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DNA Methylation 40 Years Later: Its Role in Human Health and Disease

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A long path, initiated more than 40 years ago, has led to a deeper understanding of the complexity of gene regulation in eukaryotic genomes. In addition to genetic mechanisms, the imbalance in the epigenetic control of gene expression may profoundly alter the finely tuned machinery leading to gene regulation. Here, we review the impact of the studies on DNA methylation, the ''primadonna'' in the epigenetic scenario, on the understanding of basic phenomena, such as X inactivation and genomic imprinting. The effect of deregulation of DNA methylation on human health, will be also discussed. Finally, an attempt to predict future directions of this rapidly evolving field has been proposed, with the certainty that, fortunately, science is always better than predictions. J. Cell. Physiol. 9999: 1–15, 2004. © 2004 Wiley-Liss, Inc.

DNA METHYLATION: THE EARLY YEARS

Evidence for methylation of nucleic acids dates back to more than 40 years ago (Fleissner and Borek, 1963). These authors found that the methylated bases of tRNA were formed at the polynucleotide level by transfer of the methyl group of S-adenosyl-methionine to the complete polynucleotide chain. The finding of the non-random distribution of 5-methylcytosine in the DNA from several organism also dates back the early days of DNA methylation studies; it was obtained at LIGB by Scarano et al. (Grippo et al., 1968). They also postulated a role for DNA methylation and cellular differentiation; using the "Synchron" model, Prof. Scarano predicted that "epigenetic modifications" such as the methylation of a cytosine, followed by deamination to give a thymine, would inevitably lead to alterations of gene activity and then to differentiation (Scarano, 1973). Later in the seventies, the interest on the role of DNA methylation in biological phenomena became high and widely discussed among world leading scientists (Holliday and Pugh, 1975; Riggs, 1975). The description of CpG island (Bird et al., 1985), its definition (Gardiner-Garden and Frommer, 1987), and its role in modulating gene expression (Toniolo et al., 1988) would have to wait until the end of eighties.

DNA METHYLATION: THE STATE OF THE ART

DNA methylation, the major modification found throughout genomes, occurs in almost all living organisms, from bacteria to plants and fungi, from invertebrates to vertebrates. However, its abundance and role varies quite evidently among these genomes, from the unmethylated genome of *Caenorhabditis elegans* to the heavily methylated vertebrate DNAs. The different patterns in DNA methylation found across species are revealing the different role that this DNA modification has in their genomes. It has been determined that, across eukaryotic species, DNA methylation occurs predominantly at the dinucleotide CpG; genomes of plants and fungi show methylation also at different sites, for example, CpNpG sites. In the mammalian genome, CpGs are normally under-represented in the genome; however, they can be found at a frequency closer to the expectations in specific genomic regions, termed CpG islands, of about 1 kb (Robertson and Wolffe, 2000).

At the evolutionary level, it has been proposed that DNA methylation evolved as a generalized repression system in more complex genomes (Bird, 1995). DNA methylation is thought to be a repressing mechanism against the inappropriate expression of endogenous transposons, that may perturb genome organization and integrity, by disrupting functional genes and/or causing chromosomal rearrangements (Yoder et al., 1997). In mammals, DNA methylation is one of the main players of the long-term gene silencing involved in important biological phenomena, such as X chromosome inactivation and genomic imprinting (Li, 2002). Only recent and

This manuscript is dedicated to the memory of Prof. Eduardo Scarano, who actively worked at LIGB in Naples, until his untimely death in 1986. He was one of the first scientists to hypothesize that DNA methylation would have a profound effect on gene expression. We recognize his scientific legacy.

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limited evidence supports a role for DNA methylation in controlling tissue-specific expression in adult somatic tissue, such as the tissue-specific expression of maspin gene (*SERPINB5*), thus indicating a role for the establishment and/or the maintenance of cell-type restricted gene expression (Futscher et al., 2002).

DNA methylation is accomplished through the activity of specific enzymes, the DNA methyltransferases, which transfer a methyl group to the cytosine of CpG dinucleotides. The better characterized are DNMT3A and DNMT3B which are mainly devoted to the de novo methylation, and DNMT1, with preferential activity for hemi-methylated DNA, which acts mainly as maintenance methyltransferase. Null mutations of the mammalian DNA methyltransferases (DNMTs) are lethal at different stages, either prenatally and postnatally (Li et al., 1992; Okano et al., 1999). ES cells homozygous for Dnmt1 mutation are viable, with no growth or morphological abnormalities (Li et al., 1992). Although these cells exhibit substantial demethylation of endogenous retrovirus, they retain the 30% of genomic methylation. Additional studies on cells with total or partial loss of Dnmt1 function revealed abnormalities in transcriptional silencing of specific imprinted genes and the Xist allele on the X chromosome (see for review Ordway and Curran, 2002).

The de novo methyltransferases, Dnmt3A and 3B, have been identified as expressed sequence tags (EST) in database (Okano et al., 1998) by sequence homology with the methyltransferase catalytic domain of Dnmt1. Li et al. produced both single and double knock out mice for these genes but only the double KO was unable to methylate foreign retroviral DNA (Okano et al., 1999). Mice lacking Dnmt3A die postnatally, whereas those lacking Dnmt3B develop normally until E9.5, then die prior to term. Double mutants already show abnormality at E8.5, then die prior to E11.5. Genomic methylation abnormalities highlight both the overlapping and specific functions of these de novo methyltransferases: however, the level of genomic demethylation is rather unaffected, differently from Dnmt1 null mutants. Specific DNA methyltransferase isoforms, such as the Dnmt1o, oocyte-specific (Howell et al., 2001), or unconventional such as Dnmt3L, whose inactivation affects maternally repressed imprinted genes, but not the paternal ones (Bourc'his et al., 2001) seem to play additional, more specific roles in the physiology of genomic methylation. In addition to methylation, an important role is played by demethylation mechanisms. Passive mechanisms, by which DNMTs may be sterically excluded from a determined region have been shown (Hsieh, 1999). Protection from DNA methylation occurs at CpG islands, where a role seems to be played by promoter activity in the early development, thus "marking" future unmethylated CpG islands. Evidences for active demethylation are, as yet, scarce: the hunt for active demethylases did not yield reproducible results (Bhattacharya et al., 1999). Active demethylation has been reported at least in one case, for the interleukin-2 gene (*Il-2*) during the activation of T lymphocytes (Bruniquel and Schwartz, 2003). Active demethylation of the paternal genome occurs also in the zygote, followed by passive demethylation during the cleavage stages, and de novo methylation which is thought to happen after implantation (Santos et al., 2002).

The role of DNA methylation in non-mammalian species is quite different: it is dispensable for the proper development of the fungus *Neurospora* and, accordingly, mutations in the single DNA methyltransferase, DIM-2, has no obvious phenotype despite genome-wide demethylation. The *Arabidopsis* genome, on the other hand, has at least ten different genes encoding DNA methyltransferases, either for maintenance or de novo methylation of CpG dinucleotides (MET1 and DRM loci, respectively) and for non-CpG methylation (CMT3 locus). Mutations of met loci have a profound effect on the viability of the species, whereas DRM and CMT mutations do not have apparent phenotypes (Tariq and Paszkowski, 2004). Interestingly, in non-mammalian species de novo DNA methylation may be induced by RNA mediated mechanisms, such as RNA directed modification of DNA (RdDM), a mechanism discovered in plants, which leads to de novo methylation of a region of identity between the trigger dsRNA and the target DNA. It may act as a trigger of transcriptional gene silencing (TGS) if the dsRNA is directed against promoter regions (Mette et al., 2000). This may suggest a link between the mechanisms that initially recognize invasive DNA and direct DNA methyltransferases to unmethylated loci in order to initiate gene silencing (Chan et al., 2004).

How can DNA methylation mechanistically repress transcription? The density of methylated cytosines has been reported to be crucial for the efficiency of repression (Boyes and Bird, 1992; Hsieh, 1994). However, other authors suggested that the position of specific cytosines may be critical for repression (Chen et al., 2001a). The repressive signal of DNA methylation is interpreted in two ways (Fig. 1). It can directly interfere with the consensus sequence of trans-acting factors, such as CTCF, a zinc-finger transcription factor (CCCTC-binding factor) that has been implicated in boundaries formation and chromatin insulation in imprinted clusters (Bell and Felsenfeld, 2000).

