Epigenetic regulation in cancer development

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1. ABSTRACT

From an operational definition of epigenetic, we move to provide the reader a general but comprehensive description of epigenetic phenomena that often lead to cell transformation. The last decade has, in fact, seen novel players involved in the regulation of gene expression. Not only protein factors but also a number of chromatin modifiers and remodelling proteins, which regulate the level of compaction of the genome through a variety of post-translational modifications deposed on histone tails or on DNA itself. Meanwhile, the discovery of tiny RNAs, of only 21-23 nucleotides in length, has brought to the attention their role as key regulators in the cell, being able to direct differentiation programs and function as oncogenes or oncosuppressors. In this general compendium, we aim to describe main cellular functions that through an epigenetic or epigenetic associated mechanism have been found to be directly implicated in cancerogenesis.

2. INTRODUCTION

2.1. A comprehensive definition of Epigenetic

"An epigenetic trait is a stably inherited phenotype resulting from changes in a chromosome without alteration in the DNA sequence". This operational definition, proposed in a world conference (1), takes into account the heritability of a phenotype, which does not rely on a genetic modification of DNA sequence. Accordingly, it was proposed that three different classes of signals, operating in a concerted manner, create heritable epigenetic state. The first signal, called **Epigenator**, arises from the environment and acts triggering intracellular pathways; the second signal, the **Epigenetic Initiator**, reacts to the Epigenator determining the precise location of the epigenetic chromatin environment and, finally, the **Epigenetic Maintainer**, as an internal signal, sustains the chromatin state through subsequent generations.

The Initiator, which responds to transient external stimuli defining the region of chromosome where the chromatin state should be established, may be a DNA binding factor, a noncoding RNA or other effectors able to determine a precise epigenetic chromatin state. In the cell, the Initiator may persist and propagate its function in combination with the Maintainer, which holds a defined epigenetic state up. Maintainer involves different pathways like DNA methylation, histone modifications (PTMs), deposition of histone variants and nucleosome localization. All these features, that require Initiator and are DNA sequence-independent, need to be exactly positioned in the genome. Deregulation of such processes may lead to genetic syndromes, cellular aging or cancer transformation.

Still, definition of epigenetic may be broader than this, and not necessarily linked to hereditability. The US National Institutes of Health (2009) states that "Epigenetics refers to both heritable changes in gene activity and expression (in the progeny of cells or of individuals) and also stable, long-term, alterations in the transcriptional potential of a cell that are not necessarily heritable". Overall, we can assess that epigenetic processes lead to a stable alteration of gene expression. Collectively, they include several events like cytosine methylation, post-translational modifications of histone and non-histone proteins, chromatin remodeling, nucleosome positioning and RNA-based mechanisms (2).

3. EPIGENETIC AND CARCINOGENESIS

In the last years, a crucial issue is the comprehension of the molecular mechanisms underlying tumorigenesis. This is a multi-faceted process, mainly based on the dys-regulation of two antagonistic classes of genes: tumor suppressor genes, which inhibit cell growth and survival, and oncogenes, that promote cell proliferation and mitosis through cell signalling pathways. It is now clear that activation and repression of cancer-related genes can be due to both genetic and epigenetic alterations. The genetic changes consist of loss or amplification of cancer-associated genes, whereas the epigenetic modifications may involve various mechanisms and imply different pathways. Several studies reveal that epigenetic modifications may result in activation of oncogenes or silencing of tumor suppressors, which end with cell transformation and uncontrolled proliferation. Often, in cancer cells several mutated or translocated chromatin modifiers together with aberrant DNA methylation of single genes or genomic regions have been found. Histone marks and modifications are also often altered, and a relevant question is whether specific PTMs and chromatin states are peculiar of disease and oncogenic traits. Therefore, advances in identification of recurrent epigenetic traits occurring in human cancer will help in set up predictive tools for cancer prognosis and future disease outcome.
Recent studies indicated that also RNA-mediated mechanisms are involved in more widespread epigenetic regulation. In particular, several epigenetic mechanisms include regulation of gene expression mediated by long non coding RNAs, some of which interact with chromatin modifying complexes (3, 4, 5), and by small non coding

RNAs, as microRNAs (miRNAs) that regulate components of the epigenetic machinery. Notably, dysregulation of such RNA-regulators may have profound impact on the establishment and maintenance of the epigenetic landscapes characteristic of differentiated cells.

3.1. DNA Methylation

DNA methylation is the most widely studied epigenetic modification in mammals; it is a trait that is heritably propagated and consists of modification, namely, methylation of cytosine on DNA at CpG target sites (6). DNA methylation influences several processes like gene regulation and nucleosome positioning, imprinting and X chromosome inactivation (7). Methylation of CpG can act directly, by preventing the binding of transcription factors, or indirectly, by recruitment of methyl-binding chromodomain proteins (8) and of silencing structural proteins, like HP1, thus reinforcing a repressive function on vaste genomic regions (9). Such process stabilises a repressive heterochromatinization, which, may further silence gene expression by recruitment of HDACs (10), that induce a generalized chromatin deacetylation.

