Epigenetics and Human Health

Randy L. Jirtle
Frederick L. Tyson *Editors*

Environmental Epigenomics in Health and Disease

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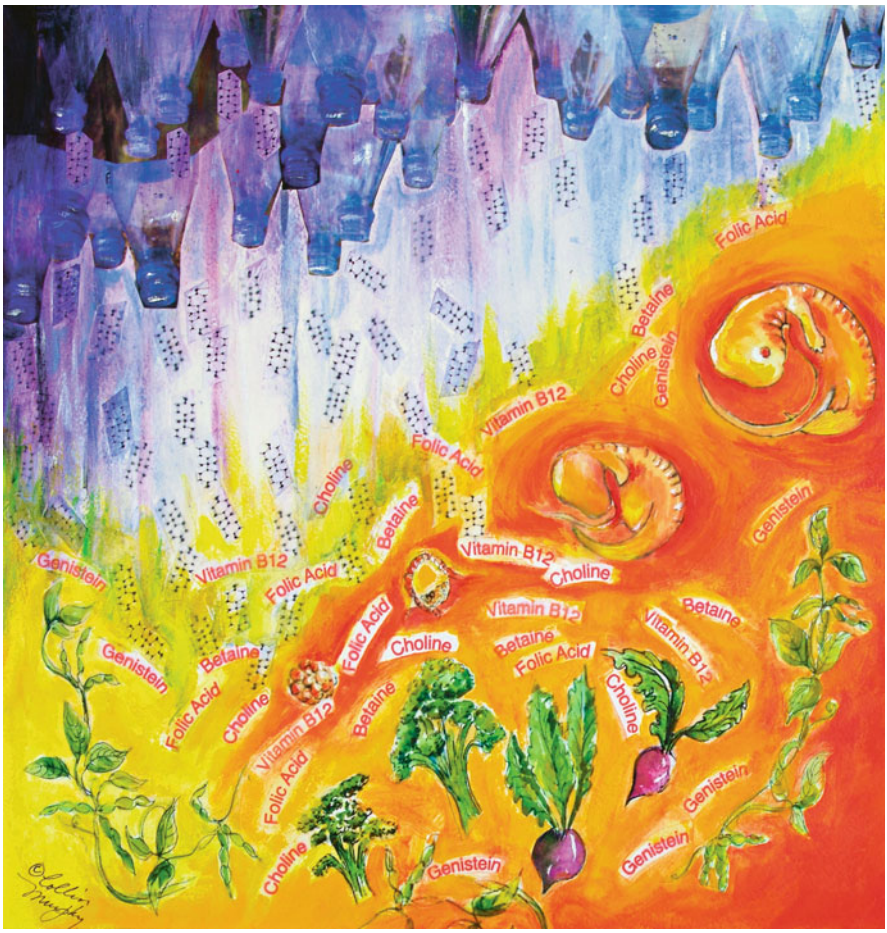

The mixed media artwork entitled *Counteracting Forces* © by Collin Murphy, Portland, Oregon, artist (http://collinmurphyart.com), depicts environmental factors counteracting each other during early development. By altering the epigenome, they can influence the incidence of chronic diseases and neurological disorders.

Preface

In the spring of 1998 my co-editor, colleague and friend, Randy Jirtle, approached me at a scientific meeting in San Diego, CA to discuss something that clearly had him excited. Initiating our discussion, Randy displayed the unbridled enthusiasm that is so characteristic of him. He advised me that he had spoken with my boss about organizing an international scientific meeting that would merge an evolving field with toxicology. That field was genomic imprinting and on that afternoon as we sat, he was effusive in his description of monoallelic expression. While this was not my first time hearing about genomic imprinting or the field of epigenetics that imprinting is a component of, I realized I was becoming fascinated with the opportunity to work with Randy and make an impact on merging aspects of toxicology and epigenetics.

In 1998, we had no way of knowing where that initial conversation would lead us, but we began crafting a vision to merge toxicology with epigenetics, as it was clear that the environment plays a pivotal role with epigenetic processes. It is now reasonable to argue that this vision is being shared globally, after organizing three extremely well attended international scientific meetings focusing on the interactions between environmental agents, epigenetics, and disease susceptibility.

What has in fact transpired with regard to developing the interface between epigenetics and environmental exposures over the last 15 years has been nothing short of remarkable. The advent of high throughput technologies such as genome-wide bisulfite sequencing, along with ChIP-Seq, RNA-Seq and technologies to map chromatin accessibility resulted in the generation of terabytes of data. Novel computational tools have been and are continually being developed to both store data and analyze it, as well. Investments in large multi-institutional consortia such as the NIH Roadmap Epigenome Mapping Consortium (REMC), the NHGRI supported ENCODE program, and the International Human Epigenome Consortium (IHEC) have spurred the development of these technologies and analytic tools.

There are compelling human epidemiological and animal experimental data that indicate the risk of developing adult-onset complex diseases and neurological disorders is influenced by persistent epigenetic adaptations in response to prenatal and early postnatal exposures to environmental factors. The epigenetic programs

are established as stem cells differentiate during embryogenesis, and are faithfully reproduced during mitosis. Moreover, they can also be maintained during meiosis. The plasticity of the epigenome allows the genome to express specific gene programs in a cell specific pattern that is spatially and temporally regulated, resulting in phenotypes. The capacity of the epigenome to interpret both internal and external stimuli and alter expression programs is a critical component in normal development, aging, and disease pathogenesis. In the past decade, our field has witnessed an explosion of unprecedented research on and support for epigenetics, epigenomics, and their interface with human health and disease. This research is in large measure an effort to generate a more precise understanding of how DNA and gene expression are regulated by DNA sequence, functional DNA elements, chromatin states, epigenomic signatures, and epigenetic processes.

It is becoming increasingly apparent that exposure to environmental toxicants can be associated with epigenetic changes, such as altered patterns of DNA methylation. These changes can affect gene expression patterns, and likely contribute to disease or other phenotypes associated with exposure. DNA methylation is thought to be one of the last steps of epigenetic gene regulation – a read-out of chromatin states established by other proteins. In order to understand the mechanism by which toxicants impact gene expression, we must examine how exposures perturb the proteins and processes upstream of DNA methylation and other epigenetic marks.

Epigenetic modifications, such as DNA methylation or post-translational modifications to histone tails, modifies the DNA and/or the way it is packaged into chromatin, making certain genes either more or less accessible to trans-acting elements, such as transcription factors. These epigenetic marks, however, represent limited facets in this complex process. Other proteins or protein complexes act as 'readers', 'writers' and 'erasers' of the epigenetic code, depositing or removing epigenetic marks or binding to them and recruiting other proteins. In addition, other factors such as non-coding RNAs, chromatin remodeling complexes, inter- and intra-chromosomal interactions and functional genomic elements play important roles in this process. Thus, to understand the mechanisms involved in the environmental control of gene regulation and the central role of epigenetics in the process, it is critical to understand all of the interacting pathways.

Exposure to environmental toxicants has been associated with changes in gene expression and DNA methylation profiles, which together likely contribute to disease or other phenotypes associated with exposure. The chapters in these volumes address a wide range of environmental exposures, such as airborne particulates, cocaine, radiation, tobacco smoke, and xenoestrogens. The health outcomes associated with these exposures include autoimmune disorders, neurodevelopmental disorders, and cancer. Importantly, dietary supplements and drugs can modify the epigenetic effects induced by these agents, thereby reducing their toxicological impact.

In the two volumes of this book, a number of leading investigators in the field of epigenetics discuss patterns of epigenomic modifications in normal cells, and how environmentally-induced changes in them are associated with disease pathogenesis.

The authors comprehensively review epigenetic processes that occur in human embryonic stem cells, as well as in differentiating cells and organs such as the brain, discussing autism, schizophrenia, and even sexual dimorphism in the developing brains of males and females. Particular emphasis is placed on the consequences of environmental exposures during development on epigenetic reprogramming that influences adult disease pathogenesis.

The overall purpose of this book is to give readers an overview of how environmental exposures can influence the development of disease by disrupting epigenetic processes and reprogramming. When Randy approached me in 1998 at the scientific meeting in San Diego, I had no idea what we would accomplish together in moving this field forward. He has been able to produce many significant contributions to the field directly from his laboratory research. Moreover, he has trained a cadre of young investigators who will continue to make an impact in enhancing our understanding of how the environment can alter epigenetic processes and influence the development of human disease. I, on the other hand, have been privileged to be among the extramural scientists at the National Institutes of Health (NIH) who develop research programs that support cutting edge science in moving this field forward.

Since that initial conversation, Randy and I have collaborated on a number of epigenetic projects. This book represents our latest collaboration to bring this field of environmental epigenomics to a growing audience. It is my desire that the readers learn as much, and have as much fun reading the chapters that constitute both volumes of this book as I did.

Frederick L. Tyson, Ph.D.

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Part I
Epigenetic Programming of the Brain

Chapter 1
Epigenetics and Maternal Brain Evolution

Eric B. Keverne

Abstract Viviparity and the evolution of a placenta have resulted in considerable hypothalamic modifications. Maternal feeding, maternal care, suspension of fertility and sexual behaviour, parturition and milk letdown are integral to hypothalamic function and have evolved under the influence of the placental hormones to meet the demands of the fetus. Viviparity has also introduced a new dimension into evolutionary genetics by providing two genomes, fetal and maternal, in one individual, the mother. The hypothalamus develops in-utero under the regulatory control of matrilineal imprinted genes that are synchronised for expression in both the placenta and hypothalamus. The two genomes, maternal and fetal, are transgenerationally coadapted to ensure the fetal hypothalamus in the next generation is genetically and epigenetically programmed for maternal care and nurturing. Advanced aspects of neocortical brain evolution in primates have emancipated maternal behaviour from hormonal determinants. The neocortex has evolved to be adaptable, and while the adapted changes are not inherited, the epigenetic predisposing processes are, thus providing each generation with the same ability to generate new adaptations while retaining a cultural predisposition to retain others.

Keywords Genomic imprinting • Hypothalamus • Maternalism • Viviparity • PEG3 • Placenta

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Abbreviations

βHLC-PAS	Beta helix loop-helix–non-palindromic sequence binding
Ag	Androgenetic
Ceas	Ceacams
ICR	Imprint control region
MPOA	Medial preoptic area
Pg	Parthenogenetic
Prls	Prolactins
PVN	Paraventricular nucleus
SON	Supraoptic nucleus
Spgs	Pregnancy-specific glycoproteins

1.1 Introduction

Viviparity has been a crucially important event in the successful evolution of mammals and is characterised by intrauterine development and the formation of a placenta. This biologically significant shift away from the production of many fertilised eggs, as seen in fish, amphibians and reptiles, avoids the vagaries of the environment and provides for a more substantial investment in the caring production of fewer offspring. Viviparity has proved to be a major determinant of mammalian reproductive success. Nevertheless, internal development is not without its problems, one of which is the oxygen supply. It is not surprising, therefore, that a key factor in the evolution of large-bodied mammalian phylogenies occurred at a time when there was an increase in atmospheric oxygen from 15 % to 20 % (Falkowski et al. 2005). Oxygen was a prerequisite of extended in utero growth, especially of metabolically demanding tissues such as the brain. Internal placental development also carries additional risks for other embryos developing in utero at the same time and for mother herself should developmental errors occur. External fertilisation and the production of many hundreds or even thousands of eggs can accommodate developmental errors and potential mortality, but for in utero development, embryos needed to achieve near perfection. Error-free development is accomplished partly by maintaining constant body temperature and partly by developing tight control over gene dosage. The latter has been achieved by monoallelic gene expression, which we now know to be particularly important in the brain (Gregg et al. 2010), and from a special type of monoallelic gene expression, genomic imprinting, which additionally ensures the same allele, is expressed in all cells of a given type.

