabdoid tumors
pRCC	–	Papillary renal carcinoma

Introduction

Cancer is a disease that arises due to the accumulation of genetic and epigenetic alterations. Genetic alterations cause permanent modifications in DNA. In contrast, epigenetic alterations are reversible modifications that alter the way gene expression is regulated without changing the underlying DNA sequence. Genetic changes are widely accepted to lead to cancer development by directly inducing mutations in the DNA sequence of oncogenes and tumor-suppressor genes. However, not all tumors contain genetic drivers that could induce malignant processes. Instead, epigenetic reprogramming was recently designated as one of the determinant mechanisms that mediate the acquisition of cancer hallmarks[1].

While both genetic and epigenetic alterations can be caused by aging and environmental exposures, their relative weight in cancer development is highly tissue-dependent[2]. For example, epigenetic alterations have shown a higher impact than genetic mutations on the risk for gastric cancer[3,4]. Furthermore, epigenetic modifications have been proposed to play a central role in the development of certain childhood cancers[5], like pediatric leukemia[6] and medulloblastoma[7]. Therefore, the study of the cancer epigenome has the potential to clarify the carcinogenesis mechanisms of certain cancer types that cannot be solely explained by genetic alterations.

During the last 15 years, advances in genomic, transcriptomic, and proteomic techniques have allowed us to dramatically increase our knowledge on the complexity of the epigenome and its role in development[8]. Conventional methods to study epigenomics estimate the abundance of epigenetic marks at a population level from bulk measurements. However, these methods do not consider epigenetic diversity at a single-cell level. Single-cell approaches are essential for cancer research, due to the high levels of tumor heterogeneity, the influence of the tumor micro-environment (TME), and the epigenetic diversity resulting from tumor evolution, among other factors[9].

While single-cell genomic and transcriptomic methods are more widely used, in the last years we have seen a surge in new single-cell methods to study epigenomics. Recent advances in the fields of molecular biology, microfluidics, imaging, and sequencing technologies now offer new and exciting possibilities for studying the cancer epigenome at a single-cell level[10]. In this review, we aim to (1) describe the epigenetic mechanisms that regulate gene expression, (2) explain how the latest single-cell methods can be used to study the cell epigenome, and (3) describe the known effects of epigenetic modifications in cancer development, as well as current opportunities in this field for single-cell approaches.

1. Epigenetic modifications that regulate gene expression

Epigenetic pathways regulate gene expression without causing permanent changes in the DNA sequence. These pathways are naturally involved in development, by controlling the expression of different genes. However, alterations in these epigenetic pathways can lead to pathological processes such as cancer. In this section, we aim to describe gene expression can be regulated by three main epigenetic processes: DNA/RNA methylation, histone modifications, and non-coding RNAs[8] (**Fig. 1**). While here we will be describing these epigenetic pathways individually, they can interact and coordinate to regulate gene expression in a precise manner.

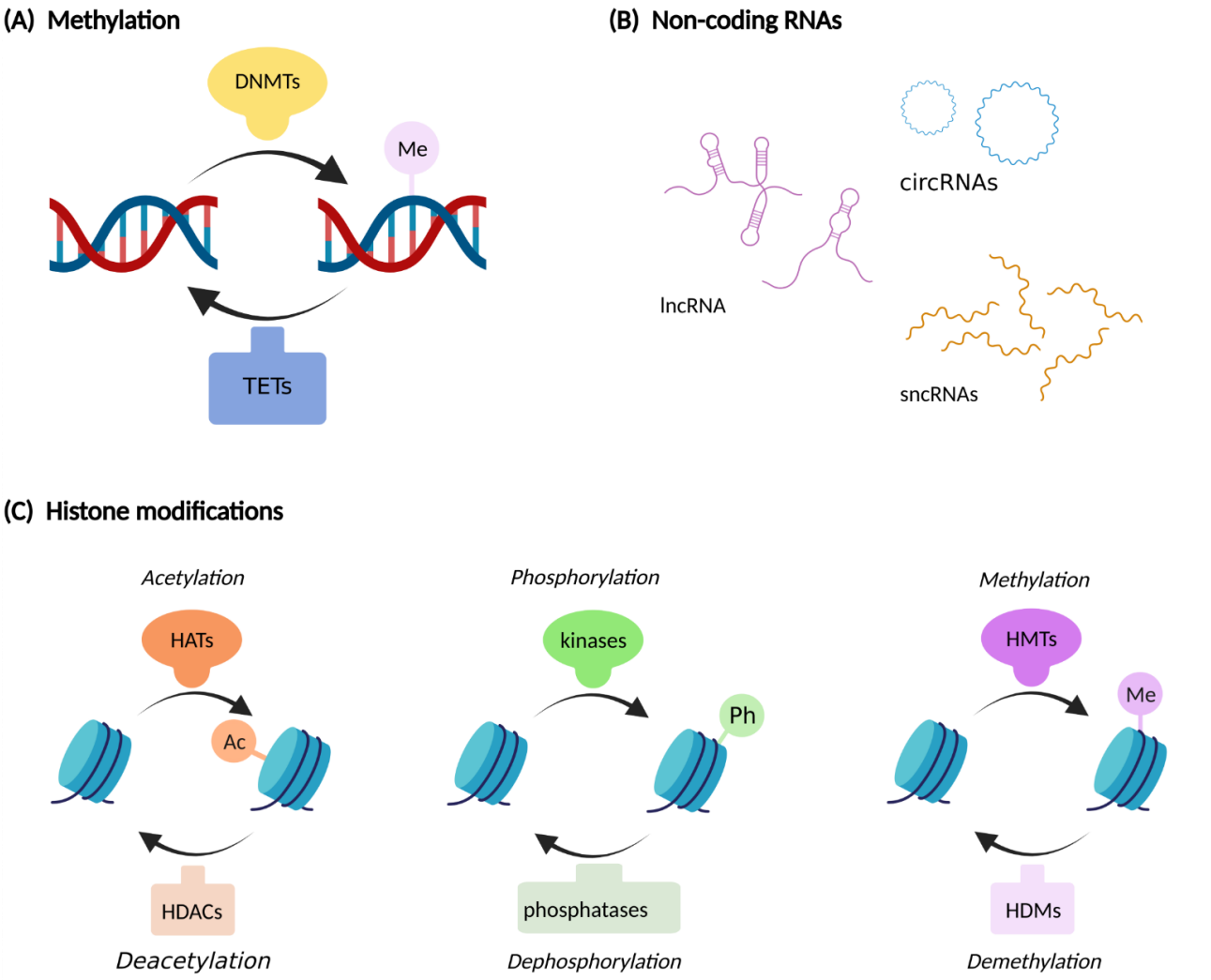

Figure 1: Epigenetic mechanisms that regulate gene expression. (A) *Methylation, the most studied epigenetic mechanism, happens in DNA and RNA via the action of DNA methyltransferases (DNMTs). The opposite process, demethylation, happens via the action of ten-eleven translocation methyl-cytosine dioxygenases (TETs).* ***(B)*** *Non-coding RNAs (ncRNAs) can be divided into two broad categories: short non-coding RNAs (snRNAs, <200 nucleotides) and long non-coding RNAs (lncRNAs, >200 nucleotides). A subtype of lncRNAs are circular RNAs (circRNAs), which have been linked to several diseases.* ***(C)*** *Three of the most studied histone modifications. Histones are acetylated by histone acetyltransferases (HATs) and demethylated by histone deacetylases (HDACs). Histone phosphorylation is carried out by kinases, and phosphatases mediate histone dephosphorylation. Histones are methylated by histone methyltransferases (HMTs) and demethylated by histone demethylases (HDMs).*

1.1 DNA and RNA methylation

The best characterized epigenetic modification is DNA methylation, which happens via the addition of a methyl group to the 5-carbon of cytosines, forming 5-methylcytosine (5mC)[11]. This induces gene silencing by preventing transcription factors from accessing DNA positions, or by recruiting methyl-binding domain proteins (MBDs), which can lead to further histone modififcations[8]. DNA methylation is carried out by the action of three DNA methyltransferases (DNMTs): DNMT1, DNMT3a, and DNMT3b[8,11]. While DNMT1 is the DNMT with the highest catalytic activity during replication and is responsible for "maintaining" the DNA methylation status, DNMT3a and DNMT3b act as *de novo* DNMTs[12]. Although DNMT3a and DNMT3b have been suspected to be partially redundant, knockout studies have shown they have distinct essential functions[13]. Enzymatic studies have shown that these functional differences are due to the different substrate preferences of DNMT3a and DNMT3b[14]. DNMT3a is essential in establishing methylation patterns during gametogenesis, while DNMT3b plays a more dominant role in early embryonic development[8].

