1.1 Historical Introduction

The modified base 5-methyl cytosine (5-mC) was first detected in DNA by Hotchkiss [1]. In subsequent years, many other modified bases were discovered in bacteria, and it became clear that they were an integral part of the modification-restriction systems that are widely distributed in prokaryotes. It was shown that restriction enzymes could distinguish between modified and unmodified DNA substrates.

In plants, approximately 80% of cytosines at CpG doublets are methylated; however, methylation also frequently occurs in CpNpG triplets [2]. Recent studies have also found that methylation of cytosines can occur in non-symmetrical sites such as CpTpA [3]. Despite the overall high global levels of 5-mC, plants contain regions of unmethylated DNA that resemble the *HpaII* tiny fragments characteristic of mammalian genomes. These areas are typically unmethylated in a wide variety of tissues, whether or not the associated gene is expressed [4].

In mammalian DNA, the major modified base is 5-mC, at a level of 2%–5% of all cytosines. Small amounts of other modified bases should not be ruled out, and there is one reliable report that 6-methyl adenine exists in mouse DNA [5]. 5-mC occurs predominantly, but not exclusively, in CpG doublets [6]. In the total mammalian genome, the CpG doublet is very significantly less than what would be expected from the overall base composition of the DNA. However, there are CpG islands associated with structural genes, where such depletion is not observed.

Riggs [7] and Holliday and Pugh [8] proposed that 5-mC in mammalian DNA might have an important role in the regulation of gene expression. They argued that, since restriction enzymes can distinguish between modified and unmodified sites, there might also be regulatory proteins that could make the same distinction at specific DNA sequences in promoter regions. Instead of cutting the DNA, they would recognize the specific methylation signal and would thereby control the presence or absence of transcription at an adjacent gene. These authors also proposed that the pattern of DNA methylation could be inherited, if there was a maintenance DNA methyltransferase that recognized hemimethylated DNA just after replication and methylated the new strand. The same enzyme would not act on nonmethylated CpG doublets. This provided a basis for the epigenetic inheritance of a given pattern of DNA methylation and therefore also of the specific controls of gene expression in given cell types. It could also account for those cases, such as X-chromosome inactivation in female eutherian mammals, where only one of two homologous genes in a diploid cell is active, while the other is inactive. It was already known that these states of activity are very stable in dividing and nondividing cells. In 1975, when these papers appeared, there was no direct evidence for the hypotheses proposed, and the authors therefore did not suggest that methylation, or lack of methylation, was associated with gene activity. However, it later became clear that methylation is associated with the inactivity of genes in almost all cases. For example, in the inactive X chromosome, CpG islands adjacent to genes are methylated, and these same islands are unmethylated on the active X chromosome. It also became clear that the pattern of DNA methylation could be stably inherited, so DNA methyl-maintenance

late with the time to flowering. *FLC* mRNA and protein are downregulated by exposure of germinating seeds to low temperatures, the extent of downregulation being proportional to the duration of cold treatment, as is the promotion of flowering. Consistent with the idea that vernalization is regulated via methylation signals, plants containing antisense methyltransferase constructs flower early and have a reduced level of *FLC* transcript. Thus, the downregulation of *FLC* suggests that methylation may block expression of a repressor of *FLC* or perhaps the binding of a repressor to the *FLC* promoter [53].

If we assume that the pattern of DNA methylation changes in a controlled, orderly way throughout development, then it would not be surprising that fully differentiated cells of any one type would have an invariant pattern. This is precisely what has been observed [54, 55; Doerfler, see Chap. 2]. The aim of the human epigenome project (HEP) is to uncover the fine detail of DNA methylation in different cells and tissues. What is also needed is an analysis of the dynamic changes in DNA methylation during gametogenesis, early embryogenesis, and subsequent development. We believe that this will illuminate in many ways the innumerable interactions between, and changes within, cells and tissues, all of which are an integral part of the developmental process.