Alternatively, DNA methylation can act indirectly through transcriptional repressors, such as the so-called methyl binding proteins (MECP2 and MBD 1–3; the fourth MBD protein, MBD4, does not act as a transcriptional repressor), a family of five proteins that share a common motif, the methyl binding domain (Hendrich and Bird, 1998). These proteins seem to convert the DNA methylation signal into the repressive state of chromatin, recruiting large complexes that include histone deacetylases and methyltransferases (Nan et al., 1998; Jones et al., 1998). A suggestive hypothesis proposed that, in order to increase the fidelity of DNA methylation silencing, an increase arose in the number and functional diversity of the methyl binding proteins during

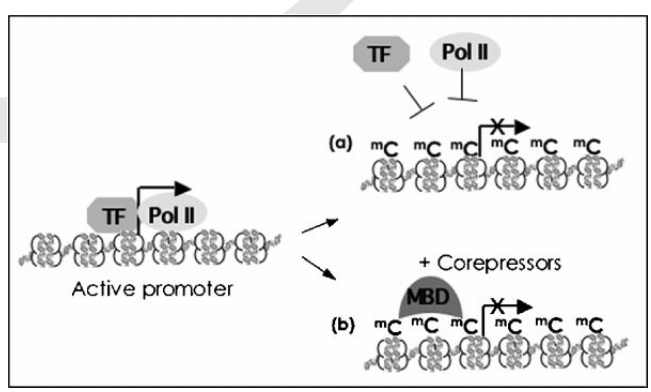

Fig. 1. Direct (**a**) and indirect (**b**) suppression of gene expression by CpG methylation. TF, transcription factor(s); MBD, methyl binding domain protein.

vertebrate evolution (Hendrich and Tweedie, 2003). According to this hypothesis, the putative ancestral *MBD2/3* gene, predicted to be the original methyl CpG binding protein, is encoded by a single gene in invertebrate genomes (Lyko et al., 1999). The first member of the mammalian MBD family to be characterized was MeCP2 (Meehan et al., 1992). MeCP2 is a multidomain protein, containing, besides the MBD, a transcriptional repression domain (TRD) that overlaps a nuclear localization signal (NLS). MeCP2 is able to bind to a single symmetrically methylated CpG dinucleotide, regardless of sequence context. MeCP2 has been shown to repress transcription by at least two different mechanisms. The TRD of MeCP2 interacts with mSin3A, a co-repressor that exists in a complex with histone deacetylases (HDACs) (Nan et al., 1998; Jones et al., 1998). Interactions of MeCP2 with DNA and histone methyltransferases has been reported (Fuks et al., 2003). Moreover, MeCP2 may also be capable of interfering with the transcriptional machinery, as suggested by the interaction of the TRD with TFIIB (Yu et al., 2000). It was recently hypothesized on the basis of MeCP2 biochemical properties that MeCP2 form stable complexes only when bound at DNA, in a template-specific manner, recruiting Sin3A but also a cohort of largely still unknown co-repressors (Klose and Bird, 2004). Thus its interactions may be more complex than thought, as well as its physiological role. MeCP2 interactors, however, are beginning to be unraveled: an example is the *X. laevis* p20 or WAP protein, which stabilize, through direct interactions, both frog and human MeCP2 (Carro et al., 2004). The other MBDs, MBD1–MBD4, have been all discovered as EST clones with sequence similarity to the MBD motif of MeCP2. Like MeCP2, MBD1 is able to repress transcription from methylated promoters; such activity requires both the TRD and MBD motifs and is sensitive to HDACs inhibitors (Fujita et al., 1999). In contrast to MeCP2, no physical association between MBD1 and HDAC proteins has yet been observed. However, MBD1 forms a stable complex with histone H3-K9 methylase SETDB1. A model has been suggested in which H3-K9 methylation by SETDB1 is dependent on MBD1 and is heritably maintained through DNA replication to support the formation of stable heterochromatin at methylated DNA (Sarraf and Stancheva, 2004). MBD2 and MBD3 are more closely related to each other than to the other MBD proteins. MBD2 binds CpG-methylated sequences in vitro and has been found to be a component of the firstly identified methyl binding repressor complex, MeCP1, together with NuRD, a co-repressor complex having both nucleosome remodeling and HDAC activity (Feng and Zhang, 2001). Despite extensive sequence similarity, MBD3, also belonging to the NuRD complex, is functionally very different from MBD2, being the only member of this protein family that lacks the capacity to selectively recognize methylated DNA (Zhang et al., 1999). MBD4 is a DNA glycosylase belonging to a complex that binds methyl-cytosines that spontaneously deaminate to uracyls. It recognizes mismatches CG/TG, suggesting that it acts as repair protein. Another protein, KAISO, structurally unrelated to the MBD proteins, has also recently been shown to be a DNA methylation-dependent transcriptional repressor, belonging to the N-CoR complex in HeLa cells (Prokhortchouk et al., 2001; Yoon et al., 2003).

MeCP2 mutations are responsible for Rett syndrome (RTT), an X-linked dominant neurological disorder affecting mainly heterozygous females (Amir et al., 1999). Genetic dissection of the role of every MBD protein has been performed thanks to the phenotypic analyses of mice lacking different MBD genes (Guy et al., 2001; Hendrich et al., 2001; Chen et al., 2001b; Shahbazian et al., 2002; Zhao et al., 2003). The phenotypic consequences of MeCP2 deletion in mice are strongly similar to the symptoms of Rett syndrome. In contrast to MeCP2, *Mbd1*(−/−) mice have no detectable developmental defects and appear healthy, even if *Mbd1*(−/−) neural stem cells exhibited reduced neuronal differentiation and increased genomic instability. Adult Mbd1−/− mice, however, show decreased neurogenesis, impaired spatial learning, and a reduction of the hyppocampal dentate gyrus long-term potentiation (Zhao et al., 2003). Mutations in MBD2 have subtle effects: these mice have a normal methylation pattern and do not show any defect in genomic imprinting or silencing of endogenous transposable elements. They show, however, broad behavioral defects (Hendrich et al., 2001). Interestingly, deficiency of MBD2 impairs intestinal tumorigenesis, with a direct proportionality between MBD2 dosage and adenoma formations (Sansom et al., 2003). On the contrary, MBD4 deficient mice show a three times higher frequency of C–T transitions and, in a cancer susceptible background, accelerate tumor formation (Millar et al., 2002).

From the above information, it is increasingly clear that DNA methylation is closely linked with various aspects of cellular physiology. In this review, we will discuss the molecular details of the involvement of DNA methylation in the long-term silencing mechanisms occurring in X inactivation and genomic imprinting. Furthermore, we will examine the effect of disturbances on the regulation by DNA methylation, in specific human genetic syndromes and in cancer.

DNA METHYLATION AND ITS ROLE IN X CHROMOSOME INACTIVATION

DNA methylation has long been thought to be an important component of X chromosome inactivation, particularly as a mechanism for stably maintaining the inactive state of X-linked genes and for transmitting it as a "memory" through every cell division. Historically, its role in this process was hypothesized by Riggs [1975] who predicted that protein–DNA interactions underlying the silencing of X chromosome were sensitive to methylated cytosines, and introduced the "maintenance methylase" model to explain the somatic inheritance of methylation patterns. A maintenance methylase was defined as a DNA methyltransferase that preferentially acts on hemimethylated sites (Riggs, 1975). It is now well known that the methylation patterns of the genome are generated and maintained by a combination of de novo and maintenance-type DNA methyltransferases and their targeted disruption clearly revealed the importance of genomic methylation profile in the normal mammalian development (Li et al., 1992; Okano et al., 1999). Some new findings on the role of DNA methylation and of the enzymes involved in this process during the main steps of mammalian X chromosome inactivation (initiation, propagation, and maintenance) will be outlined. Because of the double level of X-linked gene products in females, compared to males, represents an impediment to normal development, female mammals transcriptionally silence one X chromosome in somatic cells. Several strategies have been developed by different species, such as *Drosophila melanogaster* and *C. elegans*, for dosage compensation (Cline and Meyer, 1996). Mammalian X inactivation, in comparison with other mechanisms, is unique in that silencing of one X

chromosome occurs while its homologue in the same nucleus remains genetically active. The initiation of inactivation correlates with cellular differentiation from early pluripotent lineages and occurs randomly with the identical probability of either the paternally or maternally inherited X being inactivated. In some extraembryonic lineages, as well as in marsupials, it is always the paternal X that is inactivated, giving an imprinted form of X chromosome inactivation. The onset of this process during early development is regulated by a region on the X chromosome called X-inactivation center (Xic), a complex locus which establishes how many (counting step) and which (choice step) X chromosomes will be silenced and produces the main player of X inactivation, the *Xist* (*X-i*nactive *s*pecific *t*ranscript) RNA. Results from deletions and transgenesis studies, as well as *Xist* targeting, have widely conferred to the Xic the role of master regulatory locus (Lee et al., 1996; Penny et al., 1996; Clerc and Avner, 1998). Several additional non-coding RNAs have been also recently mapped in Xic (Johnston et al., 2002; Ogawa and Lee, 2003). Among them, the antisense transcript of *Xist*, *Tsix*, plays a crucial role as potential negative regulator of *Xist* in *cis*. At the moment of X inactivation, *Xist* transcription is up-regulated on the chromosome that will become the inactive X, while *Tsix* expression is lost but continues on the future active X. How the *Tsix* expression molecularly interferes with *Xist* RNA is still unknown. Some evidences link *Tsix* also to the "choice step," and the prominent CpG island at 5′ end of this gene has been proposed to play an essential role, considering the occurrence of multiple CTCF binding sites (Bell et al., 1999). The involvement of methylation status of this region remains to be identified (see details in Fig. 2a).