In contrast, DNA demethylation "recovers" genes silenced by DNMTs. DNA demethylation is carried out by ten-eleven translocation methyl-cytosine dioxygenases (TETs) like TET1, TET2, and TET3. These enzymes can turn m5C into 5-hydroxymethylcytosine (5hmC), or turn 5-hmC into 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC). DNA demethylation and oxidized 5mC forms (5hmC, 5fC, and 5caC) are involved in several developmental stages, like pre-implantation, germ cell development, pluripotency, and differentiation[15].

While here we will focus on DNA methylation, RNA can also be methylated. The most studied form of RNA methylation is the methylation of 6-carbon of adenosine, forming N6-methyladenosine (m6A). This RNA modification is commonly enriched near the stop codon of genes, as well as 3'-UTR and within internal long exons, and affects almost every aspect of RNA processing such as RNA transcription, degradation, splicing, and translation[8,16].

1.2 Histone post-translational modifications

DNA is wrapped around histone octamers called nucleosomes, which are composed of a tetramer of two H2A and two H2B, flanked by dimers of H3 and H4. Each histone contains a globular C-terminal domain and an extended N-terminal tail, which can be subjected to post-translational modifications (PTMs)[8]. Several distinct histone PTMs regulate gene expression, but in this review we will focus on histone acetylation, methylation, and phosphorylation.

The most studied histone PTM is histone acetylation. Adding an acetyl group to the histone removes its positive charge, loosening up the tight chromatin regulation and enabling the access of transcription factors. Histone acetylation is mediated by histone acetyltransferases (HATs), while the removal of acetyl groups, *ie*. histone deacetylation, is mediated by histone deacetylases (HDACs)[17].

Phosphorylation happens on the serine, threonine, and tyrosine residues of histones. Histone phosphorylation sites can broadly be divided into two categories: (1) those that play a role in transcriptional regulation and (2) those that play a role in chromatin condensation. Adding a phosphate group to a histone confers it with a negative charge, opening up the chromatin conformation. This is carried out by kinases, while the removal of the phosphate groups is performed by phosphatases[18]. Histone phosphorylation has been found to play a role in mitosis, apoptosis, DNA

repair, replication, and transcription[8]. Several assays take advantage of this, such as the γH2AX-based assays, which rely on the detection of the phosphorylated form of H2AX histone, a variant of histone H2A, as a DNA damage biomarker[19].

Lysine methylation happens in histones on the arginine, lysine, and histidine residues. While histone acetylation and phosphorylation can change the histone's electric charge, methylation doesn't. Instead, it directly affects the recruitment and binding of regulatory proteins. Histone methylation is carried out by histone methyltransferases (HMTs), while histone demethylases (HDMs) mediate histone demethylation[18].

Other existing histone PTMs not described here include ubiquitylation, sumoylation, and ADP ribosylation, among others[18,20]. Furthermore, mutations directly on histone genes like can lead to the development of pathologies like cancer[21].

1.3 Non-coding RNAs

A large portion (98%) of the genome is never translated into proteins[22], but can be transcribed into non-coding RNAs (ncRNAs). These ncRNAs can be divided into two broad categories based on their size, distinguishing between "small" non-coding RNAs (sncRNAs) and large non-coding RNAs (lncRNAs). sncRNAs are highly conserved across species and take part in transcriptional and post-transcriptional gene silencing. One of the most studied sncRNAs are micro RNAs (miRNAs), which are smaller than 20 nucleotides. In humans, miRNAs regulate the expression of 60% of protein-coding genes[8].

In contrast, lncRNAs are less conserved across species and have a more varied mechanism of action[8]. They can influence target sites both in the nucleus and the cytoplasm and can act as regulators, enhancers, sponges for other ncRNAs, molecular scaffolding, etc. A subset of lncRNAs are circular RNAs (circRNAs). Both lncRNAs and circDNAs can directly bind to miRNAs and act as miRNA sponges, interfering with miRNA-mediated gene silencing[23].

2. Single-cell methods to study epigenomics

In general, single-cell methods offer several advantages over traditional bulk methods, as bulk methods are unable to detect cell-to-cell variability and require more starting material. Single-cell methods are very useful when the starting material is limited and valuable, and therefore not suitable for bulk approaches. Furthermore, single-cell approaches are ideal for subpopulation studies, as they allow for the characterization of rare cell populations that would be typically missed by bulk methods. When studying epigenetic regulation, single-cell methods allow us to study the different epigenetic landscapes present in heterogeneous cell populations. Furthermore, single-cell multi-omic approaches allow the study of interactions between the different layers of the epigenome. When combined with transcriptomic data, they can be used to link epigenetic characteristics to specific phenotypes[24].

However, single-cell epigenomic methods also provide disadvantages compared to studies in bulk. While in single-cell genomics and transcriptomics we can directly study sequencing data, epigenomic methods rely on chemical conversions, antibody binding, and enzymatic cleaving to translate epigenetic information into sequencing data. In addition, data analysis procedures for epigenetic data are less established than that for genomic and transcriptomic data. There is a lack of guidelines to choose what methods might be more suitable for what kind of studies and what kind of analyses might be more appropriate. On top of that, single-cell epigenomic methods are more labor intensive, have a higher cost, and require more complex analysis, which can lead to misinterpretation of results and wrong conclusions[24].

2.1 Tagging of single cells

Single-cell epigenomic methods require to individually sequence cells. There are three main categories of methods to isolate or tag single cells. The first category requires to physically isolate and compartmentalize single cells. The most popular way of doing this is via Fluorescence Activated Cells Sorting (FACS), which allows to separate individual cells in a highly specific manner and with high throughput. However, the cells need to be dissociated into suspension before sorting, and a large amount of starting material is required[25]. Another method for individual cell isolation and compartmentalization is manual cell picking or micromanipulation, which allows to isolate cells from intact live tissue at the cost of lower throughput[26]. Other possible single-cell isolation techniques are MACS, LCM, and microfluidics[25]. All these techniques require a substantial infrastructural investment and skilled operators for the machinery. Furthermore, the physical isolation of single cells can result in a lot of noise due to single-cell amplification bias.

The second category of methods is droplet barcoding. This approach relies on microfluidics equipment to encapsulate single cells into droplets together with barcoding oligonucleotide primers, lysis reagents, and other needed compounds. After lysis, barcodes are attached to the extracted DNA/RNA fragments so there is a unique sample barcode per cell. Afterwards, material from all cells is pooled and sequenced, and the barcodes in the resulting reads can be used to identify sequencing material from individual cells[27,28]. Compared to the physical isolation and compartmentalization of cells, this method allows for a smaller single-cell amplification bias, lower cost, and higher throughput[29].