Often, DNA methylation may occur not only at CpG, but also at repetitive DNA sequences like centromeres, transposons and telomeres. In these cases, such DNA modification leads to genome instability causing chromosome rearrangements and translocations. Notably, alteration of DNA methylation induces the aberrant activation or repression of various signaling pathways triggering cell transformation. The "two hits" model (11) proposes that, in cancer, the genetic mutation of one allele is reinforced by methylation of the second resulting in loss of heterozygosity. Since epimutations are inherited, they can survive and selectively expand in a rapidly growing cell population, thus conferring a selective advantage over the normal propagating cells. This epigenetic event may occur very early in cancer development and represents the first hit for uncontrolled cancer cell expansion (12).

CpG dinucleotides are clustered in short CpG islands, often localized at 5' end of genes, and are associated to almost 60% of human promoters (13). While some CpG are methylated, the vaste majority remain unmethylated; however, the dynamic equilibrium between methylated and unmethylated DNA states is crucial for development and differentiation programs (14).

DNA methylation works in concert with silencing processes acting through repressive histone marks and produces a higher ordered compaction of chromatin. In addition, histone methyl marks may provide a signal for further recruitment of chromatin associated repressors. Histone H3-K9me3, is, for example, the target for the recruitment of HP1 or MECP2 (15), whereas, histone H3-K27me, interacts with Polycomb Repressive Complexes (PRCs) and recruits HDAC to silence gene expression. Hypomehtylation leads to the activation of growth-promoting oncogenes such as R-Ras and MASPIN in gastric cancer, S-100 in colon cancer and MAGE in melanoma (16). In Wilms' tumor, loss of heterozygosity is associated to an early event of DNA hypomethylation

inducing a further loss of imprinting of IGF2 gene (17). The hypermethylation also contributes to tumorigenesis by repressing suppressors. Several genes have been associated to heavy CpG island hypermethylation; from the first report on Retinoblastoma, Rb (18), various other tumor suppressor genes, including p16, MLH1 and BRCA1, have been shown to undergo tumor-specific silencing by hypermethylation (19). DNA methylation can also exert its function through repression of transcription factor genes involved in the activation of oncosuppressors, like RUNX3 in esophageal cancer (20) and GATA-4 and GATA-5 in colorectal and gastric cancers (21). Oncogenic transcription factors, like the aberrant PML–RAR fusion protein found in acute promyelocytic leukemia (22) may recruit DNA Methyl-Transferases (DNMTs) to specific genes where they induce a tumor specific CpG island methylation. The presence of methylation on a subset of CpG islands corresponding to known polycomb targets involved in differentiation, was found in colorectal cancer providing a direct link between the process of DNA methylation and cellulare differentiation (23).

3.2. Chromatin signalling

Chromatin is organized in nucleosomes, nucleoprotein complexes, which are the fundamental units that contribute to the structural dynamic of the genome. Distinct chromatin structural states, affecting the accessibility of DNA to protein complexes and transcriptional machinery, regulate gene expression. Chromatin is, therefore, fluctuating in a dynamic equilibrium between heterochromatin, the compacted, silenced and transcriptionally inactive state, and the euchromatin, the decondensed and active state bearing spaced nucleosomes. In the nucleosome, the DNA is wrapped around the fundamental repeat of chromatin, represented by the histone octamer. The canonical octamer is formed by histones (H3, H4, H2A and H2B)$_2$ and consists of a central globular region from which protrude prolonged external N- termini; these represent the main place of deposition of a variety of post-translational modifications (PTMs) (24), like acetylation and methylation of lysines, methylation of arginines and phosphorylation of serines. Histone tails are, therefore, signal platforms where key epigenetic marks are deposed producing subsequent structural modulation and variation of the chromatin compaction. Overall, PTMs (25) work through in cis activity, which mainly affects processes like the chromatin remodelling, nucleosome positioning and regulation of gene transcription, or in trans activity, dealing with the interaction of chromatin associated proteins. Such proteins interact at specific regions of the genome with PTMs through chromatin reading modules that recognize single modified residues on histone tails (26).