When the decision to inactivate has been made, *Xist* coats the X chromosome in *cis* and triggers its inactivation, recruiting the silencing complexes and inducing a cascade of chromatin changes in order to further lock and stably maintain an inactive state that is initially labile. One X chromosome is thus transformed in facultative (developmentally regulated) heterochromatin, resulting in a compact structure cytologically visible at the nuclear periphery, called the Barr body. Other peculiar marks of the inactive X have been well described and the temporal order of their occurrence has been shown analyzing the kinetics of events in differentiating female ES cells. From the earliest to the latest, just after accumulation of *Xist* on the X chromosome, transcriptional silencing, and late replication during S-phase, association with hypoacetylated histones H3 and H4, dimethylated H3 in Lys-9 (H3K9), tri-methylated H3 in Lys-27, and lack of di- and tri-methylated H3 in Lys-4 (H3K4), enrichment with macroH2A variants and finally DNA methylation at the 5′ ends of genes, have been observed (Heard et al., 2001; Chaumeil et al., 2002; Heard, 2004). The identification of the molecules responsible for these histone modifications resulting in a heritably repressed chromatin conformation along the X chromosome is currently under investigation. Recent studies showed that a polycomb group (PcG) protein complex containing Eed and Enx1, which harbors an activity of methylating histone H3 at lysine 9 and 27 is localized to the X chromosome transiently in *Xist*-dependent manner in the early phase of inactivation (Silva et al., 2003) (Fig. 2b).

However, it is not completely clear how each of the epigenetic marks discovered contributes to the X inactivation process, they seem to act synergistically together with *Xist* RNA (Csankovszki et al., 2001). In addition, the partners (proteins or nucleic acids) mediating the binding of the *Xist* transcript to the X chromosome and the exact chromatin remodeling that is induced by this association are still unclear. Because the developmental window during which cells are both responsive to, and dependent on, *Xist* expression (Wutz and Jaenisch, 2000) is tight, it has been suggested that the recruitment of factors that mediate this transcriptional silencing, is developmentally regulated and within this defined window of time. It is possible that either the partners of *Xist* that generated silencing are subsequently not available and/or competent or that the chromatin of the X chromosome has become remodeled to resist their effects. In this light, irreversibility might

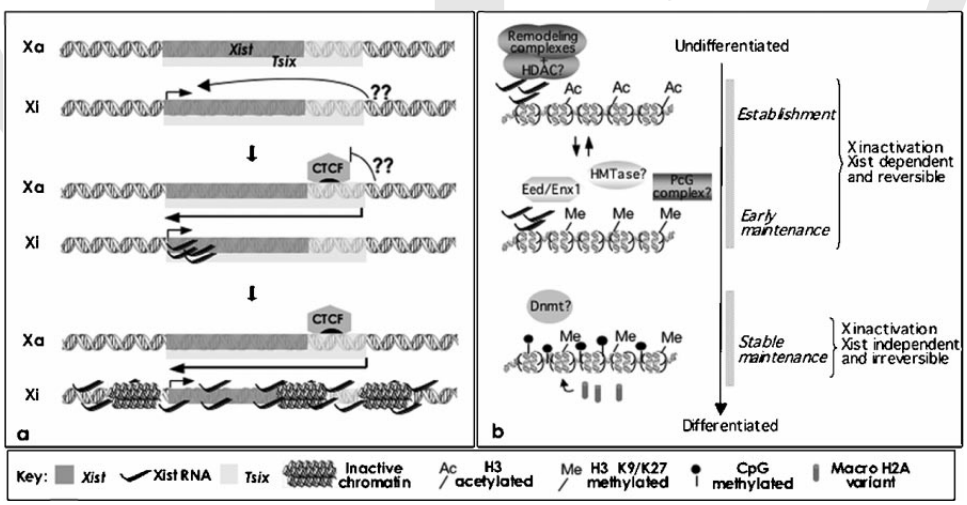

Fig. 2. **a**: A model for the epigenetic switch of the *Xist* locus mediated by CTCF and *Tsix*. On the inactive X *Xist* expression is upregulated by the activity of a putative enhancer (??), which is blocked on the active X by CTCF binding to *Tsix* CpG island. This permits *Tsix* transcription and the further *Xist* repression. **b**: Proposed mechanisms regulating the establishment and maintenance of X chromosome inactivation derived from experiments in differentiating female ES cells. Recruitment of histones modifications/remodeling complexes by *Xist* RNA leads to an initial level of chromosomal silencing, reversible and *Xist*-dependent. Other late epigenetic changes, such as a shift in the replication timing, incorporation of MacroH2A variant and DNA methylation of CpG islands, result in a self-maintained, irreversible X inactivation.

also reflect the involvement of promoter DNA methylation, as widely postulated. Consistent with this, the relative instability of X inactivated genes in the marsupials has also been associated to hypomethylation of CpG islands of X linked genes (Kaslow and Migeon, 1987). In addition, treatment of somatic cells with demethylating agents can release X inactivation, strongly indicating that methylation clearly contributes to reinforce inactivation (Graves, 1982).

The functional importance of maintenance and de novo DNA methylation during the process of X inactivation has been addressed with studies of mouse knockout mutants defective in the various DNA methyltransferases. The first implication of DNA methylation in the control of *Xist* expression has been the evidence that its promoter was unmethylated on the inactive X and highly methylated on the active X (Norris et al., 1994). In differentiating ES cell lines and embryos lacking the Dnmt1 the process of X inactivation can still normally occur, but maintenance-type methylation at the *Xist* promoter seems to be necessary for its stable silencing at the active X. *Xist* de-repression in some differentiated cells and initiation of inactivation of X-linked genes in *cis* had been in fact observed (Beard et al., 1995; Panning and Jaenisch, 1996). Because some residual methylation is still detected at the *Xist* promoter in Dnmt1−/− embryos, the role of de novo methylation in the establishment of this differential pattern and its correlation with the monoallelic expression of *Xist* have been studied. ES cell deficient for both Dnmt3a and 3b showed that *Xist* locus is widely demethylated, confirming that its promoter is one of the targets for these de novo methyltransferases. Despite this alteration in the level of DNA methylation at the *Xist* CpG island, however, its expression remains monoallelic and, consequently, X inactivation can initiate and propagate properly along the chromosome in the female mutants, suggesting that the de novo methylation is dispensable for X chromosome silencing (Sado et al., 2004). Similarly to the Dnmt1 deficiency, the effect caused by the failure of methylation of the *Xist* locus in Dnmt3a and 3b mutants, is its progressive reactivation in a differentiated cell population. But, in contrast with the previous report, the ectopic expression of *Xist* results in no induction of silencing of X-linked genes; this fact would be also consistent with the existence of a critical developmental window for the silencing of the chromosome hypothesized by Wutz and Jaenisch (2000).

Moreover, based on the same targeting studies, it has also been suggested that maintenance DNA methylation is critical for "locking" the repression of X-linked genes, given that a partial reactivation of X-linked lacZ transgenes in the Dnmt1−/− embryos has been observed. On the contrary, the lack of de novo methylation does not seem to reactivate the inactive copy of four genes in female Dnmt3a−/− and 3b−/− embryos analyzed at the same stage of development. This incongruence could be also due to the difference between exogenous and endogenous genes. However, the implication of the defect of Dnmt3a and 3b in the maintenance mechanism of the inactivation state has not been completely understood. The insights of these targeting studies on the initiation step of X inactivation are clearer, providing the important evidence that a mechanism(s) other than DNA methylation is responsible for generating and controlling the differential expression of *Xist*. In this light, it has been suggested that earlier events leading to modifications in chromatin structure such as specific lysine methylation of the histone H3 tail, might induce differential expression of *Xist* on their own. DNA methylation would be responsible for stabilizing the already established transcriptionally repressive state of *Xist*.

Useful clues on the role of DNA methylation in the initiation and spreading steps of X inactivation came also from studies of the human ICF syndrome, caused by DNMT3B mutations (Hansen et al., 1999). Several X-linked genes show hypomethylation in their CpG islands but only two of them are improperly inactivated (Hansen et al., 2000). Because of the expression and localization of *Xist* RNA in ICF cells, as well as the overall process of X inactivation, occur properly, the establishment and propagation of X inactivation appear to be normal in this DNMT3B-deficient background. This is consistent with the mentioned evidence provided by the targeting mouse studies, where de novo DNA methylation seems to be a secondary silencing event in the regulation of *Xist*. Recently, the effect of the loss of DNMT3B on the methylation status of LINE-1 (L1) repeats of the X chromosome in ICF cells has been analyzed (Hansen, 2003), in order to study possible implications of the de novo methylation in the spreading mechanism of the inactivation signal. As the "Lyon Repeat Hypothesis" suggests, L1s might function to promote spreading along the X chromosome (Lyon, 1998). These elements would thus be interesting candidates for this role also in the light of the L1-rich nature of the human and mouse X chromosomes compared with the autosomes. A clear hypomethylation of only L1 CpG islands of the inactive X chromosome, and not of the ones on active X and autosomes, has been demonstrated in ICF patients, suggesting that these sequences are specific targets of the DNMT3B methyltransferase. Consequently, the correct X inactivation in this context led to the hypothesis that at the beginning of X inactivation L1 repeats were probably unmethylated (Hansen, 2003). Thus, in the spreading of X inactivation the unmethylated state of L1 elements rather then methylated L1s could be involved. Further studies in this direction would surely be significant to reach a complete view of the contribution of the DNA methylation in the X inactivation puzzle.

