Chapter 7

DNA Methylation, Stem Cells and Cancer

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1. Introduction

Cancer has been traditionally seen as a disease characterized by many genetic alterations, but recent studies have proven the implications of epigenetic abnormalities along carcinogenesis [1, 2].

The fundamental base of carcinogenesis is described by two major models: clonal evolution and cancer stem cell (CSC) model [3-5].

In the past few years 'cancer stem cells' (CSCs) area has become an interesting field of cancer research. In 19th century, Durante and Conheim [6] and after one hundred year Sell and Pierce [6, 7] issued the hypothesis that stem cells could induce cancer in all type of tissues. Unlike normal tissue stem cells, cancer stem cells are characterized by an abnormal differentiation rate, which can lead to tumor [8, 9].

The five principal factors, reported to be involved in carcinogenesis are:

- chemicals – John Hill, in 1761, was the first who showed that the chemicals agents produce cancer of the nasal cavity [6];
- infections – Francis Peyton Rous was the pathologist awarded the Nobel Prize in Medicine for his research that reported that the viral agents are involved in the origin of cancer [10];
- mutations – Theodor Heinrich Boveri and Von Hansemann argued the association between development of cancer and abnormal mitoses [11];

- teratocarcinomas – the field theory, which explains that in pathology of cancer are implicated a mixture of mature and differentiated cells and also embryonic tissue [12];
- epigenetic alterations – coerce to development of abnormal phenotypes, without any structural changes of DNA [13];

The term epigenetic was introduced by Conrad Waddington in 1942, to explain the relationship between environment and genome. Model of „cancer stem cells" indicates that epigenetic changes occurred in stem or precursor cell are the earliest events that take place in cancer [14].

There are two primary mechanisms involved in the epigenetic process: methylation of DNA and covalent modification of histones [15].

DNA methylation is an inheritance mechanism, fundamentally important in normal development and cellular differentiation in mammalian organisms. This is a post-replication DNA modification, by the addition of a methyl group to carbon 5 (C5] of the pyrimidine ring of cytosines, predominantly in cytosine-phospho-guanine (CpG) dinucleotides.

In the eukaryotic cells, the pattern of methylation is the result of complex interactions between three types of normal methylation processes: *de novo* methylation, the maintenance of existing methylation and demethylation. DNA-methylation is catalyzed by several DNA cytosine-5-methyltransferases (DNMTs), which can catalyse cytosine methylation in different sequence context. DNMT family include: DNMT1, which is responsible for methylation maintenance and DNMT3a and DNMT3b, which are responsible for the *de novo* DNA-methylation [16].

The decisive developmental effect of DNA-methylation on gene expression is the long-term silencing of gene expression. In human, the process of DNA-methylation is associated with transcriptional silencing imprinted genes and X-chromosome inactivation. Both genomic imprinting and X-chromosome inactivation are suggested to regulate gene expression in embryonic and fetal growth.

Dysregulated normal imprinting is supposed to induce embryonic death and to impair fetal growth. Defects in DNA-methylation process may also have major consequences for embryonic development and are associated with congenital defects, autoimmunity, aging and malignant transformation.

In recent years, the human methylation profile of the whole genome has been investigated by DNA methylomic studies and altered DNA methylation has been found in cancer DNA [2, 17] The transformation of normal cells into dysplastic and cancerous cells is due to a broad range of genetic and epigenetic changes. Some of the epigenetic mechanisms of initiation and progression of cancer are strongly related to post translational modifications of histones, among which the methylation process is highly involved. The resistance of various types of cancer to therapy led to the hypothesis that cancers present some cells, able to self renew and differentiate into all types of cells that compose a tumor, named cancer stem cells [8]. During embryonic development, characteristic patterns of CpG methylation are produced in the different cell lineages that are then well conserved in normal adult cells, while

in tumor cells, DNA methylation patterns become altered. A family of germline-specific genes that use DNA methylation as a primary silencing mechanism, has been indicated as a stem cells signature. These germline-specific genes expression in tumors, has also been hypothesised to reflect the expansion of constitutively expressing cancer stem cells [8].

Post-translational modification of histone proteins is an important area of regulation epigenetic. The post-translational modifications of the N-terminal tail domains include: methylation, acetylation, phosphorylation, citrullination, ADP-ribosylation, sumoylation and ubiquitination [18-20]. The most studied of these modifications are methylation and acetylation. Modifications of histone N terminal ends by methylation and acetylation processes are closely related to cancer and are done by the competition of two families of enzymes: histone acetyltransferases (HAT) and histone acetylases (HDAC). Lysine residues acetylation in the histone H3 and H4 by (HAT and lysine 4 methylation in the histone H3 (H3K4me) by histone methyltransferase (HMT) are generally correlated with active transcription of chromatin. In contrast, methylation of lysine 9 and lysine 27 (H3K9, H3K27me) in the histone 3, have been considered as markers in transcriptionally silenced-chromatin [21, 22].

Dysregulation of epigenetic mechanisms in stem cells may induce alteration in stem cells function, (i.e. self-renewal and differentiation potential), leading to cancer initiation and progression. During the last years, a major challenge in cancer biology is to elucidate how the histone modifications in stem cells influence carcinogenesis.

Thus, epigenetic control of gene expression patterns in embryogenesis, stem cells and cancer stem cells is a very important aspect for our understanding of human cancer development, progression and therapy.

2. DNA methylation

According to the cancer stem cell theory, aberrant epigenetic changes may allow the transformation of stem cells in cancer stem cells [14].

Epigenetic regulation is realized by modifications that consist in four important mechanisms: DNA methylation, covalent modification of histone, nucleosome positioning and changes of microRNA expression [23].