3.3. Epigenetic effectors and histone PTMs

The epigenetic effectors or modifiers are enzymes, DNMTs, HMTs, HATs, HDACs, often member of multi-protein complexes, whose catalytic activity is responsible for post-translational chemical modifications. DNMT family includes three different enzymes: DNMT1 (maintenance DNMT), which preserves the methylation pattern throughout each cell division, and DNMT-3a and 3b (de novo DNMTs), which transfer a methyl group to previously unmethylated genomic regions (27). Acetyltransferases (HATs) are divided into three main families, GNAT, MYST, and CBP/p300. In general these enzymes modify more than one lysine but some limited specificity can be detected for some enzymes. There are three distinct families of histone deacetylases (HDACs): the class I and class II histone deacetylases and the class III NAD-dependant enzymes of the Sir family. They are involved in multiple signaling pathways and are present in numerous repressive chromatin complexes. Lysine methyltransferases (HMTs) have enormous specificity compared to acetyltransferases. They usually modify one single lysine on a single histone and their output can be either activation or repression of transcription. Like lysine methylation, arginine methylation can be either activatory or repressive for transcription, and the enzymes protein arginine methyltransferases, (PRMT's) are recruited to promoters by transcription factors. PTMs are able to change the functional properties of the chromatin fiber, which, in turn, affects cellular processes leading to activation or silencing of gene expression, recombination, repair and replication. A histone code of PTMs (28), deposed at specific sites on the same (intra) or different (inter) histones tails, is finely regulated and is often cross regulated by reciprocal activity of PTMs, like acetylation, phosphorylation, methylation ubiquitination, sumoylation, proline isomerization and ADP-ribosylation (29). As an example of an inter-nucleosomal cross-talk is the requirement of histone H2B-K123 monoubiquitination, for methylation of histone H3-K4 and H3-K79 during transcription. Also histone H3 acetylation was shown to stimulate the other positive mark H3-K4 methylation. From these and other data it has been, therefore, proposed a histone code hypothesis where activating marks may reciprocally regulate the deposition of other modifications on the same histone tail or across tails of different histones (Figure 1).

Among histone PTMs, acetylation is a bona fide positive mark; it neutralizes the positive charges of lysine side chains, thus reducing the strength of the binding of histone tails to DNA. This mechanism leads to the opening of chromatin structure, enabling nucleosome eviction and/or repositioning. Methylation of lysines like histone H3-K4, K36 (30), K79 and H4-K20, and arginines H3-R2, R17, R26 and H4-R3, represent activating marks, whereas methylation of H3-K9, K27 and H4-K20 are associated to repression and may be classified as repressive marks (31, 32).

In gene silencing, the nucleosomes must be deacetylated to prevent cryptic initiation of transcription. It was reported that methylation of histone H3-K36me provides the signal for the recruitment of the HDAC Rpd3 complex (33), which through deacetylation compacts chromatin and represses gene expression. In sum, histone PTMs regulate the expression of disease genes by modifying individual promoters like that of the cell cycle dependent cyclin E gene, whose downregulation is linked to ipoacetylation though aberrant recruitment of HDAC1 by retinoblastoma (Rb) (34).
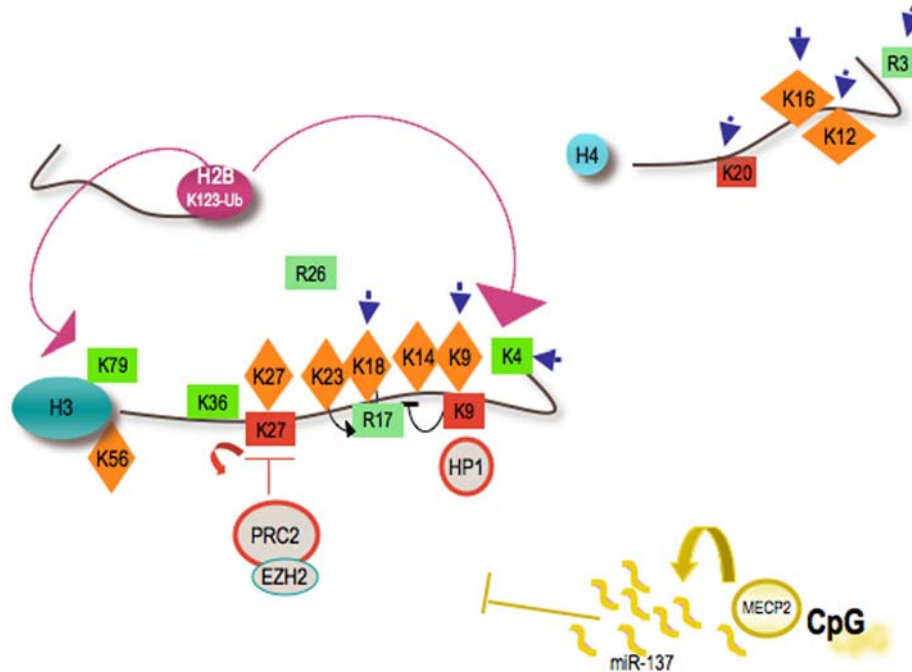
Figure 1. Schematic representation of cross regulation between epigenetic marks and miRNAs. Methyl-marks (rectangle: activating, green; repressing, red). Acetyl-K (orange rhombi). Epigenetic marks found altered in tumors are indicated by arrows.

