

REVIEW

The growing role of gene methylation on endocrine function

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Abstract

DNA methylation is the best studied epigenetic factor, playing a key role in producing stable changes in gene expression, thus defining cell identity and function and adapting cells to environmental changes. DNA methylation has also been recently shown to mediate cell responses to physiological endocrine signals. Moreover, alterations of the normal DNA methylation pattern can also contribute to the development of endocrine and metabolic diseases and can explain the relationship between an individual's genetic background, the environment, and disease. It should be remarked that although DNA methylation and demethylation are active processes, epigenetic changes produced during development can impact adult processes, establishing the idea that endocrine function can be persistently affected by events occurring in early life. Given the complexity of the endocrine system, both genetic and epigenetic processes, including DNA methylation, must be involved in its proper development and functioning. In this study, we summarize the recent knowledge in the field of DNA methylation and endocrinology. Given that DNA methylation can be involved in a number of endocrine and metabolic disorders, understanding and manipulating this modification opens a new door for preventing and treating endocrine diseases.

Journal of Molecular Endocrinology (2011) **47**, R75–R89

What is epigenetics?

The term 'epigenetics' has classically been used to explain phenotypic events that cannot be described by genetic mechanisms. Waddington (1942) coined the term 'epigenetics' in the 1940s, defining epigenetics as 'the branch of biology that studies the causal interactions between genes and their products, which bring the phenotype into being'. An updated definition now considers epigenetic conditions to be those that affect gene expression without changing the nucleotide sequence, in a way that can involve mitotic, or, less frequently, meiotic inheritance (Holliday 2006), conferring a certain stability on epigenetic events through cell generations. Very recently, Skinner *et al.* (2010) have defined epigenetics as 'molecular factors and processes around DNA that are mitotically stable and regulate genome activity independent of DNA sequence'. Although most of the cells in a multicellular organism share the same genetic information, an organism produces many different cell types during its development, each characterized by a typical gene expression profile and by specific functions. Consequently, cell differentiation might be considered as a

largely epigenetic phenomenon. The first epigenetic modification identified was DNA methylation in the 1970s (Holliday & Pugh 1975). However, epigenetics also addresses covalent modifications of histones and the mechanisms through which they alter chromatin structure (Turner 1998). Other gene regulation mechanisms such as non-coding RNA are also included among epigenetic pathways (Aguilera *et al.* 2010). Epigenetic processes are likely to be expanded in the future. For example, the recent identification of hydroxymethylcytosine residues is a new epigenetic mark whose function remains to be elucidated (Kriaucionis & Heintz 2009). In this review, we will focus on only one of these epigenetic modifications, namely DNA methylation.

DNA methylation

DNA methylation is the most extensively investigated epigenetic modification because more than two decades ago it was shown to play a critical role in cancer. Feinberg & Vogelstein (1983) demonstrated for the first time that tumor cells can lose DNA methylation

at specific DNA regions. Subsequently, many laboratories identified a number of tumor suppressor genes that often become aberrantly hypermethylated and repressed in cancer cells (Feinberg 2007). Later on, investigations moved outside of the cancer field and today it is well established that DNA methylation is essential for normal development and differentiation in mammals (Fraga 2009). DNA methylation consists of the covalent addition of a methyl group at the 5-position of cytosines. This normally occurs on a cytosine followed by a guanine, called a CpG dinucleotide. This dinucleotide normally occurs at low frequency in vertebrate DNA. However, stretches of DNA with a high C+G content and a high frequency of CpG dinucleotides relative to the bulk genome have been defined as CpG islands by Gardiner-Garden & Frommer (1987). Most CpG dinucleotides are methylated, but those located in CpG islands are usually unmodified. Although CpG islands are located preferentially in gene promoters or regulatory regions, most of these dinucleotides are nonetheless found in repetitive DNA elements. The DNA methylation pattern is removed in the early embryo and then reconstituted at the time of implantation (Kafri *et al.* 1992). DNA methylation is also responsible for genomic imprinting, securing monoallelic gene expression by repressing expression of the paternal or maternal genes, for X-chromosome inactivation in females, and for silencing of parasitic elements (Payer & Lee 2008, Kacem & Feil 2009).

Although methylation of cytosine bases (mCs) in CpG dinucleotides is the principal form of DNA methylation, other modifications such as methylation of cytosine in CpNpG trinucleotides (where N denotes any base) or hydroxymethyl cytosines (hmCs) also exist, and all these modifications define the DNA methylome of a cell. A full methylome analysis should include these modifications in addition to mCG, although the biological function of mCGs and hmCs has not yet been clarified (Beck 2010). Methylome analysis, as well as the analysis of other epigenetic marks, will be needed to define the epigenome map characteristics of normal development and differentiation, including development of endocrine glands, and for the identification of genomic regions involved in endocrine and non-endocrine disorders. Another unresolved issue in DNA methylation is that of bisulfite sequencing, the currently used method of analysis that relies on the conversion of unmethylated cytosine residues to uracil cannot distinguish between methylation and hydroxymethylation, and densely hydroxymethylated regions of DNA may be underrepresented in quantitative methylation analyses (Huang *et al.* 2010). In 2009, the first genome-wide, single-base resolution maps of methylated cytosines in a mammalian genome have been resolved (Lister *et al.* 2009). Surprisingly, and in

contrast with differentiated cells, a significant fraction (almost one-quarter) of the methylation found in embryonic stem cells occurred in a non-CG context. This modification was lost on induction of differentiation and was restored in induced stem cells. These interesting results provide a foundation for future studies exploring this key epigenetic modification in human disease and development and to analyze its role in endocrine function.

DNA methyltransferases (DNMTs): hormonal control of DNMT expression

S-adenosylmethionine acts as a methyl donor for addition of the methyl group to cytosine residues within CpG dinucleotides. This reaction is catalyzed by DNA methyltransferase (DNMT) enzymes. In mammals, DNMTs include four members, in two families that are structurally and functionally distinct. The DNMT3 family establishes the initial CpG methylation pattern, whereas DNMT1 maintains this pattern during DNA replication (Mortusewicz *et al.* 2005, Chen & Li 2006). The DNMT3 family has three members: DNMT3A, DNMT3B, and DNMT3L (DNMT3 like). DNMT3L does not have enzymatic activity but may function as a regulator of DNA methylation. Inactivation of DNMT1, DNMT3A, and DNMT3B by gene targeting was found to cause lethality in mice, either pre- or post-natally. DNMT3A and DNMT3B act basically as *de novo* methyltransferases establishing the DNA methylation patterns at early developmental stages, although they have also a function at later stages maintaining global DNA methylation levels. In contrast, DNMT1 lacks *de novo* methyltransferase activity but serves as a maintenance for methyltransferase by copying the DNA methylation pattern from the parental strand to the daughter strand after DNA replication (Robertson 2005; Fig. 1).