The biological process of DNA methylation is found in both eukaryotic and prokaryotic cells and it can be involved in pathogenesis of several diseases, especially in cancer. This is the most studied mechanism of epigenetic regulation, consisting in addition of a methyl group to the carbon-5 position of the pyrimidine base cytosine (C) from the nucleotide structure cytidine-5′-monophosphate (CMP). S-adenosyl-L-methionine (SAM), the active form of amino acid methionine, is the donor of methyl group resulting S-adenosylhomocysteine (SAH) [23].

In the mammalian genome, cytosine is coupled to guanine (G) to form a base pair, commonly called cytosine-phosphate-guanine (CpG) dinucleotides. These dinucleotides are general-

ly methylated (CpG-poor regions) with the exception of GC-rich regions known as the CpG islands [24, 25]. In human normal cells, it was observed that CpG islands are often hypomethylated. In oncogenesis CpG islands suffer a hypermethylation process whereas the entire pool CpG-poor regions are hypomethylated. DNA hypermethylation and hypomethylation coexist in cancer cells, both processes demonstrating the importance of DNA methylation in sustaining a normal gene expression pattern, genomic imprinting and silencing of genes involved in X-chromosome inactivation [1, 23, 26-29].

The DNA methylation process is catalyzed by specific DNA methyltransferase (DNMTs). In human cells, five types of DNMTs enzymes have been reported [23, 30-33]. DNMT1 - DNA (cytosine-5-)-methyltransferase 1, with role in regulation of normal tissue-specific methylation; unusual methylation is related to the appearance of human cancer. DNMT2 has an uncertain role in human health and illness [34]. Grant A Challen et al. have shown that DNMT3A and DNMT3B are implicated in embryonic stem cells differentiation [35] and DNMT3L was reported to stimulate the activity of both DNMT3A and DNMT3B [36].

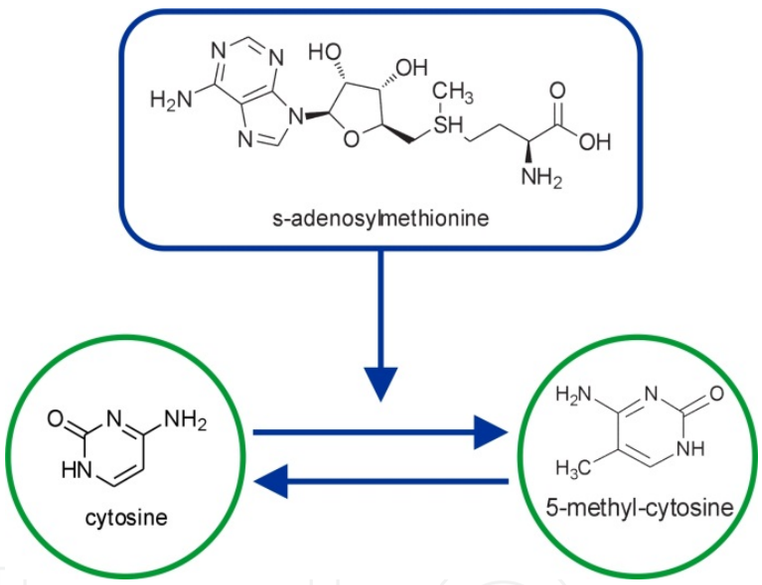

Figure 1. Methylation of cytosine. Cytosine methylation is one of the most extensive studied epigenetic processes. The donor of the methyl group is the active form of methionine, S-adenosyl-L-methionine (SAM) and its addition to cytosine is realised at the carbon-5 position.

Over the past two decades, it was shown that DNA methylation plays a major role in the regulation of the specific gene expression, during mitotic cell division, in the normal mammalian cell, as well as in the stem cell [13, 37, 38].

A new hypothesize about tumorigenesis consists in dysregulation of the stem cell self-renewal process. Dissemination of cancer stem cells is suggested to be induced by gene mutations and epigenetic modifications that may lead to metastasis [39].

DNA hypomethylation was found to activate cancer-germline (CG) genes or cancer-testis (CT) gene family in tumours. The promoter region of CG genes is demethylated in a several

tumour types, inducing genes transcriptional activation. In their study, Costa et al. hypothesized that expression of CG genes may be indispensable for stem cell biology [40, 41].

In addition to DNA hypermethylation and hypomethylation, a DNA demethylation process was also described. While the active DNA demethylation process takes place in presence of enzymes that catalyzed specific reactions, passive DNA demethylation can occur during replication cycles and operates on DNA methyltransferases [42, 43]. Although the demethylation process is not fully elucidated, there are many studies showing transient involvement of this process in various types of tumors, especially in advanced stages of their development [44]. The mechanism of DNA demethylation in cancer has been relatively less studied. Until just a few years ago, scientists believed that the hypomethylation affects the whole genome, randomly [45]. Dysregulations in embryonic normal development and also in normal stem cells development, are generated by many signaling pathways, which can be associated with cancer. Pathways signaling implicated in regulation of normal stem cell evolution are also involved in stem cells self-renewal and carcinogenesis. The most common signaling pathways in all processes mentioned are: Wnt, Notch and Sonic hedgehog (Shh) [46, 47]. Other signaling pathways reported to be involved in stem cell maintenance and pluripotency are: TGF-beta, MET, MYC, EGF, p53, BMI, etc [5].

It is well-known that Wingless gene encodes the Wnt protein family that control the self–renewal and tumorigenesis processes. Wnt protein is well preserved from Drosophila melanogaster and has an essential role during normal embryonic development. Wnt protein is a part of a particularly signalling pathway, common to humans and Wnt protein dysregulation was reported to be involved in the development of tumors. It has been demonstrated that some genes implicated in the Wnt signaling pathway, are inactivated by promoter hypermethylation, generating lung metastasis from primary tumors [48].

The Notch signaling pathway was also suggested to have an important role in stem cell differentiation, proliferation and oncogenesis as well. Scientists have shown that in humans, exist four Notch paralogs (i.e. Notch 1, 2, 3, and 4) and five ligands (i.e. Delta-like 1, 3, 4 and Jagged 1 and 2). Activation of these Notch paralogs are found in stem cell self-renewal but also in many types of cancers [49].

