Gene Silencing and DNA Methylation

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ABSTRACT

This review revealed a new mechanism for gene regulation through "gene silencing" at the transcriptional level (TGS) or at the post-transcriptional level (PTGS), which play a key role in many essential cellular processes. Today dsRNA is used as a powerful tool to experimentally elucidate the function of essentially any gene in a cell. The immense impact of the discovery of RNA interference (RNAi) on biomedical research and its novel medical applications in the future are reviewed in this article, with particular stress on therapeutic applications of radio-labeled antisense oligonucleotides (RASONs) for diagnosis and treatment of various cancers and neurodegenerative diseases by "gene silencing". Antisense oligonucleotides (ASONs) can also modulate alternative splicing which 74% of all human genes undergo. Epigenetic changes affect chromatin structure and thus regulate processes such as transcription, X-chromosome inactivation, allele-specific expression of imprinted genes, and inactivation of tumor suppressor genes. Treatment with inhibitors of DNA methylation and histone deacetylation can reactivate epigenetically silenced genes and has been shown to restore normal gene function. In cancer cells, this results in expression of tumor suppressor genes and other regulatory functions, inducing growth arrest and apoptosis.

Keywords: RNAi, X-chromosome RASONs, DNA methylation, Gene silencing.

INTRODUCTION

Gene silencing is a general term describing epigenetic processes of gene regulation. The term gene silencing is generally used to describe the "switching off" of a gene by a mechanism other than genetic[24] modification. That is, a gene which would be expressed (turned on) under normal circumstances is switched off by normal circumstances is switched off by machinery[1] in the cell. Genes are regulated at either the transcriptional or post-transcriptional level. (See fig. 1.)

Transcriptional gene silencing is the result of histone modifications, creating an environment of heterochromatin[26] around a gene that makes it inaccessible to transcriptional machinery (RNA polymerase, transcription factors, etc.).

Post-transcriptional gene silencing is the result of mRNA of a particular gene being destroyed. The destruction of the mRNA prevents translation to form an active gene product (in most cases, a protein). A common mechanism of post-transcriptional[28] gene silencing is RNAi.

Both transcriptional and post-transcriptional gene silencing are used to regulate endogenous genes. Mechanisms of gene silencing also protect[3] the organism's genome from transposons and viruses. Gene silencing thus may be part of an ancient immune system protecting from such infectious DNA elements.

RNA interference

The enzyme dicer trims double stranded RNA, to form small interfering RNA or micro RNA. These processed RNAs are incorporated into the RNA-induced silencing complex (RISC), which targets messenger RNA to prevent translation.

RNA interference (RNAi) is a mechanism that inhibits gene expression by causing the degradation of specific RNA molecules or hindering the transcription of specific genes. The targets are often RNA from viruses and transposons[14] (probably a form of innate immune response), although it also plays a role in regulating[16] development and genome maintenance. Key to the RNAi processes are small interfering RNA strands (siRNA), which have complementary nucleotide sequences to a targeted RNA strand. The siRNA "guides" proteins[5] within the RNAi pathway to the targeted messenger RNA (mRNA) and "cleaves" them, breaking them down into smaller portions that can no longer be translated[24] into protein. A type of RNA transcribed from the genome itself, miRNA, and works in the same way.

The selective and robust effect of RNAi on gene expression makes it a valuable research tool, both in cell culture and in living organisms because synthetic dsRNA

introduced into cells can induce suppression of specific genes of interest. RNAi may also be used for large-scale screens that systematically shut down each gene in the cell, which can help identify the components necessary for a particular cellular process[7] or an event such as cell division. Exploitation of the pathway is also a promising tool in biotechnology[6] and medicine.

DNA methylation

DNA methylation is a biochemical process that is important for normal development in higher organisms. It involves the addition of through cell a methyl group purine rin to the 5 position of the cytosine pyrimidine ring or the number 6 nitrogen of the adenineg (cytosine and adenine are two of the four bases of DNA). This modification can be inherited division.

