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DNA Methylation as a Regulatory Mechanism for Gene Expression in Mammals

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A B S T R A C T

Epigenetics refers to the study of heritable changes in gene expression that occur without a change in DNA sequence. In the last decade, it has been shown that epigenetic mechanisms provide an »extra« layer of transcriptional control that regulates genes expression. Three distinct mechanisms appear intricately related in initiating and sustaining epigenetic modifications: RNA-associated silencing, DNA methylation and histone modification. These mechanisms are critical components in the normal development and cell growth. DNA methylation is involved in transcriptional silencing of genes, regulation of expression of imprinted genes, a number of tumour suppressor genes in cancer and silencing of genes located on the inactive X chromosome. In this review, we are focused on the basic principles of DNA methylation as the main epigenetic mechanism for normal embryonic development and epigenetic alterations that contribute to carcinogenesis.

***Key words**: DNA methylation, genomic imprinting, cancer*

Introduction

Genomic DNA represents the base of cellular genetic information contained in a sequence of four nitrogen bases: adenine, guanine, thymine and cytosine[1]. The sequence of these four bases determines the primary structure of DNA, but it still does not mean that the existing sequence is really going to be transcribed and translated, i.e. the sequence would undergo expression[2]. Despite the fact that every single cell of our body contains the identical set of chromosomes, there are big differences among these cells both in their shape and in their functions. So, the cells of a multicellular organism are genetically homogeneous, but structurally and functionally they are heterogeneous due to differential gene expression[3]. It implies that these cells activate only those genes which are indispensable for their functioning and survival, while the rest of genes remain inactive[4]. Such a pattern of differential gene activation has been established very early during the period of embryonic development, and further on it is stably inherited from one cell generation to the next[5]. It is clear therefore, that there must be an additional level of control over the observed genetic activity, totally independent from the primary DNA structure[6]. Such variability of gene expression which is not the consequence of a change in DNA sequence is the topic of interest for epigenetics, the term used for the first time in the fifties by Conrad Waddington[7,8]. Epigenetic inheritance may be described as the transmission of information from a cell or multicellular organism to its descendants without that information being encoded in the nucleotide sequence of the gene. All of our cells contain the same number of genes; however, in a given tissue and at a given state, owing to an epigenetic code, only some of these genes are expressed, giving rise to the phenotype. Three systems, including DNA methylation, histone modification and RNA-mediated action, are considered today of having roles in regulation of gene expresion[9]. Upsetting any single factor of this interactive system could bring about inadequate gene expression and consequently present epigenetic diseases[10].

Here we are going to concentrate primarily on DNA methylation as one among the essential mechanisms for regulation of gene expression in mammals, and the other stakeholders in this story will be mentioned only for sake of better understanding of the mechanisms of action of methylation on gene expression.

The Process of DNA Methylation

Methylation process is one among the key mechanisms for modification of DNA molecule, and hence the epigenetic control of gene expression in vertebrates11. Such a regulatory mechanism allows a cell to stop transcription, ensures inactivity of the majority of genes of one X chromosome of the female organism, and enables the process of genomic imprinting, as well as protection of the endogenous genome from eventual intrusion of a parasitic genome[12,13]. The very mechanism of methylation refers to the binding of a methyl group to the 5th carbon atom of the cytosine ring, and is carried out with the help of the enzyme DNA methyltransferase (Dnmt). The result is the formation of a new base, 5-methylcytosine (5mC)[14,15]. Addition of the methyl group is a process which takes place immediately after replication, and is completed within 1 minute after completion of replication[16,17]. The result of such modification changes the affinity for particular transcription factors towards DNA molecule which prevents the formation of the transcriptional initiation complex, or elongation of those already initiated, that is gene silencing[18].

DNA methylation typically occurs in a CpG dinucleotide regions of DNA[19]. Assuming random distribution of nucleotides the probability of a cytosine and guanine lying next to each other is very high. However, there are actually very few CpG sites in eukaryotic genomes[20]. This is due to the action of DNA methyltransferase, which recognizes these CpG sites and methylates the cytosine, turning it into 5-methylcytosine. Following spontaneous deamination, the 5-methylcytosine converts into thymine. If this has no effect (as in most cases), the error is not recognized by the repair machinery, thus resulting in the loss of the CpG site. CpG sites thus tend to be eliminated from the genomes of eukaryotes[21].