Recent results suggest that DNMT expression is under hormonal control. For instance, DNMT3A, DNMT3B, and DNMT1 are under the regulation of female sex steroid hormones during the menstrual cycle (Yamagata *et al.* 2009, van Kaam *et al.* 2011), and aberrant expression of DNMTs was observed in endometriosis (Wu *et al.* 2007, van Kaam *et al.* 2011). On the other hand, estradiol increases DNMT3A and DNMT3B expression in the dorsal hippocampus, and increased methylation of memory suppressor genes (e.g. reelin) may be crucial for estradiol to enhance memory consolidation (Zhao *et al.* 2010). Paradoxically, even though congenital hypothyroidism is associated with impaired cognitive development, increased methylation of the reelin and *BDNF* genes is also found in the hippocampus of hypothyroid rats (Sui & Li 2010), showing the complex effects of thyroid hormones in brain development.

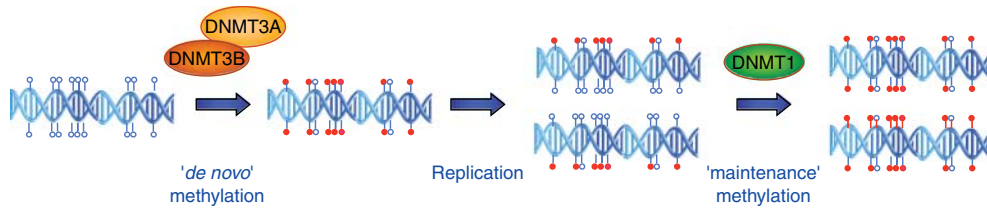


Figure 1 The DNA methyltransferases DNMT3A and DNMT3B are responsible for *de novo* methylation, whereas DNMT1 is in charge of maintaining methylation after DNA replication by modifying the daughter strand. The daughter strands of DNA following replication are unmethylated. Hemi-methylation status is recognized by DNMT1, which is recruited to the replication fork, and then restores the parental methylation pattern. Unmethylated CpGs are shown by empty circles and the methylated nucleotides by red circles.

Alterations in male reproductive tract development and postpubertal prostatitis observed after exposure to the antiandrogenic compound vinclozolin during gonadal sex determination could also be related to changes in DNMTs expression in the testis and prostate (Anway & Skinner 2008, Anway *et al.* 2008, Cowin *et al.* 2010). Furthermore, changes in DNMT1 expression have been shown to contribute to both prostate cancer initiation and promotion (Esteller 2005, Feinberg *et al.* 2006).

Peptide hormones can also regulate DNMT expression. For instance, autocrine human GH (hGH) increases expression of DNMT1, DNMT3A, and DNMT3B in mammary carcinoma cells and it has been postulated that the oncogenic effects of hGH in these cells is secondary to the increase in DNMTs that in turn causes silencing of the plakoglobin gene by hypermethylation of its promoter CpG island (Shafiei *et al.* 2008).

Although DNMT expression and function can be regulated by hormones, the cited studies show mostly correlative effects and the molecular mechanisms involved are still poorly understood.

Gene repression by DNA methylation: link to histone modifications

In general, DNA methylation leads to repression of gene expression. Normally, methylated genes are either not transcribed into mRNA or transcribed at a markedly reduced rate (Esteller 2005, Feinberg *et al.* 2006). Cytosine methylation alters the structure of the major groove of DNA, and this can block the recruitment of transcription factors to their binding sites and inhibit gene transcription. However, although DNA methylation can affect association of several transcription factors (including E2F, MLTF, or CTCF) to their recognition sites, other transcription factors, such as Sp1, can still bind to methylated DNA. In addition to altering transcription factor binding, DNA methylation allows the recruitment of proteins with high affinity for methylated CpGs (methyl binding proteins or MBDs)

that can repress gene expression. Five MBDs have been identified (MeCP2, MBD1, MBD2, MBD3, and MBD4), and excluding MBD4, they induce transcriptional silencing (Bird & Wolffe 1999). Histone deacetylation is linked to chromatin compaction and transcriptional repression, and after binding to methylated CpG dinucleotides, the MBD MeCP2 interacts with corepressor complexes containing histone deacetylases (HDACs) and Sin3a inducing gene silencing (Jones *et al.* 1998, Nan *et al.* 1998). Interestingly, the same corepressor complexes are recruited to hormone nuclear receptors and mediate ligand-independent repression of gene expression by some unliganded non-steroid receptors and by antagonist-bound steroid receptors (Aranda & Pascual 2001). Other MBDs interact directly with DNMT1 and with one or more HDACs (Fuks *et al.* 2000, 2001, Robertson *et al.* 2000, Rountree *et al.* 2000, Aapola *et al.* 2002, Kimura & Shiota 2003), suggesting the existence of a functional cooperation between CpG methylation and histone deacetylation that silences gene expression in a stable manner.