Some epigenetic changes like histone methylation and downregulation of gene expression, which collaborate with the Notch developmental pathway during oncogenesis, were also described [50].

3. Chromatin dynamic and histones modifications

Chromatin is represented by the mandatory association between nuclear DNA and proteins. Chromatin presents different compression degrees during to cell cycles. It exists in two different types: euchromatin and heterochromatin.

Euchromatin has more non repetitive DNA with prevailing of guanine and cytosine bases and nonhistonic proteins; it is also less condensed and represents the active and transcrip-

tional part of the chromatin; it replicates early at the beginning of S faze, being R positive bands from bands marked chromosomes [51].

Heterochromatin has more repetitive DNA with predominating adenine and thymine bases and histones; it is very compact, genetically inactive, with late replication. Heterochromatin functions are to stabilize the centromere and the telomeres of the chromosome, playing an important role in meiosis and in cellular differentiation [52]. Heterochromatin is expressed as constitutive or facultative chromatin. Constitutive heterochromatin is constantly found in a condensed form. It doesn't have functional genes and it is made of highly repetitive DNA (satellite DNA).

Facultative heterochromatin is a chromosome region, densely packed and inactive in a particular cells, having lost gene expression [53]. Both constitutive and facultative heterochromatin, are regulated by the DNA silencing in the mammalian cells. Constitutive heterochromatin is mandatory transcriptional silenced while facultative heterochromatin is conditionally silenced [53].

Electronic microscope analysis shows a hierarchical system of chromatin fibers with different dimensions, made of DNA, histones and nonhistone proteins. The supramolecular organization of DNA has four different levels. The first level is represented by four core histone proteins (H2A, H2B, H3 and H4) which form an octamer wrapped around 1.75 times by 146 base of DNA, making together nucleosomes [54]. Nucleosomes are linked by a short fragment of free DNA (approximately 60 pairs of bases), closed tight by histone H1. The compactness of DNA at this level is 10:1 [54]. Nucleosomes seem to be dynamic structures, since they have to suffer structure modifications during transcription, replication or recombination of DNA. The second level of chromatin economy is represented by the chromatin fiber of 30 nm, creating a solenoid aspect. A fundamental unit in the interphase, this chromatin plays an important role by putting together regions of linear DNA, stimulating genic interaction. The solenoid has a heterogeneous structure, characterized by an alternation of spiral and not spiraled areas, creating a proper configuration for RNA polymerase action, during transcription. The third level of chromatin organization results from creation of lateral loops of 300 nm diameter, attached to a protein nonhistonic matrix. At the beginning of prophase, a matrix will be formed by a 20 times compaction a chromatid, the highest level of chromatin organization [55]. So, the basic DNA will suffer an overall 10000 times compaction, being able to fit a small place into a nucleus. This conformation offers sterically occlusion for nucleosomes, which will be there for protected against nucleases cleavage, while the linker DNA doesn't have this kind of protection [56].

Many cancers are associated with translocations which can be explained by mutual rearrangements due to misfit of two unrepaired double stranded breaks, determined by the close proximity of some genetic regions, thus suggesting the dynamic properties of chromatin [57, 58]. Translocation that characterizes tumorigenesis may depend on the physical distance between individual genetic elements. Chromatin is a dynamic structure, with its own mobility that influences either gene regulation (local diffusion of chromatin) or genomic stability (global chromatin immobility) [57]. In normal cells, as well as in tumor cells, there are similar nuclear layers, defined as center of nucleus-to-locus distance, with a random distri-

bution of genetic loci inside it [59]. Polarization of some chromosomes, with their genes located in the interior of the nucleus and their centromeres located at the nuclear periphery, along with all the other data already presented, strongly support the existing relation between the chromatin pattern and tumor development, but still many aspects remain to be proved.

The debate concerning chromatin remodeling as a cause or a consequence of tumorigenesis is still on.

Many works indicate that DNA methylation and chromatin remodeling are in reciprocal causal relationship: DNA methylation may cause chromatin modifications and specific chromatin modification may induce DNA methylation [1]. Recent data suggest that chromatin remodeling is a combination between a CIS effect determined by the action of a proximal genetic sequence and a TRANS effect induced by sequence independent complexes, most likely by ATP dependent nucleosomes remodeling complexes [60, 61].

Alu sequences are a class of repetitive DNA characterized by a pattern of CG dinucleotides (CpG) repeating every 31-32 bases. They may modulate the nucleosome strength when the CG elements are methylated. Thus, epigenetic nucleosomes within Alu sequences may have methylation-dependent regulatory functions [62].

Emerging data are suggesting that since genome regulation might be influenced by nucleosome positioning and their compositional modifications, nucleosomes are regulating the initiation of transcription, therefore nucleosomes positioning is leading to cancer or developmental effects [17]. Nucleosomes adopt preferential positions near promoter regions and random positions inside genes [63]. Transcription needs exposed binding sites consisting in nucleosome free regions at the 5′ and 3′ ends of the genes, so any change in nucleosome positioning at this level might determine gene activation [64, 65].