Mammals are also characterised by the massive and indeed rapid evolution of their brain, with a 200-fold increase from reptiles to mammals, taking account of body weight, and even within mammals themselves, there is a tenfold variance

across different orders (Striedter 2005). In order to minimise the vast energy demands of brain tissue, in utero growth is relatively slow, and in order to achieve such a large brain size, much of its development occurs after birth. Such postnatal development has been fundamentally dependent on maternal care and maternal lactational nurturing. Not surprisingly therefore, almost all of the morphological phenotypes that characterise the evolution of mammals relate in one way or another to viviparity and the matriline. Indeed, females spend most of their reproductive life either pregnant or lactating and caring for offspring, and less than 1 % of the lifetime budget of small-brained rodents is spent engaged in sexual behaviour. Males spend most of their adult life engaged in searching for the 1 % of oestrous females and competing with other males for this scarce resource. It is hardly surprising therefore that key regulatory genes for placental and intrauterine fetal growth are themselves regulated by imprinted control regions (ICRs) which mainly originate in the matriline (17 of 20 known ICRs) (Ferguson-Smith and Surani 2001; Schaefer et al. 2007). This parental nonequivalence of imprinting control regions (ICRs) during mammalian development has resulted in maternal ICRs being qualitatively focused on genes which regulate the fetal-maternal interface, while paternal ICRs weakly influence non-convergent processes (Schultz et al. 2010). Genes that are regulated by maternal ICRs indirectly influence genes regulated by paternal ICRs while the converse is not observed. This functional dominance of maternal imprints over early embryonic development is linked to methylation-dependent control of maternal over paternal ICRs. The rate of CpG loss at the paternal ICR has been higher than at the maternal ICR during mammalian evolution. Maternal ICRs have gained CpGs compared with the CpG-rich promoters of non-imprinted genes. From an evolutionary viewpoint, this matrilineal control can be best understood by the need for selection pressures to ensure the tight regulation of transcription and dosage of genes affecting the maternal-fetal interface while avoiding the mutagenic environment of the paternal germ line (Schaefer et al. 2007).

Further evidence for functional dominance of maternal epigenetic marks is seen in certain germ line genes that resist global demethylation after fertilisation and inherit promoter DNA methylation from the oocyte. In this study, all tested genes were shown to have methylated alleles at E2.5 preimplantation stages, suggesting that methylation is inherited from gametes. These genes differ from imprinted genes because none of them maintain allele-specific methylation after implantation (Borgel et al. 2010). Such inherited maternal epigenetic transmission might be important for the earliest stages of development and may be of significance for avoiding allogeneic mismatch proteins that could trigger immune rejection at implantation.

1.2 Maternal-Fetal Interface

Viviparity has introduced a new dimension into evolutionary genetics by creating two genomes, fetal and maternal, in one individual, the mother. These two genomes interact at the fetal-placental interface with the mother, not just in the transfer of

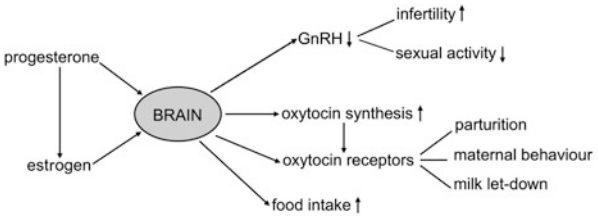

Fig. 1.1 Placental regulated steroids – effects on maternity. Progesterone acts on the brain to change GnRH, fertility and sexual behaviour, to promote feeding and to induce oxytocin synthesis in the hypothalamic neurons. As term approaches, the fetal adrenal matures to convert progesterone to oestrogen which induces oxytocin receptors. Oxytocin release induced by parturition and suckling acts on these receptors with positive feedback for parturition and milk letdown and at the same time promotes maternal behaviour

oxygen and nutrient resources from the mother but also in the transfer of hormonal signals from the fetoplacental interface to the mother. Placental hormones act on the maternal brain (hypothalamus in particular) to shut down reproduction by regulating control of the maternal hypothalamus over fertility and in turn sexual receptivity (Keverne 2006). They also regulate the maternal brain to increase food intake and provide fat reserves for the later stages of pregnancy when the energy demands of the fetus increase. Nest building and maternal care are likewise dependent on placental hormone priming of the hypothalamus. These same hormones also act on the mother's body to induce milk production, increase body fat stores and indirectly determine the timing of parturition, cornify the birth canal and promote milk letdown following birth (Fig. 1.1).

By the time the female becomes pregnant, her hypothalamus has long since fully developed, and yet, without any prior exposure to offspring, this hypothalamus is capable of responding to hormonal signals from the placenta and successfully executing all aspects of maternalism. In this way, the fetal genome determines its own destiny, and while the outcome for good maternalism is dependent on the adult brain, the evolutionary selection pressures must have operated during development, logically at the same time as the placental giant cells mature to produce their hormones. Moreover, if the genes successfully regulating this placental communication with the maternal hypothalamus are at the same time regulating development of the fetal hypothalamus, a genetic template is available on which selection pressures may operate to rapidly establish the evolutionary process. Thus, while the outcome for successful maternalism is dependent on the adult brain, the proximate evolutionary selection pressures will have acted on the developing brain. The better the coadaptation of placenta and brain, the more successful is maternalism in the next generation. Successful mothering begetting successful mothering can thus be viewed as an epigenetic process, but, because of the fundamental contribution of imprinted genes to this process, it has become heritable through the matriline ICRs. Regulating these coadapted genes through a maternal epigenetic mark has all the advantages of combining evolutionary flexibility with

genetic stability. Maternal imprints (ICRs) resulting in paternal expression provide stability through the maternal allele serving as a template for gene repair, while the imprint mark retains a flexibility for other genes to be recruited to the imprinted locus.

1.3 Genomic Imprinting

Genomic imprinting is heritable and requires germ line reprogramming to reset the parental specific imprints (Sasaki and Matsui 2008). The first genes expressed in this way probably arose in the metatherian marsupials, but increased substantially with the evolution of placental eutherians (Coan et al. 2005; Killian et al. 2001). Indeed, genomic imprinting is thought to have coevolved with placental development (Hore et al. 2007; Renfree et al. 2008), although many of these imprinted genes are also expressed in the brain, and brain evolution has itself been influenced by the developing placenta under selection pressures regulating maternalism (Curley et al. 2004). The developing brain is a key target for imprinted genes as first shown by studies with androgenetic (Ag) and parthenogenetic (Pg) chimeric cells introduced into the developing blastula and subsequently accumulating in different regions of the brain (Keverne et al. 1996).

Molecular genetic studies of brain development fail to take into account the placenta, and likewise, investigations of the placenta rarely take into account the brain. Nevertheless, there are a number of imprinted genes which are expressed in the placenta and in those hypothalamic regions of the brain that regulate mammalian maternal physiology and behaviour. Moreover, all of these genes are maternally imprinted (Keverne 2009), and imprint reprogramming occurs in the female germ line, when the imprints are removed by active demethylation between days E10.5 → E12.5 of oocyte development (Sasaki and Matsui 2008). This coincides with the timing for placental hypothalamic developmental programming of the next generation. Thus, maternal imprinting extends its influence across three generations: the maternal generation where the adult mother's hypothalamus responds to placental signals, the next generation where the developing hypothalamus is genetically coadapted for placental signalling and a third generation when the key regulatory genes responsible have their imprint reprogrammed in the developing female germ line. The removal of imprints occurs between E10.5 and E12.5 of mouse development by active demethylation in the oocyte, and they are restored prior to fertilisation throughout oocyte growth which also occurs within the matriline (Sasaki and Matsui 2008). In this way, the matriline retains transgenerational control over the epigenetic marks for maternally imprinted genes. It is also the case that some maternally expressed genes (Igf2r, H19DMR, Grb10 and p57kip2) actually receive their methylation mark in the oocyte and only three ICRs are known to be methylated in spermatozoa (Feil 2009).

1.4 The Imprinted Peg3 Gene

Detailed studies with mice carrying a mutation in maternally imprinted gene (Peg3) have provided some insights as to how the functioning of the hypothalamus, which develops in utero, is adapted to respond as an adult brain to the placental hormones that determine maternalism (Keverne and Curley 2008). When this gene is silenced in the maternal hypothalamus but not in the placenta or fetus, many aspects of the phenotype have similar functional consequences to the gene being deleted exclusively in the placenta and fetus, but not the mother's hypothalamus (Curley et al. 2004). The consequences of maternal brain deletion of Peg3 are manifest through reduced cell numbers in the basal forebrain (PVN, SON and MPOA) which results in impaired maternal care, milk letdown and difficulty for infant suckling which, in turn, affects the onset of puberty and adult reproduction (Curley et al. 2005a). Gene deletion in the placenta reduces placental growth which impairs the placental hormonal priming of maternal care and the mammary glands. Low birth rate impairs postnatal growth which also delays puberty onset. Indeed, the phenotypic outcomes are so functionally convergent as to suggest the action of this gene is coadapted in the hypothalamus and placenta (Fig. 1.2).