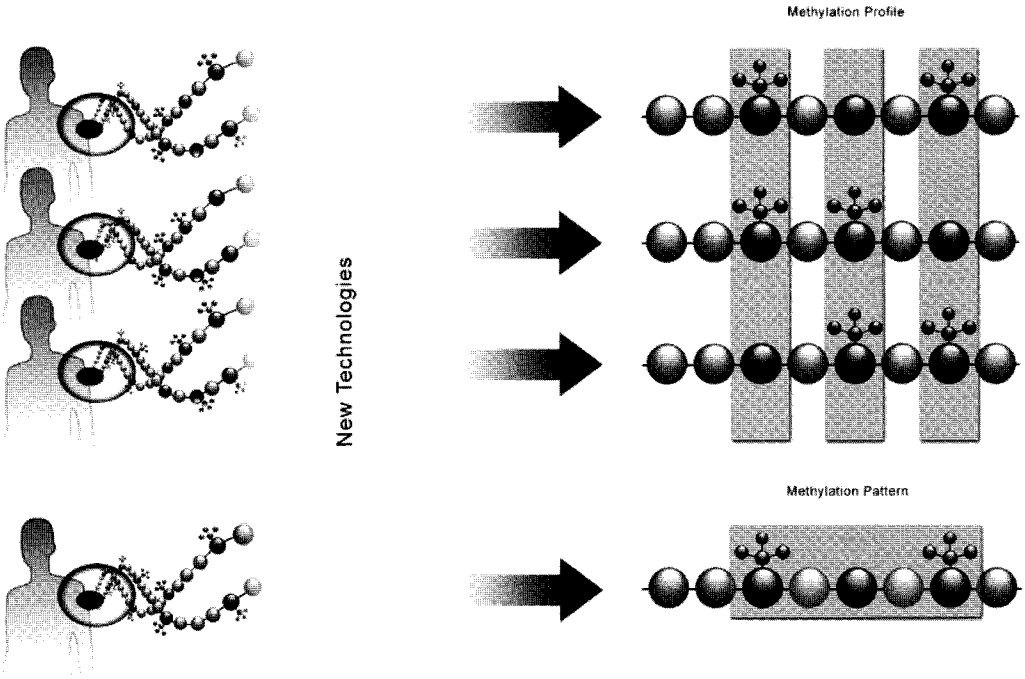
1.5
Abnormal DNA Methylation in Cancer Cells

It was first proposed nearly a quarter of a century ago that DNA methylation may be an important contributor to the process of carcinogenesis [56]. The first direct evidence for an alteration in methylation levels in cancer was the observation that global methylation levels in cancer were lower than the levels in normal cells [57]. Fienberg and Volgelstein further demonstrated that the decrease in global methylation levels was associated with the activation of cellular oncogenes such as *K-ras* in lung and colon tumors [58]. The hypomethylation in tumor cells most commonly occurs in repetitive and parasitic DNAs, which are characteristically hypermethylated in normal cells. This phenomenon could also correlate with the genetic instability seen in cancer due to the activation of endogenous transposable elements and other mobile elements found in the human genome.

In addition to hypomethylation, cancer cells exhibit regional hypermethylation. Frequent targets of hypermethylation are promoter regions within CpG islands. In 1986 the gene for calcitonin was the first to be identified as being abnormally methylated in cancer cells but not in normal tissues [59]. To date, at least 60 genes have been shown to be abnormally hypermethylated in cancers. A selection of these genes are listed in Table 1.1. This table is by no means exhaustive, since new genes are being identified with an ever-increasing frequency.

Several studies have demonstrated a direct role of abnormal CpG-island promoter methylation in gene silencing in cancer. Gene silencing had been shown in *Von-Hippel Landau* in renal cancer [60], in retinoblastoma [61], for GSTP1 in prostate cancer [62], as well as many in other cancer types as the primary inactivation event, since

3
Epi Meets Genomics: Technologies for Finding and Reading the 5th Base



Tab. 1.1 A selection of genes found to be hypermethylated in cancer (see http://www3.mdanderson.org/leukemia/methylation/cgi.html for updates to this list)