DNA METHYLATION AND GENOMIC IMPRINTING

Genomic imprinting is a system of non-Mendelian inheritance that is unique to eutherian mammals, marsupial, and higher plants. It is an epigenetic mechanism of gene regulation that determines the parent-of-origin-dependent expression of a number of genes during development. Imprinted genes are marked in the male and female germlines and retain the molecular memory of their parental origin, resulting in allelic expression differences. DNA methylation is a basic mechanism for the establishment and maintenance of the imprinting status. Genomic imprinting plays a critical role in embryogenesis as evidenced by certain aberrations of human pregnancy such as hydatiform moles or ovarian dermoid cysts. A large number of imprinted genes identified in humans and mice are involved in pre- and postnatal development. A number of evidences suggests that imprinted mammalian genes influence complex neuropsychological behavior and neuropathological disorders (Falls et al., 1999).

The concept of genomic imprinting as a possible mechanism of explaining functional differences between maternal and paternal genomes in mammals was proposed in 1984 (McGrath and Solter, 1984; Surani

et al., 1984). Two different groups demonstrated that gynogenetic or androgenetic embryos that had either two maternal or paternal pronuclei showed early embryonic lethality and never developed to term. Few years later, a role of imprinting in human diseases [Prader–Willi syndrome (Nicholls et al., 1989)] was suggested. In the early 1990s, the first imprinted gene to be identified was Igf2 (DeChiara et al., 1991). Systematic screening methods have contributed to the identification of a large number of imprinted genes and to the precise localization of imprinted chromosomal regions. To date, about 60 imprinted genes have been isolated from the human and mouse genomes, and it has been estimated that there are roughly 100 imprinted genes in the mouse genome (Reik and Walter, 2001). Several hypotheses have been proposed to explain the evolution of such a peculiar regulatory mechanism of gene expression. The more generally accepted is the so-called "parental conflict hypothesis." According to this, the evolutionary force that propagates genomic imprinting in mammals might be a parental conflict for the maternal resources, promoting imprinted expression of genes that function in growth regulation (Walter and Paulsen, 2003). The mammalian genes that undergo parental-specific regulation and the chromosomal region where they map, share an interesting range of physical, genetic, and epigenetic features that may be summarized in some main relevant points. First of all, imprinted genes tend to be clustered. This clustered organization is thought to reflect coordinated regulation of the genes in a chromosomal domain. In some clusters, an imprinting control element (ICs) has been discovered: the ICs seem to be necessary for the regional control of imprinting and/or imprinted expression. Deletions and inappropriate DNA methylation of these regions are associated to loss of imprinting (LOI) and human diseases. Imprinted gene products show no notable common features; however, it is possible to underline functional similarities between genes with a role in fetal growth and development. It is interesting to note that, within the imprinted gene clusters, genes that encode for untraslated RNA and antisense RNA transcripts possibly involved in the control of imprinted expression (Jaenisch and Bird, 2003) were found. The analysis of nucleotide sequences also shows two elements of functional significance. First, they are unusually rich in CpG islands (in mouse, 88% of imprinted genes are associated with CpG islands vs. the 47% in the whole genome). Second, the CpG islands are frequently surrounded by direct repeats. The repeats, and the associated CpG islands, are involved in conferring or maintaining the differential methylation level of the imprinted allele (Reik and Walter, 2001). The parental alleles of an imprinted gene show differences in the methylation pattern. As described below, the differentially methylated regions (DMRs) probably have different properties and methylation patterns during development and in germ and somatic cells. Deletion of DMR results in LOI. DMRs are generally CpG rich and often fulfil the criteria of a CpG island. Some DMRs (e.g., H19, Igf2r) also contain repeat elements. As expected, imprinted genes can differ with respect to bulk chromatin structure for specific modifications such as the accessibility of DNA to the transcriptional machinery and histone covalent modifications. They also often reside in chromosomal regions that undergo asynchronous replication (Kitsberg et al., 1993). Methylation imprints are associated to chromatin imprints (Li, 2002).

The mechanism of genomic imprinting is complex and not completely understood. It is well established that DNA methylation plays a leading molecular role. Cytosine methylation marks imprinted genes differently in gametes and the inheritance of these epigenetic marks leads to differential gene expression. Genomic imprinting changes during the life cycle of the organism and proceeds in a different way in germ and somatic cells (Fig. 3). In somatic cells, imprinting may be established in a tissue-specific manner. Imprints are established during the maturation of germ cells to sperm or eggs. After fertilization, the differential mark of paternal and maternal alleles in somatic cells is maintained during the organism development. In the germ cells of the new organism, imprints are erased at an early stage in the primordial germ cells, and at a later stage of development, in the inner cell mass, they are established again in a sex-specific manner. The fine molecular mechanism of imprint determination during the development is not yet clear. According to present knowledge, it may be schematized in three main steps: erasure, establishment, and maintenance.

In mouse germ cells during erasure there is a marked and apparently genome-wide demethylation. First demethylation occurs in the male pronucleus, and seems to be independent of DNA replication. After the formation of the zygote, at the stage of blastocyst, both paternal and maternal chromosomes undergo progressive demethylation erasing most but not all of the marks that are inherited from the gametes. Whether this mechanism is passive or active is still unknown. If the methylation marks on imprinted genes are protected from genome-wide demethylation is also discussed (Li, 2002). The general DNA demethylation process is accompanied by chromatin modifications that seem to facilitate the genomic reprogramming.

The timing of the imprints re-establishment seems to be quite different in oocytes and sperm (Geuns et al., 2003). After erasure, de novo methylation of cytosine begins in both sex germ lines at late fetal stages and continues after birth. The maternal or paternal imprints established in germ cells lead to the formation of the

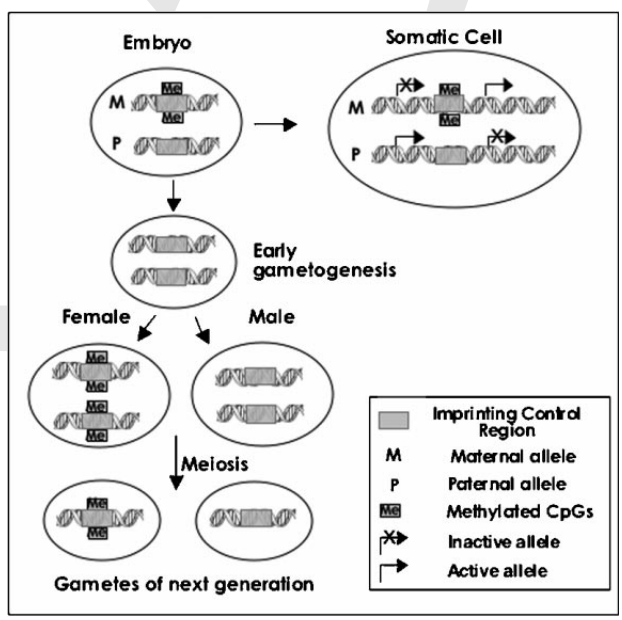

Fig. 3. Schematic representation of the imprints cycle during gametogenesis and somatic cell differentiation.

primary DMRs. How DMRs are recognized and marked is still under discussion. De novo and maintenance methyltransferases seem to be needed for remethylation in sperm and oocyte. Dnmt1 and its germ cell-specific isoform (Dnmt1o; see Howell et al., 2001) are candidates; but also the known somatic de novo methyltrasferase, Dnmt3a and Dnmt3b may carry out the same function in germ cells (Jaenisch and Bird, 2003). It is also yet unclear how Dnmts specifically target DMRs in eggs and sperms. The regulation of the imprinted status also involves histone acetylation and possibly histone methylation (Li, 2002). When the methylation and chromatin imprints are established they have to be converted into differential transcription. The reading mechanisms that have been explored so far are: (1) differential silencing by CpG island or promoter methylation; (2) regulation by antisense transcripts associated to CpG island or promoter methylation (i.e., air gene); (3) regulation by silencing factors that repress the promoter in *cis*; (4) allele-specific regulation of neighboring genes by differential methylation of boundary elements within a CpG island. A well-described example is the role of CTCF in recognition of unmethylated boundary in H19/Igf2 system (Jaenisch and Bird, 2003). The same binding sites are recognized by Boris (Loukinov et al., 2002), another protein which seems to act in a tissue restricted manner (testis). Recent reports suggest that Boris and CTCF may serve as counteracting genes by manifesting and reprogramming states, respectively (Feinberg et al., 2002).