The N-terminal tails of histones can undergo different posttranslational modifications that seem to have a crucial influence on chromatin structure and are linked to either transcriptional repression or activation (Kouzarides 2007). Whereas histone acetylation is normally associated with transcriptionally active chromatin, methylation of histone H3 at particular residues can lead to local formation of heterochromatin, which in contrast with DNA methylation that causes stable transcriptional repression is fully reversible. The existence of a cross talk between DNA methylation and histone modifications, which can be mediated by interactions between histone methyltransferases and DNMTs, has been recently demonstrated (Fig. 2). Relationships between DNA methylation and histone modification have implications for understanding normal development as well as somatic cell reprogramming and tumorigenesis (Cedar & Bergman 2009). Thus, histone deacetylation facilitates methylation of H3K9 by the histone methyltransferase G9a. Methylation of this residue facilitates the recruitment of the

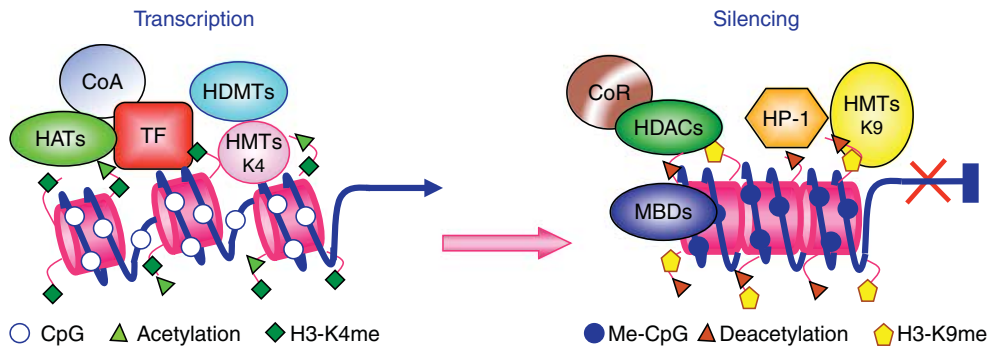


Figure 2 DNA methylation causes gene silencing. Transcriptionally active chromatin is characterized by unmethylated DNA and binding of transcription factors (TF) with recruitment of coactivators (CoA) and histone-modifying enzymes such as histone acetyltransferases (HATs), methyltransferases (HMTs), and demethylases (HDMTs), which induce among other modifications of histone acetylation and H3 di- or tri-methylation in lysine 4 (H3-K4me, a marker of active chromatin). CpG methylation (Me-CpG) silences gene expression by recruitment of methyl-binding proteins (MBDs), the heterochromatin protein HP-1, corepressor (CoR) complexes containing histone deacetylases (HDACs), and histone methyltransferases leading to deacetylation of the histone tails and methylation of H3 in lysine 9 (H3-K9me). These silencing complexes produce chromatin compaction. Many other epigenetic factors are also known to control gene transcription and silencing and therefore this figure illustrates only an example of regulation of gene expression.

heterochromatin protein 1 (HP1), allowing local heterochromatinization. The histone methyltransferase G9a also recruits DNMT3A and DNMT3B, inducing *de novo* DNA methylation, at the final step of silencing of pluripotency genes (Feldman *et al.* 2006). These functions of the histone methyltransferase-containing complexes appear to play an important role in post-implantation gene repression by DNA methylation and represent a basic example of the cross talk between DNA methylation and histone posttranslational modifications.

DNA methylation and endocrine diseases

Epigenetic changes are not only responsible for normal development, but they are also involved in disease. Genetic lesions, including mutations, deletions, or breakages, are well known to produce disorders in humans, but there is increasing evidence that diseases can also be caused by epigenetic alterations. Changes in DNA methylation can cause silencing of normally active genes or activation of normally silent genes. This could occur in cancer, in hereditary disorders resulting from DNA methylation defects, and in late-onset diseases caused by the interaction of genomic, epigenetic, and environmental changes (Robertson 2005, Feinberg 2007).

The Beckwith–Wiedemann syndrome, an overgrowth disorder characterized by an increased risk of cancer and different malformations, is an example of a single-gene epigenetic disease. Some patients with Beckwith–Wiedemann syndrome show loss of imprinting of insulin-like growth factor 2 (IGF2), leading to an

increase in the levels of this growth factor (DeBaun *et al.* 2002). A human disorder with multiple hormone resistance, the pseudohypoparathyroidism type IA (PHPIA), is also caused by tissue-specific differential imprinting of splice variants of the guanine nucleotide regulatory protein (encoded by GNAS1; Lalande 2001). Other class of monogenic diseases involves mutation of genes involved themselves in methylation machinery. For instance, Rett syndrome is caused by mutations that affect the methyl-CpG-binding protein MeCP2. Rett syndrome is a neurodevelopmental disorder that affects mainly females. Among other symptoms, affected patients can have seizures, present intellectual disability with learning difficulties, and have no verbal skills. This phenotype argues for the primacy of MeCP2, as opposed to other MBDs, in the silencing mechanism associated with DNA methylation, at least in the central nervous system. In Rett syndrome, DNA methylation is not altered, but gene silencing is abnormal because DNA methylation goes unrecognized (Bienvenu & Chelly 2006).

Gene hypomethylation is a common event in cancer, although site-specific hypermethylation and other epigenetic chromatin modifications are also found in tumors (Cedar & Bergman 2009). A large number of cancer-related genes that are silenced or activated by aberrantly methylated CpG islands have been identified in different types of tumors indicating that epigenetic events play a key role in tumorigenesis (Costello *et al.* 2000). Many growth-promoting genes are activated in tumors due to DNA hypomethylation and, not surprisingly, some major hypermethylated genes in cancer include tumor suppressors (Feinberg & Vogelstein 1983). In the case of an imprinted tumor suppressor

gene, hypermethylation of only one allele is sufficient to cause a total loss of its tumor suppressor function because the other allele is already inactive (Feinberg *et al.* 2006). On the contrary, loss of imprinting of an oncogene can lead to overexpression of the oncogenic protein and consequently to increased risk of tumor formation. Importantly, lymphocytes in ~10% of the human population have loss of imprinting at the IGF2 locus. Interestingly, the IGF2 receptor (IGFR2) gene that functions as a tumor suppressor is not imprinted in humans but is imprinted and has a maternal expression in mice. Therefore, it has been predicted that the incidence of tumors resulting from IGFR2 inactivation should be lower in humans than in mice (Jirtle & Skinner 2007).

DNA methylation of tumor suppressor genes is common in endocrine cancer. To give a few examples, epigenetic gene silencing of tumor suppressor genes, including E-cadherin, PTEN, RASSF1A, and FGFR2, and of differentiation-related genes, such as TSH receptor and the sodium-iodide symporter, has been reported in thyroid cancers (Kondo *et al.* 2006, 2007, Xing 2007). Frequent promoter hypermethylation of the APC, RASSF1A, or Rap1GAP tumor suppressors is found in parathyroid tumors (Juhlin *et al.* 2010). von Hippel-Lindau inactivation by promoter methylation is an important pathway for the development of malignant sporadic pancreatic endocrine tumors (Schmitt *et al.* 2009) and pheochromocytomas (Cascon *et al.* 2004), and loss of pRb and neuronatin expression is associated with promoter hypermethylation in pituitary adenomas (Simpson *et al.* 2000, Dudley *et al.* 2009, Revill *et al.* 2009).