Gene	*Location*	*Cancer*	*Comments*
14–3-3 Sigma	1p	Breast/gastric	
ABL1	9q34.1	CML/AML	Only methylated when part of bcr-abl translocation
ABO	9q34	Cell lines	
APC	5q21	Colon, gastric, and esophageal	Invasion tumor architecture
Androgen Receptor	Xq11–12	Prostate/colon	Growth factor
BLT1		Various cell lines	
BRAC1	17q21	Breast/ovarian	DNA damage repair
Calcitonin	11p15	Colon, lung. and hematopic	One of first genes hypermethylated in cancer
Caspase 8	2q33–34	Neuroblastoma	Apotosis inhibitor
Caveolin 1	7q31.1	Breast cell lines	
CD44		Prostate	
CFTR	7q31.2	Cell lines	Not primary tumors
COX2	1q25.2–25.3	Colon, breast. and prostate cell lines	Correlates with expression
CSPG2	5q12–14	Colon	Regulated by RB
CX26	13q11–12	Breast cell lines	
Cyclin A1	13q12.3–13	Cell lines	
DBCCR1	9q32–33	Bladder	Also low level in normal bladder
E-cadherin	16q22.1	Breast, gastric, thyroid, SCC, leukemias. and liver	Cell–cell adhesion
Endothelin Receptor B	13q22	Prostate	Growth factor response
EPHA3	3p11.2	Leukemias	
Estrogen Receptor	6q25.1	Colon, liver, heart, lung. and leukemias	Growth factor response
FHIT	3p14.2	Esophageal	
Glypican 3	Xq26	Mesothelioma/ovarian	
GSTP1	11q13	Prostate, liver, colon, breast, and kidney	DNA damage repair
H19	11q15.5	Wilm's	Imprinted gene
H-cadherin	16q24.1–2	Lung/ovarian	
HIC1	17p13.3	Prostate, breast, brain, lung, and kidney	Candidate tumor suppressor gene

Tab. 1.1 (continued)

Gene	*Location*	*Cancer*	*Comments*
hMLH1	2p22	Colon, endrometrial, and gastric	DNA mismatch repair
HOXA5	7p14.2–15	Breast	
IGF2	11p15.5	Colon/AML	Imprinted gene
IGFBP7	4q12	SV40-induced hepatocarcinoma	Normal and primary tumors?
IRF7		Cell lines	
LKB1	19p13.3	Colon, testicular, and breast	Serine/threonine protein kinase
MDG1	1p33–35	Breast	
MDR1	7q21.1	Drug-sensitive leukemias	Primary tumors?
O^6MGMT	10q26	Brain, colon, lung, and breast	DNA damage repair
MUC2	11p15.5	Colon cell lines	Primary tumors?
MYOD1	11p15.4	Colon, breast, bladder, and lung	One of first cancer related found
N33	8p22	Colon, brain, and prostate	Oligosaccharyl transferase
NEP	3q21–27	Prostate	
NIS	19p13.2–12	Thyroid cell lines	Not primary tumors
P14/ARF	9q21	Colon cell lines	Cell cycle control
P15	9q21	AML/ALL	Cell cycle control
P16	9q21	Lung, colon, lymphoma, bladder, and many others	Cell cycle control
P57/KIP2	11p15.5	Gastric cell lines	Cell cycle control
PAX	11p13	Colon	
PgR	11q22	Breast	Effect on transcription?
RASSF1	3p21.3	Lung	Growth factor response
RB1	13q14	Retinoblastoma	Cell cycle control
TESTIN	7q31.2	Hematopoetic	
TGFBR1	9q33–34	Gastric	
TIMP3	22q12.1–13.2	Brain/kidney	
VHL	3p25	Renal/common in solid and liquid tumors	Angiogenesis stimulator
WT1	11p13	Breast, colon, and Wilm's	Correlation with expression?

patterns to the donor cells [72]. A follow-up study found that the inefficient demethylation observed in the nuclear-transfer embryos could be reprogrammed by the presence of oocytic nuclei [73]. These results indicate that some factors provided by the oocytic nuclei may assist the demethylation of satellite sequences in normal development. Supporting evidence for this comes from a recent study in which Tada et al. [74] fused adult thymocytes with embryonic stem cells (ES). The inactive X chromosome derived from the female thymocyte adopted some characteristics of the active X chromosome [74]. In addition *Oct 4*, which is normally silenced in the adult thymocyte, was reactivated after fusion. Interestingly, the somatic DNA methylation pattern of the imprinted genes *H19* and *Igf2r* was maintained in the hybrids but erased in hybrids between ES cells and embryonic germ cells [74].

When the epigenetic reprogramming in pig embryos generated by nuclear transfer and IVF technology was analyzed, it was found that, unlike in bovine embryos, the methylation patterns of the centromeric satellite and Pre-1 SINE elements were similar in the nuclear-transfer and IVF-derived embryos. These results could indicate that species-specific differences in modifying the epigenetic status of cloned donor genomes may exist [75].