The final category is combinatorial barcoding of single nuclei. These methods rely on tagging the nuclei with a combination of barcodes to dramatically increase the throughput. A significant advantage of this method is that nuclei can be isolated from preserved tissue samples and samples that are difficult to dissociate[30], allowing the study of otherwise inaccessible samples. While this method hasn't been widely applied yet, in the last couple of years pre-existing single-cell epigenomics methods have been adapted for combinatorial barcoding to increase throughput[31,32]

The most suitable method for tagging single cells should be picked based on the nature of the cells being isolated, the desired scale of the experiment, and the budget allocated for cell tagging. Physical cell isolation via FACS can be used to select specific cell populations based on antibody-binding, the presence of transgenic fluorescent constructs, or even cell characteristics such as size and granularity[33]. This makes it ideal for the study of specific cell populations in transgenic animal models. However, this method requires a large amount of starting material. In contrast, droplet-based methods reduce the single-cell amplification bias, increases the throughput, and have a lower cost per cell. Nonetheless, the number of cells studied is limited by the number of unique barcodes available. Overall, this method can be used when the starting material is rare and too scarce for physical cell isolation. Combinatorial barcoding allows for the dramatic increase of throughput, as well as the study of tissues and cells that cannot be suspended. It is therefore the best method for tagging single cells from solid tumors that are not easily dissociated. However, it should be noted that an increase in the number of cells studied results in an increase in the cost of sequencing. One of the main limitations of most single-cell tagging methods is that they require significant investments in infrastructure.

2.2 Existing single-cell methods for the different epigenomic layers

During the last two decades, several single-cell methods to study epigenomics have been developed. Some of these methods focus on one epigenomic layer (DNA methylation, histone PTMs, ncRNAs, chromatin accessibility, or chromatin architecture), while some others try to study several layers at the same time and/or other omic layers (transcriptomics, genomics). For a brief overview of the advantages and disadvantages of some mono-omic single-cell epigenomic methods, see **Table 1**.

Target	Method	Pro	Con
Methylation	scBS-seq[34]	Less starting material than BS-free methods	Misses C>T mutations, Low mapping
	scRRBS[35,36]	High resolution at CpG islands, Low cost	Misses C>T mutations, Only CpG islands, Low mapping
	sciMET[32]	Increase read alignment, Higher throughput (combinatorial indexing)	Misses C>T mutations, Low coverage per cell
	scPBAT[37]	Repeat-specialized approach, Lower cost, No amplification bias	Misses C>T mutations, Targeted approach, Adaptor-ligand bias
	scCGI-seq[38]	BS-free, High coverage, Accurate profile CpG islands	Requires more starting material, No non-CpG data
Histone PTMs	Drop-ChIP[39]	High throughput	Background noise from nonspecific antibody pulldown, Low number reads per cell, Large amount starting material needed
	scDamID[40] + EpiDamID[41]	Doesn't rely on antibody binding	Only suitable for disease models (not patient samples), Limited by GATC motif
	scChIC-seq[42]	High number reads per cell	Low throughput
	scCut&Tag[43]	Cost-effective, High throughput	Repetitive reads, Open chromatin bias (Tn5)
	COBATCH[44]	High throughput, High number reads, Low background	Unsuitable for detecting repressive marks, Open chromatin bias (Tn5)
	ACT-seq[45]	High throughput, Simple & straightforward protocol	Low unique reads, Open chromatin bias (Tn5)
	scChIL-seq[46]	Low background	Time-consuming & complex workflow, Open chromatin bias (Tn5)
ncRNAs	scRNA-seq[47]		Only sequence polyadenylated RNA
	MATQ-seq[48]	Sequences both polyadenylated and non- polyadenylated RNA, Transcriptome-wide coverage	No detection of mature miRNAs
	Smart-seq-total[49]	Sequences both polyadenylated and non- polyadenylated RNA, Transcriptome-wide coverage, Detect both lncRNAs and sncRNAs	Loss of information about polyadenylation status, Misses circRNAs
	VASA-seq[50]	Sequences both polyadenylated and non- polyadenylated RNA, Transcriptome-wide coverage, Scalable throughput, Detect both lncRNAs and sncRNAs	Loss of information about polyadenylation status
Chromatin accessibility	ScATAC-seq[51]	Straight-forward and quick protocol, High coverage per cell	Sequence bias, Low throughput
	sciATAC-seq[52,53]	High throughput	Low coverage per cell
	scDNase-seq[54]	High coverage per cell	Sequence cleavage bias, Depends on enzymatic efficiency, Low mapping efficiency & throughput
	scMNase-seq[55]	No sequence cleavage bias	Indirect approach, Depends on enzymatic efficiency, Background noise due to non-specific binding
Chromatin architecture	scHi-C[56]	High coverage per cell	Low throughput
	sciHi-C[31]	High throughput (combinatorial indexing)	Low coverage per cell

Table 1: Comparative overview of mono-omic single-cell epigenomic methods. *This table expands on previous comparisons made by Mehrmohamadi et al (2021)[29].*

DNA methylation

DNA methylation is one of the best characterized epigenetic layers. As such, several different approaches have been developed to study it.

Bisulfite conversion (BS) is the basis of many single-cell DNA methylation assays, like scBS-seq (single-cell bisulfite sequencing)[34]. BS converts all unmethylated cytosines into uracil via deamination. After fragmentation, the fragments are tagged with Illumina primers, amplified, indexed, and sequenced. However, traditional BS-based methods cannot distinguish between 5mC and 5hmC, which requires more specialized methods. Furthermore, the deamination of unmethylated cytosines into uracil reduces the complexity of the sequence, complicating read alignment, which and can lead to missing individual SNPs, as we cannot distinguish between nucleotide changes resulting from BS and those resulting from C>T mutations[29]. This needs to be considered when researching the methylome of cancer patients, as C>T mutations are characteristic in mutational signatures from temozolomide-treated patients or those with defective DNA mismatch repair mechanisms[57]. On top of that, scBS-seq is a costly method and has a bias for CpG sites[29]. Sc-RRBS (single-cell reduced representation bisulfite sequencing)[35,36] combines BS with restriction enzymes to produce sequence-specific fragmentation. This method allows the study of methylation specifically at CpG sites, especially CpG islands, which are more likely to be representative of the overall methylome. However, it still misses a large portion of CpG sites and does not cover non-CpG islands, like those containing enhancers. Furthermore, it has a very low mapping efficiency (~50%). As read alignment is a big issue in BS-based methods, sciMET[32] tries to improve it by using transposomes (transposase-adaptor complexes) with adaptors depleted from cytosines, so they are not affected by BS. This method uses combinatorial indexing to increase the throughput of the assay. Still, assessing methylation in repetitive regions is challenging, which lead to the development of scPBAT[37], which uses post-BS random priming and primer extension to effectively target methylation in repetitive regions.

To avoid BS, the BS-free approach scCGI-seq was developed[38]. This method relies on the comparison of sequencing data from untreated cells and cells treated with methylation-sensitive restriction enzymes to identify methylated CpG islands. However, this method requires a larger amount of starting material, as each sample will need to be divided between a test and methylation control subsample.

The choice of single-cell methylation assay should be based on the specific aim of the study. For genome-wide methylation studies, either scBS-seq or sciMET are good approaches, depending on the desired throughput and coverage per cell. ScRRBS is an alternative for a lower-cost method. If repetitive regions are the focus of the study, scPBAT offers better performance than other methods. If there is abundant starting material and we want to avoid BS, a BS-free approach like scCGI-seq can be used.

Histone PTMs

Three main approaches are used to study histone PTMs: immunoprecipitation-based, DamID-based, and cleavage-based. Drop-ChIP[39] is a single-cell method that combines droplet barcoding with

classical chromatin immunoprecipitation (ChIP). Antibodies specifically bind to the histone PTM of interest, and the subsequent pulldown of the chromatin regions attached to these antibodies allows to separate regions harboring specific histone PTMs.