Nucleosome positioning is also influenced by another protein complex that activates or represses transcription through biochemical processes, such as octamer transfer, nucleosome remodeling or nucleosome sliding: switch/sucrose nonfermentable (Swi/Snf) complex. It consists in approximately 10 subunits of 2 MDa, with many variants of combinations, first discovered in Saccharomyces cerevisiae [16, 66]. The multiple varieties of Swi/Snf complexes exist in many cell types [67]. Swi/ Snf performs a crucial function in gene regulation and chromosome organization by directly altering the contacts between nucleosomes and DNA [68], using the energy of ATP hydrolysis [69]. The *in vitro* studies revealed that two subunits of the complex, Brg1 or Brm, are able to remodel nucleosomes, with maximal results when subunits BAF155, BAF170 and Ini1 presents a 2:1 stoichiometry relative to Brg1 [70]. The activity of chromatin remodelers appears to be gene specific [71]. The subunits of Swi/ Snf complex seem to have a broad range of functions: BAF155 and BAF170 regulate the protein levels and ensure framing functions for other SWI/SNF subunits [72, 73]; BAF53 is an actin and β-actin related protein signaling, through phosphatidylinositol 4,5- bisphosphate, which binds to Brg1, stimulating the binding to the actin filaments [74-76]; Ini1 is involved in rare and aggressive pediatric cancers [77, 78] as well as in HIV 1 infection [79-81]. The role of SWI/SNF components in cancer stem cells and tumor suppression is still vaguely under-

stood, but their transcriptional pathways are already described, including the cell cycle and p53 signaling [82], insulin signaling [83], and TGFb signaling [84], or signaling through several different nuclear hormone receptors [85]. The biological roles of Swi/Snf components and their involvement in human disease remain to be completed. It is also known that loss of Snf2h impairs embryonic development and differentiation [86] and contributes to tumor development [87]. These tumors are also characterized by polyploidy and chromosomal instability [88]. Since Swi/Snf complex plays also an important role in DNA double strand brake repair, alteration of its function may lead to genomic instability [89]. Critical subunits of Swi/Snf complex miss or are disrupted in approximately 17% of all human adenocarcinomas [90]. Another tumor suppressor gene is Ikaros, a molecule that plays a central role in lymphocyte development through its association with chromatin remodeling complexes [91]. Fusion protein BCR-ABL from preB lymphoblastic leukemia mediates an aberrant splicing of Ikaros, with consequences on cell differentiation [92].

Radiations have the ability to paradoxically induce or cure tumors. It seems that chromatin structure might be influenced by UV and gamma radiation. To study the changes in chromatin pattern under irradiation conditions, Fluorescence In Situ Hybridization (FISH), combined with high-resolution confocal microscopy has been used [93, 94]. FISH studies were performed in leukemia cells for tumor suppressor gene TP53, revealing that TP53 genes are getting closer to each other, as well with the nuclear center within 2 hours of exposure to gamma-radiation, returning during the following 2 hours to its pre-irradiation conditions [95, 96]. There is increasing evidence that CSCs have a higher intrinsic radioresistance than non-CSC tumor cells [97], explaining the difference of CSCs and non-CSC in their response to cancer therapy.

Such repressive complex for chromatin is “Nucleosome Remodeling and Histone Deacetylase” (NuRD) [98], suggested to play a role in acute promyelocytic leukemia (APL) [99]. Human APL is characterized by PML-RARa translocation, which represses gene transcription through several distinct epigenetic mechanisms: DNA methylation, chromatin compaction, heterochromatinization, histone deacetylation, histone modification. NuRD complex is strongly implicated in the epigenetic silencing, by PML-RARa. Earlier findings regarding carcinogenesis, such as the combination of both genetic and epigenetic factors, were confirmed. in this case, PML-RARa oncogenic fusion protein recruiting, induces DNA hypermethylation [100] and result in blocking of hematopoietic differentiation [101].

Covalent modification of histones is an important mechanism, involved in the epigenetic processes. Five types of histones are known to be involved in chromatin building: H1/H5, H2A, H2B, H3, and H4 [15, 102]. In their structure, histones have three distinct domains: a central globular conserved domain and two terminal domains; one short N-terminal tail and one longer C-terminal tail [54].

Generally, histone modifications affect gene transcription, DNA replication and DNA repair mechanisms. The post-translational modifications of the N-terminal tail domains include: methylation, acetylation, phosphorylation, citrullination, ADP-ribosylation, sumoylation and ubiquitination [18-20]. The most studied of these modifications are methylation and acetylation. Lysine residues acetylation in the histone H3 and H4 by histone acetyltransferase

(HAT) and lysine 4 methylation in the histone H3 (H3K4me) by histone methyltransferase (HMT), are generally correlated with active transcription of chromatin. In contrast, methylation of lysine 9 and lysine 27 (H3K9, H3K27me) in the histone 3 have been reported as markers in transcriptionally silenced-chromatin [21, 22].

During the last years, a major challenge in cancer biology is to elucidate how the histone modifications in stem cells, influence carcinogenesis.

Histones methylation is a post translational modification that occurs at the lysine residues and is considered a reversible process [103]. Transcriptional activation or repression, correlates with different degrees of methylation of histones. The binding of one to three methyl groups at each lysine amino acid in the histone structure, give rise to unmethylated, monomethylated, dimethylated and trimethylated degrees of methylation [104, 105]. The monomethylation state of histone has been reported to be associated with an open chromatin structure that lead to transcriptional activation. In contrast, the trimethylation state was shown to be associated with a condensed chromatin structure, which in turn inhibits transcription [106].

Some important exceptions from this rule have been reported by Strahl BD et al., they showed that H3K4 histone methylation state (mono-, di-, or tri-methylated level) is invariably associated with active chromatin, while H3K9 trimethylation can be connected to both transcriptionally active and inactive chromatin [107]. To explain this exception from the general rule, Vakoc et al., described a mechanism by which an association between meH3K9 with RNA polymerase II complexes induces chromatin modification and transcriptional activation [108]. However, it is not fully understood why these markers differs from the general rule.

The binding of the methyl group of each lysine 4, 36 or 79 in the histone 3 (H3K4, H3K36, H3K79) and H4 (k20, H2BK5) induces trnascriptional activation. In contrast, the binding of three methyl group of lysine 9, 27 in the histone 3 (H3K9, H33K27) AND h4k20 was show to be associated with inhibiton of transcription [109, 110]. The histone modifications are arising from the action of enzymes which are responsible for methylation/demethylation activity in the pattern of histone H3 and H4. The enzymes involved in histone modifications are histone acetyltransferases (HATs) and histone deacetylases (HDACs), histona methyltransferases (HMTs) and histone demethylases (HDMs). These enzymes add or remove acetyl or methyl groups, respectively [111, 112]. Several enzymes, like histone methyltransferase (HMTs), histone demethylases (HDMs) and histone deacetylases (HDACs), are connected with each other to create a strong link between chromatin state and transcription.

