Dynamic and reversibility of heterochromatic gene silencing in human disease

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ABSTRACT

In eukaryotic organisms cellular fate and tissue specific gene expression are regulated by the activity of proteins known as transcription factors that by interacting with specific DNA sequences direct the activation or repression of target genes. The post genomic era has shown that transcription factors are not the unique key regulators of gene expression. Epigenetic mechanisms such as DNA methylation, post-translational modifications of histone proteins, remodeling of nucleosomes and expression of small regulatory RNAs also contribute to regulation of gene expression, determination of cell and tissue specificity and assurance of inheritance of gene expression levels. The relevant contribution of epigenetic mechanisms to a proper cellular function is highlighted by the effects of their deregulation that cooperate with genetic alterations to the development of various diseases and to the establishment and progression of tumors.

Keywords: DNA methylation, chromatin remodeling, epigenetics, retinoic acid, acute myeloid leukemia.

MOLECULAR MECHANISM OF DNA METHYLATION REACTION

Among all epigenetic mechanisms involved in gene expression regulation, DNA methylation has been the most widely studied subject. DNA methylation results from the transfer of a methyl group from a methyl donor substrate, namely S-adenosyl-L-methionine (AdoMet), to the 5' position of a cytosine in the palindrome dinucleotide CpG [1]. Non CpG DNA methylation has also been observed in eukaryotic genomes on CpNpG or CpA and CpT sequences [2], which account for almost 20% of the global methylation in mouse embryonic stem cells [3]. The function of non CpG methylation however needs to be further elucidated. To date, cytosine methylation represents a unique chromatin modification that can be faithfully propagated through cell divisions [4]. In eukaryotes the CpG methylation reaction is catalyzed by enzymes known

as DNA cytosine-5-methyltransferases (DNMTs) [5, 6] (Fig. 1) including:

DNA cytosine-5-methyltransferase 1

DNA cytosine-5-methyltransferase 1 (DNMT1), known as a maintenance methylase, copies the parental DNA methylation patterns onto daughter DNA strand during replication. The maintenance role of pre-existing DNA methylation patterns is attributed to DNMT1 based on its activity on hemimethylated substrate and its nuclear localization during DNA replication. DNMT1 activity is 5-50 folds higher in presence of a hemimethylated substrate as compared to that in the presence of an unmethylated DNA sequence [7, 8]. Nonetheless, the specific activity of DNMT1 on unmethylated DNA substrates is greater than that of DNMT3A and DNMT3B, which are considered as "de novo" DNMTs instead [6].

Moreover, DNMT1 localizes in association with PCNA proteins to the replication foci during the S phase [9]. DNMT1 through its amino terminal domain interacts with the DNMT1 associated protein (DMAP1), histone deacetylases (HDACs) and retinoblastoma protein (Rb) [10-

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Fig. 1 Schematic representation of DNA-methyltransferases functional/structural domains in relation to their activities. (—) indicates the binding sites for: DMAP1, PCNA, Rb, HDAC and MeCP2. The nuclear localization signal (NLS), the conserved cysteine-rich region (Cys-rich), which shares homology with the X-linked ATRX gene of the SNF2/SWI family (ATRX Hom) and the catalytic motifs are also indicated.

12]. DNMT1 is constitutively expressed in proliferating cells and ubiquitously in somatic tissues throughout mammalian development. A number of studies have been conducted to evaluate the biological consequences of losing DNMT1 activity. In 1992 Li *et al* [13] have shown that insertion of a mutant DNMT1 gene into mouse germ line produces a recessive lethal phenotype. The m5C levels in homozygous mutant mouse ES cells were one-third of that in wild-type cells but the cells were still viable. In addition, loss of a functional DNMT1 also determines the following:

- 1) mice or embryonic stem (ES) cells homozygous for null alleles of DNMT1 are generally demethylated, although there is a basal methylation level [14];
- 2) biallelic expression of most imprinted genes in homozygous embryos [15];
- 3) an increase of mutation rates for exogenous marker genes in ES cells and an increase of losing heterozygosity due to mitotic recombination [16];
- 4) enhanced microsatellite instability in mouse embryonic stem cells [17];
- 5) demethylation and expression of transposons of the intracisternal A particle (IAP) class, the LTR retrotransposons [18-20] attributing to the specific role of DNMT1 in gene silencing on transposable elements;
- 6) very high rates of lethal T-cell lymphomas [21].