In addition, other studies have shown that the epigenetic information established during gametogenesis, such as certain imprints, cannot be restored after nuclear transfer experiments. The fact that animals derived from nuclear transfer survive through birth and beyond suggests that a certain level of abnormal epigenetic reprogramming is tolerated during mammalian development. Although cloned animals may appear normal, the data suggest that these animals have an epigenome that is more similar to the adult donor cells than is the epigenome of IVF-derived progeny.

1.7 Aging

The stability of differentiated cells is an essential feature of higher organisms. Specialized post-mitotic cells, such as neurons, or dividing cells, such as fibroblasts or keratinocytes, have uniform, unchanging phenotypes. The question arises whether, during normal aging, some cells acquire altered phenotypes. Changes in DNA methylation could produce such effects. For example, de-novo methylation of a CpG island could switch a gene off, and if it has an important regulatory or cell-specific function, the effect would be deleterious. Similarly, loss of methylation might turn on a gene that is normally inactive. This is known as the ectopic expression of an inappropriate gene. In both situations it is likely that only a small minority of cells in a given tissue would be affected, which makes it difficult to assess the possible contribution of such cells to the overall senescent phenotype.

So far, the evidence for methylation changes during the aging of organisms is somewhat inconsistent. However, age-related methylation changes have been documented in IGF2 [76] and in N33 and Myc in colon cancer [77]. E-cadherin has been identified as methylated in normal bladder tissues from elderly patients [78], the *tau* gene in human cortex tissue [79], and the estrogen receptor in cardiovascular tissue

- **Step 2** – Restriction-digested DNA (100–200 ng) is amplified in the presence of radiolabeled tracer (α^{32}P-dCTP) using AP-PCR with a single primer or a combination of 2 or 3 primers. The methylation fingerprints generated by AP-PCR are highly dependent on the sequences of the arbitrary primers. Our studies showed that GC-rich primers are more successful at amplifying GC-rich DNA fragments, because there is a high probability that they preferentially anneal to sequences associated with CpG islands. We have designed primers between 10 and 20 bases in length. For 20-base primers, a GC rich sequence is used at the 3′ end. For 10-base primers, a very high GC content (80%–100%) is used. Primer length also affects the number of bands, with shorter arbitrary primers yielding better results.
- **Step 3** – PCR products are separated in 5% polyacrylamide gels. Radiolabeled DNA fragments are identified after exposure to X-ray film. Candidate bands that are differentially methylated are excised from polyacrylamide gels and eluted in sterile water. The eluate is then used in a PCR reaction with the same primer(s) used in the original AP-PCR to generate sufficient amounts of template for vector cloning and DNA sequencing.
- **Step 4** – The resulting nucleotide sequences are then compared with the GenBank sequences, using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). Often, Southern blotting analysis is necessary to confirm that the cloned band corresponds to the band visualized by AP-PCR.

3.3.3 Applications

In a recent study, Liang and colleagues used methylation-sensitive AP-PCR to detect and isolate many regions of genomic DNA that had undergone methylation changes during tumorigenesis [18, 19]. Fig. 3.7 shows a detailed example of a DNA band con-

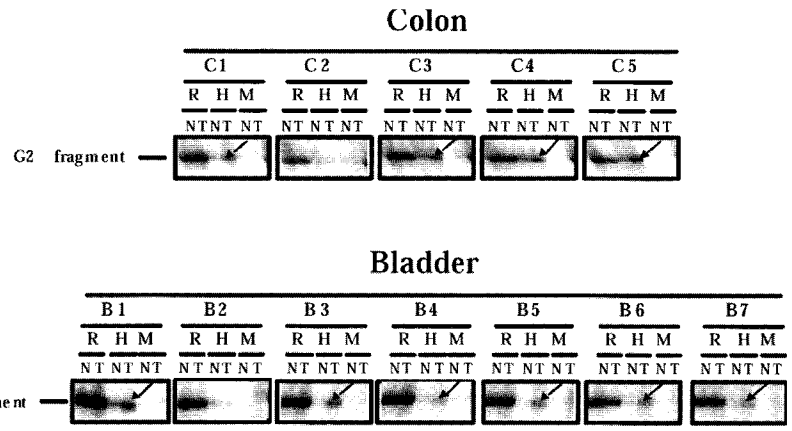


Fig. 3.7 Details of typical methylation-sensitive AP-PCR gels, showing bands (indicated by arrows) of a hypermethylated fragment, G2, present in colorectal and bladder tumors (see text for explanation). N, DNA from adjacent normal tissue; T, tumor DNA; R, *Rsa*I digestion; H, *Rsa*I + *Hpa*II digestion; M, *Rsa*I + *Msp*I digestion.

taining hypermethylated sites in four of five (80%) colon tumors and six of seven (85%) bladder tumors compared with their paired normal controls. As of today, ~100 novel CpG islands that are frequently hypermethylated in bladder tumors have been isolated. Using this approach, several novel genes such as *TPEF*, *PAX6*, and *endothelin receptor B* have been shown to be differentially methylated in many cancers [20–22].