DNA methylation is a crucial part of normal organismal development[2] and cellular differentiation in higher organisms. DNA methylation stably alters the gene expression pattern in cells such that cells can "remember where they have been" or decrease gene expression; for example, cells programmed to be pancreatic islets during embryonic development remain pancreatic islets throughout the life of the organism without continuing signals telling them that they need to remain islets. DNA methylation is typically removed during zygote formation and re-established through successive cell divisions during development. However, the latest research shows that hydroxylation of methyl group occurs rather than complete removal of methyl groups in zygote. Some methylation modifications that regulate gene expression are inheritable and are referred to as epigenetic regulation.

In addition, DNA methylation suppresses the expression of viral genes[42] and other deleterious elements that have been incorporated into the genome of the host over time. DNA methylation also forms the basis

of chromatin structure, which enables cells to form the myriad characteristics necessary for multi cellular life from a single immutable sequence of DNA. Methylation also plays a crucial role in the development of nearly all types of cancer.DNA methylation at the 5 position of cytosine has the specific effacing gene expression and has been found in every vertebrate examined. In adult ct of redu somatic tissues, DNA methylation typically occurs in a CpG dinucleotide context; non-CpG methylation[9] is prevalent in embryonic stem cells.

Mechanism of DNA methylation

In mammalian DNA, 5-methylcyto-sine is found in approximately 4% of genomic DNA, primarily at cytosine-guanosine dinucleotides (CpGs). Such CpG sites occur at lower than expected frequencies throughout the human genome but are found more frequently at small stretches of DNA called CpG islands. These islands are typically found in or near promoter regions of genes, where transcription is initiated. In contrast to the bulk of genomic DNA, in which most CpG sites are heavily methylated, CpG islands in germ-line tissue and promoters of normal somatic cells remain un-methylated, allowing gene expression to occur. (See fig. 2.)

DNA methylation helps to maintain transcriptional silence in non expressed or non coding regions of the genome. For example, pericentromeric[11] heterochromatin, which is condensed and transcriptionally inactive, is heavily methylated. Hyper methylation thus ensures this DNA is late-replicating and transcriptionally quiescent, and suppresses the expression of any potentially harmful viral sequences or transposons that may have integrated into such sites containing highly repetitive sequences. By contrast, these sites are generally un-methylated in promoter regions of euchromatin, regardless of the

transcriptional state of the gene. Exceptions to this rule, however, can be found in mammalian cells where these regions are methylated to maintain transcriptional inactivation. Thus, CpG islands in promoters of genes located on the inactivated X chromosome of females are methylated, as are certain imprinted genes in which only the maternal or paternal allele is expressed.

Epigenetic effects such as hypermethylation can also induce inevitable alterations in gene xpression. Methylation of the DNA repair genes *MLH1* and *MGMT* can lead to their inactivation, methylation may affect the transcription of genes in two ways. First, the methylation of DNA itself may physically impede the binding of transcriptional proteins to the gene, and second, and likely more important, methylated DNA may be bound by proteins known as methyl-CpG-binding domain proteins (MBDs). MBD proteins then recruit additional proteins to the locus, such as histone deacetylases and other chromatin remodeling proteins that can modify histones, thereby forming compact, inactive chromatin, termed heterochromatin. This link between DNA methylation and chromatin structure is very important. In particular, loss of methyl-CpG-binding protein 2 (MeCP2) has been implicated in Rett syndrome; and medimethyl-CpG-binding domain protein 2 (MBD2) ates the transcriptional silencing of hypermethylated genes in cancer. Research has suggested that long-term memory storage in humans may be regulated by DNA methylation.

Regulation of DNA methylation

DNA methylation is controlled at several different levels in normal and tumor cells. The addition of methyl groups is carried out by a family of enzymes, DNA methyltransferases (DNMTs). Chromatin structure in the vicinity of gene promoters also affects DNA methylation and

transcriptional activity. These are in turn regulated by various factors such as nucleosome spacing and histone acetylases[13], which affect access to transcriptional factors.

DNA methyltransferases

In mammalian cells, DNA methylation occurs mainly at the C5 position of CpG dinucleotides and is carried out by of enzymatic activities–maintenance methylation and *de novo* methylation. Two general classes.