DNA cytosine-5-methyltransferase 3A and 3B

DNA cytosine-5-methyltransferase 3A and 3B (DNMT3A and 3B), known as *de novo* methylases, methylate unmethylated CpG dinucleotides [6]. DNMT3A and DNMT3B are highly expressed in ES cells, early embryos, and developing germ cells where *de novo* methylation takes place, but expressed at lower rate in somatic tissues of postnatal animals [22]. These enzymes have no preference for hemimethylated DNA [22, 23] and can methylate *de novo* unmethylated CpGs when ectopically expressed in mammalian cells or transgenic flies [24, 25]. DNMT3A has also been reported to methylate CpA sites [3]. DNMT3A-deficient mice survive to adult but die early in adulthood with global methylation patterns maintained [6]. DNMT3A^{-/-} DNMT3B^{-/-} double knock-out mouse embryos die at 8.5 dpc with global demethylation in genomes [6]. Point mutations in human DNMT3B are responsible for the autosomal recessive human disorder known as ICF (immunodeficiency, centromere instability, and facial anomalies) syndrome [23].

DNA cytosine-5-methyltransferase3 Like

DNA cytosine-5-methyltransferase3 Like (DNMT3L) was originally identified by database analysis of the human genome sequences and subsequently a mouse homolog was characterized [26-28]. This enzyme lacks amino acids essential for the catalytic activity of methyltransferase. DNMT3L is thus unable to perform enzymatic activity. The gene is however essential for the establishment of maternal methylation imprints and for the correct expression of maternally imprinted genes [28]. A mechanism of forming protein complex with DNMT3A and 3B and thus modulating the enzymatic activities of DNMT3A and 3B is probably involved [27, 29].

The DNMT1, DNMT3A and DNMT3B cooperate to establish and maintain DNA methylation patterns. Genetic and biochemical analyses have shown that double knock-out of DNMT1 and DNMT3b diminishes most of the genomic methylation while single knock-out has limited effect on DNA methylation pattern [30]; that DNMT1 alone is insufficient to restore DNA methylation after 5-aza-CdR treatment [31] and that DNMT1 can form complex with DNMT3A and 3B [32] (Fig. 1).

CpG DINUCLEOTIDES LOCATION AND FUNCTION

DNA methylation levels are not equal in eukaryotic genomes with a great disparity found in the four eukaryotic groups (protists, plants, fungi, and animals). Furthermore, CpG dinucleotides are not equally distributed along the genome; most of them are gathered in regions named "CpG islands" [33].

In mammals CpG islands ranging from 0.4 to 4 Kb in length, have CG content higher than 55%, which is five folds higher than that in bulk DNA and are unmethylated [34]. The unmethylated status of CpG islands is preserved during replication and maintained during following cell cycles despite of their accessible localization in decondensed chromatin for enzymes and transcription factor. Although maintenance of unmethylated CpG status has been studied by several groups [35-40], the detailed mechanism is still not very clear. CpG islands usually span the 5'-end region-promoter, untranslated region and exon 1 of 40% mammalian genes [41].

The CpG dinucleotides dispersed along the genome and located in non regulatory DNA regions on the other hand are largely methylated. A great majority of these CpGs resides in transposable elements, which are interspersed repeated sequences that constitute more than 45% of the human genome [42]. Transposable elements can be reactivated by demethylation of methylated CpGs [20, 43]. These observations strongly suggest a role for DNA methylation in the protection of eukaryotic genome from viral and parasitic sequences. The de-silencing of transposable elements affects the genome stability and the expression of genes through different mechanisms including the activation of RNAi pathways [8, 44].