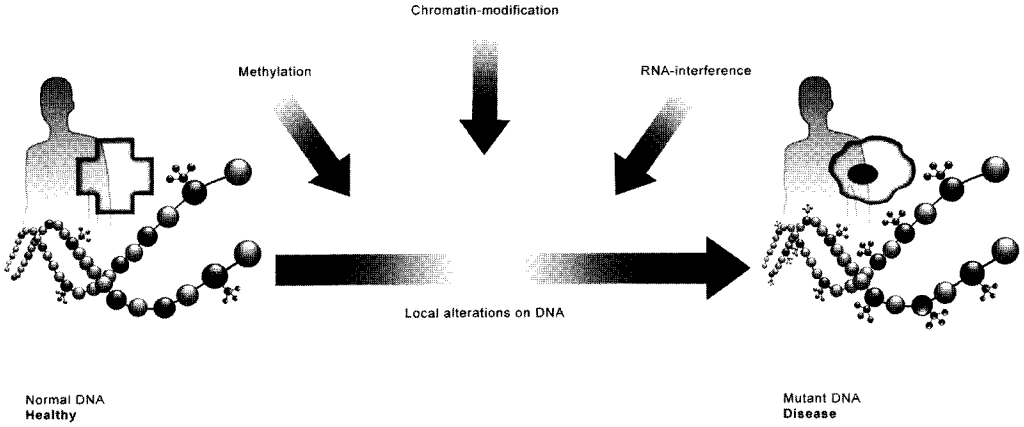
In addition to isolating unknown sequences associated with methylation changes in genomic DNA, methylation-sensitive AP-PCR was used to rapidly quantitatively estimate the variability in methylation at multiple sites between different cell lines or between normal and tumor tissues [18, 19, 23]. White blood cells (WBCs), tumor tissue (colon, bladder, and prostate cancer) tissue, and adjacent normal tissue from 17 patients were screened by methylation-specific AP-PCR [19]. DNA methylation pattern analysis based on 45 positive bands showed little interindividual differences in WBCs and adjacent normal tissue samples, but showed some tissue-specific differences. However, cancer cells showed marked methylation changes that varied considerably between different tumors, suggesting different methylation patterns in patients. Based on the number of putative methylation sites detected by methylation-sensitive AP-PCR in matched sets of normal and tumor DNAs, the major differences observed were bands representing putative regions of hypo- or hypermethylation in tumors relative to normal samples. Regardless of the primer combination used for methylation-sensitive AP-PCR, hypomethylated regions of DNA were consistently associated with CpG-poor sequences, whereas hypermethylated regions of DNA correlated with CpG-rich sequences. These findings are consistent with the types of methylation changes associated with tumorigenesis [24]. These studies demonstrated that methylation-sensitive AP-PCR can therefore be used to identify new CpG islands that may become differentially methylated during tumorigenesis.

3.4 Differential Methylation Hybridization (DMH): Identifying the 5th Bases in the Genomic Crossword Puzzle

3.4.1 Principle

DMH is an array-based approach for screening methylation changes of CpG islands in the genome [25, 26]. The array consists of many short CpG island fragments spotted at specific locations on an "affinity matrix" coated on a glass slide surface. This type of nucleic acid array (often called a DNA microarray or chip) can contain more than 5000 DNAs per square centimeter, dramatically increasing the experimental efficiency and information content. The microarray hybridization works in a manner similar to northern or Southern blotting. In this new approach, the immobilized nucleic acid, arrayed on the surface, is called the "probe", and the free nucleic acid in solution used for array hybridization is called the "target." The hybridization is, in effect, a highly parallel search by each target for a matching probe attached to

6
Epigenetic Trouble: Human Diseases Caused by Epimutations



have to be performed in an integrated fashion, and probably on one and the same technological platform, slashing platform investments for cost-constrained clinical institutions in half. Methylation analysis, reading expression information from DNA, is the only method that could possibly achieve this.