Maintenance methylation activity is necessary to preserve DNA methylation after every cellular DNA replication cycle. Without the DNA methyltransferase (DNMT), the replication machinery itself would produce daughter strands that are un-methylated and, over time, would lead to passive demethylation. DNMT1 is the proposed maintenance methyltransferase that is responsible for copying DNA methylation[15] patterns to the daughter strands during DNA replication. Mouse models with both copies of DNMT1 deleted are embryonic lethal at approximately day 9, due to the requirement of DNMT1 activity for development in mammalian cells.

It is thought that DNMT3a and DNMT3b are the *de novo* methyltransferases that set up DNA methylation patterns early in development. DNMT3L is a protein that is homologous to the other DNMT3s but has no catalytic activity. Instead, DNMT3L assists the *de novo* methyltransferases by increasing their ability to bind to DNA and stimulating their activity. Finally, DNMT2 (TRDMT1) has been identified as a DNA methyl-transferase homolog, containing all 10 sequence motifs common to all DNA methyltransferases; however, DNMT2 (TRDMT1) does not methylate DNA but instead methylates cytosine-38 in the anticodon loop of aspartic acid transfer RNA.

Histone deacetylation

Although methylation controls gene activity, it alone is insufficient to repress transcription. The local chromatin structure also contributes in determining whether genes are transcribed or repressed. For example, DNMTs may regulate gene silencing accompanying promoter DNA methylation by recruiting histone deacetylases (HDACs) and other chromatin-binding proteins to promoter sites to maintain histone deacetylation.

The state of histone acetylation is important in regulating chromatin[17] structure and gene transcription. The chromatin structure surrounding un-methylated, transcriptionally active genes differs from that of methylated, silenced genes. Both nucleosome structure and histone acetylation affect chromatin structure and thus regulate gene transcription. Nucleosomes are composed of 146 base pairs of DNA wound around complexes of eight histones within the nucleus. In CpG islands that are un-methylated, the nucleosomes are in a more open configuration that allows access of factors that favor transcription When CpG islands are hypermethylated, as in heterochromatin, the nucleosomes become more tightly compacted, inhibiting the access of regulatory proteins that promote transcription. Interestingly, recent studies have determined that methylation of specific residues on the tails of histone H3 are critical for maintaining the appropriate chromatin configuration. Methylation of lysine 9 residues on H3 facilitates transcriptional repression, whereas methylation of lysine 4 on H3 is associated with transcriptionally active euchromatin. (See fig. 3.)

Histone deacetylation inhibitors

Given that HDACs are integral to maintaining transcriptional silence, they can increase gene expression in the absence of hypermethylation[19]. A variety of HDAC inhibitors have been shown to inhibit histone

deacetylation in human tumor cells, leading to accumulation of acetylated histone proteins. In leukemic cells, treatment with HDAC inhibitors results in growth arrest, differentiation, or apoptosis. These drugs hold promise for cancer therapy, especially when used in combination with methylation inhibitors, other differentiating agents, or cytotoxic compounds. HDAC inhibitors undergoing clinical evaluation for treatment of hematopoietic malignancies include the natural HDAC inhibitor trichostatin, hydroxamic acid derivatives (such as suberanilohydroxamic acid), the cyclic tetrapeptide depsipeptide[50], benzamide derivatives like MS-275 and CI-994, and aliphatic acids such as valproic acid and phenylbutyrate.

It should be noted that methylation is dominant to histone deacetylation, so transcription cannot occur without first inhibiting methylation. Initial treatment with azacitidine or decitabine, followed by HDAC inhibitors, can produce additive or synergistic effects for re-expression of transcriptionally silenced genes These preclinical findings, coupled[21] with the above model of chromatin structure and its regulation by both methylation and acetylation, provide the rationale for therapeutic approaches in which the two processes are simultaneously targeted[22].

Gene methylation in cancer

According to the widely accepted 'two-hit' hypothesis of carcinogenesis proposed by Knudson, loss of function of both alleles in a given gene (e.g. a tumor suppressor) is required for malignant transformation. The first hit typically is in the form of a mutation in a critical gene, followed by loss of the wild-type allele through deletion[32] or loss of heterozygosity. In familial cancers this first hit may occur through germ-line mutations, while in noninherited sporadic cancers somatic

mutations are more commonly observed. Subsequent loss of the remaining allele through deletion, point mutation, or loss of heterozygosity can eliminate the remaining functional gene. Inactivation of regulatory genes in this manner can lead to the development of cancer.