The existence of hormone-independent tumors is a substantial problem for the treatment of several endocrine tumors with hormone antagonists. Hypermethylation can selectively silence multiple promoters of steroid receptors in hormone-dependent cancers, including ovarian, endometrial, breast, and prostate cancer. Aberrant DNA methylation of the promoter region of estrogen (ER), progesterone (PR), and androgen (AR) receptors can play an important role in the loss of hormone dependence, although resistance to hormonal treatment can appear even in the presence of steroid receptor expression (Lapidus *et al.* 1996, Jarrard *et al.* 1998, Sasaki *et al.* 2003, Asada *et al.* 2008). Interestingly, DNMT inhibitors such as 5-aza-2'-deoxycytidine and HDAC inhibitors such as trichostatin A have been successfully used to induce ER expression in ER-negative breast cancer cells (Ferguson *et al.* 1995) and to sensitize hormone-resistant ER-negative breast cancer cells to tamoxifen (Jang *et al.* 2004). It is increasingly recognized that epigenetic gene silencing plays a major role in tumor initiation and progression in hormone-dependent cancers. Indeed, in the first genome-wide DNA methylation profiling according to

the receptor status of breast cancer, it has been found that ER/PR status affects the global DNA methylation profile (Li *et al.* 2010).

Expression of other non-steroid hormone nuclear receptors has also been found to be inactivated by promoter DNA methylation in tumors. This is the case for the thyroid hormone receptor β isoform (TR β), which can function as a tumor suppressor (Martinez-Iglesias *et al.* 2009, Garcia-Silva *et al.* 2011). The TR β promoter is methylated in early stage breast cancer tumors (Li *et al.* 2002). The same occurs with the retinoic acid receptor β (RAR β). Retinoic acid (RA), an active metabolite of vitamin A, plays a key role in development and has important antitransforming actions in many types of cancer cells. RAR β is one of the tumor suppressor genes that are more often epigenetically inactivated in tumors (Esteller *et al.* 2002). Very recently, it has been found that RA plays a key role in the fate of intestinal cells through regulation of DNA demethylase activity (Rai *et al.* 2010). DNA demethylation has been proposed to involve the cooperative actions of proteins from the cytidine deaminase family (activation-induced deaminase and Apobec2), the G:T mismatch-specific glycosylase family (MBD4), and a DNA repair protein family (GADD45; Rai *et al.* 2008). However, the role of GADD45 in promoting DNA demethylation is still controversial (Jin *et al.* 2008) and DNA methylation is conserved in genetically modified mice lacking GADD45 (Engel *et al.* 2009). Loss of the adenomatous polyposis coli (APC) tumor suppressor gene is the key initiating step in a model of genetic and epigenetic events that lead to colorectal cancer. Loss of APC causes upregulation of a DNA demethylase system and the concomitant hypomethylation of key intestinal cell fating genes, whereas RA strongly downregulates demethylase components, thereby promoting DNA methylation of key genes and helping progenitor cells commit to differentiation (Rai *et al.* 2010).

Hormonal control of DNA methylation/demethylation at target promoters

Although the mechanisms of DNA demethylation are not yet totally understood and remain a controversial issue (Ooi & Bestor 2008), recent data obtained studying the effect of hormones, mainly ligands of nuclear receptors, on transcription of their target genes indicate that they can mediate active DNA demethylation occurring independently of DNA replication. One of the first evidence was obtained with the glucocorticoid receptor (GR). Glucocorticoids cause local DNA demethylation of the tyrosine aminotransferase (*Tat*) gene around a glucocorticoid response element that is located 2.5 kb upstream of the

transcriptional start site. Kress *et al.* (2006), showed that demethylation is a consequence of an active mechanism that involves the creation of DNA nicks 3' to the methylcytosine and the participation of a demethylase initiating a base excision repair.

GR-dependent demethylation was slow since it was detected after a few hours of stimulation. However, a rapid methylation/demethylation at the ER target gene pS2 has been observed in breast cancer cells (Kangas-peska *et al.* 2008, Metivier *et al.* 2008). It had been previously shown that ER is recruited in a cyclic manner to the pS2 promoter and that this is followed by the assembly of coactivators, which in turn provokes local structural changes in chromatin. This allows the engagement of the basal transcription machinery and the activation of the RNA polymerase II, which is then followed by the recruitment of corepressor complexes and the initiation of the next transcriptional cycle (Metivier *et al.* 2003). Remarkably, these changes are accompanied by changes in the methylation of CpG dinucleotides in the pS2 promoter and breaks in the sugar–phosphate backbone of DNA. Cyclical methylation and demethylation of CpG dinucleotides had a periodicity of around 100 min, implying the existence of an active demethylation process. Methylation takes place at each transcriptional cycle after the occupancy of ER and polymerase II and is connected with the recruitment of MeCP2 and DNMT1 to the pS2 promoter. Recruitment of MBD2 and MBD3 occurs later, simultaneously with the recruitment of the remodeling complex NuRD. CpG methylation at the pS2 promoter is strand specific, occurring primarily in the transcribed strand. DNMTs appear to exhibit dual actions during these cycles, being involved in CpG methylation and also in active demethylation of 5mCpGs through deamination. As stated above, demethylation of the *Tat* gene involves the formation of DNA nicks, and CpG methylation/demethylation at the pS2 promoter also involves a base excision repair process with base substitution (Metivier *et al.* 2008). The following process has been suggested: deamination of 5mC by DNMT3A or DNMT3B would result in a T/G mismatch, which would be repaired by a thymine DNA glycosylase by excision of the mispaired cytosine, generating an abasic residue. This would be followed by the action of apurinic/aprimidinic endonuclease I, which would create a single nucleotide gap by cleaving the phosphodiester bond 5' to the excised base. Then, DNA polymerase β would replace the missing nucleotide and finally a DNA ligase would end the repairing process (Reid *et al.* 2009; Fig. 3).