4. ALTERED EPIGENETIC PATTERNS IN CANCER

Cancer may evolve from combinatorial convergence of genetic and epigenetic abnormalities (35). Altered epigenetic landscape is characterized by aberrant CpG island methylation and deacetylation and/or methylation of histones (36, 37). In case of mutation of a single allele, the silencing of the second one may induce the hit, resulting in loss of heterozygosity and inactivation of tumor suppressor genes. In addition, silencing of oncosuppressors may cooperate with oncogenic mutations and induce tumor development. Accordingly, loss of heterozygosity has been recently considered as a relevant marker for diagnosis and prognosis of cancer. The epigenetic aberration linked to chromosome instability may also contribute to the loss of heterozygosity increasing the frequency of aneuploidy, which is an hallmark of many cancer types.

In several tumours, dysregulation of epigenetic modifiers produce alteration in PTMs pattern on critical promoters or at bulk genomic level with consequent uncontrolled proliferation of undifferentiated totipotent cell sub-populations. Immunostaining of cancer nuclei showed great heterogeneity in bulk levels of histone modifications in tissue specimens (38); accordingly, the identification of aberrant epigenetic states of chromating may represent novel predictive tools for human cancer.

In leukaemia, translocations involving Mixed Linear Leukaemia (MLL) gene leads to the loss of H3K4 methyltransferase activity, which contributes to transform haematopoietic into leukaemia stem cells with poor prognosis (39). Similarly, negative regulators like Polycomb Group (PcG) complexes are engaged in the targeted repression of genes during cell cycle. Two different Polycomb complexes, PRC1 and PRC2 have been shown, in fact, to be involved in different cancers. Up-regulation of EZH2, the PRC2-H3K27 methyltransferase, is found in mantle cell lymphoma (40), breast (41) and prostate cancers (42). In addition, PRC complex can be aberrantly recruited, by PML-RAR alpha oncofusion protein, at tumour suppressor genes thus inducing their repression in leukemia (43). Another component of PRC1, RING1, which monoubiquitylates histone H2A-K119, is upregulated in prostate cancer (44).

Acetylation counteracted by deacetylation is achieved by a fine equilibrium between opposing HAT and HDAC catalytic activity crucial for cell regulation. Deregulation of HATs activity is often found in cancer; this is the case of the chimeric fusion of two HAT catalytic domains MOZ-p300 involved in leukemogenesis in Acute Monocytic Leukemia (AML) (45). Collectively, these and other reports demonstrate that untargeted aberrant acetylation and unbalanced deacetylation are recurrently found, and become negative regulators of tumor suppressor genes (46).

4.1. Analysis of global histone PTMs in cancer tissues

Aberration of PTMs generally occur at single gene promoters where they regulate transcription and affect the binding of transcription factors. Nonetheless, a global genomic assay of bulk post-translational modifications of histones might represent an innovative approach for screening normal and cancer tissues. In the clinical practise it is, in fact, not easy to evaluate and classify cancer cells.

Several cell types often elude the molecular analysis or lack of a clear classification method, as in the case of prostate cancer which is difficult to classify but still represents the second cause of death in US.

It was reported that the pattern of histone modification was indeed a prognostic feature in some tumors like prostate, kidney, lung, gastric ovarian and breast cancer (Figure 1). Locus specific changes in histone acetylation or methylation was correlated to the expression of critical genes in highly aggressive pancreatic adenocarcinoma (47).

In human breast carcinomas, global histone modification profile represented a tool for the identification of cancer phenotype and a prognostic sign of patient outcome (48). It was reported that H4K16ac was very low or absent in the majority of breast cancer cases. High relative levels of global histone acetylation and methylation were associated with a favorable prognosis. On the other hand, low levels of lysine acetylation (H3-K9ac, K18ac, and H4-K12ac), lysine methylation (H3-K4me2 and H4-K20me3), and arginine methylation (H4-R3me2) were found in carcinomas of poorer prognostic subtypes, including basal carcinomas and HER-2-positive tumors. Other reports demonstrated that also the level of acetylated H3-K9, K18 and H4-K12 and dimethylated H4-R3 and H3-K4 in prostate cancers were prognostic signatures (38). Loss of histone H4-K16ac and K20me3 were clearly associated to DNA hypomethylation at non coding repetitive sequences (49). In prostate cancer, lower to higher risk subtypes were compared and grouped on the basis of a statistical analysis of histone PTMs revealed by immunostaining (38). Although this type of analysis must be further extended, we may assume that this approach might be useful not only for cancer classification but also for predicting response to a specific drug-treatment.

5. miRNAs TAKE THEIR PLACE IN THE EPIGENETIC WORLD

For their mode of action, miRNAs provide an important layer of epigenetic information according to the broader definition of epigenetics (2): they modify gene expression without modifying DNA sequences.