In addition to changes in histone acetylation, widespread changes in histone methylation patterns are described in cancer. Accordingly, in cancer, aberrant gene silencing was shown to be associated with changes in H3K9 and H3K27 methylation patterns [113].

A recent analysis in the context of histone modifications in cancer, illustrates different scenarios such as histone methylation and its consequences, describing the role of histone methyltransferases (HMTs) and histone demethylation (HDMS) by adding or removing a

methyl group. It has been reported that the level of transcriptional activation is largely maintained by HMTs and HDMs which are involved in the histone methylation [103].

The histone lysine methyltransferase (HMT) that is responsible for the histone methylation, has a catalytically active site known as SET domain, which is formed by de 130 amino acid sequence. The major function of the SET domain is to modulate gene activity [114].

The binding of the methyl group at several lysine sites in histone H3 (H3K9, H3K27, H3K36, H3K79) and loss of acetylated H4 lysine 16 and H4 lysine 20 trimethylation have been reported to be associated with changes that occur during tumorigenesis[115]. The enzymes HDACs and HATs have been suggested to be responsible for these changes and are commonly found to be altered in various forms of cancer [116].

Various observations suggest the presence of a novel chromatin pattern in embryonic stem cell, which consists of lysine 27 and lysine 4 tri-methylation superposition, termed "bivalent domains" [117].

The bivalent domains have been analysed by the genome mapping of histone methylation profiles in embryonic stem cell and was reported to include both active and repressive chromatin marks. Developmentally, the "bivalent domains" is responsible for maintaining epigenomic plasticity, enabling embryonic stem cells to regulate gene expression [117]. Bivalency is lost during stem cell differentiation, allowing epigenetic plasticity and lineage commitment. Epigenetic plasticity in association with bivalent gene promoters is suggested to induce a transcriptionally repressive and permissive histone mark in embryonic stem cells [117, 118].

In cancer, bivalency has been suggested to stigmatize specific genes for DNA methylation, inducing aberrant reprogramming [119-121]. In analogy with embrionic stem cells, bivalent gene promoters were reported to be DNA-methylated in cancer cells, suggesting the provenience of cancer cells from embryonic stem cells [122]. In absence of DNA methylation, the repressive H3K27 trimethylation mark was also demonstrated to induce gene silencing in cancer cells.

Chromatin regulating complexes are commonly observed in cancer, and is hypothesized to involve multiple mechanisms, including DNA methylation and Polycomb repressive complexes (PRCs). Chromatin regulating complexes including two families of Polycomb repressive complexes (PRC1 and PRC1), mediate trimethylation on H3K27 in cancer cells [123, 124]. PRC2 complex has also been reported to intermediate H3K27 trimethylation in embryonic stem cell [125].

4. miRNA and DNA methylation in cancer stem cells

MicroRNAs (miRNAs) was first discovered in 1993 by Victor Ambros, Rosalind Lee and Rhonda Feinbaum. The recent definition of miRNA is: small non-coding RNA molecules (21-24 nucleotides long), implicated in posttranscriptional gene expression, regulation by

two different mechanisms: splitting and subsequent degradation of targeted RNAm or inhibiting translation, both determining the stop or stimulation of cell reproduction [126-130].

However, the entire mechanism of miRNA is not yet fully understood [131, 132].

The study on Caenorhabditis elegans (C. elegans) has permitted the clonation of first miRNA, lin-4 and let-7 and their targets [133, 134]. This study discovered that the gene lin-14 was able to transcribe a precursor that matured to a 22 nucleotide mature RNA, which contained sequences partially complementary to multiple sequences in the 3' UTR of the lin-14 mRNA, ensuring inhibition of translation of lin-14 mRNA. In addition, in 2000, along with the discovery that gene let-7 repressed the genes lin-41, lin-14, lin-28, lin-42 and daf12 mRNA during transition in developmental stages in C. Elegans, it was also established that non-coding RNA identified in 1993, was part of a wider phenomenon [133].

More than 700 miRNAs have been identified in humans and over 800 more are predicted to exist. These molecules have an important role in cellular physiological processes (e.g. cell cycle, cell proliferation, apoptosis, cell differentiation and development), by implication in gene regulation; miRNA has been found to control about 30% of all human genes.

In human embryonic stem cells and the differentiated embryonic bodies, over 100 miRNAs have been already described [101]. The self-renewal and pluripotency of embryonic stem cells are regulated by an array of protein-coding genes in a regulatory circuitry [135], which includes OCT4, SOX2, and KLF4 genes. Extensive studies have indicated the importance of OCT4 in self-renewal and pluripotency of embryonic stem cells [136, 137]. Multipotent cell lineages in early mouse development, have also been reported to be dependent on SOX2 function [138, 139] in the process of embryonic stem cells self-renewal and pluripotency. The miRNA genes are also connected to the transcriptional regulatory circuitry of embryonic stem cells [140] and are overexpressed in their differentiating processes.

The three key proteins of pluripotent cells, Oct4, Nanog and SOX2, and TCF3 were found in the promoters of miRNA specific stem cells, but also in promoters of miRNA, which controls cell proliferation (mir 92 si let7g) and differentiation (e.g., mir-9 or mir-124a for neutral line). OCT4 was reported to bind and repress miR-145 promoter in human embryonic stem cells. On the other hand, inhibition of Oct 4 increases the activity of these miRNAs that in turn inhibit the stem cell renewal.

miRNAs, occasionally causes DNA methylation of promoter sites and can regulate other epigenetic mechanisms. An altered miRNA gene methylation patterns in human cancers was reported to sustain in tumorigenesis. Half of these genes are associated with CpG islands and several studies indicated that miRNA gene methylation was often detectable, both in normal and malignant cells. Recent works have identified many types of miRNA, which allow cancer cells to multiply indefinitely by avoiding natural cellular aging mechanisms, thus suggesting a close relation between cancer development and miRNA expression [141].