Another example of rapid methylation/demethylation in a hormonally regulated promoter has been provided by the cytochrome p450 27B1 (CYP27B1), the final enzyme in vitamin D biosynthesis (Kim *et al.* 2009). CYP27B1 gene expression is regulated by two calcemic

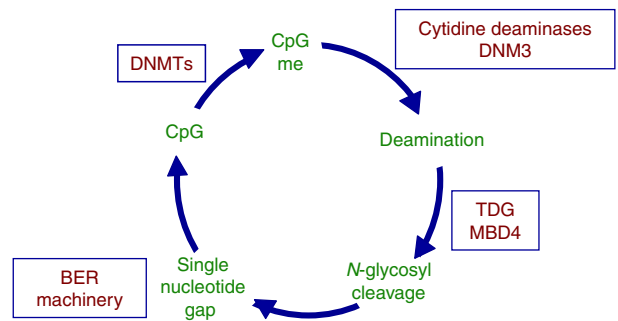


Figure 3 Proposed model for active DNA methylation/demethylation at steroid hormone target promoters. The process would involve deamination to thymidine resulting in T/G mismatch. The action of a thymine DNA glycosylase (TDG) such as MBD4 would initiate a process of repair that would be completed by the base excision repair (BER) machinery leading to unmethylated CpG, which can then be again methylated by DNMTs starting a new cycle.

hormones: parathyroid hormone (PTH) and vitamin D. PTH activates CYP27B1 transcription through stimulation of protein kinases A and C (PKA and PKC), whereas vitamin D through binding to its nuclear receptor, VDR, represses CYP27B1 transcription. A basic helix–loop–helix transcriptional activator (VDR-interacting repressor, VDIR) binds to the CYP27B1 promoter and VDR interacts with VDIR and represses transcription by means of recruiting a HDAC corepressor complex (Murayama *et al.* 2004). Reflecting vitamin-D-mediated transrepression of the CYP27B1 gene, rapid methylation of CpG sites is induced by vitamin D in this gene promoter. This methylation step requires DNMT1 and DNMT3B. Conversely, treatment with PTH causes active CpG demethylation of the CYP27B1 promoter. Purification of a VDIR-associated complex showed the presence of both DNMTs and the DNA glycosylase MBD4 in the complex. Activation of PKC by PTH causes MBD4 phosphorylation and activation. The DNA glycosylase would cause the excision of the mispaired nucleotide and a base excision repair process would achieve CpG demethylation of the promoter (Kim *et al.* 2009). Thus, the results obtained with the CYP27B1 promoter further suggest that DNA methylation/demethylation plays an important role in hormonal regulation of transcription.

Other member of the CYP superfamily, oxysterol 7 α -hydroxylase (CYP7B1), is also regulated by DNA methylation. The nuclear receptor peroxisome proliferator-activated receptor α (PPAR α) represses liver expression of CYP7B1 in females but not in males. This inhibition appears to involve a sex-specific sumoylation of the receptor and is mediated by methylation of a Sp1 site in the CYP7B1 promoter, which contains a CpG dinucleotide. Methylation of this CpG inhibits binding of Sp1 to the promoter. Furthermore, complexes containing MBDs, NCoR,

HDACs, and histone methyltransferases are recruited to methylated DNA (Yoon *et al.* 2003), and it was found that these complexes are involved in inhibition of *CYP7B1* gene transcription by PPAR α (Leuenerger *et al.* 2009).

DNA methylation and the persistent neonatal effects of glucocorticoids in the brain

Exposure to stress during neurodevelopment has an effect on the quality of physical and mental health. Maternal care might influence hypothalamic–pituitary–adrenal (HPA) function in the rat through epigenetic programming of GR expression (Weaver *et al.* 2007). In humans, childhood abuse alters HPA stress responses and increases the risk of suicide. Decreased levels of *GR* mRNA and increased methylation of the GR promoter were found in hippocampus from abused suicide victims that also showed a decrease in NGFI-A transcription factor binding. These findings translate previous results from rat to humans and suggest a common effect of parental care on the epigenetic regulation of hippocampal GR expression (McGowan *et al.* 2009). The most normally used technique to induce early life stress is periodic infant–mother separation during the neonatal period. This causes an irreversible increase in glucocorticoid secretion with disruption of the activity of the HPA axis and increased sensitivity to stress later in life, which are related to disorders of mood and cognition. Murgatroyd *et al.* (2009) have examined the expression of AVP and CRH in early life stress. These hypothalamic secretagogues regulate HPA axis activity by increasing ACTH expression. They found that neonatal stress induces lifelong hypomethylation of the *AVP* gene, causing increased AVP expression, activation of the HPA axis, and behavioral alterations. They found that hypomethylated CpG residues serve as DNA-binding sites for MeCP2 that is phosphorylated by CaMKII after depolarization of hypothalamic cells. Thus, this study defines this methyl CpG-binding protein as an important component in the epigenetic programming of neuroendocrine and behavioral functions.

There is evidence for hypercortisolemia playing a role in the generation of psychiatric symptoms and for epigenetic variation within HPA axis genes mediating behavioral changes also in the adult. When mice are treated with corticosterone, they exhibit anxiety-like behavior together with a significant decrease in the hippocampal mRNA levels of GR and an increase in the stress-related gene *Fkbp5*. This gene encodes a co-chaperone of hsp90 that binds to GR and promotes cytoplasmic localization of the receptor, regulating GR sensitivity. Differences were seen in *Fkbp5* methylation

in hippocampus and hypothalamus of glucocorticoid-treated animals. The same occurs in a mouse hippocampal neuronal cell line exposed to corticosterone. This suggests that DNA methylation plays a role in mediating effects of glucocorticoid exposure on FKBP5 function, with potential consequences for behavior (Lee *et al.* 2010).

Lower weight at birth is associated not only with risk of metabolic syndrome but also with cardiovascular disease and hypertension in adulthood (Stein *et al.* 1996, Heijmans *et al.* 2008). The renin–angiotensin system appears to play a role in this process since a maternal low-protein diet results in undermethylation of the *At1b* angiotensin receptor promoter and early overexpression of this gene in the adrenal of offspring (Bogdarina *et al.* 2007). Furthermore, maternal glucocorticoids modulate this effect on fetal DNA methylation since treatment of rat dams with the 11- β -hydroxylase inhibitor metyrapone prevents the epigenetic change and hypertension in the offspring (Bogdarina *et al.* 2010). Collectively, these studies suggest that DNA methylation might have an important role on the long-term effects of glucocorticoids in neonatal brains. However, as the absolute changes are low and the cell-type composition was not always systematically analyzed, further studies are needed to determine the functional role of epigenetic factors in the long-term effects of early development.