The effectiveness of these small non-coding RNAs (approximately 21-nucleotide-long) in silencing gene expression at the post-transcriptional level in eukaryotic cells resides in their ability to act through very simple but highly specific mechanisms. miRNAs recognize their mRNA targets by sequence-specific base-pairing and, depending on the degree of complementarity, they can induce mRNA degradation (perfect pairing) or translational repression (imperfect pairing). Through this procedure, they function as molecular guides that address the multi-protein effector complex (miRNP) on the specific mRNA targets, preventing gene expression. Recent studies using high-throughput proteomics approaches (50; 51), highlighted the ability of individual miRNAs to affect hundreds of proteins in humans. This pleiotropic action allow them to simultaneously tune up the activity of multiple genes, which in turn often control a biologically coordinated genetic program. For this feature, miRNAs may globally affect entire pathways exerting a significant impact on cell fate.

On the other hand, it has been observed that a single mRNA can be controlled by multiple miRNAs, which can act combinatorially. Therefore, even if individual miRNAs suppress their targets only moderately, they can exert broad and strong effect on gene expression.Recently, more than five hundred miRNAs have been identified in humans, but at least a thousand have been predicted by bioinformatic analyses (52; 53). Functional studies indicate that they are crucial players in vital processes such as development, differentiation, cell proliferation and cell death, regulating the activity of approximately 30% of all protein-coding genes in mammals. Notably, dys-regulation of miRNA expression has been associated to developmental defects and to the onset and/or progression of carcinogenesis in humans. Accordingly, miRNAs are aberrantly expressed in a variety of cancers (54)

6. DYSREGULATION OF miRNA EXPRESSION IS ASSOCIATED TO CARCINOGENESIS

The first correlation between miRNA aberrant expression and cancer derived from the observation that miR-15a and miR-16-1, which are down-regulated in the cancer samples relative to the normal tissues, were clustered at chromosome 13q14, a region that is frequently deleted both in chronic lymphocytic leukemia (CLL), the most common human leukemia, and other cancers (55). Supporting such correlation, a large body of evidence indicate that over 50% of miRNA genes are positioned in cancer-associated genomic regions, such as fragile sites, minimal regions of loss of heterozygosity, minimal regions of amplification, or common breakpoint regions (56).Thanks to the development of high-throughput approaches, such as microarray and quantitative RT-PCR (qRT-PCR), global miRNA gene expression profiling has been successfully developed. Such analyses produced atlas of global miRNA expression patterns, in both cancers and normal tissues and definitely linked miRNAs to cancer. Following this discovery, significant changes in miRNA levels were also observed in several tumours without evident cytogenetic abnormalities, suggesting that the altered levels of miRNAs may be due to dysfunction in their biosynthesis or processing events. Such dysfunctions can result in the loss or gain of miRNA function, both contributing to initiation, progression and metastasis of human malignancies (57).

At the moment, it is current opinion that alterations in miRNA expression are the rule in human cancers and several studies assess that miRNAs can function as oncogenes or as tumour-suppressor genes in carcinogenesis (58). In particular, if a specific miRNA is over-expressed in primary tumours with respect to the normal tissue it may be considered a potential oncogene; on the contrary, if the miRNA is down-regulated in the tumoural tissue with respect to the normal one it may be regarded as a potential onco-suppressor gene. Notably, some miRNAs may act both as oncogenes and as tumour-suppressors, depending on the cellular context. This finding render more complex the functional classification of individual miRNAs, and

makes essential to refer to specific tumours for describing their contribution to cancerogenesis.

The effect of the altered expression of miRNAs in a large number of malignancies, result in functional consequences by targeting main players in the eukaryotic survival, cell cycle and differentiation programs. Some miRNAs are deregulated in many cancers, suggesting that they may be involved in the control of basal processes as cell proliferation and apoptosis that are commonly deregulated in cancer; other miRNAs are, instead, deregulated in a tumour-specific manner according to their tissue-specificity.

6.1. Tumor suppressor and/or oncogenic miRNAs

Among the more studied miRNAs with tumour suppressor functions are the members of the let-7 family, which map to different human chromosomes; they are differentially expressed in different tissues and are involved in different tumours. let-7 is almost absent during developmental stages, whereas it is highly expressed in most differentiated tissues. Its oncosuppressive role has been largely studied in lung cancer where it is poorly expressed: it has been shown that let-7 directly controls cell proliferation by repressing the human RAS gene, which is overexpressed in lung cancer (59), and other cell cycle oncogenes as HMGA2 (54) and c-Myc (60). Among the most significant miRNAs with a putative role of oncogene, miR-21 that is overexpressed in almost all kinds of cancers, including colorectal cancer, pancreas endocrine and exocrine tumours, glioblastoma, ovary, lung. In breast carcinoma, miR-21 mediates cell survival and proliferation directly targeting the oncosuppressor genes PTEN, PDCD4 and TPM1 (61). Furthermore, it has been associated with advanced clinical stage and poor patient prognosis.