In mammals there are so far discovered 5 members of the family of DNA (cytosin-5) methyltransferases, enzymes involved in the methylation of CpG sequences: Dnmt 1, Dnmt 2, Dnmt3a, Dnmt 3b and Dnmt3L[22,23]. Dnmt1 is the proposed maintenance methyltransferase that is responsible for copying of the already established methylation pattern by recognizing the hemimethylated sites in DNA helix, and it is present always in replication forks of the cells undergoing division[24,25]. Inactivation of this enzyme in mice brings about the global loss of methylation and abnormal biallelic expression of imprinted genes[26,27]. In contrast to Dnmt1, the biological activity of Dnmt2 does not demonstrate affinity towards CpG sequences, and knock-out mice for this gene do not show recognizable abnormalities[28]. It is assumed that this enzyme plays some role in DNA methylation, but it appears not to have any DNA methyltransferase activity[29]. Dnmt3a and Dnmt3b participate in processes of *de novo* methylation that sets up DNA methylation patterns early in development.. Kaneda et collaborators reported the disruption of Dnmt3a and Dnmt3b in germ cells, with their preservation in somatic cells, by conditional knockout technology. Offspring from Dnmt3a conditional mutant females died in utero and lacked methylation

and allele-specific expression at all maternally imprinted loci examined. Dnmt3a conditional mutant males showed impaired spermatogenesis and lacked methylation at 2 of 3 paternally imprinted loci examined in spermatogonia. By contrast, Dnmt3b conditional mutants and their offspring showed no apparent phenotype. The phenotype of Dnmt3a conditional mutants is indistinguishable from that of Dnmt3L knockout mice, except for the discrepancy in methylation at 1 locus (IG-DMR). The conclusion was drawn that both Dnmt3a and Dnmt3L are required for methylation of most imprinted loci in germ cells, but other factors are probably involved[30].

Beside these randomly distributed CpG sites there are regions of the genome which contain extremely high concentration of CpG sites. The concentration of these dinucleotides is more than ten times higher than in the rest of the genome[31]. These regions, known as CpG islands, are found at the promoters of 50–60% of all human genes[32,33]. Surprisingly, these CpG sites are unmethylated, and therefore any spontaneous deaminations of cytosine to uracil are recognized by the repair machinery and the CpG site is restored.

The question is being raised on how does the DNA methylation influence gene expression, i.e. how is the gene repression acquired by the mechanism of DNA methylation? It is considered that the repression proceeds in two ways: (1) directly – the methylation of CpG sequences changes the recognition site for a particular transcriptional factor, so that it does not recognise it any more and does not bind to it, and (2) indirectly – by binding of specific proteins which have affinity for methylated CpG sequences[34]. These are the proteins which bind to methylated CpG groups (methyl-CpG binding proteins, MeCPs), via a domain responsible for binding to CpG sequences (methyl-CpG binding domain, MBD), and are consequently called MBD1, MBD2, MBD3, MeCP1 and MeCP2[35,36]. MeCP2 is a transcription factor that recognizes and binds to a symmetrically methylated CpG dinucleotide[37]. It is also a component of the histone deacetylase complex[38]. MBD1 is included in histone deacetylation, too, while MBD2 is a part of MeCP1 protein complex which mediates the methylation-dependent repression of transcription[39,40]. The indirect repression of transcription which includes the mentioned proteins is undoubtedly connected with the degree of histone acetylation[41]. Every histone contains a domain which is responsible for mutual histone interactions and winding of DNA around them, and an aminoterminal end which protrudes out of the nucleosome, by which histones communicate with other regulatory proteins[42]. The aminoterminal domain is rich in lysine, which are the most frequent targets for acetylation, which in turn markedly decrease the affinity of histone H4 for DNA[43]. It is being considered that the histone acetylation is the molecular mechanism by which DNA becomes generally available for trans-regulatory factors, and at the same time retaining further the nucleosomal architecture[44]. DNA methylation together with such chromatin organisation contributes to repression of transcription by stimulating