Monitoring. Monitoring patients for relapsing cancer relies on the same technological principles as the screening of asymptomatic people for early-stage disease, i.e., selective amplification from blood serum of fragments carrying a particular diagnostic methylation pattern against a background of normal, differently-methylated background fragments. For "simple" diagnosis of relapsing cancer, the same sets of methylation markers may be used as in screening for early-stage cancer.

However, for monitoring, additional questions are important and may be answered along with the diagnostic issues. For example, knowing whether a tumor is becoming chemoresistant could be of great value: the therapeutic regimen could be changed or adapted even before macroscopically visible metastases occur. One indicator of emerging drug resistance would be the overexpression – and concurrent hypomethylation – of multiple drug-resistance genes such as *MDR1* or other drug-resistance genes, a change in the hormone receptor status of cancer cells, or the appearance or disappearance of therapeutically useful surface antigens. Using methylation analyses, we expect that it will be possible to make such diagnoses in many cases.

Taken together, a comprehensive set of tests as described above, all of which could be based on methylation testing, has the potential to significantly change or even revolutionize the management of cancer.

9.2.3
Methylation, the Environment, and Lifestyle Diseases

A large body of evidence suggests that many diseases that are strongly modulated by environmental factors, as well as age-related diseases, are associated with, if not caused by, altered DNA methylation patterns in particular tissues. Aberrant DNA methylation in these diseases may be caused by three different mechanisms:

- Tissue-specific methylation patterns are established during cellular differentiation. In general, previously methylated genes become demethylated upon stimulation with transcription factors, thereby creating a repertoire of genes that are "ready to go" for later expression in this cell and, being a heritable signal, in successive cell generations. Indirect evidence suggests that environmental influences during this period may cause a failure to establish correct expression patterns in some genes, e.g., in hormone-responsive genes, through overstimulation or a lack of stimulation in the critical period. Although in theory reversible, these erroneous patterns may be difficult to erase and lead to predisposition of individuals to certain diseases later in life.
- Once established, tissue-specific methylation patterns are maintained quite stably throughout life. However, there is growing evidence that changes in the environ-

ment of cells or tissues leaves traces in the DNA methylation pattern which are likely to contribute to development of disease later in life. The traces of environmental influences may be targeted to specific genes, if specific metabolic pathways are stimulated or down-regulated with a subsequent change in their methylation patterns, or may occur randomly in the genome, e.g., through factors influencing the availability of methyl donor groups.

- A third mechanism may be the accumulation of methylation errors with age, which does happen [18–20], probably due to imperfect copying of methylation patterns to the daughter strands after replication by maintenance methyl transferase.

One archetypal disease which, albeit under the influence of genetic factors, is also influenced by the lifestyle of patients is type 2 diabetes mellitus (T2D). Indeed, type II diabetes is high correlated with obesity. Strict diet and exercise are excellent preventive measures against type II diabetes, even against the background of a genetic pre-disposition for obesity, further strengthening this notion. A range of circumstantial evidence suggests that all three proposed mechanisms contribute to the development of insulin resistance in type 2 diabetes mellitus:

- The level of prenatal glucose to which a person is exposed (e.g., in comparisons of siblings born before and after development of any type of diabetes in the mother) influences the likelihood of the child getting T2D [14]). This suggests the presence of a "cellular memory" in insulin target tissues such as adipose tissue, skeletal muscle, and liver.
- The promoters of several genes involved in glucose metabolism exhibit differential DNA methylation, e.g., the genes for facilitative glucose transporter 4 (GLUT4), the major glucose transporter in adipose and muscle tissues [15], and uncoupling protein 2 (UCP2) [16], a major candidate gene for the development of type 2 diabetes and an important regulator of energy expenditure. Establishment of correct methylation patterns of these genes may be disturbed in a critical period, or the patterns may change after environmental influences later in life.
- A general defect in DNA methylation in diabetes is suggested by the recent observation that S-adenosylmethionine (SAM), the main physiological donor of methyl groups, is decreased in the erythrocytes of diabetic patients. In addition, decreased erythrocyte concentrations of SAM and other alterations are associated with the disease progression [22].
- Recent insights into the pathogenesis of transient neonatal diabetes (TND), a rare subtype of diabetes that is characterized by transient hyperglycemia in the neonatal period and a predisposition to diabetes in adult life, provide a link between methylation, gene-dosage effects, and diabetes. Transient neonatal diabetes results from a doubling of the dosage of genes on chromosome 6q24. Paternal uniparental isodisomy, duplication of the respective band on 6q24, and loss of methylation in this imprinted region all result in phenotypically undistinguishable TND [17]. Interestingly, these individuals have an increased risk of developing type 2 diabetes later in life. Type 2 diabetes is age-related: not only is its incidence increased in older populations, but also the metabolic condition of individual patients dete-