DNA methylation patterns are established during embryonic development and should be maintained unchanged during adult life for proper cellular function [45]. Germ cells of females are less methylated than those of males, and after fertilization gamete methylation patterns are erased by a wave of genome wide demethylation [46] that is active in the sperm derived male pronucleus and passive in the female pronucleus [47]. During the implantation stage, methylation patterns are established by a wave of *de novo* methylation and they probably serve to compartmentalize the genome into transcriptionally active and inactive zones. The methylation status of CpG sites within gene regulatory regions strongly affects the expression of the downstream genes. In particular the unmethylated status of a 5' promoter CpG island is associated with the transcription of the downstream gene, whereas CpG island methylation inhibits gene transcription; genes on inactive X chromosome, imprinted genes and tumor suppressor genes in tumors are silenced and have regulatory elements largely methylated.

If methylation of regulatory CpG islands is critical for gene expression, intragenic methylation in mammalian genes never blocks transcription and RNA elongation. Very recently Lorincz *et al* [4] have shown that the efficiency of transcription can be seriously affected by methylation of non regulatory CpGs dispersed in the body of the gene.

We may assert that DNA methylation exerts its func-

tions through an inhibitory effect on gene expression. For instance, functioning DNA methylation machinery is fundamental for a correct expression of imprinted genes. While the great majority of genes are expressed from both maternal and paternal alleles, few genes are specifically expressed only from one allele. In these cases the promoter CpG islands associated with the silent genes are largely methylated [48]. In addition, in mammalian females the inactivation of X-chromosome is dependent on DNA methylation, CpGs sites in promoters of the genes on the inactive X chromosome are methylated and the genes are transcriptionally silent [49]. A role for DNA methylation in tissue specific gene expression and differentiation has also been proposed. First data on DNA methylation and tissue specificity were obtained by Walsh and Bestor [18]. These Authors investigated the 5' methylation status of seven tissue-specific genes and found no correlation with tissue-specific expression. Recent results, mostly obtained by genome wide approaches to study DNA methylation, do confirm a role for DNA methylation in tissue specific gene expression. One of these genome wide approaches known as Restriction Landmark Genome Scanning (RLGS) has enabled to identify in six mice tissues a large number of differentially methylated regions (TDMs) which exhibit a tissue-specific expression pattern that is consistent with methylation status and a role in tissue differentiation [50]. Furthermore, Ching *et al* [51], by using BAC microarray have identified CpG island loci that are methylated in a tissue-specific manner in normal human tissues, including SHANK3 whose differential methylation in different tissues was also evolutionary conserved.

MECHANISM OF GENE SILENCING BY DNA METHYLATION

Two mechanisms have been proposed to explain the inhibitory effect of CpG methylation on gene expression. First, CpGs methylation inhibits binding of transcription factors to their CpG containing recognition sites [52]. The second mechanism proposes the involvement of proteins with high affinity for methylated CpGs. MeCP1 and MeCP2 (methyl CpG binding protein 1 and 2) were the first proteins with high affinity for methylated DNA containing a "methyl binding domain" (MBD) described [53]. MeCP1 targets DNA sequences containing a minimum number of methylated CpGs, whereas MeCP2 binds a single symmetrically methylated CpG pair. Both proteins were found to bind chromosomes at sites known to contain methylated DNA. Mutations in MeCP2 cause the human female neurodevelopmental disorder called Rett syndrome and a closely related disorder in MeCP2 mutant mice [54]. On the basis of the ability to bind methylated sequences *in vitro* other methyl CpG binding proteins have been identified:

Fig. 2 Epigenetic modifications leading to gene transcriptional regulation. TF: transcriptional factors; DNMT: DNA methyltransferase; HAT: Histone acetyltransferase; HDAC: Histone deacetylase; MBP: Methyl-CpG binding protein; HMT: Histone methyltransferase. Modifications of the histone H3 tails, Lys4 (K4) and Lys9 (K9), are indicated. Ac: acetylation; Me: methylation.