DNA methylation is an important regulator of gene transcription and a large body of evidence has demonstrated that aberrant DNA methylation is associated with unscheduled gene silencing, and the genes with high levels of 5-methylcytosine[23] in their promoter region are transcriptionally silent. DNA methylation[49] is essential during embryonic development, and in somatic cells, patterns of DNA methylation are generally transmitted to daughter cells with a high fidelity. Aberrant DNA methylation patterns have been associated with a large number of human malignancies and found in two distinct forms: hypermethylation and hypomethylation compared to normal tissue. Hypermethylation is one of the major epigenetic[25] modifications that repress transcription via promoter region of tumour suppressor genes. Hypermethylation typically occurs at CpG islands in the promoter region and is associated with gene inactivation. Global hypomethylation has also been implicated in the development and progression[27] of cancer through different mechanisms. (See fig. 4.)

Demethylation and gene reactivation

The demethylating agent azacitidine and its deoxy derivative, decitabine, are powerful inhibitors of DNA methylation. Preclinical studies have shown that after cellular uptake and phosphorylation, azacitidine is incorporated into RNA, where it suppresses RNA synthesis and has cytotoxic effects. After conversion to 5-aza-2'-deoxycytidine diphosphate[47] by ribonucleotide reductase and subsequent phosphorylation, the triphosphate form is

incorporated into DNA in place of the natural base cytosine. Because of the substitution of the 5' nitrogen atom in place of the carbon, the DNMTs are trapped[29] on the substituted DNA strand and methylation is inhibited. Thus, in the presence of these analogs, a significant proportion of the DNA becomes hemimethylated[41]. A second round of DNA synthesis in the presence of these drugs results in full double-stranded DNA demethylation while both azacitidine and decitabine inhibit methylation, they do differ in some respects. Because it contains a ribose moiety, azacitidine can be phosphorylated by uridine-cytidine kinase, and when this happens it is subsequently incorporated into RNA. Since decitabine contains a deoxyribose[31] group, it is incorporated only into DNA. While some have suggested that differential incorporation into RNA and DNA may account for differences in methylation and toxicity profiles between these two agents, there are no direct clinical data at present to support this proposition.

Inhibition of Methylation with azacytidine
See figure 5.

Detectors
DNA methylation can be detected by the following assays currently used in scientific research:

- Methylation-Specific PCR (MSP), which is based on a chemical reaction of sodium bisulfite with DNA[39] that converts un-methylated cytosines of CpG dinucleotides to uracil or UpG, followed by traditional PCR. However, methylated cytosines will not be converted in this process, and primers are designed to overlap the CpG site of interest, which allows one to determine methylation status as methylated or un-methylated.
- Whole genome bisulfite sequencing, also known as BS-Seq, which is a high-throughput genome-wide analysis of

DNA methylation. It is based on aforementioned sodium bisulfite conversion of genomic DNA, which is then sequencing on a Next-generation sequencing platform. The sequences obtained are then re-aligned to the reference genome to determine methylation states of CpG dinucleotides based on mismatches resulting from the conversion of unmethylated cytosines into uracil.

- The HELP assay, which is based on restriction enzymes' differential ability to recognize and cleave methylated and unmethylated CpG DNA sites.
- Now rarely-used assay based upon restriction enzymes' differential recogni-tion of methylated and unmet ChIP-on-chip assays, which is based on the ability of commercially prepared antibodies to bind to DNA methylation-associated proteins[33] like MeCP2.
- Restriction landmark genomic scanning, a complicated and ylated CpG sites; the assay is similar in concept to the HELP assay.
- Methylated DNA immunoprecipitation (MeDIP), analogous to chromatin immunoprecipitation, immunoprecipi-tation is used isolate methylated DNA fragments for input into DNA detection methods such as to DNA microarrays (MeDIP-chip) or DNA sequencing (MeDIP-seq).

Future challenges
- The age in which epigenetics becomes an essential component in the clinical management of the clinical management of the oncology patient is getting closer
- Treatment of age macular degeneration
- Prevents brain damage caused by huntingtons disease
- Treatment of alzimers disease and spinal cord injury

- Treatment of developmental abnormalities.