1.5 Brain and Placenta Genetic Coadaptation

Genetic adaptation to underpin the convergent phenotypes revealed by transcriptional silencing of Peg3 would require co-expression of genes in the fetal hypothalamus and placenta coincident with the timing of placental hormone production acting on the maternal brain (E11–12–13). Between E11 and E12, most of the developmental changes are unique to the placenta (56 %) or hypothalamus (34 %) with only 10 % of gene transcription changes being co-expressed in the brain and placenta. Peg3 inactivation suppresses all the co-expressed gene changes at this stage and further induces significant changes to new co-expressed genes. The induced changes to co-expression in the Peg3 mutant are, however, not in synchrony; 90 % are upregulated in the placenta, but in the brain they are downregulated. Days E12–13 demonstrate a marked increase in brain and placental gene co-expression (up to fourfold from E11 to 12), 59 % of which are also synchronised for direction of changed expression (Fig. 1.3a). At this same developmental time period, the inactivation of the Peg3 suppresses 42 % of these co-expressed gene transcription changes and induces changes to a further 39 % of brain and placenta co-expressed genes (Fig. 1.3b). Days E12–13 thus represent a period for major changes in transcriptional synchrony for brain and placenta and are also the period when the Peg3 inactivation has maximal effects, not only suppressing many of these transcriptional changes but also inducing synchronised co-expression of new genes. Peg3 inactivation is thus particularly effective at desynchronising the expression of genes that are simultaneously co-expressed in the fetal hypothalamus

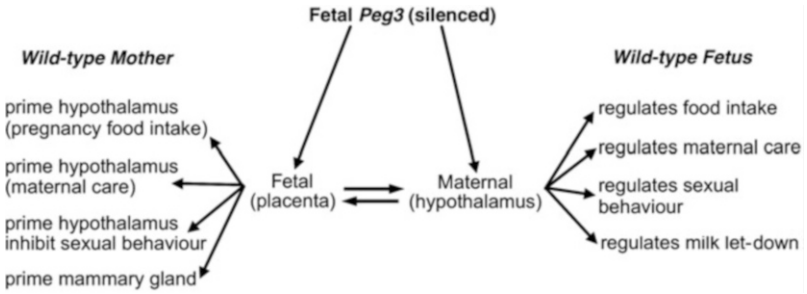

Fig. 1.2 Comparison of phenotypic effects brought about by *Peg3* mutation in the fetal placenta and maternal hypothalamus. The maternally imprinted Peg3 gene acts on development of the fetal placenta enabling the production of hormones that prime the mother's hypothalamus to be maternal. At the same time, *Peg3* acts on the development of the fetal hypothalamus to ensure that when adult, this hypothalamus responds to the fetal hormones of the next generation

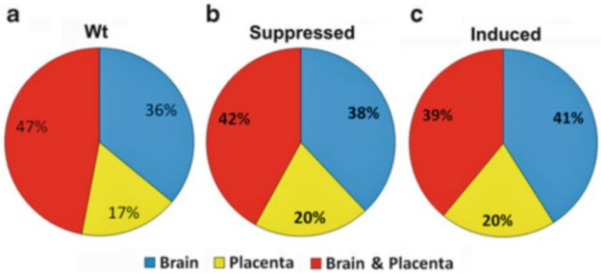

Fig. 1.3 Changes in developmental gene expression and effects of *Peg3* mutation (days 12–13). Coincident with the timing of hypothalamic development and maturation of placental hormone-producing giant cells, most of developmental gene changes are co-expressed in the hypothalamus and placenta with relatively few changes restricted to placenta (**a**). The *Peg3* mutation has its greatest impact on these co-expressed genes, suppressing 42 % (**b**) and inducing changes to 39 % new genes (**c**)

and placenta as well as disrupting the coordinated functional interactions between the mother and the fetus of this and the subsequent generation of wild-type offspring.

It might be expected that genes which have played such an important role in mammalian evolution might show considerable variance and high accumulation of synonymous to non-synonymous mutations across species. In fact, Peg3 is remarkably conserved. A conserved YY1 binding sequence is located in the ICR of Peg3 and is integral to maintaining the DNA methylation status of Peg3 (Kim et al. 2007). Within a 3kb genomic interval of the first intron of mouse Peg3 are 7 YY1 binding sites, and these may vary from 4 to 14 across different mammalian species (Kim 2008). Although YY1 binding sites appear to have been repeatedly duplicated to different extents across mammalian lineages, the spacings and positions not

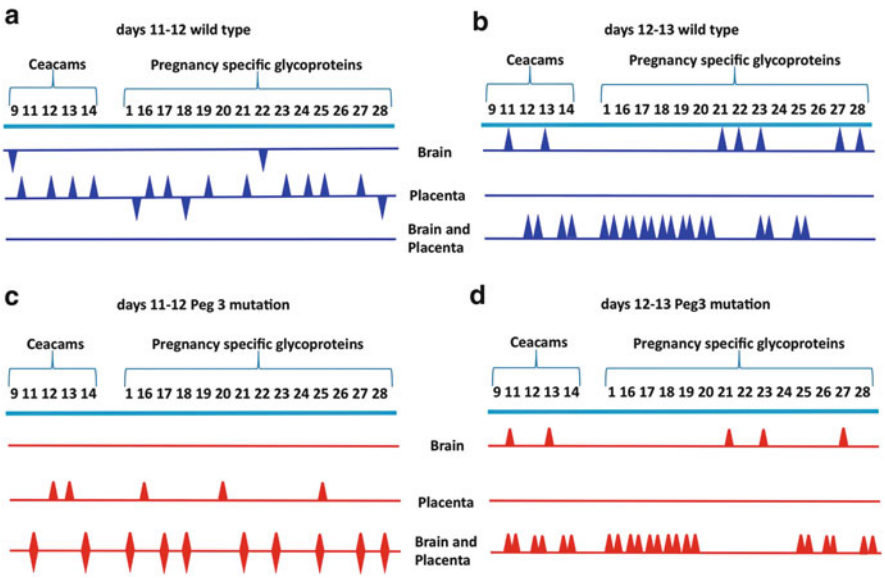

Fig. 1.4 Expression changes in Psgs and Ceas in the brain, placenta and brain and placenta and effects of *Peg3* mutation. Pregnancy-specific glycoproteins (*Psgs*) and Ceacams (*Ceas*) are expressed primarily in the placenta between E11 and E12 (**a**), while between E12 and E13, none are expressed in the placenta alone and ten of these genes are upregulated for co-expression in the brain and placenta (**b**). The Peg3 mutation induces co-expression of ten of these genes in the brain and placenta on E11–12, but they are desynchronised with upregulation in the brain and downregulation in the placenta (**c**). On E12–13, Peg3 mutation changes the expression profile of Psgs and Ceacams that are co-expressed, with most effect on brain and placenta co-expressed changes to gene transcription (**d**) (Modified after Broad and Keverne 2011)

conserved, but they have nevertheless retained high levels of sequence homogeneity during mammalian evolution with identical orientation.

In the mouse, Peg3 resides on chromosome 7, and it is notable that a substantial disruption (E11–12 60 %; E12–13 47 %) of synchronised co-expressed gene transcriptional changes in the brain and placenta affected by the Peg3 inactivation is also on chromosome 7. Particularly noteworthy are the two large families of ceacams (Ceas) and placenta-specific glycoproteins (Psgs). Between E11 and E12, none of these 19 genes (chromosome 7) show significant changes for co-expression in the brain and placenta, but (76 %) increase their expression in the placenta, and 2 also independently change in the brain (Fig. 1.4a, b). The effect of the Peg3 inactivation over E11–12 results in 67 % of the Psgs and Ceas, whose gene expression was not coordinated in the wild-type brain and placenta, now becomes co-expressed in the brain and placenta (Fig. 1.4c, d). However, these co-expression transcriptional changes are not in synchrony; they are all significantly upregulated in the placenta but are all significantly downregulated in the brain. This premature induction of desynchronised changes to Cea and Psg's co-expression is in contrast to their co-expression in the wild-type brain and placenta at the later developmental

stage, E12–13 when transcription of these genes are also synchronised, but all with upregulated changes in both brain and placenta (100 %).

Coadaptation of hypothalamus and placenta in the context of maternalism has notable effects for both synchronised gene expression and for the coordinated development of both the maternal and offspring phenotype. Genes which change their expression in these tissues between days E12 and E13 primarily belong to functional categories which promote growth and vascularisation. Eleven of the 23 prolactin gene family, five of the ceacam family and twelve of the pregnancy-specific glycoprotein family genes show synchronised developmental changes for hypothalamus and placental co-expression between E12 and E13. They also represent genes which have undergone multiple duplications, a powerful method of creating new genetic material that can drive evolution (Ohno 1999). The vast majority of the mouse Prl family members are thought to have diverse functions in promoting maternal adaptations to pregnancy (Rawn and Cross 2008). These include effects on vascular development, hemopoesis and immune cell differentiation (Soares et al. 1998). The pregnancy-specific glycoproteins (Psgs)- and Cea-related cell adhesion molecules belong to the immunoglobulin superfamily. They are transcribed in placental structures where Peg3 is expressed, namely, the giant cells and spongiotrophoblast of the mouse hemochorial placenta. They are thought to have an immunomodulatory role in preventing rejection of the allotypic fetus (Harris et al. 1984; Majumdar et al. 1982) and bind to the CD9 receptor of natural killer cells (Wynne et al. 2006). Anti-Psg antibodies terminate mouse pregnancy (Hau et al. 1985), and in humans, they are associated with interuterine growth restriction and fetal hypoxia. Psg expression is also localised to the endothelial lining of vascular spaces and is thought to modulate angiogenesis and vascular remodelling. Moreover, Peg3 inactivation silences a substantial number of these genes (9 Psgs, 1 Ceacam, 3 Prls) at E12–13, and its inactivation over E11–12 prematurely induces co-expression of 8 Psgs, 2 Ceacams and 2 Prls in the brain/placenta, all of which are only normally expressed at the later E12–13 of wild-type mice.

The brain and placenta are genetically co-regulated during developmentally important periods (E11–12–13) by sets of genes whose expression changes are synchronised in the placenta and hypothalamus. This co-expression is substantially disrupted by mutation to a maternally imprinted gene (Peg3) which also has significant developmental consequences for a range of phenotypes integral to successful growth and maternalism. An increase in atmospheric oxygen appears to have played a significant role in early mammalian evolution (Falkowski et al. 2005), and hypoxic conditions are a notable signal for Peg3 transcription in both developing placenta (Vaiman et al. 2005) and neural cells (Yamaguchi et al. 2002) and for induction of vascularisation. Interestingly, the mammalian Peg3 gene is structurally different from that of other vertebrates, but across mammalian lineages, this gene and its promoter are highly conserved (He and Casaccia-Bonnefil 2008; Song et al. 2008). The evolutionary significance of this kind of imprinting must, therefore, be underpinned by its ability to regulate those large gene families (Prls, Psgs and Ceacams) which have themselves undergone multiple duplications and

differential expansion across different mammalian species (McLellan et al. 2005; Zebhauser et al. 2005).

Like many of the imprinted genes, the transcriptional influence of Peg3 is largely focused at the pre- and early postnatal stages of development. An accumulating amount of evidence now shows that compromised in utero environments can influence the epigenetic status of imprinted genes with consequences for health and wellbeing (Lim and Ferguson-Smith 2010). Delays to placental growth relative to the developmental programme for the fetal brain could be a possible explanation for such pathologies. Retardation of developmental placental vascularisation by the Peg3 mutation (Hiby et al. 2001) could induce brain hypoxia, the signal for Peg3 activation, resulting in dysregulation of synchronised gene expression by prematurely activating members of the Psg, Cea and Prl gene families, as shown in this study. Delays or advances to this timing could desynchronise neural connectivities resulting in programmed cell death. This could explain the reductions in the neurons of the hypothalamus seen in the adult brain and the increased apoptosis in the developing hypothalamus (Broad et al. 2009; Li et al. 1999). Brain hypoxia is known to activate a number of transcription factors, including Peg3, which we have now shown has the capacity to deregulate the synchronised expression of hypothalamic and placental genes by prematurely inducing Psgs, Ceas and Prls.

In addition to the first generation of infants and mothers considered in this study and shown to have poor maternalism, there are also second generation epigenetic consequences of this poor mothering in itself (Champagne and Meaney 2007; Champagne et al. 2009). Thus, synchronised genetic regulation of the developing placenta and hypothalamus which has beneficial consequences for optimal nourishment and maternal care also provides an additional epigenetic predisposition for the next generation to undertake good mothering.