The pro-opiomelanocortin (*POMC*) gene plays an important role not only in the regulation of the HPA axis and adrenal development but also in obesity. The *POMC* gene is activated in ACTH-dependent Cushing's syndrome. This disorder may be a consequence of activation of the highly tissue-specific *POMC* promoter in pituitary and non-pituitary sites. This promoter contains a CpG island, which is methylated in normal non-expressing tissues, but is specifically demethylated in expressing tissues and tumors (Newell-Price *et al.* 2001). Methylation near the response element for the tissue-specific *POMC* activator PTX1 abolishes binding of this transcription factor that plays a key role in pituitary development. It has been suggested that the *POMC* promoter could show different degree of methylation in *POMC*-expressing hypothalamic neurons, thus influencing food intake and obesity (Newell-Price 2003).

DNA methylation and gonadal hormones in the developing brain

Many brain sex-specific features arise from effects of the gonadal steroid hormones, which are exerted during the perinatal period. Testosterone is converted into estradiol within developing neurons, and estradiol mediates several developmental sex differences in cell

anatomy and physiology. These differences are responsible for dimorphic regulation of pituitary gonadotropin secretion and for sex-specific behavior (McCarthy 2008). Alterations in DNA methylation of genes essential for sexual brain differentiation, including ER α , ER β , and the PR themselves, have been described to be sex and hormonally regulated. Methylation of the ER α promoter within the preoptic area, which is crucial for sexual behavior, is influenced by maternal care during the neonatal period in rodents. Thus, maternal licking and grooming has been demonstrated to alter ER α promoter methylation, and consequently ER α expression, in a sex-specific manner (Champagne *et al.* 2006). In addition, methylation of the ER α gene in the preoptic area is higher in newborn females than in males or estradiol-treated females, and sex and hormone-mediated differences in methylation were also observed at later stages (Kurian *et al.* 2010). While ER α activation is required for neonatal brain masculinization, ER β activation appears to be involved in brain defeminization and in the regulation of neuroendocrine functions, since this receptor colocalizes with neuroendocrine hormones such as GnRH, CRH, oxytocin, vasopressin, or prolactin. CpG methylation of the ER β gene in the preoptic area, hypothalamus, or hippocampus of newborns is not significantly influenced by sex or hormonal treatment. However, there are differences in ER β methylation in these brain areas in the adult (Schwarz *et al.* 2010). On the other hand, whereas in hypothalamus of newborn animals no sex differences in PR promoter methylation are detected, significantly lower levels are found in adolescent females than males. It has been suggested that gonadal female hormones promote PR methylation in the hypothalamus, silencing PR expression during the critical period of sexual differentiation in the male, a process that is essential for masculine behavior in the adult.

Whereas DNA methylation can be profoundly influenced by gonadal hormones during brain development, the effect of these hormones in the adult brain has received less attention. However, it has been recently reported that testosterone regulates expression of vasopressin (AVP) within the bed nucleus of the stria terminalis (BST) in the adult brain. Castration of male rats strongly inhibits AVP expression in this nucleus, and this inhibition is reversed on testosterone treatment. It was found that castration results in AVP promoter methylation at specific CpG sites in the BST. Conversely, castration significantly increased ER α mRNA levels by decreasing ER α promoter methylation. These results suggest that the DNA methylation pattern of some steroid responsive genes is actively regulated by gonadal steroid hormones in the adult brain. It is intriguing the opposite regulation of ER α and AVP promoter methylation in response to changes in steroid

hormone levels in the same brain region, and how this specificity is regulated remains to be elucidated. In any case, these results indicate that regulation of DNA methylation in the adult brain could play a role in the hormonal control of behavior (Auger *et al.* 2011).

Endocrine disruptors and their possible transgenerational effects

Epigenetic factors can be altered by the environment (Fraga *et al.* 2005, Baryshnikova *et al.* 2008, Stidley *et al.* 2010), and increasing evidence suggests that a large variety of environmental and dietary chemicals can interfere with normal endocrine functions and result in adverse consequences. Initial animal studies demonstrated that compounds with estrogenic activity can disrupt reproductive tract function. However, it is now evident that different chemical compounds, referred to as 'endocrine disruptors', can either mimic or interfere with the normal actions of hormones including not only sexual steroids, but also thyroid, hypothalamic, and pituitary hormones (Newbold *et al.* 2006). The effect of endocrine disruptors is particularly strong when exposure occurs during fetal or neonatal periods. If exposure occurs during these critical stages, it can produce permanent effects that may be observed much later in life. For instance, exposure of newborn mice to environmental estrogens causes uterine lesions and uterine tumors in adults. Mechanistic studies provided support that estrogens cause both genetic and epigenetic alterations in developing target tissues. Thus, the estrogen-responsive genes lactoferrin and c-fos are permanently upregulated in the uterus after developmental exposure to the estrogen-like compound diethylstilbestrol (DES) due to hypomethylation of the promoter region of these genes after exposure to this chemical (Li *et al.* 1997, 2003). DES was prescribed during many years to pregnant women to prevent spontaneous abortions. More recently, Tang *et al.* (2008) identified 14 genes whose methylation patterns are altered after neonatal treatment with DES or genistein (other estrogenic compound), among them the gene encoding the nucleosomal binding protein 1 (NSBP1), a nucleosome core particle binding protein that plays a role in chromatin remodeling. On the other hand, bisphenol-A is a non-steroidal estrogen that is ubiquitous in the environment. Bromer *et al.* (2010) have recently demonstrated that methylation of the *Hoxa10* gene was decreased in the reproductive tract of mice exposed *in utero* to bisphenol-A. Decreased DNA methylation led to an increase in binding of ER α to the *Hoxa10* promoter and to increased estrogen-dependent transcription. Permanent epigenetic alteration of sensitivity to estrogen may then be a mechanism through which endocrine disruptors exert their action.