DamID-based technology relies on the fusion of *E. coli* deoxyadenosine methylase (Dam) and a protein of interest (ID). Subsequently, Dam methylates DNA on the adenine residues in GATC sequences close to the binding site of the protein of interest[58]. It should be noted that this approach is limited by the occurrence of GATC sequences near the protein of interest. While the original DamID approach does not allow for the study of histone PTMs, the recently developed EpiDamID[41] extends its use to detect histone PTMs. Already existing single-cell DamID-based methods like scDamID[40], can be combined with EpiDamID to study histone PTMs at a single-cell level.

Cleavage-based methods make use of antibodies to target a specific DNA-bound protein. The cells then undergo specific enzymatic treatments to cut the DNA bound to the protein of interest, releasing short DNA fragments. At bulk level, two main methods use this approach: Cut&Run[59] and Cut&Tag[60]. Cut&Run uses Mnase-pA as a cleaving enzyme and has yet to be adapted for single-cell histone profiling, although a Cut&Run approach was recently developed to study transcription factors binding at a single cell level[61]. However, scChIC-seq[42] uses a similar approach to study histone PTMs at a single-cell level. On the other hand, Cut&Tag[60] uses a pA-Tn5 fusion protein loaded with sequencing adapters to cleave the DNA and has been recently adapted for single-cell use via droplet barcoding as scCut&Tag[43]. Before this adaption, other methods like CoBATCH[44], ACT-seq[45], and scChIL-seq[46] used a Cut&Tag-like strategy for single-cell histone PTM profiling. However, methods reliant on Tn5 can introduce biases via non-specific cleaving to open chromatin.

The choice of a method to study histone PTMs at a single cell level should be made according to the nature of the cells, the amount of starting material, and the histone PTM of interest. While immunoprecipitation- and cleavage-based methods rely on the existence of commercial antibodies that target the protein of interest, DamID-based methods do not. Therefore, they are more suited for the study of rare histone PTMs for which there are no specific antibodies available. However, EpiDamID requires the system studied to express the Dam-fusion protein construct. Consequently, this method is suitable to study histone PTMs in animal models and cell lines, but not in patient samples[41]. To study patient samples, immunoprecipitation- and cleavage-based methods should be used instead. All these methods only allow to study one histone PTM at a time and rely on data integration approaches to study interactions between different histone PTMs. In the future, the development of methods allowing for multiple histone PTM profiling at the same time should provide us with essential information about interactions between histone modifications within the same cell in a more direct manner.

Non-coding RNAs

ncRNAs can be detected at the single-cell level using traditional scRNA-seq[47], however, this method only captures short (~400-600 base pairs) sequences adjacent to the poly(A) tail of the RNA molecule, ignoring more distant sequences and non-polyadenylated RNA molecules. As a consequence, the fraction of ncRNAs lacking poly(A) tails will be ignored[50]. In the last 5 years, some single-cell methods that allow to sequence both polyadenylated and non-polyadenylated RNA have been developed, increasing the sensitivity of ncRNA detection. MATQ-seq[48] uses random primers to

sequence both polyadenylated and non-polyadenylated transcripts. Smart-seq-total[49] adds a poly(A) tail to all transcripts before reverse transcription and uses the MMLV reverse transcriptase to convert the full RNA sequence into cDNA. VASA-seq[50] also adds poly(A) tails to all transcripts before reverse transcription. Both Smart-seq-total and VASA-seq can detect a broad spectrum of ncRNAs, VASA-seq is more sensitive and detects a larger number of lncRNAs[50].

Other methods that can be used to capture ncRNAs are qRT-PCR and microarrays. However, qRT-PCR is labor-intensive and requires a targeted approach, while microarrays rely on transcriptomic probes and can contain noise due to low abundant transcripts[47].

Chromatin accessibility

The level of compaction of chromatin of a region, also known as chromatin accessibility, is the result of the interplay between the different elements of the epigenome. The study of changes in chromatin accessibility can be used to identify regulatory regions that may play a role in development and disease[62]. The most popular method for measuring chromatin accessibility at single-cell level is scATAC-seq, which uses Tn5 to insert sequencing adapters in open chromatin regions after isolating and tagging single cells[51]. An improved version of scATAC-seq, sciATAC-seq, was developed to combine scATAC-seq with combinatorial barcoding[52,53].

Other single-cell methods for assaying chromatin accessibility are scDNase-seq[54] and scMNase-seq[55]. scDNase-seq uses digestion with DNase to allow the sequencing of accessible chromatin regions[54]. In scMNase-seq, MNase cleaves DNA regions not protected by nucleosomes, indirectly allowing for the measurement of open chromatin and the position of nucleosomes[55]. However, these methods have more complicated workflows than Tn5-based methods.

Chromatin architecture

The three-dimensional organization of the DNA in the cell nucleus is determined by epigenetic marks and can provide essential information when studying development and disease, as specific spatial nuclear domains regulate gene expression. Several methods based on chromatin conformation capture have been developed to study chromatin architecture at a single-cell level by cross-linking and ligating chromosomal regions that physically interact[29]. The first of these methods was scHiC, which allows for the detection of simultaneous chromatin contacts at single-cell level[56]. A recently published version of scHi-C, sciHi-C[31], improves the throughput of traditional scHi-C by combining it with combinatorial barcoding.

Multi-omic / multi-modal methods

Most of the above-described methods only allow studying one layer of epigenetic alterations at a time. As each method requires specific chemical conversions, antibody binding, or enzymatic cleaving, it is not possible to use two different approaches on the same cell. If two different approaches were to be used on the same cell, the corresponding reactions might interact and interfere with each other, making the results not reliable. However, several methods have been developed specifically to study several epigenomic layers at once. scNOME[63] (Nucleosome Occupancy and Methylome Sequencing) allows for the joint study of methylation, chromatin accessibility, and nucleosome phasing at a single cell level. Furthermore, there are two methods to study jointly methylome and chromatin conformation: methyl-HiC[64] and sn-m3C-seq[65].

There are also methods to simultaneously obtain information about epigenetic markings and genomic, transcriptomic, or proteomic data. Obtaining both epigenetic and gene expression data from the same cell can provide very valuable phenotypic information on the consequences of epigenetic alterations. The first single-cell method combining epigenomic and transcriptomic data was scM&T-seq[66], which allowed for joint methylome and transcriptome profiling. An alternative would be smart-RRBS[67], a method combining RRBS and smart-seq to provide both methylation and gene expression data. scDam&T-seq[68] is a method developed to study both DNA-protein contacts and gene expression. If combined with EpiDamID, it can be used to study histone PTMs and their effect on gene expression[41]. Phenotypic information can also be obtained from proteomic data, as shown by scCut&Tag-Pro[69], which allows the joint study of histone PTMs and surface proteins. Furthermore, there are methods to jointly study methylation and genetic modifications, like scCOOL-seq[70] and epi-gSCAR[71]. scTrio-seq[72] was developed to allow the joint study of the DNA methylome, transcriptome, and genome.

2.3 Data analysis

Analysis of single-cell data is more complicated than bulk data analysis due to data sparsity and high levels of background noise. It is even more difficult to analyze single-cell epigenomic data, as there is a lack of established pipelines when compared to single-cell genomics or transcriptomics. On top of that, missing data is a big problem for data integration approaches, as the missing data might follow different patterns within the same cell[73].

Data pre-processing and clustering

Before analyzing the epigenetic characteristics of each cell, sequencing data pre-processing is necessary to identify individual cells. The DNA barcodes introduced during the single-cell tagging steps are here used to allocate reads to individual cells. After filtering low-quality cells according to the read depth and the signal-to-noise ratio, a quality check is performed both in bulk and individual cells. The remaining reads can then be converted to a read count matrix, which can be used to select subsets of cells according to their features. While dimensionality reduction is difficult in single-cell data due to its high dimensionality and the sparsity of the data, it can be done via approaches like latent semantic indexing, spectral embedding, and topic modeling. Data clustering via algorithms like k-means clustering or Louvain can be used to identify cell subpopulations, which can be visualized using tSNE or UMAP. This step is essential to remove low-quality and doublet cells[73].