DNA METHYLATION DISORDERS AND HUMAN DISEASES

The list of human diseases caused by or involving DNA methylation disturbances has become quite long. These pathologies are often syndromic, given the pleiotropic effect of mutations on the phenotype (Table 1). We can subdivide all these human pathologies into three classes.

1. Defects of imprinting;
2. defects of DNA methylation level and/or interpretation; and
3. methylation and cancer.

DEFECTS OF IMPRINTING

Angelman syndrome and Prader–Willi syndromes

The Angelman syndrome (AS, OMIM #105830) and the Prader–Willi syndrome (PWS, OMIM #176270) are autosomal dominant disorders. These syndromes together with the Beckwith–Wiedemann, are the best-characterized examples of diseases associated with alterations in imprinting maintenance. These disorders show parent-of-origin effects since the inherited diseases are transmitted from only one of the parents. The AS and PWS are clinically distinct but are associated with same chromosome region, the 15q11-q13. They have a frequency of about 1:10,000/1:40,000. Both syndromes are neurodevelopment disorders associated with deficiencies in sexual development and growth; the PWS and AS patients show behavioral and neurological problems including several learning disabilities. The most common genetic feature associated to Prader–Willi and Angelman syndromes is an interstitial de novo deletion of 3–4 Mb in the chromosome 15q11-q13. The deleted region contains the *HERC2* gene, a highly conserved gene which affects development and fertility in mice (Clayton-Smith and Laan, 2003). These deletions are of maternal origin in AS patients and of paternal origin in PWS patients. Imprinted expression is co-ordinately controlled by an imprinting center (IC), which is functional in the germline and in early post-zygotic development. The IC regulates the establishment of parental-specific allelic differences in DNA methylation, chromatin structure and expression (Brannan and Bartolomei, 1999; Nicholls and Knepper, 2001). The 15q11-q13 IC has a bipartite structure. One part seems to be involved in switching the paternal imprinting in the maternal one; the other seems to be responsible for the opposite switch (Buiting et al., 1995). Alterations in IC methylation status have been identified in a number of patients. The main genes involved in the AS development have been identified. Deletions and other alterations in the UBE3A and ATP10C have been shown to be associated to AS. The *UBE3A* gene encodes for an ubiquitin protein ligase, mainly expressed in the hippocampus and in Purkinje cells. This gene has a maternal imprinting in brain cells and is biallelically expressed in other tissues (Albrecht et al., 1997).

Recent evidences support the existence of at least 30 candidate genes for PWS occurrence (Bittel et al., 2003). Several genes have been identified and characterized, and a paternal expression was demonstrated. In contrast to AS which is often associated to single gene mutations, PWS is always associated to chromosomal abnormalities affecting multiple genes. The finding of many paternally imprinted genes has contributed to the identification of several candidate genes for PWS. The SNRF/SNRPN locus is the main candidate. It is noteworthy that the 5′ end of the SNURF/SNRP maps in the IC. This locus is highly complex with a long variable policistronic precursor transcript with multiple functions. At least four transcripts are associated to this locus: SNURF, IPW, PAR5, and multiple small nucleolar RNAs (snoRNAs) (Runte et al., 2001). The snoRNAs have been proposed to confer most of the PWS phenotype (Gallagher et al., 2002).

The Beckwith–Wiedemann syndrome

The Beckwith–Wiedemann syndrome (BWS, OMIM #130650) is an autosomal multigenic syndrome. As

TABLE 1. DNA methylation defects and associated genetic diseases

Disease	Locus/protein	Main genetic defect	Molecular phenotype
Angelmann syndrome	15q11-q13	Deletions, UPD, IC methylation abnormalities	Loss of imprinting (LOI)
Prader–Willi syndrome	15q11-q13	Deletions, UPD, IC methylation abnormalities	LOI
Beckwith–Wiedman syndrome	11p15.5	Deletions, UPD, IC methylation abnormalities	LOI
ICF syndrome	DNMT3B	Point mutations	Genomic hypomethylation
Rett syndrome	MeCP2	Point mutations	
Fragile X syndrome	FMR1	CGG repeat expansion, CGG and promoter methylation	Protein inactivation, promoter silencing
ATR-X	ATR-X	Point mutations, splicing alteration	
FSHD	4qter?		

described above, this syndrome shows a clear parent-of-origin phenotype with a mainly sporadic inheritance. Clinically it is characterized by pre- and post-natal overgrowth and anterior abdominal wall defects. Peculiarly, about the 5% of affected children show embryonal tumors, such as Wilms' tumor. The molecular bases of BWS are very complex. It is associated to the 11p15.5 locus. This region is organized into two imprinted domains with separate imprinting control regions. Both paternally and maternally expressed genes have been associated to the BWS development. Oversimplifying, in the BWS pathogenesis two main mechanisms seem to be involved: uniparental disomy (paternal) and LOI at the insulin-like growth factor (IGF2) locus, and maternally derived translocations and specific mutations in the *CDKN1C* gene, a cyclin dependent-kinase inhibitor (Weksberg et al., 2003). These two genes contribute to the BWS phenotype together with a number of other imprinted or not imprinted genes, which are localized in these two domains (Maher and Reik, 2000).

The distal domain 1 contains a differentially methylated region, the DMR1, supposed to be an imprinting center. DMR1 is methylated on the paternal chromosome while it is unmethylated on the maternal allele. The genes *IGF2* and *H19* are closely linked and opposite imprinted. The DMR1, is located 2 kb upstream H19 and regulates the reciprocal imprinted expression of IGF2 and H19. IGF2 encodes a paternally expressed fetal growth factor; H19 codes for an untranslated pol III transcript. The expression profile of IGF2 in normal development resembles the spectrum of tissues and organs involved in BWS. In mouse, IGF2 and H19 seem to compete for a cluster of enhancers located at the 3′-end of the *H19* gene. In this region, a chromatin boundary, recognized by CTCF, has been identified. The binding of CTCF to this region seems to avoid IGF2 promoter interaction with downstream enhancers inducing H19 promoter transcription (Sparago et al., 2004). The proximal domain 2 contains several imprinted genes. A well-defined role in BWS syndrome is described for CDKN1C, which is maternally expressed. Mutations in this gene are associated with the rare cases of dominant transmission of the syndrome. Other genes have been identified in this region. The intron 10 of *KCNQ1* gene contains the DMR2. KCNQ1 is maternally expressed and encodes for a number of transcripts. One of these codifies for part of a potassium channel, which is associated to other human disorders (i.e., long QT syndrome) (Neyroud et al., 1997). The paternal DMR2 is not methylated and permits the transcription of a long antisense transcript known as KCNQ1OT1 or LIT1. DMR2 seems to have insulator activity in mouse (Kanduri et al., 2002) and insulator and silencer activity in man (Weksberg et al., 2003).

DEFECTS OF DNA METHYLATION LEVEL AND/OR INTERPRETATION

ICF syndrome

Disturbance in the methylation level is seen in a rare autosomal recessive disease, the ICF syndrome (ICF, OMIM #242860). This acronym states for Immunodeficiency, Centromeric decondensation, and Facial anomalies. ICF patients invariably show two peculiar signs: a severe immunodeficiency and a peculiar decondensation of the pericentromeric heterochromatin, especially of chromosomes 1 and 16, less frequently involving chromosome 9. Other variable signs at a clinical level are facial anomalies, psychomotor and mental retardation. The syndrome was mapped on chromosome 20 by genetic analysis (Wijmenga et al., 1998), and the causative gene was identified as the one encoding for DNA methyltransferase 3B (DNMT3B) (Hansen et al., 1999; Okano et al., 1999; Xu et al., 1999). DNMT3B mutations are diverse but they are often missense mutations in the conserved DNMT motifs specifying the methyltransferase catalytic domain. Homozygous and compound heterozygous patients have been described. Because Dnmt3b null mice are not viable, some residual activity from at least one of two DNMT3B alleles have been supposed. Given that the hypomethylation of the genome in ICF syndrome is much less generalized than after treatment with DNA methyltransferases inhibitors, such as 5-azacytidine, what are the target sequences of DNMT3B enzyme activity?

Major satellite regions as well as a number of repetitive sequences that are affected by DNMT3B loss of function [D4Z4 and NBL2 (Jeanpierre et al., 1993)] are hypomethylated. Inactive X chromosome is also hypomethylated, though it does not prevent the occurrence of X inactivation (Bourc'his et al., 1999). L1 elements are hypermethylated in normal cells; in ICF patients derived cells, DNMT3B loss of function produces no effect on the Xa, but a significant hypomethylation of X inactive specific LINEs (Hansen, 2003).