MBD1, MBD2, MBD3, MBD4 and Kaiso. MBD1 was first identified as a methyl binding protein and it has been recently shown as able to bind CpG sequences independently of the methylation status [55]. MBD2 is part of the MeCP1 protein complex [56]. MBD3 binds methylated

probes *in vitro* but this interaction is not competed by an excess of methylated cold competitor making its ability to interact specifically with methylated sequences questionable [57]. MBD4 turns out to be a DNA T:G mismatch repair enzyme and may function to minimize mutation at

methyl-CpG sites [58]. The transcriptional repressor Kaiso, which binds methyl-CpGs through a zinc-finger motif [59], recognizes methylated DNA regions containing at least two methylated CpGs. The knock-down of Kaiso leads to premature zygotic gene activation and subsequent developmental arrest and apoptosis, mimicking the phenotype of hypomethylated embryos deficient in DNMT1 [60]. In HeLa cells, Kaiso is coupled with the NCoR corepressor to mediate DNA methylation-dependent gene silencing [61].

THE CONNECTION BETWEEN DNA METHYLATION AND OTHER EPIGENETIC MECHANISMS OF GENE SILENCING

Despite the exact cascade of events leading to remodeling of chromatin structure and gene inactivation or activation, DNA methylation, histone acetylation, deacetylation and histone methylation all work together to build up chromatin structures which coordinately may shift from a transcriptional permissive state to a transcriptional inactive state and vice versa (Fig. 2). The methyl CpG binding proteins exert their function as transcriptional repressors *via* chromatin remodeling. These proteins are often part of larger repressor complexes as NuRD, NoRC, mSin3A and SWI-SNF, that recruit HDACs [56, 62-67] and histone methyltransferases (HMTs) [68] on methylated targeted sequences. Acetylation of the α amino groups of evolutionary conserved lysine residues present on nucleosomal histone H3 and H4 is catalyzed by the histone acetyl transferases (HAT). The level of histone acetylation at these sites depends on the equilibrium between the histone acetyl transferase activity and histone deacetylase activity [69]. Methylation of nucleosomal histones occurs on lysine and arginine residues and the reaction is catalyzed by the HMTs [70]. Both the HDAC and HMT activities determine changes in the post translational modification of histone tails thus promoting the shift from a transcriptionally permissive chromatin state to a transcriptionally inactive chromatin state (reviewed in [71]) as schematically represented in Fig. 2.

In mammals, the decondensed transcriptionally active euchromatin is enriched for lysine 9 (K9) acetylated histone H3 and lysine 4 (K4) methylated histone H3 (mainly in lower eukaryotes), whereas K9 deacetylation, K9 (di and tri-methylation) and K27 methylation of H3 histone are common marks of a condensed transcriptionally inactive heterochromatin [70]. Furthermore, methylated K9 histone H3 recruits the heterochromatin protein 1 (HP1) allowing the stabilization of an inactive condensed chromatin [70]. A very intriguing role for K9H3 methylation has been suggested by experiments conducted on *Neurospora crassa*, *Arabidopsis thaliana* and mammals. In *Neurospora crassa*, mutations in Su(var)3-9 H3K9 methyl-

transferases (*dim-5*) gene are responsible for a genome wide demethylation of methylated CpGs [72]. The same result was obtained in *A. thaliana* by mutations of Kryptonite gene, a relative of Su(var)H3-K9 methyltransferase, in this case the CpNpG methylated sequences were largely demethylated while methylation status of CpGs remained unchanged [73]. In mice, mutation of the two Su(var)3-9 homologs has a relatively minor effect on cytosine methylation and causes partial demethylation of major satellite regions of pericentromeric heterochromatin [74]. These data suggest a clear dependence of DNA methylation on histone H3 methylation.

Literature provides evidences that support the current debate on the exact cascade of events leading to gene inactivation through chromatin remodeling. The DNA methylation may represent the *in cis* input able to induce the recruitment of chromatin remodeling factors and the following shift of chromatin structure from a transcriptional active to a transcriptional inactive state. On the other hand H3K9 methylation may represent the *in trans* input as shown by his role in targeting DNA methylation in fungi, plants and mammals. In this case the histone modifications and the successive chromatin remodeling precede changes in DNA methylation pattern.

PUTATIVE REGULATORY ROLE OF THE RNAi PATHWAYS IN THE EPIGENETIC CONTROL OF GENE EXPRESSION.