1.6 Other Imprinted Genes

There are a number of imprinted genes expressed in the developing hypothalamus and placenta that influence neuroendocrine function (Davies et al. 2008), a function that is relevant to successful maternalism. Necdin and Magel2 are maternally imprinted genes dysregulated in Prader-Willi syndrome which is characterised by a failure to suckle and subsequent development of a voracious appetite in early childhood (Davies et al. 2008). Magel2 belongs to the Mage/necdin family of proteins which have roles in cell cycle, differentiation and apoptosis (Mercer et al. 2009). Mutation of Magel2 produces a mouse phenotype characterised by neonatal growth retardation, presumably due to poor suckling or milk letdown since later in life, after weaning, the offspring show excess weight gain (Bischof et al. 2007). Necdin-deficient mice have a reduction of oxytocin neurons (Muscatelli et al. 2000) as does the Peg3 mutant (Li et al. 1999), which produces hormones that are important for parturition, milk letdown and maternal behaviour in the adult female. The Magel2 mutant also has a significant reduction in oxytocin and a single

injection of oxytocin soon after birth can rescue the phenotype and the survival of Magel2 mutants pups (Schaller et al. 2010). The maternally imprinted gene Mest, like Peg3, also regulates hypothalamic development and maternal behaviour, and Mest-deficient females show abnormal maternal behaviour including a failure in placentophagia (Lefebvre et al. 1998). Peg3 and Mest are also expressed in the placenta, and the mutants show impaired placental growth development (Lefebvre et al. 1998; Li et al. 1999). There are no reports on the function of Magel2 or Necdin in the placenta, but since they both code for the Mage family of proteins which have roles in cell cycle, differentiation and apoptosis, it is likely their expression in the placenta will have similar effects on growth as Mest and Peg3. Thus, all four maternally imprinted genes have common components to their mutant phenotype (milk letdown, suckling, feeding, maternal care). Furthermore, Peg3 and Necdin bind Hif1-α and Arnt (Friedman and Fan 2007), the latter producing an HLH-PAS protein which dimerises with Sim1 in the development of hypothalamic oxytocinergic neurons (Duplan et al. 2009). Hif1-α and Arnt are hypoxia induced and also play an important role in placental development, especially for differentiation of giant cells (Gultice et al. 2009) which produce the placental hormones that prime the maternal hypothalamus for regulation of maternalism. In this context, it is significant that the fetal hypothalamus and placenta are developing at the same time which is a critical period for placental vascular supply and thereby provides nutrition to the fetus and transport of placental hormones which regulate the maternal hypothalamus.

The placenta is, therefore, in a pivotal position for enabling fetal hypothalamic development at the same time as commandeering the adult maternal hypothalamus to provide the resources. Thus, genomic imprinting provides the same allele to be simultaneously engaged in development of the placenta and fetal hypothalamus at the same time as the former interacts with the adult maternal hypothalamus. Interestingly, placental stem cells can differentiate into neurons (Portmann-Lanz et al. 2010). Such linked coadaptation in the development of placenta and maternal brain ensures that offspring which have extracted "good" maternal nurturing will also be well provisioned for and genetically predisposed towards developing a hypothalamus that determines good mothering in the next generation (Keverne and Curley 2008). Moreover, the rates of propagation for paternally expressed genes are greatly enhanced by capitalising on the greater reproductive success of advantaged males (Swaney and Keverne 2009).

1.7 Social Complexity and Evolution of the Neocortex

Primates differ from other mammals in the complexity of their social interactions, and unlike small-brained mammals, these interactions are not restricted just to periods of prime biological significance, such as parturition. Throughout primate evolution there has been a general trend of increasing brain size relative to body size, driven largely by the requirement to process this increasingly complex social

information (Dunbar 1998). Among the highly social Old World monkeys, it is the females, rather than males, who determine stable cohesive groups that are maintained over successive generations, with the social rank of daughters, but not sons, being inherited from mothers (Bergman et al. 2003; Wrangham 1980). Thus, the matriline is pivotal for social evolution, and not surprisingly, the neural mechanisms subserving this social evolution engage pathways also involved with mother-infant bonding (Curley et al. 2005b). Moreover, females that have higher infant survival and reproductive success are also the females that are more social (Silk et al. 2003). Such social behaviour is linked neurologically to the biology of brain reward, and the affiliative behaviour underpinning reward, such as grooming, releases the same neuropeptides (oxytocin, beta-endorphin) that are also released at parturition. These neuropeptides have acquired the distinctive function of rewarding positive social encounters, thereby forming the "social glue" of these complex societies (Keverne et al. 1989) as well as mother-infant bonding (Broad et al. 2006).

In order for social reward to have gained such power from cortical expansion, it has been necessary for maternal care in particular to become emancipated from hormonal determinants. Older sisters and maternal relatives participate in offspring care without having undertaken pregnancy or parturition. Mothers themselves are thus not required to delay their next pregnancy until the offspring brain development is fully mature. The extended matriline fulfils this role, and under the watchful eye of the matriarch, they gain experience in mothering before undertaking pregnancy themselves.

Understanding brain evolution is crucial to the realisation of what hormonal influences on small-brained mammals can and cannot inform us about primate/hominid brain and behaviour. Epigenetic processes are activated by prosocial mothering in rodents (Champagne 2008) and produce offspring that display the same prosocial mothering in the next generation, even when cross-fostered from a strain of mothers that score low on these behaviours. However, synchronising primate neocortex development with the extended postnatal social environment is especially important in large-brained mammals. This ensures a second phase of development when the brain is exposed to a social environment that epigenetically equips it to function for a lifetime of social interactions.

Studying the genetic basis of mammalian brain evolution and genes which underpin primate brain differences has met with little success. For human/chimpanzee comparisons, the strong sequence similarities between these two species produce high stochastic uncertainly in estimating evolutionary rates of change for nervous system genes (Dorus et al. 2004). The vast majority of changes in the human versus chimpanzee genome are thought to represent neutral drift, and despite the human brain being three times the size of the chimpanzee brain, some 44 % of brain-expressed genes show evidence of some form of differential regulation (Johnson et al. 2009). Recent studies have shown that non-coding regions of the human genome contain evolutionary acceleration of substitutions that diverge from chimpanzee to human (Xu et al. 2010). One of these accelerated regions is part of a novel RNA gene (HAR1F) that is co-expressed with reelin, a known regulator of cortical development (Pollard et al. 2006).

In the brain, developmental "timing" is of great importance, and slowing down the developmental process, neoteny, is thought to have contributed to an evolutionary increase in human brain size. Thus, it has been shown that the frontal cortex, which is late to develop in human and undergoes reorganisation post-pubertally, also shows a transcriptome that is remodelled in development and is delayed relative to other primates (Somel et al. 2009). This delay or neotenic shift affects a subset of genes that play an important role in neural development and which diverge more rapidly than genes in other categories during early adolescence. Similar studies on the late mid-fetal stage of human neocortical development have found conserved non-coding sequences that act as cis-regulatory elements (Pennacchio et al. 2006). A subset of these elements have undergone human-specific accelerated evolution. A large number of the genes in close proximity to these human-specific accelerated regions are differentially expressed, providing a significant enrichment of accelerated evolution in gene regulatory regions (Johnson et al. 2009).

The complexity of the neocortex raises the important question as to how the billions of neurons and trillions of synapses are brought together as functional units using the fairly restricted number of genes available. Clearly, it is not possible without making mistakes, and these errors need to be accounted for during development. In a comparison of the mouse cortex with the monkey cortex and deploying the common basic developmental constructs which include size of the neurogenic pool, the number of symmetric and asymmetric divisions and cell cycle interval, the monkey cortex should theoretically be three times larger than it actually is (Rakic 2009). Timing, spatial relationships, directional cues, target cues and growth rate of axons all require integration when developing the neocortex. Moreover, all cortical neurons make contact with other cortical neurons that are undertaking the same process. Correct timing is therefore critical at all stages, and while a genetic programme is fundamental, an epigenetic programme embracing timing and cell death is equally crucial to normal brain development. Bearing in mind the primate brain develops in a socially meaningful, complex environment, it is quite possible, at the synaptic level, that no two brains are exactly the same. For all these reasons, it would seem likely that epigenetics has played a particularly important role in cortical evolution through development.

1.8 Conclusions

Epigenetics epitomises brain development more than that of any other structure. The billions of neurons exponentially magnified by the trillions of synaptic interconnections probably mean no two human brains are alike. This is hardly surprising when we consider that some 76 % of all human gene transcripts are expressed in the developing brain, and of these 33 % are differentially expressed and a further 28 % show different exon usage or alternative splicing (Johnson et al. 2009). Even monozygotic twins show differences in behaviour (Haque et al. 2009)

and in psychiatric disorders (Ptak and Petronis 2010), differences which become more marked with age.

The larger the mammalian brain, the more adaptable are its capabilities. Nevertheless, parts of the brain and some aspects of behaviour are so fundamental to mammalian survival that gene regulatory changes instrumental in their success have become heritable through genomic imprinting. Hence, the importance of these imprinted genes for developing a hypothalamus capable of successfully regulating maternal behaviour, sexual behaviour, feeding behaviour and metabolism. Since the greatest burden for mammalian reproductive success depends on maternalism, it is not surprising that imprint control regions are primarily under matrilineal regulation. In small-brained mammals, much of adult behaviour is in harmony with physiological homeostasis, and the mouse brain generates such motivated behaviour by responding epigenetically to the dictates of the body's hormones. The sex hormones oestrogen, testosterone and progesterone are epigenetic regulators of puberty, oestrus, maternal behaviour and sexual behaviour and are primarily concerned with the timing and activation of primary motivated behaviours.

The neocortex, probably more than any other brain tissue, has evolved to be adaptable and sustains both long-term and short-term synaptic connections that underpin complex learning and memory. Here, the adapted changes are not themselves inherited but the predisposing epigenetic processes are, thus providing each generation with the same ability to generate new adaptations while retaining a predisposition to preserve others. Humans can sit, stand, walk and feed themselves, all predisposed motor activities that take years for the newborn to achieve, and are dependent on a social environment that is instructive in this achievement. Moreover, "once learned never forgotten" applies to these and many other human memories and experiences, including traumatic memories which we might prefer to forget.

The evolutionary enlargement of the neocortex has enabled the process of human decision making to override the primary motivated sexual, parental and even feeding behaviour. Emancipating such survival behaviour from basic physiology has required a great deal of intelligent brain power and social knowledge, much of it acquired over successive generations of social living and only made possible by the neocortex of the largest of mammalian brains.