There are several mechanisms which may lead to modification of mi RNA in cancer [142-145].

- *Chromosomal changes* - quantitative gene changes have been identified in approximately 283 miRNA, determined by either loosing heterozigotism by the action of a suppressive gene or by amplification of a chromosomal region of an oncogene either by chromosomal ruptures or translocations [146];
- *miRNA biosynthesis abnormalities*, mainly represented by gene amplification for proteins as Drosha (implicated in miRNA maturation process) or Ago2 (responsible for the interaction with target messengers);
- *Epigenetic changes*- recent data suggest the implication of DNA methylation in the disorder of miRNA expression. Gene analysis for miRNA established that these genes are usually associate with CpG islands and thus represent candidate targets of the DNA methylation machinery. A high level of miRNA genes methylation exists both in normal and malignant cells. Epigenetic changes of chromatin, for instance histone deacetylation, cause important alteration of miRNA expression as well [141];
- *miRNA as oncogenes or tumor suppressors* - miRNA always acts as negative regulator of gene expression. In cancer, miRNA were classifies as miRNA with oncogenic effect (oncomirs) and miRNA with suppressor effect (supressor mirs) [147-149].

Oncogenic activity of miRNA, initially determined for mir-17-92 and mir-155, was further sustained by the discovery of other potentially oncogenic miRNA [150]. Therefore it is logical that this classification of miRNA in oncogenes or tumor supressor genes may facilitate the identification of different tissues where they are expressed [151].

Embryonic stem cells gene expression of Oct4, Sox2, Klf4, and Nanog was observed in highly aggressive human tumors [152]. It has been reported that miR-200 known to mediate transcriptional repression, also play an important role in both cancer stem cells andembryonic stem cells [153, 154].

Several miRNAs have been reported to be overexpressed in human cancer. The mir-17-92 polycistron (cluster) is overexpressed in B-cell lymphoma [155, 156] and in testicular germ cell tumors miR-372 and miR-373 were identified as possible oncogenes [157].

Another theory supports the idea that some cancers such as Kaposi `s sarcoma, were induced by viral oncogenic miRNA [158]. It is clear that discovery of miRNA involvement in cancer stem cells function will be a crucial step in elucidating the process of oncogenesis [159].

5. Epigenetic targeting in cancer stem cells

Recently, several epigenetic drugs targeting epigenetic mechanisms have been tested *in vivo* and *in vitro*. The epigenetic mechanisms comprise modifications of histones and DNA methylation. Histone modifications includes several post translational modification of the: methylation, acetylation, phosphorylation, ubiquitination, sumoylation; commonly found in tumor cells. Thus, epigenetic modifications targeting is an important event in cancer thera-

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py. Epigenetic targeting may be realized by two classes of substances with antitumor effect in malignancies: the hypomethylating agents and histone deacetylase inhibitors [160].

5.1. Targeting DNA methylation

DNA methylation is the most studied epigenetic marker. Abnormal DNA methylation of several regulatory genes is usually associated with cancer. The methylation process is reversible, therefore the reactivation of silenced genes can be realized using substances with hypomethylating activity [161].

The new development cancer therapies are based on molecules that can inhibit the classes of DNA methyltransferases (DNMT), histone deacetylases (HDACs), histone acetyltransferases (HATs) and new substances that target chromatin and nucleosome remodeling proteins. DNMT inhibitors (DNMTi) can be natural or synthetic compounds [148, 162].

As mentioned before, DNMTs are enzymes that catalyze the reaction between methyl groups and pyrimidine base cytosine. The methyl group donor is S-adenosyl-L-methionine (SAM), which is the active form of amino acid methionine.

The DNA hypomethylating agents are divided into two categories*:* nucleoside analogs drugs and non-nucleoside analogs drugs. The first description substances are 5-azacytidine (azacitidine, Vidaza™) and 5-aza-2′-deoxycytidine (decitabine, Dacogen™) that have the most powerful effect from nucleoside analogs drugs [1, 163-165].

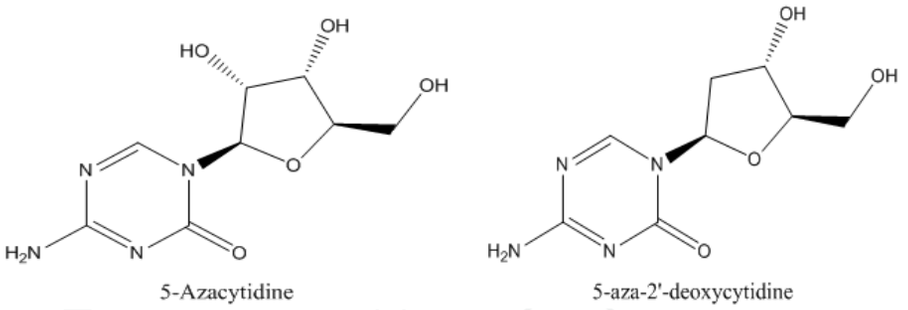

Figure 2. Nucleoside analog drugs. Their structure allows incorporation into the DNA and subsequent hypomethilation.

Other substances from this group are: 1-β-D-arabinosyl-5-azacytidine (fazarabine) [166], dihydro-5-azacytidine (DHAC), 5-fluoro-2′-deoxycytidine (FCDR) and zebularine [163]. Incorporation into the DNA structure of nucleoside analogs is facilitated by their similar chemical structure.