The demonstration that many estrogenic compounds show lifelong effects in animals has raised the concern that fetal and neonatal exposure to these compounds in humans could also produce epigenetic changes and impact negatively human health. For instance, human fetuses can be exposed to high estrogen levels, due to unintentional continuation of birth control pill intake by the mother before detection of pregnancy. On the other hand, exposure of the mother to environmental estrogen disruptors or intake of high levels of phytoestrogens can affect the fetus and even the infant during breastfeeding, with possible adverse consequences (Prins 2008).

Although epigenetic transgenerational inheritance is a controversial issue at the moment, results obtained with experimental animals have suggested that adverse effects caused by endocrine disruptors may be transmitted to subsequent generations. Environmental factors may induce epigenetic changes in the germ line, which would be passed to the progeny (Jirtle & Skinner 2007). In agreement with this hypothesis, the susceptibility for tumors after treatment with DES was transmitted to the descendants through the maternal germ cell lineage (Newbold *et al.* 1998), and prenatal exposure to the endocrine disruptors vinclozolin (an antiandrogenic compound) or methoxychlor (an estrogenic compound) induced an adult phenotype in the F₁ generation of decreased spermatogenic capacity and increased incidence of male infertility. Furthermore, these effects were described to be transferred through the male germ line to nearly all males of several generations (Anway *et al.* 2005). However, the transgenerational effects have not been reproduced by other groups (Renner 2009), casting some doubts on the transmission of the effects of vinclozolin beyond the F₁ generation. In contrast, a recent study has examined genome-wide promoter DNA methylation alterations in the sperm of F₃ generation rats whose F₀ generation mother was exposed to vinclozolin (Guerrero-Bosagna *et al.* 2010). This study has identified 52 different regions with altered methylation in the sperm promoter epigenome, suggesting again that an endocrine disruptor could have the ability to induce epigenetic transgenerational changes. As the DNA methylation differences reported in this study are very marginal, further investigations are needed to fully characterize the putative heritability of epigenetic factors.

Type 2 diabetes, metabolism, and DNA methylation

Insulin plays a key role in metabolic control, and therefore, regulation of insulin gene expression has been extensively analyzed. However, only recently it has

been shown that the insulin promoter is demethylated specifically in pancreatic β cells both in humans and in mice. The insulin gene is methylated in mouse embryonic stem cells and becomes demethylated on differentiation into insulin-expressing cells. Methylation of a specific CpG located in a cAMP response element (CRE) of the insulin promoter inhibits association of the transcription factors CREB and ATF2 that bind to the CRE, while inducing MeCP2 recruitment, leading to a strong reduction of promoter activity (Kuroda *et al.* 2009). Therefore, promoter demethylation may play an important role in the specific expression of the insulin gene in pancreatic β cells.

Abnormal nutrition during embryonic development has been shown to influence disease susceptibility in the descendants. The global prevalence of obesity and type 2 diabetes is increasing, and parent obesity is a risk factor for developing obesity in childhood (Whitaker *et al.* 1997). In rodents, it has been shown that when mothers are fed with a high-fat diet (HFD), male offspring exhibit increased body weight and are diabetic and insulin resistant. Furthermore, the offspring of these males also present insulin resistance, showing that fathers can start intergenerational inheritance of metabolic diseases (Dunn & Bale 2009). Accordingly, paternal HFD alters gene expression in pancreatic β cells of adult female offspring. The *I13ra2* gene, a gene belonging to the Jak–Stat signaling pathway, presented the highest difference in gene expression. An epigenetic mechanism appears to contribute to the altered *I13ra2* expression, since methylation at CpG –960 of *I13ra2* was significantly reduced in HFD offspring with respect to controls. This CpG is located in a putative recognition site for the transcription factor TCF-1A and for the methylated DNA binding protein NF-X. These results show that paternal HFD could affect metabolism of the offspring by epigenetic regulation of genes important for pancreatic β cell function (Ng *et al.* 2010).

Offspring of males fed a low-protein diet also exhibit metabolic disturbances. Epigenomic profiling of offspring livers reveals changes in cytosine methylation depending on paternal diet, including reproducible changes in DNA methylation of PPAR α , a key lipid regulator. These results indicate that parental diet can affect cholesterol and lipid metabolism in offspring and define a model system to study environmental reprogramming of the heritable epigenome (Carone *et al.* 2010).

Intrauterine growth restriction (IUGR) also increases susceptibility to age-related diseases, including type 2 diabetes. In a rodent model of IUGR, which develops diabetes in adulthood, global decreases in DNA methylation concomitant with a decrease in DNMT1, MeCP2, and HDAC1 is observed in tissues such as liver

or brain (Lillycrop 2011). Furthermore, it was found that expression of PDX1, a pancreatic and duodenal homeobox 1 transcription factor critical for β cell function and development, was permanently reduced in IUGR β cells and underwent epigenetic modifications: throughout development, there were epigenetic histone modifications, but after the onset of

diabetes in adulthood, the CpG island in the proximal *Pdx1* promoter was methylated, resulting in permanent silencing of the *Pdx1* locus (Park *et al.* 2008). Thompson *et al.* (2010) have generated the first DNA methylation map at almost 1 million unique sites throughout the rat genome in normal pancreatic islet cells, allowing the identification of the changes that