Gene expression is also affected in ICF syndrome. In earlier experiments, no demethylation was observed in the single Dnmt3B mouse mutant, whereas the double mutant Dnmt3A/3B showed demethylation of the 5′ region of *Xist* and the DMR2 of *Igf2* genes. A generalized hypomethylation of X inactive genes has been reported in ICF patients: however, only genes showing also an advanced replication time reactivate silent alleles (Hansen et al., 2000). One of these genes, the pseudoautosomal gene *SYBL1*, shows DNA hypomethylation accompanied by histone H3/4 hyperacetylation, differential methylation of H3K4 and H3K9, with enrichment in the former and a decrease in the latter (Matarazzo et al., 2002). This phenomenon is mechanistically linked with MBDs function on SYBL1 promoter, which is also affected in ICF syndrome cell lines (Matarazzo et al., in process[Q2]). Moreover, since the main system affected in ICF syndrome is the immune system, the effort of Ehrlich and Hanash teams, to derive the expression profiling of these patients, was very interesting. Over 30 genes were found to be deregulated, many of them with specialized functions in lymphocyte signaling, maturation and migration (Ehrlich et al., 2001).

Rett syndrome

Rett syndrome (RTT, OMIM #312750) is a neurological disorder that affects females almost exclusively (Rett, 1966.), occurring with a frequency of up to 1/10,000 live female births. After an early period of apparently normal or near normal development (until 6–18 months of life), this disorder results in profound mental disability, reduction in speech, and purposeful hand movements and reduced brain growth. The RTT locus has recently been mapped to Xq28, allowing subsequent identification of mutations in the *MECP2* gene (encoding for a Methyl-CpG-binding protein type 2; (Amir et al., 1999). Approximately 75% of RTT females have been found to carry heterozygous mutations in the X-linked *MECP2* gene (Wan et al., 1999; Vacca et al., 2001a). Recently, large deletions in a deletion prone region (DPR) of the *MECP2* gene have been reported in an additional 7%–16% of the patients analyzed (Laccone et al., 2004). Moreover, *MECP2* mutations have been found in roughly 2% of patients

with nonspecific X-linked mental retardation (Couvert et al., 2001).

Interestingly, it has been recently reported that a splicing isoform of MECP2 (MECP2B) has been identified, which accounts for a small part of RTT cases (Kriaucionis and Bird, 2004; Mnatzakanian et al., 2004).

Three mouse models have been described for functional deletion of *MECP2* gene (Chen et al., 2001b; Guy et al., 2001; Shahbazian et al., 2002). The phenotypes obtained, as well as the timing of their occurrence, are very similar to those exhibited by the RTT patients. Genetic analysis also reveals an effect on neuronal maintenance rather than development of brain functions. MECP2 mutated mice show hyperacetylation of histone H3 in brain tissues, which may alter chromatin structure. Interestingly, the deletion of *MBD2* gene causes a behavioral phenotype (Hendrich et al., 2001). MBD2 and MECP2 are expressed ubiquitously in both humans and mice; thus, functional redundancy of MBDs might be less in brain than in other tissues or brain development may be exquisitely dependent upon DNA-methylation-mediated silencing of inappropriate transcription (transcriptional noise). The identification of genes misregulated in the presence of a dysfunctional MBD could discriminate between these alternatives. So far, direct binding of these proteins to specific gene promoters have been identified only in few genes (Fournier et al., 2002; Ballestar et al., 2003). Even if expression profiling experiments did not reveal substantial differences in gene expression (Tudor et al., 2002), the role of MECP2 as transcriptional repressor and its importance for brain function has been confirmed recently, by the identification of two putative targets: the transcriptional repressor of neuron-specific genes in *Xenopus*, xHairy2a (Stancheva et al., 2003) and the brain derived growth factor (BDNF; (Chen et al., 2003; Klose and Bird, 2003; Martinowich et al., 2003)) which is thought to be essential for converting transient stimuli into long-term changes in brain activity. The upregulation of BDNF is caused by cortical depolarization that, on one hand, phosphorylates MeCP2, thus affecting its affinity for methylated DNA; on the other hand, it causes a limited hypomethylation of BDNF promoter III, thus boostering the effect. These latest results may indicate that the MBD proteins, in addition to genome-wide functions, perform a number of gene-specific functions, revealing an unsuspected functional plasticity (Klose and Bird, 2003).

Fragile X syndrome

Fragile X syndrome (OMIM #309520) is one of the most common causes of mental retardation, with an incidence of 1/4,000 in males and 1/8,000 in females. The syndrome is caused by mutations of the *FMR1* gene, located on the X chromosome. FRA-X syndrome was the first syndrome associated to trinucleotide expansion: in fact, the *FMR1* gene contains, at its 5′-UTR region, a CGG repeat which is highly polymorphic in normal individuals (between 7 and 60 repeats). Alleles with between 60 and 230 repeats are defined premutated, whereas alleles with over 230 repeats are fully mutated (Coffee et al., 2002). FMR1 encodes for an RNA binding protein, highly expressed in the brain. A step towards the understanding of its role in brain function has been made recently (Zalfa et al., 2003): by regulating specific dendritic RNAs, FMRP acts as a repressor of translation. FMRP is found in ribonucleoparticles (RNPs) together with a non-translatable RNA, BC1.

What about the role of DNA methylation in FRA-X syndrome? The onset of disease is connected with the CGG expansion at 5′-UTR; however, the expansion triggers cytosine methylation of the repeat and of flanking sequences, including the FMR1 gene promoter, thus inactivating the gene. Treatment of FRA-X cells with 5′-aza-2′-deoxycitidine causes loss of methylation, reactivating FMR1 transcription (Chiurazzi et al., 1998). A synergistic effect of the combined treatment leading to histone hyperacetylation and DNA methylation has been demonstrated to reactivate FMR1 transcription (Chiurazzi et al., 1999). A detailed analysis of the histone modifications of the *FMR1* gene has recently been published (Coffee et al., 2002), thus making it possible to use some pharmaceutical treatment for this syndrome in the near future.

Alpha thalassemia with mental retardadation syndrome (ATR-X syndrome)

The ATR-X syndome (ATR-X, OMIM #301040) is an X linked mental retardation syndrome, characterized by severe mental retardation, reduced or absent speech, delayed developmental milestones and congenital microcephaly. Alpha-thalassemia is a very frequent, but not constant feature of the syndrome. In these patients, mutations are found in the *ATRX* gene, located on Xq13 (Gibbons et al., 1995). *ATRX* codes for a putative ATP-dependent type II helicase of the SNF2 family. This protein binds the short arms of human acrocentric chromosomes, and interacts with two heterochromatin proteins, mHP1 and EZH2. The ATRX protein belongs to an ATP-dependent chromatin remodeling complex, together with the transcription factor Daxx. This complex is localized near promyelocytic nuclear bodies (Xue et al., 2003).

The effects of ATRX mutations on the chromatin structure of rDNA arrays, located at acrocentric chromosomes have been analyzed. Methylation differences have been noticed: in ATRX patients, rDNA genes were substantially unmethylated (Gibbons et al., 2000). In addition to rDNA arrays, the analysis was extended to highly repetitive sequences spread in the genome. Abnormal methylation (hypomethylation) has been found at Y chromosome-specific repeats, DYZ2 and subtelomeric repeats TelBam3.4. These features resemble those observed in ICF patients; however, in ATR-X syndrome the total amount of methylated cytosines is unaffected (Gibbons et al., 2000). α-Thalassemia also arise by the silencing of the α-globin gene (*HBA2*) by CpG hypemethylation: in this interesting case, the novel mechanism of action involves an RNA antisense transcription without affecting chromatin structure (Tufarelli et al., 2003).

Facioscapulohumeral dystrophy syndrome (FSHD)

The autosomal dominant facioscapulohumeral muscular dystrophy (FSHD1, OMIM #158900) is the third more common myopathy, which progressively affects muscles of the face, shoulder, and upper arm. It has been associated to the contraction of the 3.3 kb repeat arrays D4Z4 on 4qter region (Wijmenga et al., 1992). Repeat number varies from 11 to 150 in normal populations whereas it is greatly reduced (1–10) in patients. It was hypothesized that loss of D4Z4 repeats induces the region to adopt a more open chromatin structure, which causes the up-regulation of target genes. The increased expression of three genes in FSHD but not in the unaffected muscle has been reported. A protein complex

drives the repression, recognizing a specific 27 bp element in the D4Z4 unit: this sequence has transcriptional repression activity in vitro (Gabellini et al., 2002). However, a recent report links the marked hypomethylation of the contracted D4Z4 allele to overt disease (van Overveld et al., 2003). This correlation is supported by the finding that FSHD patients with unaltered D4Z4, show hypomethylation of this repeat.

METHYLATION AND CANCER

Tumorigenesis is a multistep process produced by the accumulation of genetic and epigenetic alterations that elicit the progressive transformation of a normal cell in a malignant and invasive derivative (Gan et al., 2003; Macaluso et al., 2003).