riorates over time. DNA methylation errors that accumulate with increasing age could provide an explanation for both phenomena.

In a similar way, environmental influences and lifestyle factors may influence the development of arteriosclerosis. For example, nicotine influences the expression patterns of endothelial cells [42, 43]. Also, gene expression in aortic endothelial cells is influenced by flow conditions [44, 45]. Occurring over long periods of time, expression patterns tend to translate into altered methylation patterns, thereby propagating the effects even in the absence of further stimuli. A specific hypermethylation has been observed in proliferating aortic smooth-muscle cells compared with their normal counterparts [18].

Random DNA methylation changes occur in several tissue types during aging of organisms [18–20]. These accumulating age-related DNA methylation changes are involved in several diseases:

- In the colon, hypermethylation often starts in normal mucosa as a function of age and leads to field defects, with an increased risk of developing colorectal cancer ("acquired predisposition to colorectal neoplasia" [20]).
- Methylation-associated inactivation of the *ERα* gene in vascular tissue seems to occur specifically when the cells switch to a dedifferentiated, proliferating phenotype, but may also be associated with aging of the cardiovascular system [18].
- DNA methylation of the promoter region of the amyloid precursor protein gene, which is involved in the development of Alzheimer's disease, is reduced in with increasing age [21].

Taken together, the evidence suggests that methylation plays an important role in regulating gene expression, most likely including the expression of genes playing essential roles in the development of the metabolic syndromes and in so-called lifestyle-associated diseases. Different expression patterns that develop in association with changes in diet [23–25], in body weight [24, 25], and in exposure to environmental factors are likely to become "locked" by DNA methylation if they are present for a long period of time. DNA methylation, therefore, is likely to be involved in mediating the deleterious effects of increased body fat and high-fat diet on insulin sensitivity of insulin target tissues and on the cardiovascular system. Being a reversible modification, DNA methylation might also be involved in the adaptation of metabolism to starvation. On the other hand, metabolism of methyl groups may be affected by diet, body weight, and environmental factors [26], thus leading to untargeted, general hypomethylation of DNA in obese patients [22]. Moreover, DNA methylation errors have been shown to accumulate over time, contributing to many age-related diseases. These errors could add to the development of type 2 diabetes and cardiovascular diseases by reducing gene responsiveness (i.e., gene expression that needs to be adjusted to rapidly changing glucose and insulin levels).

contribute to phenotype variation and might become the basis for relevant QTL-marker tests for use in animal breeding programs.

9.6 Outlook

In this chapter we discussed applications of methylation sciences that are so far in the future that no products have yet reached the market. However, millions of dollars of venture capital have been invested in all the areas treated here and are even now being used in development of mature products.

The potential of methylation science can be glimpsed if we recall that these few pages go through a list of many of the major sectors of the life sciences, diagnostics, pharmaceutical development, personalized medicine, and agriculture, in each of which we find highly attractive opportunities. At the same time, we discussed several completely different disease indications: we discussed disease management and diagnostics exclusively for oncology, and we illustrated uses in pharmaceutical research and development mainly for metabolic diseases. The tremendous potential of methylation as an applied science becomes obvious if we believe that the diagnostic applications expand into metabolic, cardiovascular, and other major human diseases. Likewise, pharmaceutical research and development can be supported with methylation information in as many disease indications. We believe that this is so, indeed attributing to methylation an importance that could be comparable to that of the other "big" genome sciences. We have argued that methylation is the only parameter that truly changes genome function in aging, as well as being affected by environmental influences. Therefore, we expect methylation to be not only competitive as a tool for diagnostics and research, but in many cases but simply irreplaceable. All in all, we expect methylation technologies to become a firm component of most, if not all, genomics-based research and development in the future.

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