binding of MeCP2 and the recruiting complex of histone deacetylation[45,46]. This suggests that specific behaviour of chromatin containing methylated DNA region forms molecular key-lock which might permanently silence down the transcriptional process[47]. Capacity of DNA methylation to increase the repression of transcription by reorganizing chromatin, largely contributes to genome division into hetero- and euchromatin in differentiated cells[48]. Covalent modifications of nucleosomal histones, like acetylation, methylation, phosphorylation and ubiquitinalization, comprise unique and sufficient gene configuration, promoting the idea which subordinates the genetic code to superior epigenetic regulatory system known as the »histone code«[49,50]. »Histone code« is represented by histone modifications like methylation of lysine 9 in H3 histone (H3mK9) and absence of acetylation of histones H3 and H4 in heterochromatin, and the other way around methylation of lysine 4 in histone H3 (H3mK4) and acetylation of histones H3 and H4 in euchromatin[51,52].

DNA Methylation and Embryonic Development

Embryonic development in mammals demonstrates the bimodal reprogramming of DNA methylation, which takes place in primordial germ cells (PGC), where the loss of methylation is connected with the creation of new methylation pattern specific for male or female gamete. After fertilization and during the preimplantation period the loss of methylation enables establishment of the totipotency of zygote[53]. Upon entrance into germinal ridge, particularly methylated PGC, undergo rapid demethylation followed by *de novo* methylation, and so the mature gametes display a high degree of methylation[54,55].

Surprisingly, recent data showed the ability of an environmental factor (for example, endocrine disruptor) to reprogram the germ line and to promote even a transgenerational disease. Namely, transient exposure of a gestating female rat during the period of gonadal sex determination to the endocrine disruptors induced an adult phenotype in the F1 generation of decreased spermatogenic capacity and increased incidence of male infertility. These effects are transferred through the male germ line to nearly all males of subsequent generations up to F4. The effects on reproduction correlate with altered DNA methylation patterns in the germ line[56].

Not only environmental factors, but even nutritional intervention is connected to altered DNA methylation patterns with transgenerational effects[57]. The finding is remarkable because it suggests that a pregnant mother's diet can affect her health in such a way that not only her children but her grandchildren and possibly great-grandchildren inherit the same health problems.

In another study in northern Sweden grandparent's prepubertal access to food was correlated with diabetes and heart disease. If food was not readily available during the father's slow growth period, then cardiovascular disease mortality of the proband was low. Diabetes mortality increased if the paternal grandfather was exposed to a surfeit of food during his slow growth period[58].

Nevertheless, there are still strong evidences that after fertilization the loss in DNA methylation takes place in both pronuclei. This methylation decline is stronger in paternal DNA which becomes demethylated several hours after fertilization, so it is considered to be the consequence of active demethylation, despite the fact that enzymes responsible for this active demethylation are not identified up to now[59]. Parallel with this process, the exchange of protamines with histones is taking place in paternal pronucleus[60]. Maternal pronucleus also undergoes demethylation, but it is more gradual and diminishes after each replication cycle for the lack of Dnmt1 responsible for the maintenance of methylation pattern[61]. Such a replication-dependent decline in methylation is called passive demethylation[62]. The second wave of methylation reprogramming takes place during the period between fertilization and appearance of blastocyst[63].

The crucial role of proper DNA methylation pattern changes during development was showed by our work with 5-azacytidine, a demethylating agent which was administered to pregnant rats in different stages of gestation. After application on day 12 and day 13 of pregnancy survival of foetuses was drastically reduced and limb malformations were present[64]. Moreover, the placentas were influenced as well when the methylation pattern was disturbed, in a way that not only the placental structure, but even the expression of different glycoprotein's was disturbed (own unpublished results).