5-azacytidine is used as single-agent therapy or in combination with other therapies in treatment of myelodysplastic syndromes (MDS), acute myeloid leukemias (AML) and solid tumor. As associated substances are utilized valproic acid, cytarabine, entinostat, etanercept etc [137, 164].

5-aza-2′-deoxycytidine (DAC) has benefited as monotherapy in myelodysplastic syndromes, chronic myelomonocytic leukemia (CMML) and has been FDA approved on May 2006. The

drug has been associated with: carboplatin useful in solid tumors treatment [167], valproic acid in acute myeloid leukemias and advanced leukemia [168, 169], imatinib mesylate in chronic myelogenous leukemia (CML) [170] and IL-2 in metastatic melanoma, renal carcinoma [171].

Zebularine (2-pyrimidone-1-β- D-riboside) is other nucleoside analog with hypomethylation activity [172] and also implicated in tumor gene expression [173].

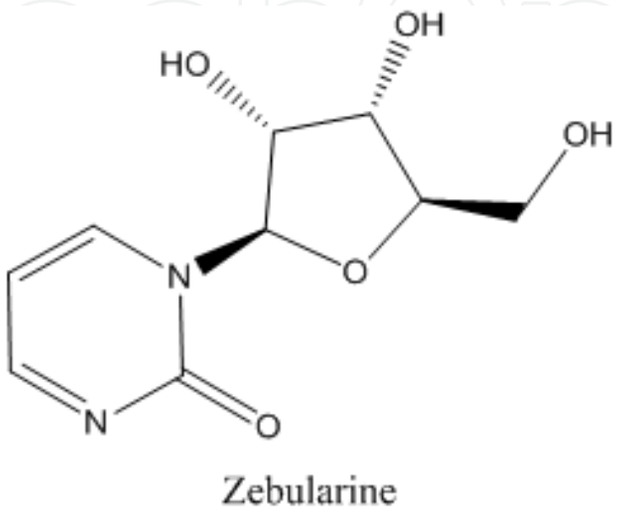

Figure 3. Structure of Zebularine. Zebularine is another nucleoside analog drug, with hypomethilation effect.

There are recent studies about another two molecules: NPEOC-DAC and SGI 110 (S110). NPEOC-DAC is the result of chemical reaction between azacytosine molecule and 2-(p-nitrophenyl) ethoxycarbonyl, with reported effect on DNA methyltransferases inhibition. Byun et al. demonstrated that NPEOC-DAC inhibited DNA methylation in two cell lines of liver cancer. The authors, also showed that SGI 110 (S110) has a pronounced effect on DNA methylation inhibition [174].

The non-nucleoside analogs category contains compounds with hypomethylation effect. This group contains hydralazine (the widely known as vasodilatator), procainamide (antiarhythmic), RG108 and SGI-1027.

Physiologically, acetylation of chromatin is realized by specific enzymes - histone deacetylases and acetyltransferases. A possible change in their normal function can promote tumors.

5.2. Targeting histone modification

At first glance, HDACs are enzymes that play a role in elimination of acetyl radical just from lysine molecules of histones, but their actions is not limited to histones, they can also act on non-histone proteins [175].

HDAC inhibitors are classified into four classes, based on their chemical structure: short-chain fatty acids, hydroxamic acids, cyclic peptides, benzamides (hybrid molecules) [176].

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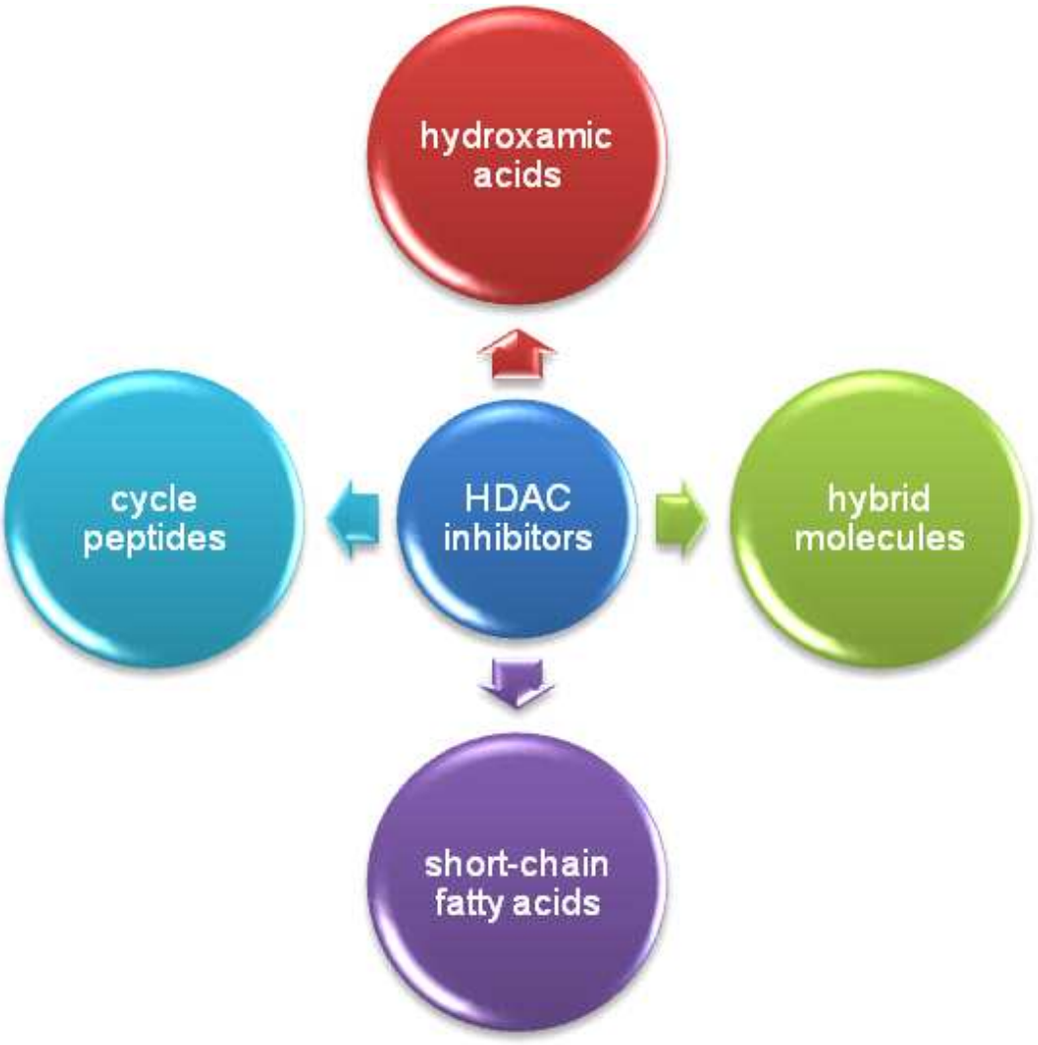