An interesting example of a miRNA that may function as oncosuppressor or oncogene, depending on the cellular context, is miR-125b, a homologue to lin-4 that is the first-identified miRNA. It has been shown that miR-125b contributes to the pathogenesis of prostate cancer displaying an oncogenic function. Shi and colleagues found that miR-125b is overexpressed in most clinical prostate cancer samples and in many cultured prostate cancer cell lines; accordingly, the ectopic expression of this miRNA stimulates androgen independent growth of prostate cancer cells, while repressing the expression of Bak1, a BCL2 family member, functioning as a proapoptotic regulator (62).

On the contrary, miR-125b exerts a tumour-suppressor function in breast cancer, where it is consistently down-regulated (61). About 25% of human breast cancers are associated with amplification and overexpression of the oncogenic receptor ERBB2; it has been demonstrated that the ectopic expression of miR-125b in a model breast cancer cell line directly repressed the expression of ERBB2, resulting in suppression of anchorage-dependent growth potential and inhibition of motility and invasive capabilities of breast cancer cells (63). Since increased expression of ERBB2 receptor was also reported in prostate cancer cell lines, the possibility that miR-125b modulates the expression of such gene in these tumour cells was investigated. Notably, miR-125b does not target ERBB2 gene in prostate cancer cells (62), indicating that

the same miRNA may behave differently in different cancer cell types. miR-125b has also been described as a tumor-suppressive miRNA in neuroblastoma (NB) and medulloblastoma (MB). NB is a tumour of the sympathetic nervous system and represents the most frequent solid tumour of childhood, whereas MB is the most frequent brain malignancy in childhood. In both cancers miR-125b is down regulated and, consistently, its ectopic expression promotes cell growth arrest and apoptosis. This action is mediated by repressing the expression of the pro-proliferative truncated isoform of the neurotrophin receptor TrkC (t-TrkC), while the full length, pro-differentiative, TrkC isoform (fl-TrkC) is insensitive (64; 65).

miR-9 represents another example of a miRNA that may exert an oncogenic or oncosuppressive function, depending on the cell context. In breast cancer cells, miR-9 is upregulated and function as an oncogene by directly repressing the expression of E-cadherin; reduction of this key metastasis suppressing protein causes an increased cell motility and invasiveness (66; 67). A tumour growth-inhibitory function has been, instead, described for miR-9 in both NB and MB tumours, where this miRNA is down-regulated with respect to normal tissues. Accordingly its overexpression in NB and MB cell lines causes a decrease of cell proliferation and triggers neuronal differentiation (64; 65). miR-221 and miR-222 inhibit the growth of erythroblastic leukaemia while targeting the oncogene KIT and, therefore, functioning as tumour suppressors in erythroblastic cells (68). Differently, they may function as oncogenes by repressing tumour suppressors, as p27, p57, PTEN and TIMP3 in several human solid tumours (69).

6.2. miRNAs as tumor diagnostic tools

A very interesting aspect emerging from these studies is that different types of cancer display unique miRNA expression profiles that can be, therefore, used for tumour classification. This is a very important point since the first step towards the best treatment for cancer diseases is an early and accurate diagnosis. Surprisingly, the miRNomes are more informative than the mRNA profiling in predicting cancer type and stage. This is particularly true in the case of poorly differentiated tumours, the gene expression program of which is very different from that of their differentiated counterparts (70). Notably, it was assessed that the expression profiles of a small set of miRNAs may allow the classification of multiple cancers more accurately than data from about 16.000 mRNAs (71).

At the moment, several studies highlighted the relevance of miRNAs as tumour signatures that can be successfully employed as diagnostic markers for tumorigenesis. As an example, it was recently reported that a signature of only 13 miRNA is able to distinguish breast cancer and normal tissue with an accuracy of 100% (61). Furthermore, common aberrant miRNA exression profile allowed to distinguish both endocrine and acinar pancreatic cancers from normal pancreas. In particular, the expression of miR-103 and miR-107 together with contemporary lack of expression of miR-155 is diagnostic of tumours; moreover, a subset of 10 miRNAs distinguish endocrine from acinar tumours , whereas the overexpression of miR-204 of miR-21

correlate with insulinomas and liver metastasis respectively. This is an example of how a small set of miRNAs provide information on neoplastic transformation and progression of malignancy (72). A differential expression pattern of 32 miRNAs has been associated to the three neuroblastoma tumor subtypes (71). Recently, Ferretti and colleagues carried out, by high throughput analysis, the first miRNA expression profiling of human primary Medulloblastoma (MB). The authors showed that specific miRNA signatures may distinguish tumours from either adult or fetal normal tissues and that typical miRNA expression patterns allow the classification of MB histotypes, correlating with disease risk stratification (65). Through a genome-wide miRNA expression profiling in a large number of normal and tumour breast tissues the existence of a breast cancer-specific miRNA signature was also demonstrated (73).