Imprinted Genes and Human Disorders

Despite such a global loss in methylation, certain genomic sequences are excepted from this process in the preimplantation embryo. It is primarily about the imprinted genes whose expression depends on the fact whether they are inherited from mother or from father e.g. they are monoallelically expressed in contrast to most genes where two alleles contribute equally to the production of gene product[65]. The first experiments which have indicated the existence of imprinted genes in the mammalian genome were carried out by McGrath and Solter, by establishing the method for transplantation of nuclei into enucleated mammalian oocytes[66]. These experiments have shown that the prerequisite for the normal embryonic development of mammals is the genetic information contained in gametes of both parents. Gynogenetic embryos obtained by combining two female pronuclei were small and morphologically normal, but without extraembryonic membranes and were spontaneously aborted[67]. On the other hand, androgenetic embryos obtained by combining two male pronuclei developed normal extraembryonic membranes, but the embryonic part was reduced, a phenomenon almost identical to that occurring occasionally in abnormal human pregnancy, when instead of normal embryo one finds formation called mola hydatidosa[68]. Mola is aggregate of

extraembryonic membranes without presence of foetal tissue, and its chromosomal composition is mostly that of the father[69,70].

The mentioned data speak in favour that the expression of each of the parental genomes is different, and it is crucial that they mutually complement each other if we want to have the normal development[71]. The question is raised whether mammalian zygotes for their normal development require the presence of total paternal and maternal genome, or only of particular chromosomes, or even some critical genes. Experiments with mouse embryos which contained rearranged parts of some chromosomes, showed that particular genes or groups of genes are active when inherited from one parent, and inactive when inherited from from parent of opposite sex[72].

The first discovered imprinted genes were insulin-like growth factor 2 receptor (*Igf2r*) gene[73] discovered in the year 1991 and a few months later *Igf2*. At the time DeChiara and collaborators have observed that targeted mutation in this gene results in dwarfed growth in heterozygotes, but only in the case when the paternal gene is involved (and not the maternal gene). The reduced growth in the newborns was identical in heterozygotes with mutant paternal gene and in recessive homozygotes, indicating that only father influences the activity of *Igf2* gene[74]. Discoveries of new imprinted genes followed, particularly after establishing the method for restriction landmark genome scanning (RLGS) which was based on the fact that the „imprinting« is connected with methylation of CpG dinucleotides identifiable using this method[75].

The imprinted genes display certain, for themselves specific rules like the fact that so far identified genes are not randomly dispersed in the genome as singular genes, but display tendency for grouping[76]. One among the largest groups is located on the distal end of the mouse chromosome 7 as well as on the proximal end of the human chromosome 11[77]. In the majority of such groups one finds interwoven maternally and paternally imprinted genes[78]. To date over 70 human imprinted genes have been identified of a total of 100–200 expected in the whole genome. These imprinted domains are regulated co-ordinately, via long-range mechanisms such as anitsense RNA interference and methylation-sensitive boundary elements. The largest group of imprinted genes is located on the X chromosome[79,80]. It is known that in the female mammals, the dose compensation for genes on X chromosome is acquired by inactivation of one X chromosome in all somatic cells[81]. Although this inactivation is random, it appears that in the extraembryonic tissues the paternal X is turned off more frequently, with the only exception of *Xist* gene, which represent the inactivation centre and is the only active gene on the inactive chromosome, and *vice versa* the only inactive one on the active chromosome[82,83]. In any case inactivation of X chromosome as well as genomic imprinting of autosomal genes generates functional hemizygotes[84].