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Chapter 2
Social Environment and DNA Methylation: A Mechanism for Linking Nurture and Nature

Moshe Szyf

Abstract During cellular differentiation cell type-specific DNA methylation patterns are formed which are conserved during life maintaining cell type identity. Cellular differentiation is an innate process. It was therefore believed that DNA methylation patterns remain stable after cellular differentiation and mitosis are completed. Recent data suggests that DNA methylation patterns could also differentiate in response to external social signals in post-mitotic cells after birth and in adults. This raises the attractive possibility that DNA methylation can serve as a mechanism for adapting genomes to changing social environments conferring upon DNA an identity that is "environment-context" specific. DNA methylation is proposed to serve as a genome adaptation mechanism, adapting genome function to changing environmental contexts including social environments. A critical time point for this process is early life when cues from the social and physical environments define lifelong trajectories of physical and mental health. We suggest that we broaden the definition of DNA methylation as a mechanism of conferring differential identities to similar gene sequences. This expands the role that DNA methylation could play beyond the traditional boundaries of cellular differentiation.

Keywords Early life • Epigenetics • DNA demethylation • DNA methylation • Maternal care • Nonhuman primates • Social environment • T cells

Abbreviations

5-HT	5-hydroxytryptamine
AID	Activation-induced cytidine deaminase

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AVP	Arginine vasopressin
BDNF	Brain-derived nerve growth factor
CAMKII	Calmodulin-dependent protein kinase II
DNMT	DNA methyltransferase
ELS	Early life stress
GABRA1	Gamma-aminobutyric acid A receptor alpha 1 subunit
GADD45A	Growth arrest and DNA-damage-inducible alpha
GC	Glucocorticoids
GR	Glucocorticoid receptor
HDAC	Histone deacetylase
HPA	Hippocampal–pituitary–adrenal
LG	Licking and grooming
MBD	Methyl-CpG-binding domain protein
MeCP2	Methyl-CpG-binding protein 2
PCDH	Protocadherin
RRNA	Ribosomal RNA
SAM	S-adenosylmethionine
TSA	Trichostatin A

2.1 Introduction

The social and physical environment influence human development after birth and during different life cycle stations. For example, social adversity early in life has a profound impact on lifelong physical health and behavior (Hertzman et al. 2001; Power et al. 1997, 2006). Maternal behavior plays a cardinal role in the behavioral development of mammals. Models of maternal deprivation in primates and rodents and natural variation in maternal care in rodents have demonstrated the significant impact of maternal care on a panel of phenotypes in the offspring that last into adulthood (Ruppenthal et al. 1976; Suomi et al. 1976). Human mental development occurs in the first years of life and is heavily influenced by external signals derived from the social environment. In addition, many studies have indicated that there is a strong environmental interaction with genetic inheritance. The first example reported is the short 5-HT transporter polymorphism. Individuals that carry two copies of this allele show depressive symptoms, diagnosable depression, and suicidality only if exposed to stressful life events (Caspi et al. 2003). This observation was repeated in nonhuman primate rhesus monkeys. Monkeys bearing the short 5-HT transporter showed lower serotonin levels only when exposed to deleterious early rearing experiences (Bennett et al. 2002). This well-established effect of external environmental signals on gene function and the phenotype begs the following questions: What is the mechanism? Why would the same gene variant

express different phenotypes because of a different history of social environments? What are the mechanisms that embed differential social environments in genome function?

Several animal models have been developed to study the impact of the environment on behavioral phenotypes later in life. The hippocampal glucocorticoid receptor (GR) controls the negative feedback of the hippocampal–pituitary–adrenal (HPA) axis by glucocorticoids. In the rat, the adult offspring of mothers that exhibit increased levels of pup licking/grooming (i.e., high-LG mothers) over the first week of life show increased hippocampal (GR) expression, enhanced glucocorticoid feedback sensitivity, decreased hypothalamic corticotrophin-releasing factor (CRF) expression, and more modest HPA stress responses compared to animals reared by low-LG mothers (Francis et al. 1999; Liu et al. 1997). Cross-fostering experiments demonstrated that the long-term behavioral phenotype of the offspring was determined by the fostering mother LG behavior, supporting a post-birth-mediated mechanism that is not germ line transmitted. Similarly, in nonhuman primates maternal deprivation early in life results in profound phenotypic effects later in life (Cirulli et al. 2009; Corcoran et al. 2011; Stevens et al. 2009; Suomi 1991). These studies provide strong evidence that early life experience could shape lifelong phenotypes. These studies beg the question of what mechanism might be mediating these effects of the early environment on the phenotype.

2.2 DNA Methylation Patterns and Cellular Identity of DNA

DNA methylation is part of the DNA molecule chemical entity. It is thus clearly differentiated from other epigenetic mechanisms such as chromatin modification and noncoding RNA which are associated with, but separate from, DNA. Cell-specific DNA methylation patterns that are formed during cellular differentiation by innate developmental programs were described almost two decades ago (Benvenisty et al. 1985; Razin and Szyf 1984) and were recently confirmed by whole-genome methylome mapping (Lister et al. 2009). The DNA molecule has thus two identities, the ancestral identity encoded in the sequence and the cell-specific identity encoded in the pattern of DNA methylation. DNA methylation is therefore a mechanism that can confer differential chemical identities to similar DNA sequences and as a consequence alter gene expression and the phenotype.

There are potentially multiple mechanisms by which DNA methylation confers different functionality on the genome. The best studied function is the impact that DNA methylation in critical regulatory regions has on regulating gene expression. There is an overall inverse correlation between DNA methylation in regulatory regions of genes and gene expression, which was discovered in the early 1980s (Razin and Riggs 1980; Razin and Szyf 1984) and was confirmed by whole-genome approaches (Rauch et al. 2009). However, the role of differential intergenic methylation and gene body methylation (Hellman and Chess 2007; Rauch et al. 2009) is still unclear, but it might be positively associated with gene expression. It is clear

nevertheless that DNA methylation confers specific functional identity to genomes at multiple levels.

The most established role of DNA methylation is in regulation of promoter activity (Stein et al. 1982). At least two mechanisms are well established for inhibition of gene activity by DNA methylation. A methyl group positioned in a recognition element for a transcriptional factor can block binding of the transcription factor to the promoter (Comb and Goodman 1990; Inamdar et al. 1991). Alternatively, methylated DNA attracts methylated DNA-binding proteins (MBD) such as the Rett syndrome protein, methyl-CpG-binding protein 2 (MeCP2), which in turn precipitate an inactive gene-silencing chromatin configuration through recruitment of chromatin-silencing proteins (Nan et al. 1997).

2.3 DNA Methylation and Cellular Differentiation and the Impact of the Early Environment

It is obvious that the differential methylation that accompanies cellular differentiation during gestation is a highly organized process and that it is driven by innate developmental programs. Faithful epigenetic inheritance is critical for DNA methylation to play a role in cellular differentiation as maintenance of the differentiated state requires accurate copying of the DNA methylation pattern (Razin and Riggs 1980). Knockdown of DNA methyltransferase 1 and the resulting demethylation could change the state of differentiation of mammalian cells (Szyf et al. 1992). If indeed DNA methylation is important for cellular differentiation, it stands to reason that changes in DNA methylation should not occur once a terminal pattern of DNA methylation is formed. Nevertheless, there is extensive data to suggest that DNA methylation patterns could be affected by external environments as well (detailed below). The main question is whether mechanisms evolved that result in an organized response of DNA methylation patterns to external signals and whether it is an adaptive response or whether the pattern of methylation is organized exclusively by innate developmental programs. Intrauterine environmental exposures, for example, could result in inhibition of DNA methylation/demethylation enzymes during critical stages when DNA methylation patterns are laid down in a stochastic manner that does not imply the presence of a specific mechanism that responds to external environmental signals.

One of the finest examples of the impact of intrauterine exposures on DNA methylation was provided by the Jirtle lab who demonstrated an effect of maternal diet during gestation on the agouti color phenotype in viable yellow agouti (A^{vy}) mice, which was mediated through methylation of a transposable element in the A^{vy} transposable element (Waterland and Jirtle 2003). The impact of methyl-rich diets during gestation or the impact of other chemicals such as bisphenol A during gestation that inhibit DNA methylation (Dolinoy et al. 2007) could be explained

just as a stochastic chemical interference in enzymatic DNA methylation reactions that are actively laying down the DNA methylation pattern during embryogenesis.

Whereas stochastic mechanisms could nicely explain effects of the environment during gestation, they do not provide explanation for the profound impact that early life experiences during the first years of life has on the development of behavior and mental capacity. If DNA methylation is involved in the response to social experience, mechanisms that translate external signals from the environment into organized DNA methylation patterns must exist.

2.4 Hypothesis: DNA Methylation Is a Genome Adaptation Mechanism That Confers Environmental Exposure-Specific Identity to DNA

DNA methylation is a mechanism for diversification of DNA identity by providing within the same chemical entity *two layers* of information: the ancestral identity encoded in the sequence and the cellular identity encoded in the DNA methylation pattern. We propose that DNA methylation pattern can respond to external as well as innate programs and therefore the same mechanism that is involved in conferring cellular identity upon DNA during cellular differentiation is also involved in the response to external experimental and experiential signals. It is hypothesized here that external signals triggered by the environment can modulate the DNA methylation pattern in an organized way to generate differential "environmental-history" DNA methylation identities. This process could occur at different time points in life and act at different timescales ranging from proximal physiological timescale to lifelong as well as trans-generational timescales if DNA methylation is reversible after birth and in adult-differentiated tissue (Szyf 2011).

2.5 A Dynamic DNA Methylation Pattern: Reversibility of DNA Methylation in Post-mitotic Tissue

In order for DNA methylation to act as a response to external environment in post-mitotic tissue, it has to be reversible. As the targets of the social environment are neurons which are predominantly post-mitotic, the DNA methylation reaction must be reversible in order to respond to these signals (Ramchandani et al. 1999). The main issue is whether the pattern of DNA methylation that is fashioned by innate developmental processes is a final state or is it in a dynamic state which is responsive to external signals and can be further programmed by these signals.

Although there was resistance in the field to accept the possibility of a reversible DNA methylation in post-mitotic tissues, there is nevertheless significant evidence for this hypothesis (Bruniquel and Schwartz 2003; Lucarelli et al. 2001; Oswald

et al. 2000; Szyf et al. 1995; Wilks et al. 1984). It has been shown that brain extracts are capable of demethylating "naked" DNA substrate in vitro (Dong et al. 2008; Fuso et al. 2011; Mastronardi et al. 2007). The strongest evidence for dynamic methylation–demethylation comes from several studies showing active demethylation in post-mitotic neurons (Feng et al. 2010; Levenson et al. 2006; Miller and Sweatt 2007; Weaver et al. 2004). Conditional knockout of DNMT1 in post-mitotic neurons results in DNA demethylation suggesting the presence of demethylation activity in nondividing neurons which is critical for a dynamic methylation pattern in the brain (Feng and Fan 2009).