7. EPIGENETIC CONTROL OF miRNA GENES

It is now becoming clear that miRNAs not only function in an epigenetic manner by post-transcriptionally regulating gene expression, but may also be targets of the epigenetic machinery (61). The study of epigenetic and transcriptional regulation of miRNA gene expression is, therefore, essential for a better understanding of their role in cancer.

Among the epigenetic mechanisms controlling miRNA gene expression, changes in DNA methylation pattern have been described and aberrant miRNA gene methylation has been associated to human tumorigenesis. In particular, DNA hypermethylation of potential oncosuppressive miRNA genes may cause their downregulation; conversely, hypomethylation of miRNA genes with potential oncogenic function may cause their upregulation. In both cases the epigenetic regulation contributes to malignant transformation. It has been shown that half of miRNAs are associated to CpG islands, further suggesting that they may be targets for this kind of gene regulation (74). For instance, miR-127 is transcriptionally inactivated by CpG island hypermethylation (75). Human miR-127 is constitutively expressed in normal tissues, whereas it is repressed in cancer cells and downregulated in 75% of primary tumours, suggesting its potential oncosuppressive role. In particular, this miRNA, as part of miRNA cluster, is embedded within a CpG island and is highly induced (49-fold) after the simultaneous treatment with the chromatin-remodeling drugs 5-aza-2'-deoxycytidine and 4-phenylbutyric acid, which inhibit DNA methylation and histone deacetylase, respectively. This suggests that miR-127 is epigenetically silenced in cancer cells (75). Another example proving that DNA hypermethylation may contribute to the transcriptional downregulation of miRNAs in human tumours is miR-124a (76). This miRNA is embedded in a CpG island that is unmethylated in normal colon tissues, whereas it is hypermethylated in a collection of human cancer cell lines as well as in primary tumours from colon, breast, lung carcinomas, leukemias and limphomas; in colorectal tumours, miR-124a hypermethylation was observed in 75% of patients. RT-PCR analyses showed that mature miR-124a was absent in the wild type colon cancer HCT116 cell lines with hypermethylated CpG islands; notably miR-124a production was restored in the same cell line after disruption of

methyltransferases (DNMT1 and DNMT3b) as well as after treatment with the DNA-demethylating agents. These results highlight the association between miR-124a methylation and loss of miRNA expression and clearly demonstrate that in cancer cells miR-124 is locked in a transcriptionally inactive state (76). Furthermore, aberrant hypermethylation was observed for miR-9-1, miR-124a-3, miR-148, miR-152 and miR-663 in 34-86% of cases in a series of 71 primary human breast cancer specimens (77). In particular, for miR-9-1 a direct correlation between methylation level and reduction of expression was demonstrated in a subset of primary human breast cancers. Also miR-34b and miR-34c, that are clustered, are downmodulated in colon cancer following DNA hypermethylation (78). Among the miRNA genes that are hypomethylated in cancer, let-7a-3, that belongs to the archetypal family of let-7 miRNA genes. The human let-7a-3 gene, with putative oncogenic function, is associated with a CpG island and is highly methylated in normal tissues but hypomethylated in some lung adenocarcinomas. Such DNA hypomethylation facilitates epigenetic activation of the gene, and the following elevated expression level of let-7a-3 in a human lung cancer cell line results in enhanced tumour phenotypes, and oncogenic changes in transcription profiles (79). Recently, Tsai and colleagues described the epigenetic control of the expression of a primate-specific miRNA cluster (C19MC), which spans about 100kb on human chromosome 19 and comprises 46 miRNAs. Such miRNA cluster is specifically expressed in the placenta, and its expression pattern is associated with methylation state of a distal CpG-rich region located at 17.6 kb upstream; in other cell types, where the miRNA cluster is poorly expressed, it is silenced by hypermethylation of the distal CpG-rich region (80).

However, methylation is not the only epigenetic mechanism that influences miRNA gene expression. In fact, also histone acetylation has been involved, as revealed by alteration of miRNA expression levels after inhibition of histone deacetylase. In this regard, Scott and colleagues explored the changes in miRNA profiles after treatment of SKBr3 breast carcinoma cells with the potent hydroxamic acid HDAC inhibitor (LAQ824). They found that the expression level of about 30 miRNAs was altered, with 22 miRNAs down regulated and 5 miRNAs upregulated (63).

8. miRNA CONTROL OF EPIGENETIC MECHANISMS

If on one hand miRNAs may be targets of the epigenetic machinery, on the other hand they may control epigenetic machinery, directly targeting its enzyme components. Such reciprocal action defines a highly controlled feed-back mechanism that contributes to the regulation of the sophisticated networks underlying the maintenance of a non pathological state.