The idea that RNAi pathways can target epigenetic modifications, such as DNA methylation and histone methylation, to genomic specific regions has gained a tremendous attention in the last decade. The first data showing a connection between DNA methylation and RNAi pathway were obtained in viroid-infected tobacco plants [75]. In viroid infected plants the cDNA copies integrated into genome undergo DNA methylation directed by RNA. Subsequently, it was shown that this *de novo* methylation activity required formation of dsRNA and of processed siRNA [76]. siRNAs direct DNA methyltransferases (MET1, CMT3), histone methyltransferases (DDM1, Kryptonite) and histone deacetylases onto targets promoter regions and induce transcriptional gene silencing in plants [77, 78]. Although not all the components of the RNA-dependent DNA methylation machinery have been identified, it seems that a similar process exists in mammalian cells.

The expression of elongation factor 1 alpha (EF1A) promoter-green fluorescent protein (GFP) integrated into the genome of human 293FT cells is inhibited following the transfection of EF1A homologous sequence EF52 siRNA and is associated with DNA methylation status shown by methylation sensitive restriction enzyme. This inhibition is

sensitive to 5-azacitidine (DNMTs inhibitor) and trichostatin A (deacetylase inhibitor) treatments. The transcription of endogenous EF1A is also regulated by and associated with DNA methylation of promoter region [79].

siRNAs targeted to promoter CpG island of E-cadherin gene in MCF-7 and normal mammary epithelial cells induce DNA methylation, histone H3K9 methylation and transcriptional repression. This siRNA mediated DNA methylation is DNMT1- or DNMT3B-dependent [80]. In addition, another group of smaller RNAs namely the microRNAs (miRNAs) might also be involved in epigenetic control of gene expression through DNA methylation and chromatin remodeling [81]. miRNAs are derived from precursors and are exported into the cytoplasm from the nucleus. Once in the cytoplasm, miRNAs interact with a target mRNA, via the RNA-induced silencing complex (RISC), to degrade the mRNA or to prevent protein translation. Bao *et al* [81] report an extensive study on DNA methylation in a region located more than 1 Kb downstream of a “miRNA-binding sites” in the PHB and PHV genes in *A. thaliana*. When the miRNAs binding sites on PHB and PHV genes are mutated, an extensive demethylation is observed within this region. The authors suggest that the demethylation facilitates gene reactivation and the methylation is mediated by miRNA [82].

ABERRANT EPIGENETIC CHANGES OF DNA AND CHROMATIN IN HUMAN DISEASE

The deregulation of epigenetic control on gene expression is frequently related to human diseases. The Rett syndrome, an X linked neurodevelopmental disorder, is linked to mutations in the MeCP2 gene. Patients affected by ICF syndrome carry mutations in the methyltransferase gene DNMT3B. The Beckwith-Wiedemann syndrome, the

Prader-Willi syndrome and the Angelman syndrome are human diseases caused by deregulation of imprinted genes or by epigenetic defects (reviewed in [83]).

Epigenetics alterations may also have effects on the onset of mental diseases such as schizophrenia and affective disorders [83]. Moreover, studies accumulated during the past two decades have proven that epigenetics is indeed involved in controlling and regulating cancer formation and progression. Aberrant DNA methylation patterns are extremely common in all types of tumors. In considerations of: i) the role of DNA methylation in the regulation of gene expression and in the maintenance of genome stability; ii) the aberrant methylation patterns observed in human specimens ahead of the onset of malignancy [84, 85]; iii) the sensitivity of cancer patients to DNMTs and HDACs inhibitors, it is plausible that an epigenetic deregulation in general and altered DNA methylation pattern in particular are not a consequence but rather a primary cause of malignant state [86].

DNA methylation machinery has been damaged seriously in cancer cells when largely aberrantly hypomethylated genomic sequences coexist with aberrantly hypermethylated sequences creating one of the most intriguing biological paradoxes [87]. The causes of such dichotomous are however not known.