Existing pipelines

The strategy required to analyze the sequencing data depends on the nature of the epigenetic features of interest. While there are no unified guidelines for the analysis of single-cell epigenomic data, several different tools have been developed. Some tools, like EpyScanPy[74], are designed to analyze several omics data types (methylation and scATAC-seq data). However, most tools are only designed to analyze one type of epigenomic data.

Pipelines like methylpy[75] and Methylstar[76] have been developed to analyze methylation data from single-cell BS-based methods. Analysis tools for BS-based approaches map the BS-converted sequences to a reference genome and determine cytosine methylation levels based on single nucleotide changes. As BS-based methods produce reads with low mappability, specific read

mapping tools like Bismark and BS Seeker are needed[36]. When using these tools to compare methylation levels across cells, we can distinguish between consistently sites, consistently unmethylated sites, and sites that are differentially methylated across cells[32]. In contrast, data from the BS-free method scCGI-seq requires the comparison of sequencing data from control and test samples to determine methylated and unmethylated sites[38].

From the bioinformatics point of view, the study of histone PTMs relies on read clustering to identify sequencing peaks that indicate the presence of the histone PTM of interest. Some of the published histone PTM profiling methods include data analysis scripts[41,43]. Data integration approaches are especially important when studying histone PTMs, as current methods only allow to study one histone PTM at a time.

Similarly, bioinformatics tools to assess chromatin accessibility from single-cell data rely on the identification of peaks of reads to detect highly accessible regions[62]. Currently, there are several bioinformatics tools available for the analysis of scATAC-seq data, like Scasat[77], snapATAC[78], and scATAC-pro[79], among others[80]. On top of that, scitools[81] was developed explicitly for the analysis of sciATAC-seq data.

Pipelines like Higashi[82] have been developed to data generated by scHi-C-based methods to study interactions between chromatin regions. Data analysis of HiC-based methods relies on abnormal mapping from paired-end sequencing data to identify chromatin contacts. Individual reads from the same pair map to seemingly distant and unrelated regions of the genome when there is contact between the two regions[83].

ncRNAs can be identified from RNA-seq data by either sequence homology with known ncRNAs, or by possessing common ncRNA features like RNA motifs and trinucleotide frequences[84,85].

Data Integration

Data integration is one of the main challenges of single-cell data analysis. With the development of multi-omic methods, there is a need to develop computational methods to integrate data from different omic levels in the same cell (intra-cell integration)[73]. The latest tools that can perform this kind of data integration are WVN analysis[86] and totalVI[87], among others. On top of that, as it is not possible to study all the different omic layers in a single cell due to cost and experimental limitations, it is necessary to develop tools that allow the integration of different omic layers between different cells of the same type (inter-cell integration)[73]. Tools that allow inter-cell data integration include Seurat v3[88] and GLUE[89].

3. Opportunities and applications in cancer research

During their progression towards neoplasticity and malignancy, cells acquire certain functional capabilities, commonly known as the hallmarks of cancer. Recently, Hanahan proposed that some enabling characteristics, like non-mutational epigenetic reprogramming, allow for the acquisition of these hallmark capabilities[1]. Widely used bulk epigenomic methods provide a snapshot of the overall epigenetic landscape of the processed tumor tissue, but fail to accurately account for cell-to-cell variability. Instead, single-cell methods are key to identifying cell subpopulations responsible for carcinogenesis, drug resistance, and progression towards metastasis.

3.1 Applications of single-cell methods in cancer epigenomics

Single-cell methods provide a unique opportunity to study intra-tumor heterogeneity and rare subpopulations (like circulating tumor cells and cancer stem cells) present in cancer, which would be missed by traditional bulk approaches[1]. Intra-tumor heterogeneity refers to the different subpopulations of cells that have distinct characteristics within the same tumor, and it has been shown to play a key role in the development of drug resistance and recurrence. Intra-tumor heterogeneity has been proposed to be caused by (1) heterogeneity in the genetic landscape of the tumor cells, as a result of mutation accumulation; (2) heterogeneity in the non-genetic mechanisms, like epigenetic marking, that regulate gene expression and (3) heterogeneity in the TME surrounding different parts of the tumor[2] (**Fig. 2**). Single-cell epigenomic studies allow for the characterization of subpopulations of cells present in the tumor and can provide a look into the epigenetic nature of intra-tumor heterogeneity.

Single-cell methods are not only useful to study tumors and their micro-environment but they can also be used to study circulating tumor cells (CTCs). CTCs are cells that enter peripheral blood from the primary tumor, and can potentially seed metastases. Thus, their characterization is essential to understand how cancer metastasize[91]. CTCs are proposed to have an epigenetic landscape distinct from that of primary tumors, as shown in a recent study by Zhao et al (2021) focusing on CTCs derived from lung cancer[92]. CTC-centric cancer epigenomic research also appears to be valuable in the search for minimally-invasive diagnostic tools, for example via the identification of characteristic epigenetic patterns in cancer cells from blood samples[91].