Among these miRNAs, called "epi-miRNAs" (81), miR-290 cluster, the loss of which in Dicer-1-deficient mouse embryonic stem cells causes downregulation of the methyltransferases DNMT3a, DNMT3b and DNMT1. Such effect is mediated by upregulation of their repressor retinoblastoma-like 2 protein (RbI2), which is a direct target of miR-290 (82; 83). The consequent aberrant DNA methylation
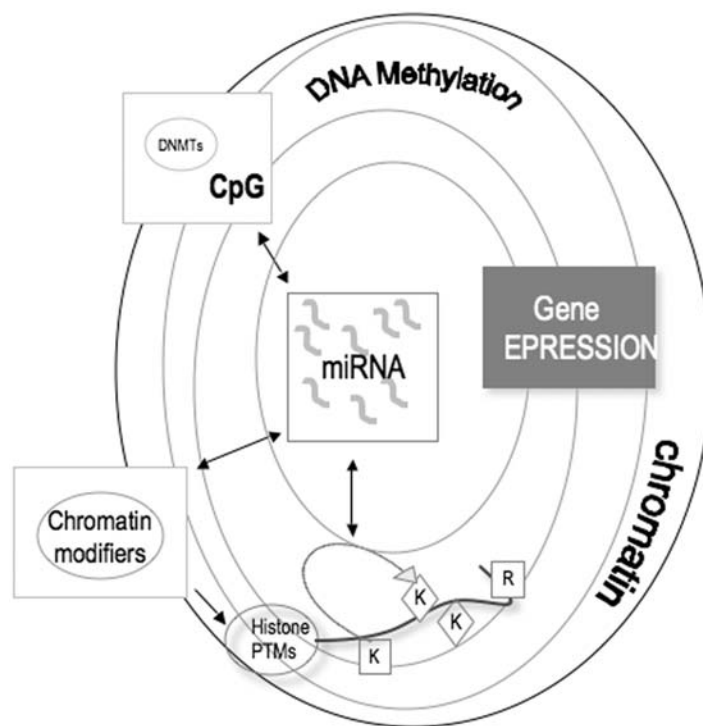
Figure 2. Crosstalk between miRNAs and epigenetic mechanisms.

impairs the embryonic stem cell differentiation program (82) and affected the length of the hypomethylated telomeres (83). Other epi-miRNAs affecting DNA methylation have been described. Among them, miR-29 family that directly regulates the "de novo" DNA methyltransferases DNMT-3A and 3B (84) and indirectly controls the maintenance DNA methyltransferase DNMT1 (85). Such enzyme is the target of other miRNA as miR-148a, miR-152 and miR301 in cholangiocarcinoma (86). Another miRNA linked to the epigenetic machinery is miR-137, which is involved in proliferation and differentiation *in vitro* and *in vivo* of adult neural stem cells. Overexpression of this miRNA promotes proliferation, whereas reduction of its level induces neuronal cell differentiation. Notably, miR-137 is epigenetically controlled by MeCP2, a DNA methyl-CpG-binding protein, and in turn represses the expression of EZH2, a histone methyltransferase and catalytic subunit of the polycomb repressive complex 2 (PRC2). Repression of Ezh2 results in a global reduction of histone H3 trimethyl lysine 27, (Figure 1). (87).

EZH2 is also a direct target of miR-101;the abnormal downregulation of this miRNA in several tumours is associated to the overexpression of EZH2, frequently observed in cancer. (88). Finally, some miRNAs controlling enzymes directing histone acetylation have been reported. Among them, miR-1 and miR140 directly target HDAC4 (89), whereas miR-449a controls HDAC1 (90).

9. SUMMARY AND PERSPECTIVES

The expression of the genome is mirrored in the transcriptome and proteome profiling and specify the fate of each cell type.The epigenetic regulation impact on the chromatin dynamic and determine the overall compaction and subsequent gene expression. Nevertheless, the extent to which epigenetic changes are heritable or if and how post-translational modifications pass on the memory of a given chromatin state to the progeny is still debated. It has been proposed that at least persistent histone marks, like H3K9me, are able to be transmitted through cell cycles and therefore represent a canonical heritable trait. Notably, epigenetic factors and miRNAs are tightly integrated in complex regulatory circuitries that underpin differentiation process. The reciprocal and concerted action between epigenetic machinery and proper expression of miRNAs may create regulatory loops that can simultaneously control hundreds of target genes, thus ensuring the harmonized gene expression and execution of differentiation programs (Figure 2). Disruption of this critical cross regulation may lead to cell transformation and cancer. A matter of future investigation and scientific challenge will be to fully dissect the close reciprocal interactions and the circuitries which cross regulate miRNAs and epigenetic mechanisms. This field of molecular biology may reveal novel precocious diagnostic tools or powerful molecules able to block uncontrolled cell proliferation and drive cell fate toward differentiative pathways.

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