CONCLUSION

The biological significance of DNA methylation in the regulation of gene expression and its role in cancer is increasingly recognized. It is now clear that these epigenetic changes affect chromatin structure and thus regulate processes such as transcription, X-chromosome inactivation, allele-specific expression of imprinted genes, and inactivation of tumor suppressor genes. Treatment with inhibitors of DNA methylation and histone deacetylation can reactivate epigenetically silenced genes and has been shown to restore normal gene function. In cancer cells, this results in expression of tumor suppressor genes and other regulatory functions, inducing growth arrest and apoptosis. Azacitidine and decitabine are clinically active in MDS and other hematologic malignancies and may have therapeutic potential in other malignant disorders. Continued clinical trials with hypomethylating agents and HDAC inhibitors, alone or in combination, may provide further advances in the treatment of hematopoietic malignancies and solid tumors.

Epigenetics is everywhere you can inherit something beyond DNA sequence that's were the real excitement in genetics now.

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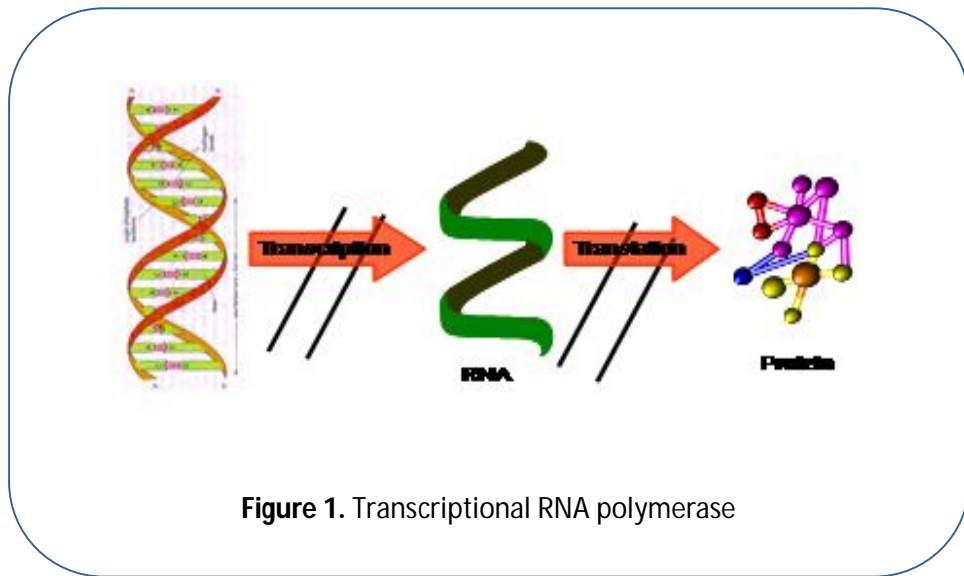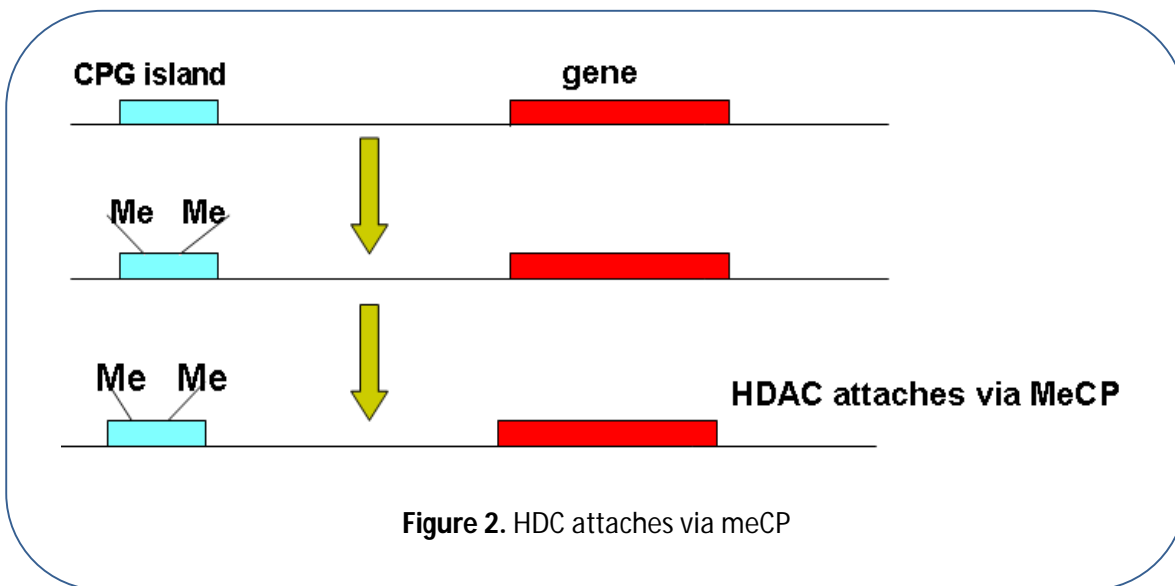
Figure 1. Transcriptional RNA polymerase