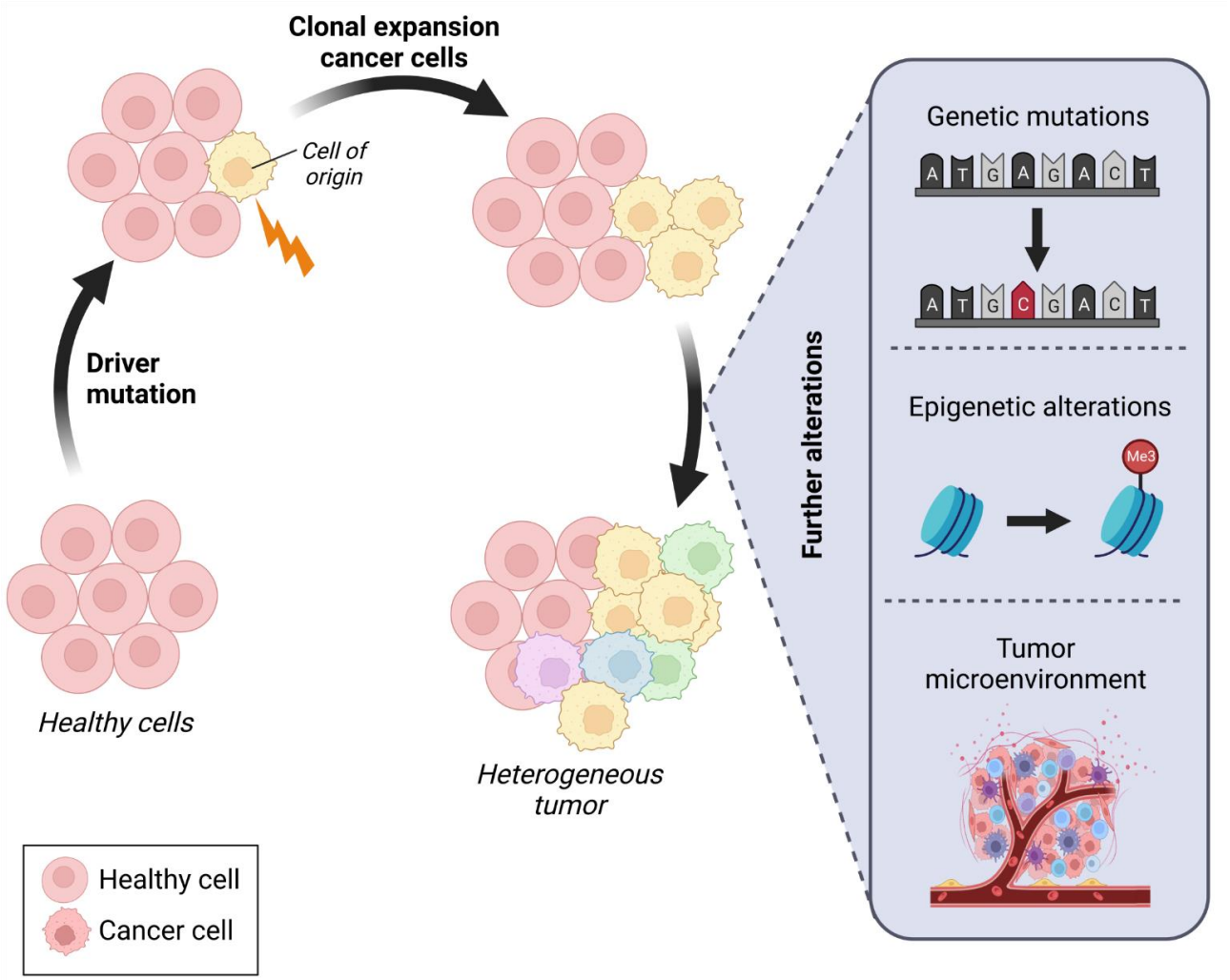

Figure 2: Mechanisms driving tumor heterogeneity. *A driver mutation (genetic or epigenetic) in a healthy cell can initiate its transformation into a cancer cell, becoming the cell of origin of the subsequent tumor. The cancerous cell clonally expands, making the tumor grow. Throughout this clonal expansion, further genetic and epigenetic alterations in individual cells, as well as the changing TME, can result in the existence of subpopulations of cells with distinct characteristics. This results in the development of a heterogeneous tumor, ie. a tumor containing distinct cell subpopulations with different genetic and epigenetic landscapes in the same tumor.*

3.2 The role of epigenetics across cancer types

Both genetic and epigenetic alterations jointly contribute to the development of cancer. While mutations in genes involved in epigenetic regulation result in changes in the epigenetic landscape, the deregulated expression of mutated genes via epigenetic mechanisms can modulate the consequences of these mutations. However, the relative level of impact of genetic and epigenetic alterations has been shown to vary across cancer (sub)types[93]. The following overview presents some cancer types characterized by high-impact epigenetic alterations. Depending on the cancer type, epigenetic alterations can affect the carcinogenesis process, progression towards metastasis, or response to treatment.

While most of the single-cell epigenomic methods are quite novel and have yet to be used to study cancer development, epigenetic studies at bulk level and single-cell transcriptomic studies already provide some insight into what types of cancer would benefit from single-cell epigenetic approaches.

Renal cancer

Genetic mutations are rare in sporadic renal cell carcinoma (RCC). RCC cases that do have genetic mutations present mostly alterations in the *VHL* and *MET* genes. In contrast, epigenetic mutations are commonly found and thought to be responsible for intra-tumor heterogeneity in these cancers[94].

This makes the recently developed single-cell epigenomic methods essential to study renal intra-tumor heterogeneity. A recent scATAC-seq analysis by Wang *et* al (2022) has shown that the epigenomic landscape of renal cells can be used to determine the cells of origin of different subtypes of papillary renal carcinoma (pRCC)[95]. The different pRCC subtypes according to the cell of origin presented distinct molecular characteristics, carcinogenic processes, and clinical behavior. However, while this method allowed the identification of differentially accessible regions, it did not identify the specific epigenetic alterations responsible for the change in chromatin accessibility. Another recently published study identified three histone acetylation signatures across hepatocellular carcinoma patients that could be correlated with different survival prognosis[96]. Single-cell approaches focusing on histone modifications, like the previously described scCut&Tag method, could be used to determine whether histone acetylation patterns are global, or whether any cell subpopulations are responsible for this difference in prognosis (**Fig. 3**). This line of research could provide us with better diagnostic and prognostic strategies than the currently available.

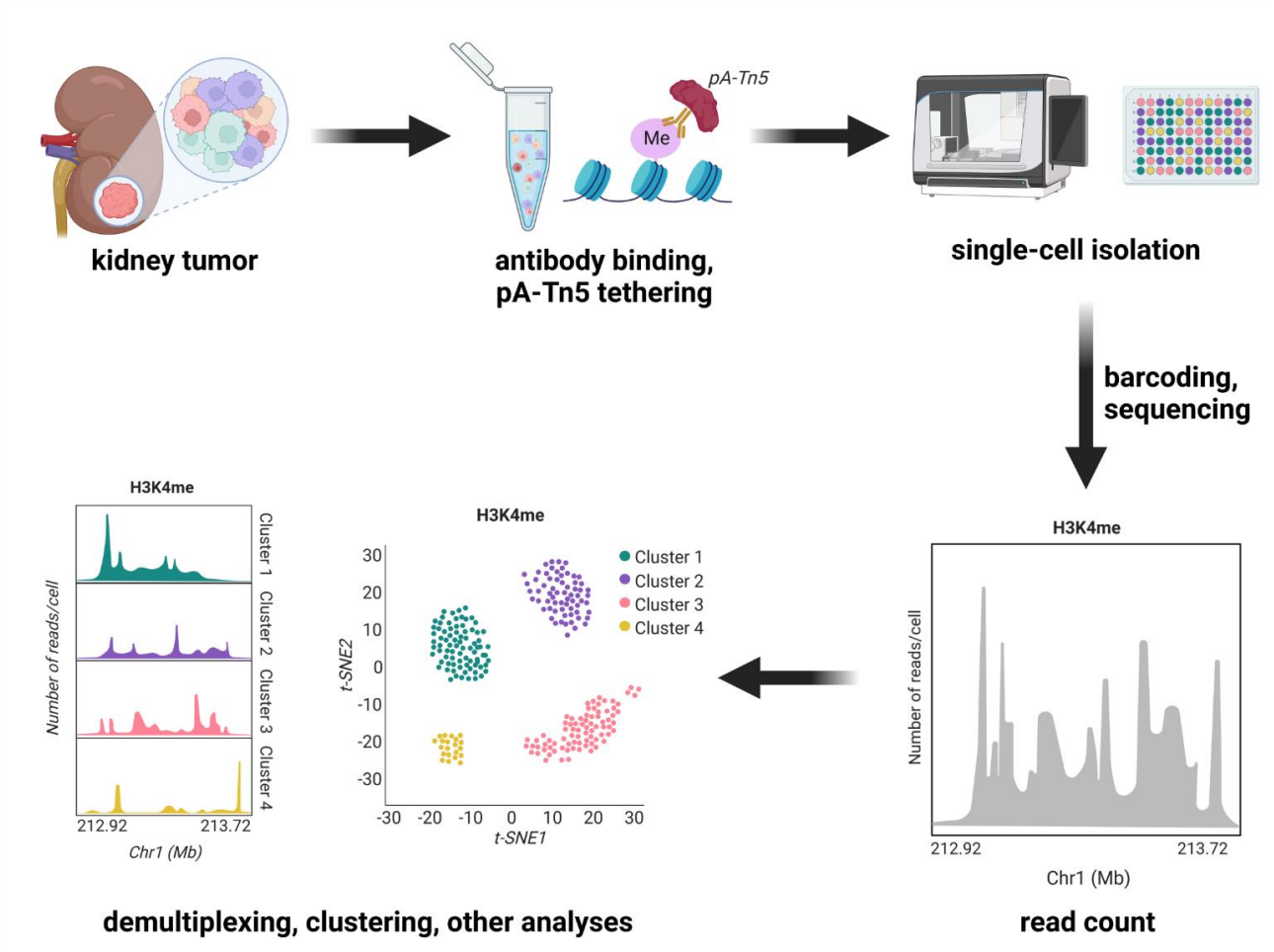

Figure 3: Example workflow of how single-cell methods could be used to study the epigenetic mechanisms behind tumor heterogeneity in renal cancer. *Epigenetic alterations are common in kidney tumors and, in particular, general levels of H3K4 methylation (H3K4me1, H3K4me2, and H3K4me3) have been shown to decrease in clear cell renal cell carcinoma as the tumor progresses towards metastasis[97]. In this diagram, we show how the previously described*

scCut&Tag method could be used to identify different subpopulations of cells from patient-derived material according to their H3K4 methylation profiles. This approach would allow us to assess whether this loss of H3K4 methylation happens tumor-wide, or whether there are specific subpopulations of cells with loss of histone methylation that are responsible for the progression of the disease.