It seems that reason for grouping of imprinted genes in the genome is that the control of their expression is executed from the single cis-regulatory sites called imprinting control regions (ICRs) or differentially methylated domains (DMDs). Their distinctive characteristic is that they are always methylated on one of the two parental alleles[85,86]. The deletion of these sites eliminates imprinting and consequently both alleles are expressed[87]. Particularly interesting and well studied is the ICR between H19 and Igf2 genes on the human chromosome 11, methylation of which may be responsible either for activation or inactivation of the above genes depending on the choice of whether it is methylated on the maternal or paternal chromosome[88]. The behaviour of these two genes illustrates how differently they may react depending on the state of methylation of the control region which is located between them[89]. This region is methylated on the paternal chromosome and consequently *H19* is inactive, while *Igf2* is expressed and active[90]. On the contrary, on the maternal chromosome the same region is not methylated, so now *H19* becomes active, and *Igf2* inactive[91]. In this case the control region behaves as an insulator. The insulators are regulatory elements which prevent the activation or inactivation of genes, and can be found between promoters and enhancers, or even on the border between transcriptionally active euchromatine and inactive heterochromatine[92,93]. When the ICR is unmethylated, it is the binding site for CTCF which belongs to the group of transcriptional regulatory proteins with the Zink finger motif, by which it prevents activation action of enhancer upon the promoter of *Igf2* gene[94]. Here we see an unusual way of regulation of gene expression, where methylation indirectly activates the gene by blocking the insulator[95]. Regulation of *H19* expression in much more common because its methylation creates inactive, i.e. imprinted site, so the methylation directly affects the activity of the promoter[96].

The second significant characteristic of imprinted genes is their temporarily uncoordinated (asynchronous) replication in relation to the active alleles on the homologous chromosome[97]. It has been observed that *Igf2* gene and gene for its receptor (*Igf2r*), as well as *H19* and SNRPN are replicating earlier in S-phase of the cell cycle if they are of paternal origin[98]. Even though the time of replication within the cell cycle very frequently correlates with the degree of gene expression, so that active genes are mostly replicated at the beginning of the S-phase, this is not the general rule, inasmuch as *H19* and *Igf2r* normally transcribed from maternal chromosome are replicated earlier on paternal chromosome[99]. In reality, it appears that, within the so far studied imprinted genes, and irrespective of the degree of gene expression, the paternal allele always replicates first, which has been demonstrated by the method of fluorescent *in situ* hybridization (FISH) suitable for visualization of particular genes during S-phase of the cell cycle[100,101].

It is surprising that almost half of the so far discovered imprinted genes does not code for proteins but for untraslated RNA. So the RNA molecule of the *H19* gene expressed only from mother is indispensable during embryonic development and required for imprinting of the

other two genes which are transcribed from paternal chromosome[102]. Imamura and collaborators have proved that RNA which is not translated may induce demethylation of the tissue specific differentially methylated domain, which might serve as a potential new tool in epigenetic manipulation of mammalian cells[103].

Importance of methylation and consequently of genomic imprinting is unquestionable for normal functioning of an organism, which is documented by numerous diseases associated with inadequate methylation or with mutation of enzymes responsible for methylation and by them mediated repression of transcription[104]. Classical examples associated with the phenomenon of genomic imprinting are two rare diseases which are a mirror image of each other[105]. Both include a disturbance in growth and behaviour, and are called Angelman and Prader-Willi syndromes[106]. They are both caused by the identical chromosomal deletion of chromosome15[107]. When the deletion is inherited from the mother it results in the phenotype of Angelman syndrome, while the same deletion inherited from father gives rise to Prader-Willi syndrome[108,109].

The loss of genomic imprinting of the chromosome 11p15.5 presents the Beckwith-Wiedmann syndrome which is characterized by embryonic tumours, excessive growth of abdominal organs, macroglossia and omphalocoela[110]. Mutation of the gene MeCP2 and enzyme included in methylation-mediated repression of transcription, brings about the neurological disease known as Rett syndrome which affects one out of 10.000 newborn girls, though up to now the effect of sex on development of this disease has not been discovered[111]. It is considered that the mutation of this enzyme brings about disturbance in differentiation of primary neurones[112]. Consequently microcephalia, ataxia, and tonic clonic seizures are developed[113].