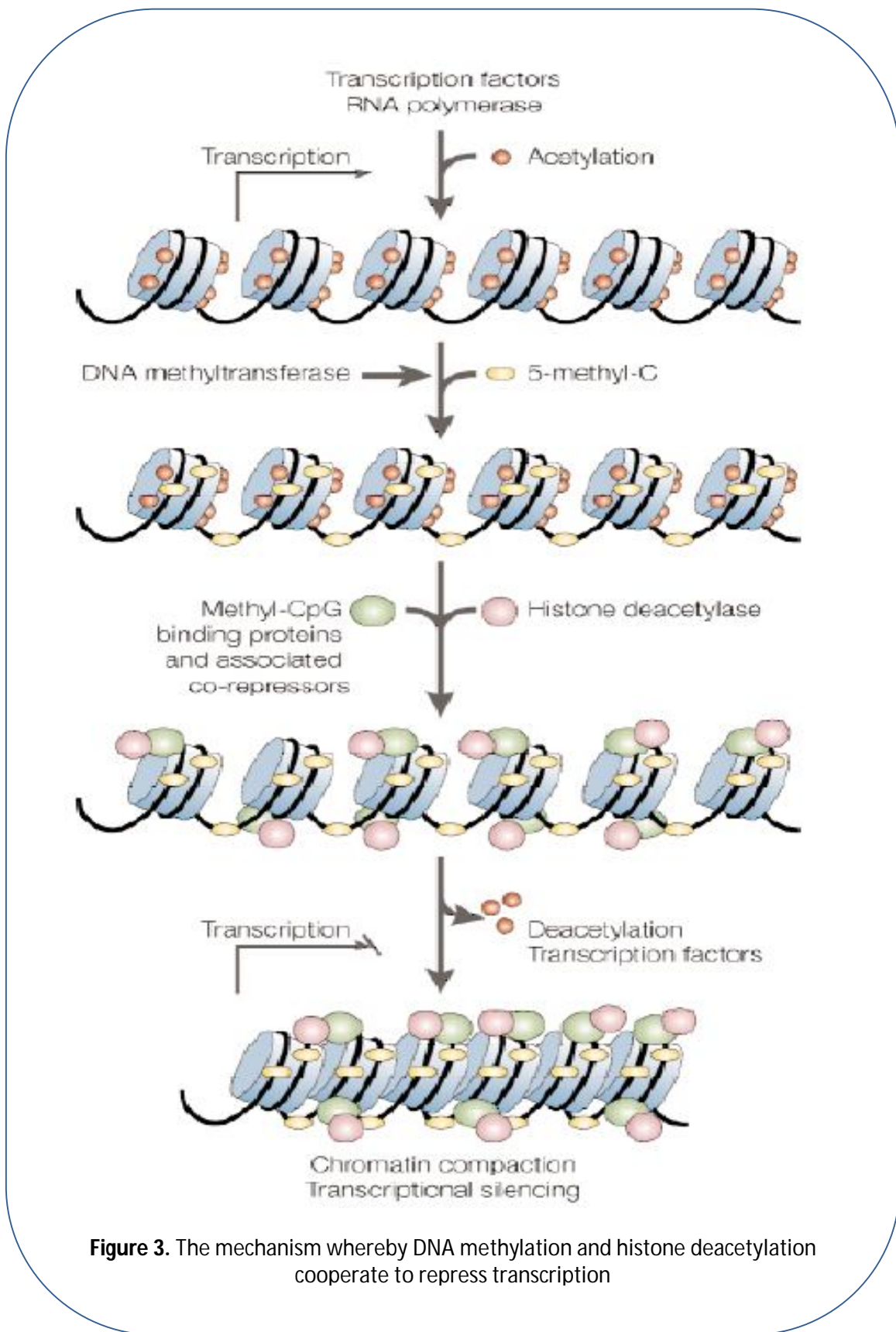**Figure 2.** HDC attaches via meCP

Figure 3. The mechanism whereby DNA methylation and histone deacetylation