Figure 4. HDAC inhibitors. There are four classes of curently known HDAC inhibitors: short-chain fatty acids, hydroxamic acids, cyclic peptides, benzamides, with a great potential use as detection and prognosis markers.

Exemples of short-chain fatty acids are: sodium n-butyrate, sodium phenylacetate, phenylbutyrate, valproate, substances that in millimolar concentrations are involved in inhibition the growth of some carcinomas but their mechanism of action is not fully understood [163, 177-179].

One of the most studied agent from class of small fatty acids, is valproic acid (VPA), an antiepileptic drug reported to target histone deacetylase. Numerous research studies *in vitro* demonstrated that VPA was implicated in hyperacetylation of histones H3 and H4 and also *in vivo* tests confirmed the drug inhibiting action of HDACs. VPA antitumor activity was demanstrated by: cell growth inhibition, apoptosis inducing, antimetastatic and antiangiogenesis effect, etc. These benefits lead to FDA approving of VPA [14, 180].

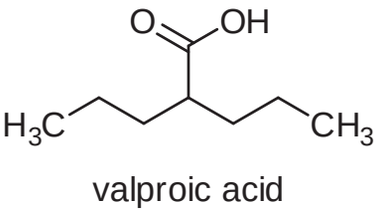

Figure 5. Structure of valproic acid. Valproic acid is a small fatty acid commonly used as an antiepileptic drug, but with recently emerged antitumor effects.

The class of hydroxamic acids include synthesized compounds such as: belinostat, panobinostat, vorinostat (SAHA) etc. Belinostat and panobinostat, have been used in clinical trials to treat solid tumors and blood malignancies [181-183]; MDL and CML [184-186], vorinostat (SAHA) that was approved by FDA for the treatment of chronic T-cell lymphoma (CTCL) and used in clinical trials for hematologic malignancies, mesothelioma, breast and ovarian cancer, etc [175].

A natural compound from cyclic peptides class is romidepsin, also known as Istodax (FK228), which was clinical tested in various lymphomas. The drug was shown to induce apoptosis in different tumor cell lines, due to blocking of HDACs [187].

Hybrid molecules (i.e. benzamides) includes two synthetic compounds: Entinostat (MS-275) and Mocetinostat (MGCD 0103). The mechanism by which Entinostat induced cytotoxic effect on tumor cells was suggested to be due to the upregulation of some tumor suppressor genes (p21). Both Entinostat and Mocetinostat are currently approved by the FDA and are used in cancer treatment. Entinostat is used in the treatment of blood and lung tumor [181, 183] and Mocetinostatin in the treatment of chronic lymphocytic leukemia (CLL) [175].

HATs are a class of enzymes discovered twenty years ago, enzymes with demonstrated role in gene transcription [188]. HATs have been reported to be implicate in numerous types of diseases (i.e. viral infection, respiratory maladies, cancer etc). It has been suggested that the HATs enzymes may be used as biological markers for cancer prediction or recurrence [14]. Four families of HATs are known that share primary-structure homology: GNAT (Gcn5-related N-acetyltransferase), p300/CBP and MYST, Rtt109 [189]. The HAT enzymes have various chemical structure and their classification is still unclear.

Histone methylation process plays an important task in epigenetic regulation, which lead to synthesizing of new target drugs for cancer therapy [163].

Researchers describe a class of enzymes called histone methyltransferases. This class of enzymes includes lysine methyltransferases and arginine methyltransferases, both of them linked to many types of cancer.

There are 8 known lysine methyltransferases (KMT1-8) with suggested role in the epigenetic gene silencing in malignancies like: prostate, liver, colon, breast cancer [190, 191].

Few of the many types of arginine methyltransferases (PRMTs), are also closely linked to cancer [191].

Thus, the importance of DNMTs and HDACs, two classes of enzymes involved in epigenetic targeted therapy of malignant diseases, is obvious. The enzymes implicated in histone methylation and demethylation are mainly attractive as validated targets for cancer therapy.

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6. Conclusions and perspectives

Epigenetic is a heritage mechanism involved in the process of stem cells differentiation to more specialized cells. According to the cancer stem cell model, dysregulation of epigenetic mechanisms (i.e. DNA methylation and histone modification) in pluripotent stem cells enable their transformation in cancer cells with high proliferation rates and poor prognosis.

DNA methylation is considered the most largely studied part of the epigenetic, but recent works associate the methylation with other epigenetic changes, such as histone modifications, chromatin remodeling and microRNA, suggesting a reciprocal relationship between them in cancer cells. The similarities between chromatin regulation process in stem cells and cancer cells have been mentioned in several studies.

It is therefore important to understand the epigenetic alterations that take place in cancer cells compared with normal cells and the importance of these modifications in carcinogenesis, according to the cancer stem cell theory. In addition, it is very useful to understand the potential of epigenetic marks in designing more effective treatment strategies that specifically target cancer stem cells.

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