Data accumulated in the last few years show that aberrant DNA methylation plays a pivotal role both in predisposition and progression of cell transformation (Fig. 4). The role of DNA methylation is complex. The analysis of the DNA methylation profile of a cell, the so-called methylome (Feinberg, 2001), suggests the existence of several differences between normal and cancer cells. Both sporadic and hereditary cancers show cytosine methylation imbalance. Recent studies (Esteller et al., 2001) show that hereditary cancers mimic the DNA methylation patterns observed in sporadic tumors. Some genes seem to be aberrantly methylated in a tumor-specific manner. These patterns suggest that methylation of specific subsets of genes may contribute to the development of a specific tumor type. For these reasons, the DNA methylation profile of a cell type may serve as a biological marker with a diagnostic and prognostic value. Aberrant methylation patterns in a cell seem contribute to gain growth advantage and to lose growth controls such as the other transforming mechanisms. Under specific cellular conditions and in particular hot-spot sequences (Murata et al., 1997), DNA methylation may give rise to point mutations, a classical genetic lesion. In these cases, the de-amination of 5′-methyl-cytosine, converts a cytosine in a thymine.

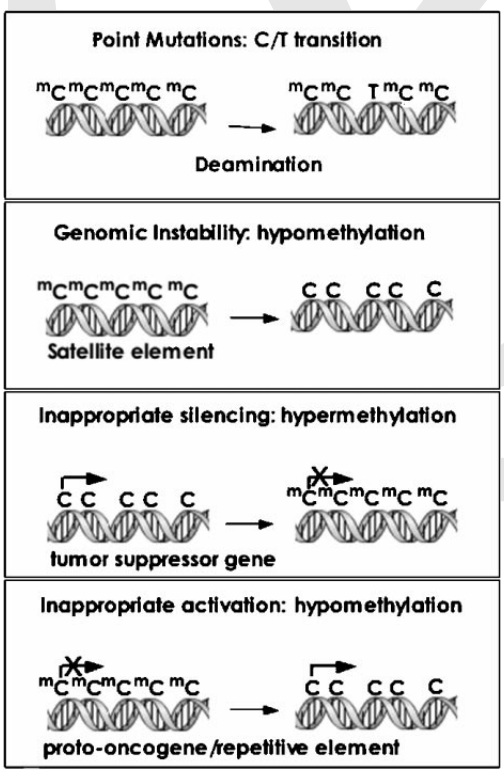

Fig. 4. Alterations in DNA methylation can lead to genome defects and/or alterations of gene expression.

DNA methylation defects include genome-wide hypomethylation and hypermethylation of specific CpG islands, both associated to alterations in other chromatin features. Together, these abnormal epigenetic mechanisms may also result in loss of genomic imprinting, which in turn induces cancer.

Hypomethylation

Historically, DNA hypomethylation was the first epigenetic modification to be identified in cancer cells. It is still debated whether this molecular change is a cause or a consequence of neoplastic transformation, and only recently its role in cancerogenesis has been clarified. DNA hypomethylation in tumors seems to be as prevalent as DNA hypermethylation and they do not seem to be mutually exclusive. In 1982, a reduced global content in 5-methylcytosine (Ehrlich et al., 1982) and a specific hypomethylation of a number of CpG islands (Feinberg and Vogelstein, 1983a) in cancer cell respect to their normal counterpart were observed by HPLC. Even if it is still under active discussion, DNA hypomethylation seems to be involved in several important pathological mechanisms. Experiments with cell lines treated in vitro with 5′-azacytidine show the formation of transformed foci and the conversion of low metastatic cell in highly metastatic ones. The best-characterized cell features associated with hypomethylation are: gene activation, repetitive elements de-repression, and chromosomal instability (Feinberg and Tycko, 2004).

Some CpG islands seem to be methylated in somatic tissue (Strichman-Almashanu et al., 2002). In some cancer cells, these islands result hypomethylated and the genes associated to these islands may be re-activated or over-expressed. Examples of gene activation associated to hypomethylation are HRAS (Feinberg and Vogelstein, 1983b) and cMYC (Ghazi et al., 1992), which are two important examples of oncogenes involved in the onset of cellular transformation and/or in tumor progression. A particular group of reactivated genes is that of tumoral antigens such as MAGE (De Smet et al., 1996), SAGE, and CAGE. The *MAGE* genes are associated to melanoma and are well investigated as potential anticancer vaccine targets. MAGE promoters analysis shows that methylation is an important regulatory mechanism; they are hypo-methylated in spermatogenic cells but heavily methylated in adult somatic tissue. The physiological function of these genes is unknown, but they have an important role in the antigenic modification of tumor cells. Another important and discussed effect of hypomethylation is the de-repression of mobile elements such as LINE or retroviral endogenous elements. A large part of these elements are inactive but a number of them may determine transposition. Transposition may be associated to activation or over-expression of cellular genes and more generally may induce genomic instability with an increase in non-homologous recombination (Feinberg and Tycko, 2004).

Hypomethylation contributes to genomic instability causing chromosomal instability as well. Demethylation of satellite sequences might predispose to their breakage and abnormal recombination. ICF syndrome shows that the loss of DNMT3b function causes the formation of abnormal mitosis figures. The ICF syndrome does not associate with an increased cancer risk, but the existence of a relationship between hypomethylation

and genomic instability is directly supported also by studies in mouse ES cells DNMT1$^{-/-}$(Chan et al., 2001). These cells show an increased mutation rate primarily involving genomic deletions.

Hypermethylation

The involvement of DNA hypermethylation in neoplastic transformation is well-characterized and experimentally sustained. It is now clear that DNA methylation may substantially contribute to the transcriptional regulation and a number of evidences show associations between CpG islands hypermethylation and gene silencing. The hypermethylation of CpG islands promoters may inactivate both alleles of an oncosuppressor gene or may act in concert with classical genetic mechanisms such as point mutations or deletions. For these reasons, CpG hypermethylation is now indicated as another possible mechanism causing tumor suppressor gene silencing according to the Knudson "two hits hypothesis." Some studies suggest that the hypermetylation of the first allele facilitates methylation of the second allele of the same gene. A number of aberrant methylation sites overlaps with recurrent sites of deletion (Fournier et al., 2002) The retinoblastoma predisposition gene (Rb) is a paradigmatic example of the class of tumor suppressor genes and also of the transforming effects of hypermethylation (Greger et al., 1989). It was the first silenced gene to be identified. Rb promoter is methylated in a significant subset of sporadic and even hereditary retinoblastomas. The identification of hypermethylation in the promoter CpG island associated to the silencing of the INK4A, a cell-cycle regulation gene, was the second important experimental evidence for the transforming role of this epigenetic mechanism. Inactivation of this gene is associated to the onset of melanomas and gliomas (Gonzalez-Zulueta et al., 1995). Another interesting example is the promoter of *hMLH1*, a mismatch repair gene. The promoter of this gene resulted methylated in colo-rectal and in endometrial carcinoma in patients showing a mutator phenotype (Strazzullo et al., 2003). Aberrant hypermethylation has been found in promoters of genes involved in almost all the important steps of cancerogenesis: cell-cycle regulation, DNA repair, drug resistance and detoxification, apoptosis, differentiation, angiogenesis, and metastasis. Mouse models, in which methylation aberrations have been induced chemically and genetically, resemble some of the events seen in human tumors. A number of "in vivo" and "in vitro" observations show that in different tumor types, modifications in cell methylation profiles may be both a pre-cancerous and progression feature (Feinberg and Tycko, 2004).

Oncosuppressor promoter hypermethylation is associated to chromatin modifications characterizing the transcriptional silencing. Recent works show altered hystone code in the promoter of tumor suppressor genes, such as INK4A (Nguyen et al., 2001) and hMLH1 (Fahrner et al., 2002). A large amount of data supports the idea that repression involves many interacting processes and mechanisms. The exact timing and dynamic between methylation and chromatin modification in the regulation of gene expression, in physiological, and pathological context, is not well understood. DNA methylation contributes to establish a silent chromatin state by interacting with proteins that modify nucleosomes organization. As described above, the discovery of proteins which bind specifically methylated CpGs (MBDs) suggests that gene silencing may be due to different mechanism: the binding of these proteins and DNA methylation itself, may interfere with the binding of other transcription factors and/or the MBDs may act as repressor factors. Therefore, MBDs would recruit corepressor and chromatin modifier such as histone deacetylases (HDAC) and histone methyltransferases (HMT) on the promoter, which would in turn convert chromatin structure from accessible to inaccessible.