The main issue in the field remains however whether DNA methylation is truly a reversible reaction that involves removal of the methyl moiety and its release as has been previously proposed (Bhattacharya et al. 1999; Ramchandani et al. 1999) or whether DNA demethylation requires excision of the methylated base and its replacement by an unmethylated cytosine through a process of DNA repair (Jost 1993; Razin et al. 1986). The predominant opinion is that DNA demethylation in post-mitotic cells is a repair process rather than a true demethylation event. Several possible mechanisms were proposed. First, DNMTs were proposed to deaminate the methyl cytosine to thymidine creating a C/T mismatch, which is then corrected by a mismatch-repair mechanism (Kangaspeska et al. 2008). Second, growth arrest and DNA-damage-inducible, alpha (GADD45A), a DNA repair protein, was proposed to participate in catalysis of active DNA demethylation by an unknown DNA repair-based mechanism (Barreto et al. 2007). However, this was disputed (Jin et al. 2008). Other studies have suggested involvement of GADD45B in demethylation in the brain (Ma et al. 2009). Third, a complex sequence of coupled enzymatic reactions of deamination and mismatch repair was shown to be involved in demethylation in zebra fish: activation-induced cytidine deaminase (AID, which converts 5-meC to thymine), a G:T mismatch-specific thymine glycosylase methyl-CpG-binding domain protein 4 (MBD4), and repair promoted by GADD45A (Rai et al. 2008). AID has been implicated in the global demethylation in mouse primordial germ cells as well (Popp et al. 2010). An open question is the role of the newly discovered modification 5-hydroxymethylcytosine as a potential intermediate in the DNA demethylation reaction (Kriaucionis and Heintz 2009). Recent data suggest that TET1, the enzyme that catalyzes the hydroxylation of 5-methylcytosine, is present and required for stem cell maintenance of inner cell mass specification (Ito et al. 2010) and for activity-driven demethylation in neurons (Guo et al. 2011). 5-Hydroxymethylation catalyzed by TET1 is followed by deamination of the 5-hydroxymethylated base by the AID/APOBEC (apolipoprotein B mRNA-editing enzyme complex) family of cytidine deaminases and base excision repair enzymes replace the deaminated base with an unmethylated cytosine (Guo et al. 2011). More recently, it has been proposed that 5-hydroxymethylcytosine is further carboxylated and this serves as a substrate for yet unknown decarboxylases that release the entire modified methyl moiety (Ito et al. 2011). Notwithstanding the precise biochemistry, the fact that DNA methylation is reversible even in post-mitotic tissue provides justification for examining the possibility that DNA methylation patterns are adapted to environmental signals including social signals in early life.

2.6 DNA Methylation Association with Early Life Social Experience: Lessons from Candidate Genes

The first line of data that showed association of early life experience with long-term changes in DNA methylation came from a candidate gene approach. Weaver et al. showed that variations in maternal care result in differences in DNA methylation and histone acetylation in the GR/NR3C1 gene encoding the glucocorticoid receptor (GR exon 1_7 promoter) that emerge early in life and remain stable into adulthood (Weaver et al. 2004). Cross-fostering experiments showed a causal relationship between maternal care and the DNA methylation differences, and reversal of the phenotypes with epigenetic drug treatments supported a causal relationship between DNA methylation differences and phenotypic variation (Weaver et al. 2005, 2006). Exposure of infant rats to stressed caretakers that displayed abusive behavior produced persisting changes in methylation of BDNF gene promoter in the adult prefrontal cortex (Roth et al. 2009). Similarly, early life stress (ELS) in mice caused sustained DNA hypomethylation of an important regulatory region of the arginine vasopressin (AVP) gene (Murgatroyd et al. 2009).

Although it is impossible to provide causal evidence for early life experience altering DNA methylation states in humans as it is ethically unfeasible to randomize in humans early life abuse, it is nevertheless possible to associate DNA variations with differences in early life experience. The state of methylation of rRNA gene promoters and NR3C1 promoter in the hippocampus was examined in a cohort of suicide victims in Quebec who were abused as children and their control group. Ribosomal RNA (rRNA) forms the skeleton of the ribosome, the protein synthesis machinery. We have previously demonstrated a critical role for DNA methylation in regulating expression of rRNA genes (Brown and Szyf 2007). Our results showed that the suicide victims who experienced childhood abuse had higher overall methylation in their rRNA genes and expressed less rRNA in a brain region-specific manner (McGowan et al. 2008). We also examined in this cohort the same promoter of NR3C1 gene that was affected by maternal care in rats. Site-specific differences in DNA methylation in the NR3C1 exon 1f promoter and its expression were detected between suicide completers who had reported social adversity early in life and suicide completers who did not experience social adversity early in life (McGowan et al. 2009).

Epigenetic modulation of other candidate genes was implicated in suicide: the gamma-aminobutyric acid A receptor alpha 1 subunit (GABRA1) promoter (Linthorst et al. 1995) within the frontopolar cortex (Poulter et al. 2008) and tropomyosin-related kinase B (TRKB) in the frontal cortex of suicide completers (Ernst et al. 2009). It is unknown yet whether these changes in DNA are also associated with early life adversity.

2.7 Reversibility of the DNA Methylation Pattern Programmed by Early Life Social Environment Using Pharmacological Agents

DNA methylation is a biochemical reaction and is therefore potentially responsive to interference with pharmacological activators or inhibitors even in adult post-mitotic cells as long as the pattern of DNA methylation remains defined by an active balance of methylation and demethylation enzymatic activities. If DNA methylation is fixed after birth as has originally been the understanding, then the pattern of DNA methylation should be resistant to any change in post-mitotic neurons. We therefore tested whether it is possible to pharmacologically reverse a phenotype that was determined by early life experience. Our results demonstrate that it is possible to reverse the phenotype of adult offspring of high-LG mothers by treating them with methionine, a precursor of the methyl donor in DNA methyltransferase reactions dependent on S-adenosylmethionine. Moreover, we can also reverse the phenotype of adult offspring of low-LG mothers by treating them with TSA, a histone deacetylase inhibitor that we have shown can trigger DNA demethylation (Cervoni and Szyf 2001; Weaver et al. 2004, 2005, 2006). The dynamic model of DNA methylation after birth is consistent with the concept that DNA methylation patterns are responsive to external experiential signals in early life as well as with the possibility that these phenotypes defined by experience are potentially reversible by pharmacology. It is tempting to speculate that not only pharmacology but cognitive and behavioral therapies as well could reverse phenotypes defined by DNA methylation.

In summary, the dynamic model of DNA methylation explains how on one hand experience could define long-term DNA methylation patterns and phenotypes as well as how this stable pattern is potentially reversible.

2.8 DNA Methylation Association with Early Life Social Experience: Involvement of Broad Genomic Regions in a Clustered and Organized Response

The response to early life experience involves multiple phenotypes suggesting a system-wide response that involves multiple genes and multiple physiological systems. Studies looking at the phenotypes associated with early life socioeconomic positioning revealed multiple phenotypes associated with early life adversity including obesity and asthma (Hertzman et al. 2001; Power et al. 1997, 2006). If indeed the response of DNA methylation states to early life adversity is an adaptation rather than a stochastic disruption, it should involve an organized change in DNA methylation across the genome. We tested this hypothesis in several studies. All studies point to the conclusion that the impact of early life adversity on the

epigenome is broad and that it involves multiple systems and is not limited to the brain. This has diagnostic and mechanistic implications. Most importantly it supports the idea that it might be worthwhile to study behavioral epigenetics in peripheral tissues. It also supports the idea that genome-wide methylation patterns should be examined for understanding of how experience shapes the methylome. We have documented several examples that support this hypothesis.

First, natural variations in maternal care in the rat are associated with coordinate changes in DNA methylation, chromatin, and gene expression spanning over a hundred kilobase pairs. Interestingly, a chromosomal region containing a cluster of the protocadherin-α, protocadherin-β, and protocadherin-γ (Pcdh) gene families implicated in synaptogenesis shows the highest differential response to maternal care. The entire cluster reveals epigenetic and transcriptional changes in response to maternal care (McGowan et al. 2011). Second, we showed that a similar pattern of response to childhood abuse is associated with DNA methylation differences throughout the genomic region spanning the six and a half million base-pair region centered at the NR3C1 gene in the hippocampus of adult humans suggesting evolutionary conservation of this adaptation (Suderman et al. 2012). Third, similar to the rat and human, the changes in DNA methylation associated with differences in rearing in rhesus monkeys are widespread in the genome, they are not limited to the brain, and occur in T cells as well (Provençal et al. 2012). Fourth, we have initiated a study of the impact of socioeconomic positioning on DNA methylation that examined blood DNA from the British birth cohort of 1958. This study detected a signature of DNA methylation that is associated with early life adversity (Borghol et al. 2011) supporting the hypothesis that social environment DNA methylation signatures are found system wide and could be examined in peripheral blood cells. The study also revealed highly organized association with DNA methylation and socioeconomic positioning that clustered across broad genomic regions (Borghol et al. 2011).

Three other studies have demonstrated that epigenetic effects associated with behavioral adversity could be detected in blood cells. First, the NR3C1 promoter was more methylated in lymphocytes in newborns exposed prenatally to maternal depression than control newborns (Oberlander et al. 2008). Second, pituitary adenylate cyclase-activating polypeptide (PACAP), a protein known to be involved in stress response in the pituitary, was found to be differentially methylated in peripheral blood cells in humans with posttraumatic stress syndrome (Ressler et al. 2011). Third, telomere length differences were identified between orphans in the Bucharest Early Intervention Project who were placed under high-quality foster care compared with those subjected to continued care in institutions (Drury et al. 2011). As discussed above, a long line of data have established that the physiological response to early life socioeconomic adversity is not limited to the brain (Cunha and Heckman 2009; Power et al. 2007, 1997). There is no reason therefore to believe that DNA methylation changes in response to adversity should not occur in the periphery as well as the brain.

2.9 Molecular Mechanisms of Experience-Driven DNA Methylation Changes

It is clear that DNA methylation patterns could be altered stochastically during gestation when DNA methylation patterns are formed by enzymatic reactions that are inherently susceptible to inhibition or activation by chemicals as no enzymatic reaction is completely free from certain degree of error. Since maintenance DNMT1 will copy new DNA methylation events from the template strand to the daughter strands, such stochastic DNA methylation events could be potentially maintained across multiple cell divisions. Stochastic alterations in DNA methylation similar to single-nucleotide polymorphisms could lead to alterations in gene function if they happen to be at critical positions, altering phenotype and causing disease. However, the data that I presented suggests that the high predictability and organization of the DNA methylation differences associated with early life adversity are inconsistent with a purely stochastic mechanism. Mechanisms should exist that mediate between the experience and targeted changes in DNA methylation. It is hypothesized here that social experience triggers signaling pathways in the brain that target DNA methylation/demethylation activities to specific genomic targets resulting in changes in DNA methylation (Fig. 2.1). For example, it was proposed that maternal LG triggered serotoninergic pathways in the hippocampus turning on cAMP-mediated signaling, that in turn activated a transcription factor NGFIA which bound specific NR3C1 promoter regions and targeted CBP, a histone acetyltransferase, to the gene resulting in changes in histone acetylation and DNA methylation (Weaver et al. 2007).