Table 1 Examples of endocrine conditions associated with DNA methylation changes

Endocrine condition	Alteration	References
Beckwith–Wiedemann PHPIA Endocrine cancers	Loss of imprinting of IGF2 Differential imprinting of GNAS1 Methylation of tumor suppressor genes	DeBaun <i>et al.</i> (2002) Lalande (2001) Cascon <i>et al.</i> (2004), Kondo <i>et al.</i> (2006, 2007), Xing (2007) and Juhlin <i>et al.</i> (2010)
Type 2 diabetes	Aberrant promoter methylation of ER α , PR, AR, TR β , and RAR β Regulation of DNA demethylase activity in intestinal cancer Paternal high-fat diet decreases <i>I13ra2</i> promoter methylation in pancreatic β cells of the offspring Paternal low-protein diet alters PPAR α methylation in livers of the offspring Intrauterine growth restriction decreases globally DNA methylation as well as DNMT1 and MeCP2 expression Permanent methylation of the <i>Pdx1</i> gene in β cells after intrauterine growth restriction Intrauterine growth restriction causes changes in DNA methylation in the proximity of genes with important roles in β cell function and development	Esteller <i>et al.</i> (2002), Li <i>et al.</i> (2002) and Asada <i>et al.</i> (2008) Rai <i>et al.</i> (2010) Ng <i>et al.</i> (2010) Carone <i>et al.</i> (2010) Lillycrop (2011) Park <i>et al.</i> (2008) Thompson <i>et al.</i> (2010)
Neuroendocrine disorders of mood and cognition	HNF4 α methylation in offspring of malnourished mothers PPAR γ C1 α methylation after overfeeding Maternal care affects brain GR methylation in rodents GR methylation in childhood abuse victims AVP hypomethylation and upregulation of HPA axis after infant–mother separation Glucocorticoids decrease GR expression and reduces methylation of the anxiety-related gene <i>Fkbp5</i>	Sandovici <i>et al.</i> (2011) Brons <i>et al.</i> (2010) Weaver <i>et al.</i> (2007) McGowan <i>et al.</i> (2009) Murgatroyd <i>et al.</i> (2009) Lee <i>et al.</i> (2010)
Cardiovascular disease and hypertension in adulthood	Maternal low-protein diet induces undermethylation of At1b angiotensin receptor promoter in the adrenal of the offspring Glucocorticoid administration prevents At1b methylation and hypertension in the offspring	Bogdarina <i>et al.</i> (2007) Bogdarina <i>et al.</i> (2010)
Cushing's syndrome	Demethylation of the POMC promoter in non-pituitary cells and tumors	Newell-Price (2003)
Sexual hormones and the brain	Maternal care alters ER α promoter methylation in the preoptic area of the brain and sexual behavior Higher ER α methylation in the preoptic area of females Sexual differences in ER β methylation in various brain areas Castration of male rats causes methylation of the AVP promoter and reduces ER α methylation in the bed nuclei of the stria terminalis Increased methylation of the memory suppressor gene reelin by estradiol	Champagne <i>et al.</i> (2006) Kurian <i>et al.</i> (2010) Schwarz <i>et al.</i> (2010) Auger <i>et al.</i> (2011) Zhao <i>et al.</i> (2010)
Endocrine disruptors	Hypomethylation of estrogen-responsive genes in the uterus after treatment with DES Changes in <i>NSBP1</i> promoter methylation after neonatal treatment with DES or genistein Undermethylation of the <i>Hoxa10</i> gene in the reproductive tract of mice exposed <i>in utero</i> to bisphenol-A Increased expression of DNMT3A and DNMT3L in testis and prostate of rats exposed to vinclozolin during gonadal sex determination Altered methylation pattern in 52 regions of the sperm in descendants of rats exposed to vinclozolin	Lee <i>et al.</i> (2003) Tang <i>et al.</i> (2008) Bromer <i>et al.</i> (2010) Anway <i>et al.</i> (2008) and Cowin <i>et al.</i> (2010) Guerrero-Bosagna <i>et al.</i> (2010)

occur as a consequence of IUGR. Comparison of growth restricted with normal rats revealed changes in DNA methylation at a number of novel loci, not limited to canonical CpG islands or promoters. The specific loci affected are in proximity to genes with important roles in β cell function and development, suggesting that that epigenetic dysregulation is a strong candidate for propagating the cellular memory of intrauterine events, causing changes in expression of nearby genes and long-term susceptibility to type 2 diabetes.

Low birth weight and unhealthy diets are also risk factors for metabolic disease including type 2 diabetes. Genetic, non-genetic, and epigenetic data propose a role of the key metabolic regulator PPAR γ coactivator 1 α (PPAR γ C1 α) in the development of type 2 diabetes. When challenged with high-fat overfeeding, low birth weight subjects develop insulin resistance and reduced PPAR γ C1 α and *OXPLOS* gene expression. PPAR γ C1 α methylation was significantly higher in low birth weight subjects during the control diet. However, PPAR γ C1 α methylation increased in only normal birth weight subjects after overfeeding. These changes are reversible, supporting that DNA methylation induced by overfeeding is reversible in humans (Brons *et al.* 2010). Moreover, in a very recent study, it has been shown that the increased risk of type 2 diabetes in the offspring of malnourished mothers was associated with the decreased expression of the orphan nuclear receptor HNF4 α , previously linked with this type of diabetes. Specifically, a pancreas-specific enhancer of HNF4 α expression is epigenetically inactivated by DNA methylation in the adult offspring of poorly nourished mothers, revealing a novel mechanism by which maternal diet and aging interact through epigenetic processes to determine the risk of age-associated endocrine diseases (Sandovici *et al.* 2011).

Conclusions and perspectives

Epigenetic mechanisms may play a key role in normal endocrine physiology, as well as in the development of endocrine diseases. A summary of the changes in DNA methylation observed to date in relation to different endocrine conditions is illustrated in Table 1. A better knowledge of the association between epigenetic mechanisms and endocrine function should lead to a better understanding of the molecular basis of endocrine disorders and could help to the development of novel therapeutic strategies. DNA methylation acts at many levels to regulate hormonal actions: during development, in response to environmental factors and endocrine disruptors, in endocrine cancer, endocrine therapies, etc. Although many aspects of endocrine system epigenetics are still unknown, the recent advances described here shed a new light on the

importance of this epigenetic modification in the functioning of the endocrine glands and on the response of target tissues to hormones. The recent development of ultra-high-throughput technologies will allow the description of the whole genome DNA modifications that occur in endocrine physiology and pathology. These data hold the potential of defining the epigenetic signatures associated with endocrine disorders for future use as diagnostic markers and can lead to the development of novel epigenetic drugs with therapeutic applications in specific endocrinological diseases.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

Funding

The laboratory of A A is supported by Grants BFU2007-62402 from Ministerio de Ciencia e Innovación, RD06/0020/0036 from the Fondo de Investigaciones Sanitarias and by the European Grant CRES-CENDO (FP-018652). The Cancer Epigenetics Unit at the IUOPA is supported by grants from the Spanish Ministry of Health (PS09/02454) and the Community of Asturias (FICYT IB09-106). The IUOPA is supported by the Obra Social Cajastur, Spain.

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Received in final form 6 July 2011

Accepted 28 July 2011

Made available online as an Accepted Preprint 29 July 2011