Loss of imprinting

If a tumor suppressor gene or an oncogene undergoes an imprinted regulation, the LOI in its regulative region may promote cell transformation. As described above, imprinted loci expression may be altered in several ways. One of these ways is an aberrant methylation of the imprinted center. Early associations between LOI and tumorigenesis started from the analyses of particular tumor types such as hydatiform moles and ovarian teratomas. These tumors arise from cells that are completely androgenetic or completely partenogenetic respectively. Confirmation of a real involvement of LOI in neoplastic transformation derives from the arising knowledge about single imprinted loci structure and function. Important examples are Wilms' tumor and embryonic rhabdomyosarcomas associated to 11p15. LOI implies biallelic expression or the loss of expression of genes that are usually expressed monoallelically in a parent-specific manner (Ohlsson et al., 1998). The *IGF2* gene is involved in the onset of Wilms' tumor and other embryonic tumors. Wilms' tumor is a childhood tumor that arises from metanephric blastemal cells. About 70% of Wilms' tumors show biallelic expression of IGF2 and the biallelic silencing of H19. Biallelic expression of IGF2 seems to induce tumor progression by the inhibition of apoptosis. H19 silencing determines the loss of growth suppressor signals. These types of tumors have a bimodal age distribution: late-arising tumors were found to involve epigenetic rather than genetic aberrations; early-arising tumors show classical genetic changes in WT1 and LOH. LOI can arise in somatic tissue giving rise, after a second hit, to a Wilms' tumor. Germinal LOI associated to the Beckwith–Wiedmann syndrome cause nephromegaly and frequently multiple tumors arise. The frequency of Wilms' tumors in BWS patients is about 1,000-fold higher than in the normal population.

Even if not well-characterized as Wilms' tumor, indications exist for the association of loss of paternal imprinting in 19q and oligodendrogliomas, whereas chromosome LOI in 9p has been associated with childhood acute lymphoblastic leukemias (Feinberg and Tycko, 2004).

FUTURE PERSPECTIVES

The disruption of DNA methylation patterns, as component of the epigenetic network, is cause of several significant disorders and the introduction of new diagnostic tools will soon allow discovering other pathologies still unknown. The common epigenetic aetiology of all these diseases has suggested the developing of new therapeutic directions taking advantage of some agents that change the methylation level and/or histone modifications of DNA. Great expectations lie in the development of clinical trials testing inhibitors of DNA methyltransferases and histone deacetylases, which reactivate the expression of genes epigenetically silenced in pathological conditions. The action of several classes of such drugs is currently under investigation. Typical examples are 5′-azacytidine and 5-aza-2′-deoxycytidine,

which act blocking the DNA methyltransferase's activity once incorporated in replicating DNA. Similarly to other modified bases, their action leads to a demethylated DNA: but, since they are covalently attached to DNA, such compounds are cytotoxic at higher doses. Even if there are still several unsolved questions regarding the clinical application of these agents, the preliminary results seem promising (Egger et al., 2004). During previous chapters, we have described modifications in DNA methylation, especially those occurring in basic phenomena and human pathologies, as if they were "purely" dependent on genetic and/or epigenetic mechanisms. However, a relationship between genetic, epigenetic, and environmental factors, is becoming more clearly defined, thanks to the elaboration of new mathematical models (Bjornsson et al., 2004).

The link between epigenetic status, environment, and cancer is sustained by DNA hypomethylation and hypermethylation occurring during aging: while the former mainly affects repeated sequences, such as transposable elements (Yoder et al., 1997), the latter is observed mainly in specific genes, for example, the hypermethylation of specific oncosuppressor genes, that could represent important risk factors for the development of cancer (Jaenisch and Bird, 2003).

There are preliminary evidences, from complex diseases, that epigenetic changes may be inherited, i.e., changes in diet and/or limitations in accessing food can leave an imprint that may pass throughout generations. The analysis of the methylome might clarify whether epigenetics may predispose to multifactorial defects, and whether these defects are heritable. Such a model has been proposed for diabetes: however, the debate is still open, since this notion is in contrast with the current knowledge of erasure of DNA methylation mark after fertilization (Pembrey, 2002). DNA methylation alterations may also arise after pharmaceutical treatments or dietary levels of methyl donor components. An example is the folate supplementation of patients with hyperhomocysteinemia secondary to chronic renal failure. Hyperhomocysteinemic individuals show DNA hypomethylation, probably mediated by increase of the methyltransferase inhibitor S-adenosylhomocysteine (AdoHcy), affecting the expression of X inactivated and imprinted genes (Ingrosso et al., 2003). Studies on enzymatic kinetics established that not all the methyltransferases are equally sensitive to this inhibition: however, most probably, histone H3 methylation efficiency could be also affected by methyltransferase inhibition, yet again affecting gene regulation (Van den Veyver, 2002). Given the increased cardiovascular risk of these patients, the hunt is open for genes affecting cardiovascular function, and these genes are probably controlled by epigenetic mechanisms.

As Jaenisch and Bird excellently pointed out in a recent review, in order "to establish a mechanistic link between environmental stimuli and epigenetic states of the genome, a well defined and sensitive phenotypic readout is required" (Jaenisch and Bird, 2003). This is what occurs for the mouse coat color locus: a diet supplemented with methyl donors affects the coat color gene agouti, which changes from agouti to mottled or yellow; these are caused by the methylation state of an intragenic IAP element (Jaenisch and Bird, 2003). A role for DNA methylation and dietary supplementation has been also proposed for behavioral disorders, such as schizophrenia. In the reeler mice, in fact, the reelin gene shows affected promoter methylation and gene expression after a diet supplemented with L-methionine. This effect is reverted by treatment with valproate (Tremolizzo et al., 2002). These data prompted researchers to further analyze epigenetic changes, especially alterations in global DNA methylation, in patients with bipolar disorder (BD) or other neurological pathologies. The dynamics of epigenetic changes better account for the fluctuating course of bipolar disorder, perhaps more so than DNA variations. In fact, despite significant effort, the understanding of the molecular causes and mechanisms of this disorder still is a major challenge. Numerous molecular genetic linkage and association studies have been conducted over the last two decades; however, the data are quite inconsistent or even controversial. Epigenetic mechanisms will be consistent with various non-Mendelian features of BD such as the relatively high degree of discordance in monozygotic (MZ) twins, the critical age group for susceptibility to the disease and the clinical differences in males and females, among many others. Epigenetic variations may explain why the onset of these pathologies is, often, late: whereas DNA variations are permanent, epigenetic changes are in a process of flux and generally accumulate over time. Efforts are in progress to virtually map all the methylation sensitive sites of the human genome.

Recent data in humans and animals suggest that assisted reproductive technology (ART) might affect the epigenetics of early embryogenesis and might cause birth defects. In sheep and cattle, epigenetic abnormalities have been shown to be involved in the large offspring syndrome (LOS) (Young et al., 1998). Affected animals exhibit various phenotypes, including large size at birth. In both species, the syndrome is caused by the in vitro exposure of embryos, between fertilization and the blastocyst stage, to various unusual environments and is related to the LOI of the IGF2. Genome-wide cytosine methylation levels were also monitored in nuclear transfer (NT) generated cattle, and results indicated reduced levels of methylated cytosine in spontaneously aborted, NT-generated fetuses (Cezar et al., 2003).

H3-K9 methylation is reprogrammed in parallel with DNA methylation in normal embryos. A study performed in bovine cloned embryos show that the majority of them exhibit H3-K9 hypermethylation associated with DNA hypermethylation, thus suggesting a genome-wide failure of reprogramming after somatic nuclear transfer (Santos et al., 2003). In humans, the first evidence that ART is associated with a human overgrowth syndrome namely, BWS, is quite recent (DeBaun et al., 2003). In a prospective study, the prevalence of ART was 4.6% (3 of 65), versus the background rate of 0.8% in the United States. A total of seven children with BWS were born after ART-five of whom were conceived after intracytoplasmic sperm injection (ICSI). Molecular studies of six of the children indicate that five of the six have specific epigenetic alterations associated with BWS-four at LIT1 and one at both LIT1 and H19. In a second study, among a large cohort of Beckwith–Wiedemann patients, a significant percentage (6/149), all born after assisted reproduction, exhibit alteration of DNA methylation of the *KVDMR1* gene. These data suggest that ART may favor imprinting alterations and, consequently, the incidence of BWS.

CONCLUSIONS

A lot of work has been carried out since Prof. Scarano developed his hypothesis on the role DNA methylation in the control of gene expression: this epigenetic

alteration has become a center of scientific attraction, especially because of its relationship to gene silencing in disease. There is currently a wide range of methods designed to yield quantitative and qualitative information on genomic DNA methylation. From all this information, it is clear that the efforts finalized by the Human Genome Project are not an endpoint, but rather a starting point to unravel the complexity of genomes, especially at the level of the fine tune of gene regulation. Epigenetic mechanisms, either modifications of DNA or the proteins interacting with it, play an essential role in genome wide, and/or locus-specific regulations, implicated in genome physiology and reprogramming. Therefore, it would be foolhardy to predict the future's specific outcomes: a lot has been discovered since Eduardo Scarano devised his pioneering theories and what we know about DNA methylation "makes us look to the future with a sense of expecting the unexpected" (Leder, 1980).

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