A different mechanism that involved direct signaling events was proposed to explain the demethylation of the regulatory region of the arginine vasopressin (AVP) gene in response to maternal stress (Murgatroyd et al. 2009). In this case, neuronal activity leads to alteration in the binding of MeCP2 to the promoter of the gene (Murgatroyd et al. 2009). It was previously shown that MeCp2 was phosphorylated by CAMKII kinase in response to neuronal activation and that this phosphorylation event altered the affinity of binding of MeCP2 to a BDNF promoter resulting in transcription activation (Zhou et al. 2006); this reduced affinity of MeCP2 to the promoter could be followed by DNA demethylation (Chen et al. 2003), perhaps through increased accessibility of the promoter to demethylases. Deciphering the mechanisms that lead from experience to DNA methylation changes is a challenge that needs to be addressed if we want to understand how experience shapes phenotype.

2.10 Summary

The scope of involvement of DNA methylation in long-lasting regulation of genome function is broader than has originally been thought. DNA methylation acts as a mechanism for providing differential identities to similar DNA sequences. Originally, it has been thought that such a mechanism is exclusive for cellular differentiation when an identical genome acquires different identities expressing different

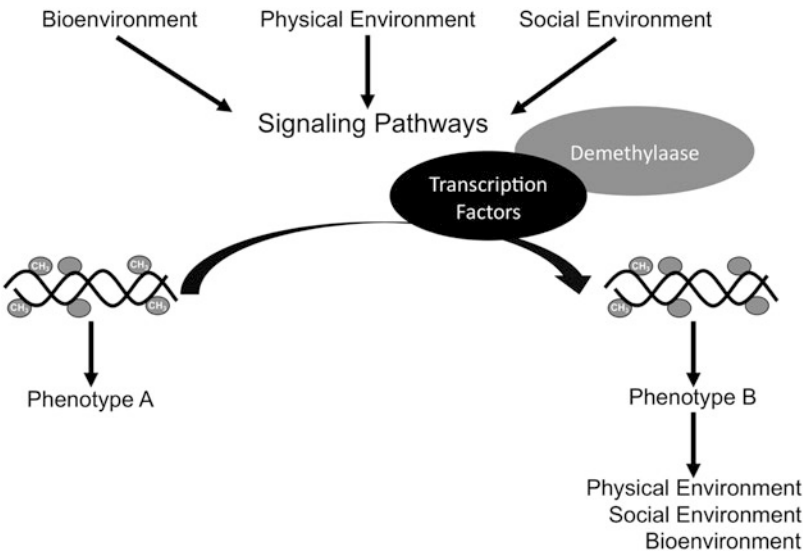

Fig. 2.1 DNA methylation, a genome adaptation mechanism: a model. Signals from the social, bioenvironmental, and physical environment act on signaling pathways to trigger changes in DNA methylation through recruitment of transcription factors and DNA-modifying enzymes to specific targets in the genome. This adapts the genome and the phenotype to the anticipated lifelong environment

phenotypes. It is proposed here that DNA methylation can also act as a mechanism for adaptation of the genome to different environments and experiences. A misfit between programmed DNA methylation in response to an anticipated environment and the real environment is a maladaptation and could result in disease.

Early life experience plays a cardinal role in these adaptations of genome function and the phenotype (Fig. 2.1) However, such a mechanism can be functional at any point in life and act in different kinds and levels of genome adaptation. There is data in both animals and humans that supports this hypothesis. However, the mechanisms that mediate between external social signals and DNA methylation changes that seem to cluster across the genome are largely unknown. Future studies are required to unravel these mechanisms.

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Chapter 3
Sex Differences in Epigenetic Programming of Brain Differentiation: Implications for Mental Health and Disease

Anthony P. Auger and Catherine J. Auger

Abstract There are multiple contributing factors that can influence one's vulnerability to disease; one of those factors is being a male or female. Specifically, disorders such as Rett syndrome, schizophrenia, Alzheimer's disease, and autism can differ in terms of age of onset, incidence, or severity based on the sex of the affected individual. As aberrant epigenetic processes are implicated in some of these disorders and emerging data indicate that there are sex differences in epigenetic processes during brain development, it is plausible that sexual differentiation of the epigenome may partly underlie sex-biased mental health disorders. This chapter will discuss recent data indicating that brief disturbances to some epigenetic processes during early neonatal brain development not only interfere with sexual differentiation of the brain but also result in atypical juvenile social behavior in a sex-specific manner. We will also discuss how the early social environment can shape sex differences in the epigenome and discuss recent data indicating that some epigenetic programming may be reversible in the adult brain. We propose that sexual differentiation of the epigenome produces a sex-biased vulnerability to mental health disorders, and understanding these differences will aid in understanding the biological pathways to resilience.

Keywords Amygdala • Coactivator • Corepressors • DNMT • Estrogen receptor • MeCP2 • Methylation • Social behavior • Sexual differentiation • Vasopressin

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Abbreviations

5caC	5-Carboxylcytosine
5fC	5-Formylcytosine
5hmC	5-Hydroxymethylation
AVP	Vasopressin
CPB	cAMP response element-binding protein-binding protein
DNMT	DNA methyltransferases
ER-α	Estrogen receptor-α
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
MBD	Methyl-CpG-binding domain
MBP	Methyl-CpG-binding protein
MeCP2	Methyl-CpG-binding protein 2
NCoR	Nuclear corepressor
SRC-1	Steroid receptor coactivator-1
SRY	Sex-determining region Y
TET	Ten eleven translocation

3.1 Introduction

The developing brain is exquisitely sensitive to endogenous and environmental cues, and subtle, brief changes in some of these cues during critical periods of brain development can produce lasting consequences on brain function. The elucidation of how gene x environment interactions program brain development at the epigenetic level will aid our understanding of how early-life experiences can profoundly alter the overall health and well-being of an individual. Specifically, emerging data indicate that differences in early-life experiences may underlie individual differences in risk and resilience to disease, including some mental health disorders. Intriguingly, an individual's sex also seems to play an important role in influencing the occurrence, severity, and age of onset in a number of mental health disorders. While sex differences in Alzheimer's disease, schizophrenia, Rett syndrome, and autism have been well documented, it is not clear what underlies the sex differences in risk and severity of these disorders. Understanding the mechanisms influencing sexual differentiation of the developing brain will provide clues about why some individuals exhibit greater vulnerability to mental health disorders. Therefore, a further refinement of investigations into gene x environment interactions in programming risk and resilience to disease may be expanded to exploring gene x environment x sex interactions (Fig. 3.1).

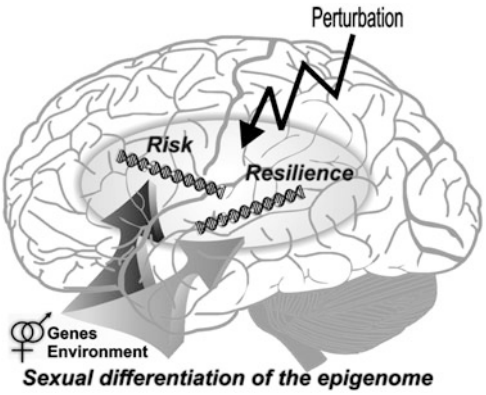

Fig. 3.1 Sexual differentiation of the epigenome may underlie sex differences in risk or resilience to mental health disorders. Several converging mechanisms program sex differences in the epigenome and thereby brain function. Genetic and environmental cues interact in the brain to differentiate the male from female brain. Sex differences in the epigenome may underlie sex differences in risk and resilience to neurological and psychiatric disorders induced by environmental or genetic perturbations

3.2 Sexual Differentiation of the Brain

Sexual differentiation of the brain is regulated by a variety of processes, ranging from genetic, hormonal, to early environmental influences (Lonstein and Auger 2009; McCarthy and Arnold 2011; Auger and Auger 2011). The classical model of sexual differentiation of the brain suggests that gonadal steroid hormones are the main factor differentiating the male and female brain. In this model, sexual differentiation is initiated by the sex-determining region Y (*Sry*) gene located on the Y chromosome. *Sry* encodes a transcription factor that triggers the development of the testes. During critical periods of development, the perinatal testes synthesize and release testosterone into the circulatory system, which then impacts a variety of steroid-sensitive tissues, including the brain. Exposure to testosterone and its metabolites, estradiol and dihydrotestosterone, organizes some of the most prominent and lasting sex differences in brain and behavior. Steroid hormones produce these lasting outcomes mostly by binding to steroid receptors located within neurons. Upon steroid binding to its receptors, the ligand-bound steroid receptor interacts with a variety of co-regulatory proteins and other transcriptional factors on response elements located within the genome (Tetel et al. 2009). While some of the outcomes of steroid receptor action at the genome are transient, steroid hormones can induce lasting changes within the brain that can be observed in alterations of cell number, migration patterns, phenotypical differentiation, as well as morphological differentiation of brain cells (Lonstein and Auger 2009). An additional pathway by which transient changes in steroid hormone can impact brain function later in life is via epigenetic reprogramming of gene function (McCarthy et al. 2009; Auger et al. 2010). Epigenetic mechanisms provide a biological mechanism

by which neurons can store a "memory" of a hormone surge, as well as other endogenous signals, at the level of the genome. It is also important to note that steroid hormone levels can be influenced by the environment, providing a signaling pathway by which environmental cues can impact brain development; therefore, neuroendocrine differentiation of the brain provides a useful model to study lasting epigenetic mechanisms that may impact mental health.

3.3 Epigenetic Mechanisms Involved in Sexual Differentiation of the Brain

While there are many epigenetic processes that can impact gene transcription, one of the commonly studied mechanisms is DNA methylation. The most widely studied pathway is when a methyl group is attached to cytosine within a 5'-CpN-3' dinucleotide to create 5-methylcytosine via an enzymatic reaction catalyzed by DNA methyltransferases (DNMTs) (Grafstrom et al. 1985; Ramsahoye et al. 2000). While methylation of DNA occurs mostly at CpG sites, methylation can occur within the promoter region of a gene, upstream from the transcriptional start site, as well as within coding and noncoding regions. It is also generally believed that methylation of CpG sites leads to gene repression (Razin and Riggs 1980); however, there are some data suggesting it may also participate in the activation of gene transcription (Metivier et al. 2008). While methylation of CpG sites can impact gene transcription by itself, it is when methyl-CpG-binding proteins (MBPs) bind to methylated DNA, causing the recruitment of chromatin remodeling corepressor complexes to DNA, that can more efficiently modify gene transcription (Klose and Bird 2006). These corepressor complexes contain histone deacetylase (HDAC) activity which removes acetyl groups from histones, resulting in chromatin condensation and gene repression (Yoon et al. 2003; Collingwood et al. 1999; Tetel et al. 2009). Recent data indicate that sex differences in epigenetic processes are apparent at multiple levels, ranging from sex differences in DNA methylation patterns, DNMTs, MBPs, histones, to co-regulatory proteins (i.e., coactivators and corepressors) within the developing brain. Sex differences in these epigenetic molecules are important in sex-specific brain and social development. They may also underlie sex differences in risk or resilience to mental health disorders.