Cancer cells undergo significant DNA hypomethylation [88] that involves methylated CpGs in non regulatory regions such as intragenic sequences and repetitive elements. Mice carrying a hypomorphic DNMT1 allele have reduced DNMT1 expression up to 10% of the wild type levels and have a substantial genome-wide hypomethylation in all tissues. These mice develop aggressive T cell lymphomas that display a high frequency of chromosome 15 trisomy [21, 89]. Hypomethylated cells carrying a hypomorphic

Tab. 1 Functional classification of putative/established tumor suppressor genes found hypermethylated and silenced in cancer cells

Function	Gene
Cell cycle regulation	<i>p16^{ink4a}, p15^{ink4b}, Rb, p27, p73, 14-3-3σ</i>
Apoptosis	<i>DAPkinase, Caspase 8, TMS1</i>
DNA repair	<i>hMLH1, MGMT, BRCA1</i>
Hormone response	<i>ER, PR, AR, PRLR, RARβ2</i>
Metastasis/invasion	<i>CDH1, CDH13, TIMP3, Maspin, FAT</i>
Signal transduction	<i>APC, PTEN, RASSF1A, NORE1A, STK11, LKB1</i>
Drug resistance/detoxification	<i>GSTP1, MDR1</i>
Transcription/transcription factors	<i>VHL, HIC-1, GATA4, GATA5</i>
Angiogenesis	<i>THBS-1</i>
Inflammation	<i>COX-2</i>
Differentiation	<i>MYOD1, PAX6, WT1</i>
Involved in other biological processes	<i>CACNA1G, CALCA, FHIT, TERT, TPEF, CSPG2</i>

allele of DNMT1 undergo an increase in loss of heterozygosity (LOH) rate, in consistent with the hypothesis that hypomethylation promotes tumor development in NPcis mice by increasing the rate of LOH [89]. In addition, patients having DNMT3B gene mutated in the germ line are prone to chromosome instability [21]. These results indicate that the tumorigenic effect of DNA hypomethylation might reside in an increase of chromosomal instability induced by the loss of methylation in specific chromosomal areas. Furthermore, loss of imprinting of specific DNA sequences may be attributed to DNA hypomethylation. Loss of imprinting of H19/IGF-2 locus in some childhood tumors is a typical example (reviewed in [90]).

DNA hypomethylation is also responsible for reactivation of transposable elements and silenced viral sequences, via erratic insertion of DNA fragments into transcribed regions or through a siRNA pathway [9, 92]. Moreover, a diet deficient in folate, a vitamin involved in AdoMet metabolism, is associated with DNA hypomethylation and hepatocarcinogenesis in murine (reviewed in [93]).

It has been hypothesized that DNA hypomethylation might also lead to re-activation of silenced oncogenes, through promoter demethylation. However, there is no supporting data available yet. Nevertheless, we cannot exclude the possibility that the promoter demethylation and the de-silencing of certain tissue specific genes at wrong place and wrong time might induce an oncogenic effect.

It is still a debate for the role of aberrant DNA hypomethylation in cancer formation. On the other hand, the role of aberrant DNA hypermethylation in tumor formation and progression has already been well established. Aberrant DNA hypermethylation can contribute tumorigenesis by different mechanisms. One way is to induce mutational hotspots. Methylation of cytosine in CpG dinucleotide favorites spontaneous deamination of cytosine that leads to C to T point mutations in transcripts as well as in promoter regions [94]. Furthermore, a less efficient mismatch repair of T/G can increase the rate of C to T point mutations [95].

Aberrant DNA methylation of regulatory CpG islands is frequently associated with gene silencing. Loss of expression of important tumor suppressor genes in cancer cells is generally attributed to genetic lesions such as mutations and deletions as well as aberrant methylation in promoter or regulatory regions (Tab. 1).

Homozygous methylation of promoter CpG islands is the prevailing mechanism of gene silencing in the majority of genes inactivated by epigenetic mechanisms. However, as shown by Zardo *et al* [96], epigenetic and genetic mechanisms may converge on specific sites and contribute to gene silencing. The aberrant DNA hypermethylation in cancer cell shows fascinating and peculiar features that

might be exploited for diagnostic, prognostic, and therapeutic purposes. For example, not all the genes are prone to DNA hypermethylation in a similar way. Some genes seem to undergo aberrant DNA hypermethylation more easily and frequently than others in the same type of tumor. Other genes undergo aberrant DNA hypermethylation in a tumor type-dependent manner. Costello *et al* [97] by using genome wide approaches have demonstrated that aberrant DNA methylation patterns are tumor type specific rather than random. The specific patterns of aberrant DNA hypermethylation (CIMP) could be used as indicators to identify and characterize different histological subtypes of the same tumor and to predict the chemo-sensitivity of cancer and the duration of patient survival.