Rhabdoid tumors

Malignant rhabdoid tumors (MRT) are childhood cancers that can arise in any soft tissue, although they tend to be found in either the kidney, the brain, or in both at the same time. MRT has a very clear genetic driver event: biallelic mutations in *SMARCB1* or *SMARCA4,* two chromatin remodeling genes. While this is the only known genetic driver, previous studies have shown that there can be variation at a transcriptomic, epigenomic and phenotypic level among MRTs[98]. In fact, MRT cases can be classified into one of three subgroups according to their global gene expression, DNA methylation, and H3K27ac profiles[99,100]. While there have been no studies focusing directly on the epigenetic landscape of heterogeneous MRTs at a single-cell level, single-cell transcriptomic studies have revealed the existence of epigenetically driven subpopulations of cells within the same tumor[101]. Epigenetic characterization of these subpopulations might allow the identification of novel targets for treatment, as well as prognostic markers.

Pediatric brain tumors

Epigenetic deregulation is one of the main hallmarks of pediatric brain tumors. Single-cell RNA studies have shown that distinct transcriptional signatures and epigenetic modifications seem to drive malignant transformation across several brain tumors[102]. While adult brain tumors have specific genetic profiles that can be used for diagnostics and prognosis, these are absent in a large number of pediatric brain tumors. However, it has been theorized that epigenetic signatures are present in both adult and pediatric brain cancers[103]. Previous studies have shown that indeed, DNA methylation data can be used to classify brain cancer subtypes[104,105] and determine the tissue of origin of the primary tumor of brain metastases[106]. Histone PTMs also play a role in the development of brain tumors. Studies have reported genetic alterations in genes that regulate histone acetylation in medulloblastoma, ependymoma, and diffuse intrinsic pontine glioma. Consequently, several studies propose HDAC inhibitors as a therapeutic approach for brain cancers[107]. However, these studies focus on global epigenetic landscapes, and epigenetic diversity within the same pediatric brain tumor remains to be studied.

Leukemia

Acute lymphoid leukemia (ALL) is one of the most common pediatric cancer types and has subtypes with distinct gene expression profiles. While the exact cause of pediatric leukemias is unknown, they arise through the acquisition of a combination of structural DNA rearrangements, DNA copy number alterations, and nucleotide mutations. Due to this heterogeneity, epigenetic signatures have been proposed as a way to differentiate leukemia subtypes at the time of diagnosis. T-cell ALLs in particular have a highly characteristic DNA methylation signature with local hypermethylation without global hypomethylation[108], and histone PTMs also play a role in leukemogenesis[109] and have prognostic value[110]. However, there is a lack of single-cell studies focusing on epigenetic heterogeneity within leukemias. These studies are essential to properly assess the accuracy of methods that rely on epigenetic patterns as diagnostic or prognostic markers.

Melanoma

Malignant melanoma is known to be resistant to most existing therapies[111]. The cutaneous subtype of melanoma is responsible for approximately 90% of skin cancer-related mortality. While most cutaneous melanomas have clear epigenetic mutations (activating mutations in either the *BRAF* or *NRAS* genes)[112], the development of drug resistance seems to stem from mainly non-genetic factors. As such, DNA methylation signatures have been used to predict patient survival and response to immune checkpoint inhibitors[111]. Furthermore, mutations in genes responsible for histone acetylation (*SIRT6*) have been identified as the basis for resistance to treatment with MAPK inhibitors[113] (**Fig. 4**). Single-cell epigenetic methods can be used before treatment to detect subpopulations of cells with alterations that serve as indicators of future treatment resistance, and subsequently decide the appropriate course of treatment. On top of that, the identification of epigenetic targets among treatment-resistant subpopulations might be the key to developing effective treatments for cutaneous melanoma.

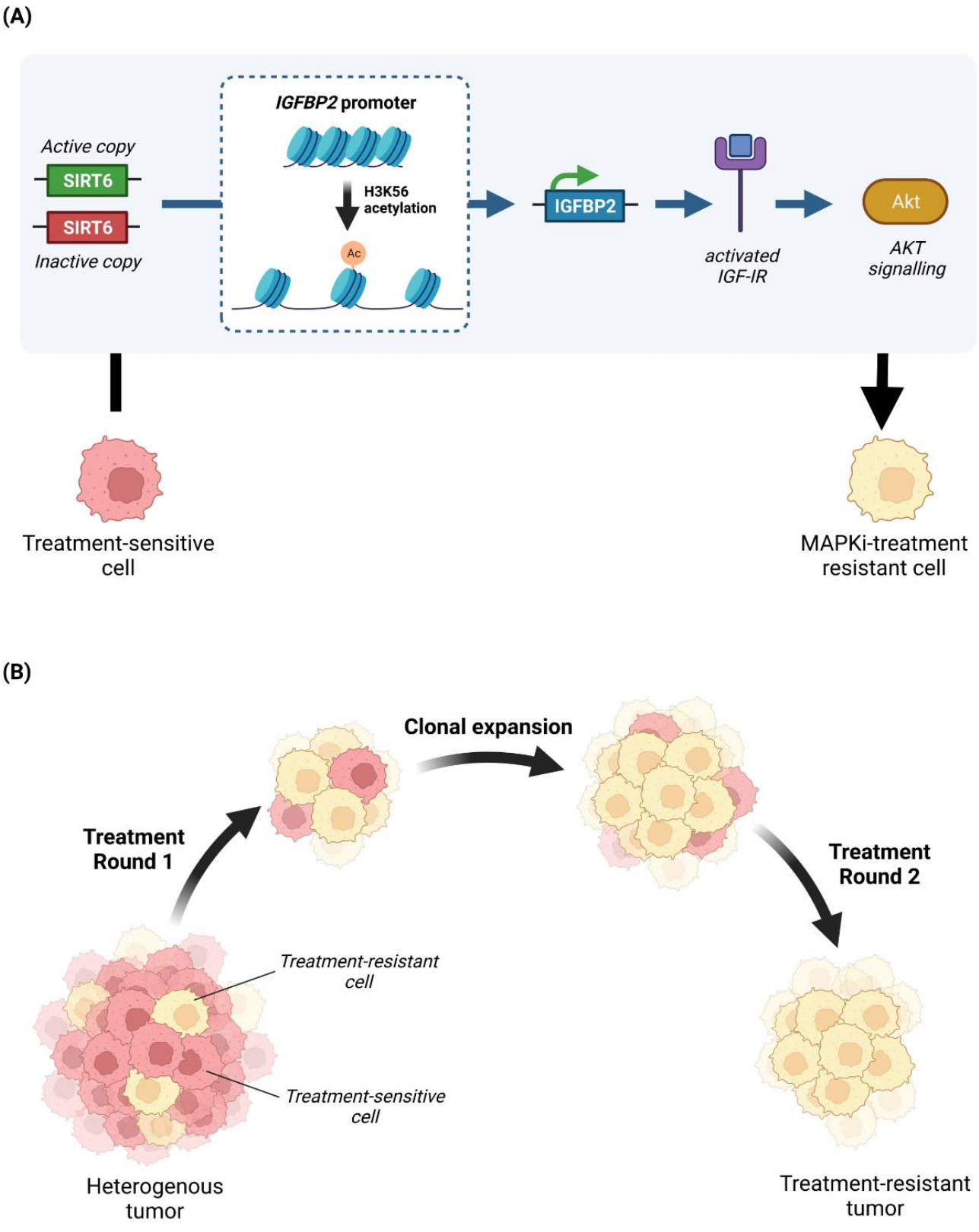

Figure 4: Epigenetic basis of drug resistance in cutaneous melanoma. (A) *SIRT6, an HDAC, regulates resistance to MAPK inhibitors (MAPKi) in cutaneous melanoma. Haploinsufficiency of the SIRT6 gene causes the acetylation of H3K56 in the promoter of the IGFBP2 gene. The open chromatin structure at the IGFBP2 promoter leads to an increase in IGFBP2 expression. This activates IGF-IR receptors, resulting in the activation of downstream AKT signaling, making the cell resistant to MAPKi treatment*[113]. ***(B)*** *Tumor heterogeneity enables the development of drug resistance. In a*

heterogeneous tumor, a subpopulation of cells can become resistant to treatment. This subpopulation of cells will be selected for during several rounds of treatment. Eventually, the tumor will become fully treatment-resistant.