cooperate to repress transcription
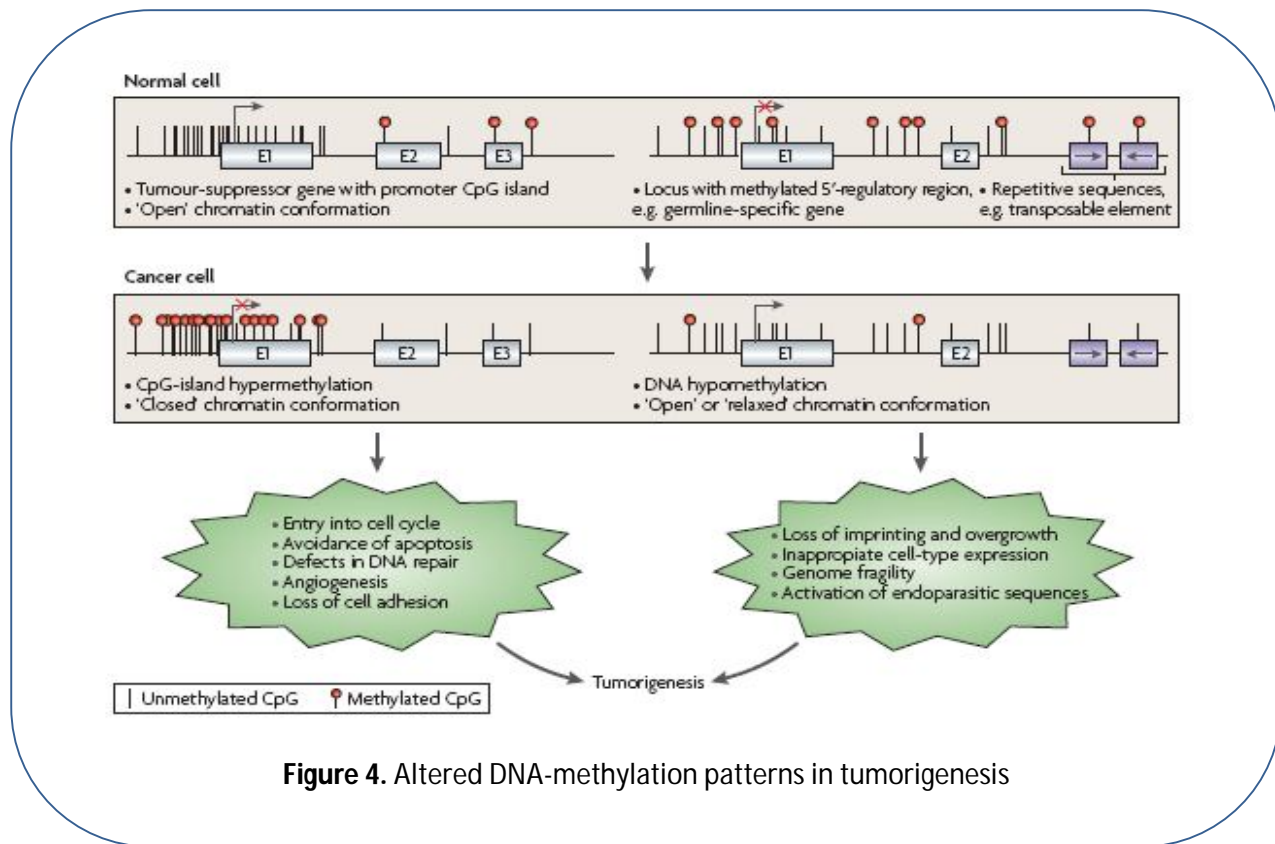
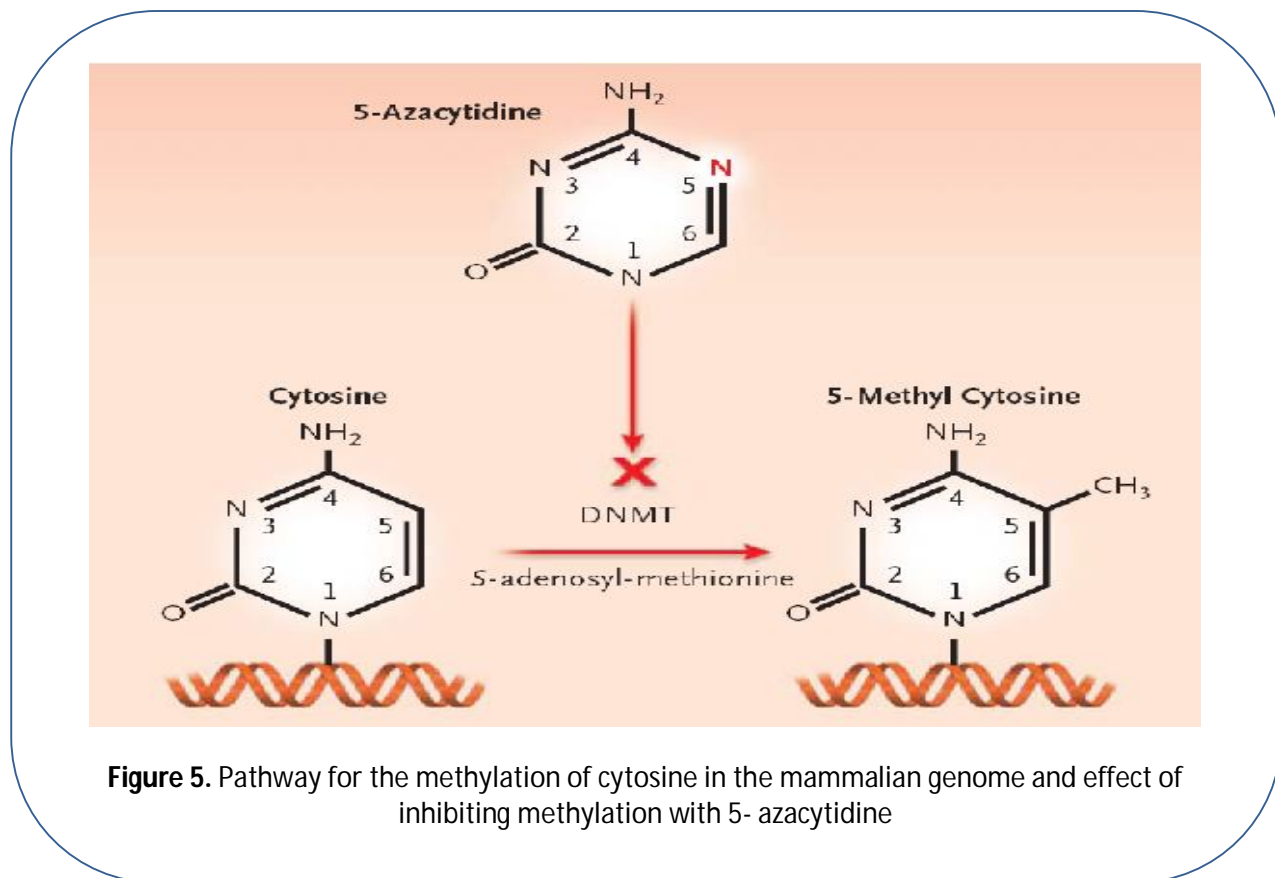**Figure 4.** Altered DNA-methylation patterns in tumorigenesis

Figure 5. Pathway for the methylation of cytosine in the mammalian genome and effect of inhibiting methylation with 5- azacytidine