TARGETING HETEROCHROMATIC GENE SILENCING AS NOVEL THERAPEUTICAL APPROACH FOR NEOPLASIA

Functional deregulation of HATs, HDACs, HMTs, DNMTs or MBDs results in abnormal transcriptional regulation of target genes that are relevant to the transformation process [98, 99]. Heritable changes in gene expression due to epigenetic modifications of chromatin by HATs, HDACs, DNMTs offer a mechanism by which upstream signaling pathways can converge to common targets associated with neoplasia. In contrast to genetic cancer causes, all these aberrant epigenetic changes can be reversed by drugs targeting chromatin regulators in cells. This highlights chromatin regulators as novel targets for the development of anti-neoplastic drugs, which might induce lasting remissions and spare the short and long term toxicity associated with high dose chemotherapy. A very well known example of the efficacy of transcriptional/differentiation therapy using chromatin remodeling agents is the acute promyelocytic leukemia (APL). APL is an acute myeloid leukemia (AML) subtype, genetically characterized by chromosomal translocations involving the retinoic acid (RA) receptor α (RAR α) and generating RAR α fusion genes and proteins (PML/RAR α , PLZF/RAR α , NPM/RAR α , NuMA/RAR α , Statb5/RAR α etc). RAR α is one of the receptors that, by regulating the expression of specific genes, mediate the effect of RA (a natural derivative of Vitamin A) on development as well as on proliferation and differentiation in a number of cell systems including hematopoietic precursors [100, 101]. Biological effects of RA are mediated by two classes of RA nuclear receptors, RAR α , β and γ and retinoid X receptors (RXR α , β and γ) [100, 102]. These receptors are members of the nuclear receptor superfamily, which includes receptors for: i) steroid hormones (e.g. estrogen and glucocorticoids); ii) non steroidal ligands (e.g. thyroid hormones), iii) diverse products of lipid metabolism, (e.g. fatty acids and prostaglan-

dins) and a large number of so-called orphan receptors for which regulatory ligands have not been identified [100, 102]. Nuclear receptors act as ligand-inducible transcription factors that exert their effects by binding to specific response elements present in the promoters of their target genes. Heterodimerization with RXRs and interaction with a number of proteins that appear to serve co-activator or co-repressor functions, are essential for effective ligand-dependent transactivation of specific RA target genes by RARs [103]. In the absence of ligand, the RAR-RXR heterodimer is tightly bound to nuclear corepressors N-CoR and SMRT that associate with adapter proteins like Sin3 and HDACs to form a transcriptional repressor complex at specific RA response elements (RARE) on RA target genes, which also include the RAR α and RAR β themselves [100]. Deacetylation of nucleosomal core histones at these sites, induces changes of the chromatin structure (local chromatin condensation), rendering the DNA inaccessible to the RNA polymerase II transcription complex resulting in transcriptional repression. At physiological ligand concentrations (1-10 nM), RA binding releases the nuclear co-repressors and HDACs from the RAR-RXR complex, whereas transcriptional adapters and coactivators with intrinsic HAT activities (such as p300/CBP, ACTR and TIF2) are recruited to the RA response element. Hyperacetylation of histones induces structural changes in the chromatin that renders DNA accessible to the transcriptional machinery and activates the transcription of RA-target genes [69, 103, 104]. Transcriptional silencing of the RA signaling pathway is the molecular event underlying the differentiation block at the promyelocytic stage and leukemogenesis in APL [105]. The APL associated RAR α -fusion proteins exist *in vivo* within oligomeric complexes [106, 107]. This causes an aberrant recruitment of both HDAC and DNMT activities on RA-target genes [39, 105, 108-112]. Paradoxically, APL also represents the most striking clinical success of a RA-based differentiation therapy in human neoplasia [105, 113]. Indeed, pharmacological doses of RA can release the HDAC repressor complex and recruit the multisubunit HAT activation complex resulting in terminal differentiation of PML/RAR α -positive APL blasts, which account for more than 90% of APLs. Treatment with HDAC and DNMTs inhibitors is able to restore RA-effect on differentiation in RA-resistant APL blasts *in vitro* and *in vivo* [39, 111, 112]. Therefore, APL has become the molecular paradigm for therapeutic approaches utilizing chromatin remodeling agents [105, 113]. Recent findings indicate that treatment with RA reprograms APL blasts to a non-leukemic phenotype also by inducing an early and coordinated decrease of DNMT1, DNMT3A and DNMT3B expression/activity in RA-responsive APL cell lines and primary blasts from