Epigenetic reprogramming is considered today as one among the most significant barriers to cloning, because when a differentiated somatic cell nucleus is put into oocyte, its genome-wide epigenetic pattern must be reprogrammed in order to restore totipotency. The difficulties associated with reprogramming in chromatin, histones, and methylation patterns along the entire length of the DNA sequence may explain why so many cloned embryos have so many developmental failures. And even after assisted reproduction there is elevated incidence of diseases associated with imprinting[114,115]. The reason for this may be because these methods, and particularly *in vitro* fertilisation (IVF) and intracytoplasmyc sperm injection (ICSI), include isolation, handling and cultivation of both gametes and embryos in early embryonic phase, when the genome methylation pattern changes dynamically, so the above mentioned cells are found themselves in a particularly vulnerable period during which this pattern might be disturbed[116,117]. The number of the up to now reported cases connected with these issues is too small to enable making any generalized conclusions, but in any case requests further scientific engagement in order to eventually eliminate the problems connected with genomic imprinting and the methods of assisted reproduction[118].

DNA Methylation and Carcinogenesis

During the past several years the interest of many scientists who are engaged in epigenetics has broadened into the field of carcinogenesis, because the status of DNA methylation changes during life, hence affecting the expression of genes associated with development of cancer[119,120]. Epigenetic silencing as the consequence of aberrant methylation of promoter regions, and CpG islands results in the loss of function of tumour suppressor genes[121,122]. Tumour cells frequently show increased activity of DNA methyltransferases, which are responsible for hypermethylation of promoters of these genes[123,124]. But there are no genetic proofs that would indicate the ectopic *de novo* methylation in cancer, neither for DNA methyltransferase genes mutations. Instead it is hypothesized that the cause is somewhere in the transcriptional machinery or in a signal transduction pathway genes[125]. So Butcher et al. consider that inactivation or disruption of these insulators may facilitate an epigenetic »hit«, in this case DNA methylation, leading to down regulation of tumor-supressor gene (e.g. BRCA1) contributing to tumorigenesis[126]. It follows from the above mentioned, that the epigenetic changes, i.e. the silencing of tumor-supressor gene promoters by methylation would trigger carcinogenesis. However, it is known that DNA methyltransferase gene mutation does not change the frequency of tumour appearance[127]. In some cell lines of colorectal carcinoma one mutant allele of *p16* gene has been found, which is therefore non-functional, but expressed, while its homologue was hypermethylated and totally silenced[128]. As the methylation can be detected very precisely with contemporary techniques, and the extent of methylation established in any sort of cells, the connection of methylation with appearance of cancer gives hope for discovery of new potential ways for prevention and treatment of these diseases[129].

Recent evidence indicates that epigenetic changes might 'addict' cancer cells to altered signal-transduction pathways during the early stages of tumour development. Dependence on these pathways for cell proliferation or survival allows them to acquire genetic mutations in the same pathways, providing the cell with selective advantages that promote tumour progression. Strategies to reverse epigenetic gene silencing might therefore be useful in cancer prevention and therapy[130]. Moreover, DNA demethylating agent 5-azacytidine has been currently used in human myelodisplastic disorders therapy[131]. Being aware that this agent has a detrimental effect upon mammalian embryonic development *in vitro*[132] and *in vivo*[133,134] one must be careful while using it on humans because of its teratogenic side effects.

Acknowledgements

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REGULACIJA EKSPRESIJE GENA U SISAVACA

S A Ž E T A K

Nasljedne promjene u ekspresiji gena koje se nasljeđuju neovisno o promjenama slijeda baza u molekuli DNA definiraju pojam epigenetike. Zadnja dekada, pokazala je da epigenetski mehanizmi omogućavaju dodatnu regulaciju ekspresije gena na razini transkripcije. Tri sistema, uključujući metilaciju DNA, modifikaciju histona i RNA-posredovano djelovanje, danas se povezuju s regulacijom ekspresije gena. Oni su kritični regulatori kako embrionalnog razvoja tako i samog rasta stanica. Proces metilacije molekule DNA predstavlja epigenetski mehanizam uključen u inaktivaciju transkripcije gena, genomski imprinting, osigurava neaktivnost većine gena na inaktivnom X kromosomu u stanicama ženskog organizma kao i tumor supresor gena u raku. Ovim pregledom iznjeli smo osnovne principe metilacije DNA molekule kao glavnog epigenetskog mehanizma regulacije genske ekspresije, s težištem na njezinoj ulozi u normalnom embrionalnom razvoju te poremećajima koji dovode do razvoja raka.