Lung cancer

Similarly, the epigenetic mechanisms behind non-small cell lung cancer (NSCLC) are of particular interest, as it represents ~85% of all lung cancer cases but only approximately half of those cases present targetable genetic mutations. However, as the genetic and epigenetic mechanisms seem to target the same cancer drivers, drugs that modulate epigenetic pathways have been proposed as therapeutic alternatives[114]. The activity of HDAC KMT9 is crucial for lung cancer proliferation, and its depletion has been proposed as a therapeutic strategy for NSCLC[115]. Nonetheless, further studies are needed to determine the exact role of KMT9-mediated histone acetylation in this type of cancer. On top of that, a recent scRNA study by Aissa *et al* (2021) indicates the presence of distinct cell subpopulations with different drug tolerance levels within the same NSCLC cell line[116]. While this study reflected epigenetic changes by looking into the differential expression of genes, the study did not detail the exact epigenetic landscapes of the cell subpopulations.

Breast cancer

Triple-negative breast cancer is known to be a very heterogeneous cancer type with poor prognosis. Epigenetic modifications seem to play a role in the development of drug resistance in breast cancer. Mutations in the gene encoding HDM KDM5 have been shown to lead to the development of resistance to anti-estrogen treatments[117]. A recent study showed that H3K27me3 depletion is key for the development of fluorouracil resistance in triple-negative breast cancer[118]. H3K27me3 prevents the transcription of genes that cells seem to need to survive the treatment, like *FOXQ1*. When cells were treated with a KM6A and KDM6B inhibitor, which depletes H3K27me3, the number of cells able to survive fluorouracil treatment reduced considerably. Other histone PTMs might also be responsible for the silencing of treatment-survival genes. The epigenetic characterization of cell subpopulations responsible for the development of drug resistance would allow to determine whether targeting epigenetic regulators is a viable therapeutic strategy to improve traditional treatment efficiency in triple-negative breast cancer.

Prostate cancer

The progression of prostate cancer (PC) seems to involve very complex epigenetic pathways. Differential DNA methylation is critical for PC progression, as DNA hypermethylation has been shown to arise at the early stages of PC development, while DNA hypomethylation happens later as cancer progresses and occurs most frequently in metastatic lesions[119]. H3K27me3 is also an important event for prostate carcinogenesis and tumor progression, as it is enriched in tumor-suppressor genes and genes involved in further epigenetic regulation (eg. *FBXO11*, *MSH6,* and *ING3*). This mark is theorized to repress the expression of these genes, mediating cancer progression[120]. Single-cell studies can be used to identify and characterize the specific cell subpopulations responsible for the progression towards metastasis.

Furthermore, some specific lncRNAs are known to be upregulated in PC tissues and be involved in the development of treatment resistance[121]. Combined testing for expression of ncRNAs and levels of PSA in blood was shown to be a better PC diagnostic biomarker than PSA alone[122]. Single-cell and

cell-free RNA studies comparing ncRNAs present in blood and CTCs could be used to further validate the accuracy of this approach.

Other cancers

Alterations within the epigenetic landscape have been implicated in other types of cancer, like thyroid cancer[123], HPV-related cervical cancer[124], gastric cancer[125], and colorectal cancer[126]. As such, these cancers might likewise benefit from further research into their epigenetic mechanisms at a single-cell level.

3.3 Clinical impact of research in cancer epigenetics

Biomarkers

Epigenetic biomarkers provide several advantages compared to genetic biomarkers, as they can provide information about the function of genes in specific cell types, the environment and the lifestyle of the patient. Epigenetic biomarkers might also be a good target for minimally invasive diagnostic methods, mostly due to their stability and detectability in biological fluids[127]. Epigenetic biomarkers could also be used as prognosis biomarkers for immunotherapy, as exhausted T-cells show a distinct epigenomic landscape[128].

As DNA methylation is the most studied epigenetic modification, several methylation-based biomarkers have been proposed for diagnostic and prognosis in certain cancer types. For example, DNA methylation marks have been proposed as biomarkers for renal clear cell carcinoma diagnostic from urine samples[129]. In the case of brain cancers, high-precision classifiers based on the methylation patterns can determine the type of brain tumor, as well as the tissue of origin of brain metastases[104].

Therapy

Alterations in the epigenetic landscape of cells have been shown to heavily correlate to resistance to anti-cancer treatment[130]. In the last couple of decades, we have seen the rise of research to develop “epidrugs”, ie. drugs that target epigenetic pathways.

There are currently two classes of FDA-approved epidrugs: DNMT inhibitors and HDAC inhibitors. DNMT inhibitors can be divided into two classes: (1) cytosine analogs like azacytidine and decitabine, which disturb methylation and induce the DNMTs degradation; and (2) non-nucleotide analog inhibitors like disulfiram, that prevent the DNMT binding to the target molecule. HDAC inhibitors like vorinostat rectify the aberrant protein acetylation status, reactivating tumor-suppressor genes. Other epidrug classes like bromodomain and extra-terminal inhibitors, and HMTs/HDMTs inhibitors are currently also in development[23].

These drugs have the potential not only to treat cancers with exclusively epigenetic drivers but also to help prevent the development of drug resistance in other types of cancers when combined with traditional chemotherapies[23].

Discussion

In recent years, we have seen the rise of single-cell methods to study several layers of the cell epigenome (methylation, histone PTMs, ncRNAs, chromatin accessibility, and chromatin remodeling). Until now, most of the research on the cancer epigenome has been done through bulk-based methods[117]. However, the newly developed single-cell epigenomic methods should allow us to properly assess the diversity of the cancer epigenome. Furthermore, single-cell multi-omic methods will allow us to study the interactions between the different layers of the epigenome, as well as phenotypic consequences at the RNA/protein level.

Single-cell epigenomic methods can be used to identify cell subpopulations according to their epigenetic landscape, accurately assessing tumor heterogeneity. These methods could be used to identify the epigenetic characteristics of the cell of origin of cancers with potential epigenetic drivers (renal tumors[95], pediatric brain tumors[102], leukemias[131], MRT[100]), and to identify the epigenetic mechanisms behind the development of drug resistance (melanoma[112], lung cancer[116], breast cancer[118]) or progression/metastasis of tumors (PC[119]). Single-cell methods can also be used to profile the epigenetic characteristics of CTCs[91], leading to an improved understanding of the mechanisms behind metastasis and the development of minimally invasive diagnostic methods. While methylation is the most studied epigenetic layer in cancer research, single-omic studies on other epigenetic layers or multi-omic approaches are essential to properly understand the cancer epigenome.

The direct application of single-cell-based epigenomic methods to diagnostics is currently impossible due to the high cost of these methods (investment in infrastructure and sequencing costs), and the lack of standard single-cell epigenomic data analysis guidelines[24]. However, single-cell studies can still provide us with novel information about the epigenetic mechanisms behind cancers. Hopefully, during the next couple of years, we will see a reduction in the cost of single-cell methods and the establishment of data analysis pipelines that will allow a more widespread application of single-cell epigenomic methods in cancer research and the day-to-day clinic.

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