APL patients [112]. This correlates with de-methylation of the promoter/exon-1 regions on RA target gene RAR β 2 in RA-responsive APL blasts cultured *in vitro*, and in blood samples from APL patients undergoing treatment with RA and chemotherapy. Of note, that: i) DNMT3B mRNA expression, which shows the earliest and largest decrease during normal myeloid differentiation [114], was mainly affected by RA treatment in the RA-responsive APL blasts *in vitro* and *in vivo* [112]; ii) down-regulation of DNMT expression/activity, of their occupancy at RARE sites and exon-1 on RAR β 2 gene and of the methylation status of the RAR β 2 promoter/exon-1 are not measurable in the RA-resistant NB4-MR4 cells following RA treatment [112]; iii) nanomolar concentrations of a selective RAR α agonist also modulated DNMT expression, and its effect is fully abolished by a RAR α selective antagonist [112]. Together, these results suggest a direct involvement of RA binding to either a functional PML-RAR α or endogenous wild type RAR α in the down-regulation of DNMT expression in APL. A decreased expression of DNMT1, DNMT3A and DNMT3B and granulocytic differentiation is also induced by the RAR α agonist AM580 in HL-60 cells, a myeloid leukemia cell line morphologically and biochemically very similar to the APL blasts lacking the t(15;17) but expressing a functional RAR α [112]. Therefore, down-regulation of DNMT expression might be a more general mechanism of RA action in cells. APL accounts for approximately 10% of AMLs, and all the other AML subtypes are not sensitive to retinoids [115]. However, an aberrant recruitment of the HDAC complexes seems not to be restricted to APL but to be present also in other AMLs. The HAT-interacting domain of the hematopoietic transcription factor AML1 is lost and replaced by the NCoR/SMRT interacting region of ETO as a result of the t(8;21) chromosomal translocation associated with AML-M2. Interestingly, the resulting AML1-ETO fusion protein also forms oligomeric structures that lead to transcriptional repression of AML1 on RA signaling pathways and block of myeloid differentiation [107, 116]. Furthermore, RA and nuclear hormone receptors transcriptional coregulators with putative HAT activities (such as p300, CBP, MOZ and TIF2), are present in chromosomal rearrangements associated with some AML cases [117-121]. Moreover, a structure function analysis of the MLL-CPB product of the t(11;16) translocation demonstrated that fusion of both the bromodomain and HAT domain of CBP to the amino portion of MLL induces full transformation and the leukemic phenotype *in vivo* [122]. All these findings indicate that common pathways of leukemogenesis could lie along the control of specific chromatin structures in terms of transcriptional activation or repression. Moreover, by using the DNMT and HDAC inhibitors in the presence or in the absence of RA we found

that all these agents are active in potentiating the effect of RA on RA-responsive promoter activities, endogenous RA target genes and myeloid differentiation in either RA-responsive or RA-resistant AML blasts *in vitro* ([112, 123, 124] and our unpublished observations). It appears, therefore, that in AMLs different genetic alterations resulting in common patterns of deregulated gene expression may lead to differentiation block and myeloid leukemogenesis. These evidences suggest a scenario where the accessibility at specific DNA-binding sites might represent the key event for generating a chromatin code coupled to specific differentiation decision in leukemic blasts. Targeting of specific chromatin remodeling activities may represent a therapeutic strategy potentially applicable also to RA-resistant APL patients and to AML in which an aberrant transcriptional repression underlies gene silencing, maturation arrest and leukemogenesis.

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