3.3.1 DNA Methylation and Brain Sex Differences

It is becoming clear that sexual differentiation of the brain is dependent upon the coordination of both gene-activating and gene-silencing mechanisms. Indeed, molecules associated with gene repression are perhaps equally important as those associated with gene activation in programming sex differences during brain

development (Auger and Auger 2011). Furthermore, some of these epigenetic mechanisms can be modified by the social environment, providing a pathway by which external social cues can shape sexual differentiation of the brain. For example, it was reported that subtle variations in maternal care during the neonatal period can modify DNA methylation patterns of nuclear receptor genes, such as estrogen and glucocorticoid receptors, within the developing offspring brain (Weaver et al. 2004; Champagne et al. 2006). Although not directly tested, it was suggested that the somatosensory stimuli associated with licking and grooming provided by the mother was the environmental cue that induced epigenetic changes within the developing brain. This suggested the exciting idea that DNA methylation patterns within the developing postnatal brain are somewhat plastic and can be altered by environmental stimuli. Intriguingly, mother rats lick and groom their male offspring more than female offspring (Moore and Morelli 1979). Based on this, it was recently assessed if the differential licking and grooming behavior of the mother toward their male and female offspring resulted in a sex difference in the epigenetic programming of gene expression (Kurian et al. 2010). Importantly, one of the major molecules in sexually differentiating the brain is estrogen receptor-α (ER-α), which is expressed at lower levels in males than females (DonCarlos and Handa 1994; Yamamoto et al. 2006; Yokosuka et al. 1997). As males receive more tactile stimulation from the mother during neonatal development, it was hypothesized that artificially increasing the somatosensory stimuli associated with maternal care in females could eliminate a sex difference in ER-α levels. More importantly, would artificially increasing maternal-like somatosensory stimulation also result in changes in ER-α promoter methylation that are consistent with changes in ER-α expression? It was found that control males had higher levels of CpG methylation than control females within the 5' flanking region of ER-α exon 1b promoter region corresponding with lower levels of ER-α mRNA. Interestingly, providing females with additional tactile stimuli associated with maternal touch increased ER-α promoter methylation and decreased ER-α mRNA expression to male-like levels (Kurian et al. 2010). The consequences of maternal touch on epigenetic reprogramming of ER-α expression appear to increase over the first 10 days of neonatal life, suggesting that there may be a sensitive period for epigenetic reprogramming of ER-α expression or that there is a critical threshold of tactile stimuli that must be reached to change DNA methylation patterns (Edelmann and Auger 2011). These data not only support the idea that DNA methylation patterns can be altered within the developing brain by the early social environment, but they also suggested the fascinating idea that maternal touch may be contributing to sexual differentiation of the brain via epigenetic reprogramming of ER-α expression (Fig. 3.2).

It is intriguing that subtle variations in maternal care or touch can alter the epigenome, as there appears to be a hyporesponsive period to stress during early postnatal brain development (Lupien et al. 2009). That is, there seems to be a mechanism in place, perhaps maternal care, that somewhat protects the neonatal brain from environmental perturbations, resulting in a subnormal response to stress during the first 2 weeks of life. Although a critical window of resistance during

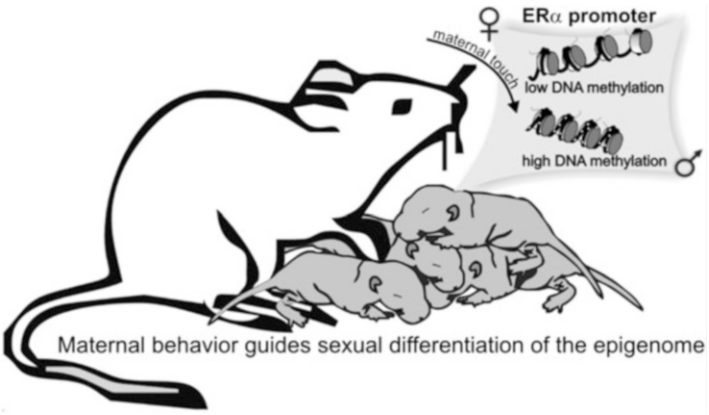

Fig. 3.2 Subtle variations in maternal behavior directed at male versus female offspring contribute to sexual differentiation of the epigenome. That is, mother rats tend to lick and groom neonatal males more than neonatal females. Within the estrogen receptor promoter region, neonatal males have higher levels of DNA methylation contrasted to neonatal females. The increased maternal touch directed at males appears to contribute to increased methylation of the estrogen receptor promoter region. Therefore, the mother rats' behavior is a contributing factor to further refining sexual differentiation of the epigenome

development appears important for typical development, this is not to suggest that other factors are constrained to this same time period or cannot override this window. For example, it is likely that the consequences of maternal neglect or drug use are less confined to this developmental window and/or can easily breach these protective mechanisms. Therefore, while there is a stress hyporesponsive period, or window of susceptibility during early neonatal life, environmental perturbations may override this protective mechanisms and cause stable reprogramming of gene function. As maternal care is suggested to be involved in this protective mechanism, sex differences in maternal care may cause sex differences in risk or resilience to environmental perturbations in the offspring. Alternatively, sex differences in maternal care may serve as a compensatory factor to blunt some of the steroid hormone-induced changes that are occurring in the neonatal male brain. While the functional role for a difference in maternal care is unclear, these data do suggest that the early social environment is a contributing factor in programming sex differences in DNA methylation patterns within the developing brain.

3.3.2 DNA Methyltransferases and Brain Sex Differences

As there are sex differences in DNA methylation patterns during early brain development, it stands to reason that there are sex differences in some of the enzymes involved in methylating DNA. Methylation of DNA is facilitated by the

DNMT family of enzymes that include DNMT1, DNMT3a, and DNMT3b. DNMT1 is generally considered important in maintaining DNA methylation patterns (Pradhan et al. 1999; Yoder et al. 1997), while DNMT3a and DNMT3b are associated with de novo methylation (Okano et al. 1998; Hsieh 1999). Interestingly, there appears to be a sex difference in the levels of DNMT3a within the developing amygdala (Kolodkin and Auger 2011). Specifically, females have significantly more DNMT3a than males at postnatal day (P) 1 within the amygdala, but not within the preoptic area or medial basal hypothalamus. This sex difference is eliminated by the second week of life, suggesting a small window in which some genes may be differentially methylated between males and females. While it remains to be determined if differences in maternal touch contributes to a sex difference in DNMT3a expression, it appears that differences in hormone exposure around birth is partly responsible for the sex difference in DNMT3a level. That is, treatment of females with either estradiol benzoate or dihydrotestosterone decreases DNMT3a levels within the amygdala. This suggests that differences in hormones levels due to endogenous or exogenous cues, such as early-life adversity, may program lasting differences in brain function by modifying the expression levels of DNMTs within the developing amygdala.

While the role of DNMT3a on programming sex differences in the amygdala has not been directly tested, the data described thus far suggest that DNMTs likely play a role in developing brain in a sex-specific manner. It will be important to assess the functional contributions of a sex difference in DNMT3a expression on the potential early developmental programming of later sex differences in mental health disorders. For example, DNMTs seem to play an important role regulating the potential risk for Alzheimer's disease (Guo et al. 2011). Oxidative stress or inflammation, which is associated with early stages of Alzheimer's disease, results in a decrease in DNMT1 and DNMT3a levels. This decrease in DMNTs is also associated with a decrease in methylation of genes that are associated with the alternative slicing and production of the pathological form of amyloid-beta, the major component in plaques found in the brains of Alzheimer's patients. This suggests that DNMTs may play an instrumental role in adult disease pathology. Taken together, it is possible that the differential DNMT signaling during development may impact the risk for developing a neurological disorder later in life. Interestingly, Alzheimer's disease is expressed differentially between males and females, with the incidence of the disease being higher in females. Although, DNMT levels are higher in females during early brain development, it is not known if sex differences in DNMT levels occur in aged populations. It will be important to determine how DNMT expression and activity is regulated throughout the lifespan.

3.3.3 *Methyl-CpG-Binding Protein 2 and Brain Sex Differences*

While changes in DNA methylation alone can impact gene transcription rates, it is when methyl-binding proteins bind to methylated CpG sites that can lead to more effective gene repression. Therefore, another way to understand the impact of DNA methylation on overall health of an individual is to understand the functional role of methyl-binding proteins within the developing brain. Methyl-CpG-binding proteins were discovered following the characterization of the methyl-CpG-binding domain (MBD) that binds methylated DNA to cause transcriptional repression (Nan et al. 1993; Hendrich and Bird 1998). Members of the MBD protein family include Kaiso, MBD1, MBD2, MBD3, MBD4, and methyl-CpG-binding protein 2 (MeCP2). Upon binding to methylated DNA, methyl-binding proteins (MBPs) interact with corepressor proteins and HDACs to modify chromatin and repress gene transcription. Of particular importance for mental health is MeCP2, as mutations in MeCP2 are believed to cause Rett syndrome, a progressive neurodevelopmental disorder (Amir et al. 1999). As MeCP2 is on the X chromosome, Rett syndrome is diagnosed mainly in females, and mutations in MeCP2 gene are generally lethal in males. In some truly groundbreaking studies, mutations of MeCP2 in mice, which results in a loss of MeCP2 expression in both neurons and glia, appear to recapitulate many of the neurological symptoms occurring in Rett syndrome (Guy et al. 2001), and re-expression of MeCP2 in these mice results in a reversal of many of these symptoms (Guy et al. 2007). These data suggest that some epigenetic consequences may be reversible later in life.

Another intriguing line of research suggests that astrocytic MeCP2 expression may also play a functional role in Rett progression. Recent data demonstrate that loss of MeCP2 in astrocytes results in a failure of these cells to support neuronal properties, including neurotoxicity and neuronal morphology (Maezawa and Jin 2010; Ballas et al. 2009). Furthermore, re-expression of MeCP2 in astrocytes results in a reversal of many of the neurological symptoms associated with MeCP2 loss within this mouse model (Lioy et al. 2011). Together, these data suggest that MeCP2 loss can impact both glia and neuronal functioning within the brain. However, these data also support the idea that some of the outcomes of MeCP2 loss can be reversed.

More recent reports have suggested that subtle reductions in MeCP2 expression along with aberrant MeCP2 methylation patterns occur in individuals with autism, another sexually dimorphic neurodevelopmental disorder (Nagarajan et al. 2006). In contrast to Rett syndrome, autism is diagnosed at higher rates in boys. If reductions in MeCP2 levels are a contributing factor in the cause of autism, then this could suggest that the male brain is less able to compensate for reductions or loss of MeCP2. In the rodent brain, males express significantly lower levels of MeCP2 mRNA within the developing amygdala compared to females (Kurian et al. 2007). It is possible that there may be a critical threshold of MeCP2 expression and that further reductions under this threshold may be detrimental to brain

development. To test the functional consequence of methyl-binding proteins in sexual differentiation of the brain and juvenile social behavior, MeCP2 was transiently reduced within the developing amygdala in both males and females. It was found that subtle, transient reductions in MeCP2 expression within the developing amygdala during the first few days of postnatal life disrupted the organization of male, but not female, juvenile social play behavior. It is important to note that males engage in higher levels of juvenile social play behavior than do females (Kurian et al. 2008). These data indicate that MeCP2 is essential in organizing typical juvenile social behavior in males and that small reductions in MeCP2 levels within the developing amygdala can disrupt some juvenile social interactions. These data also support the idea that the male brain may be more sensitive to disruptions in MeCP2 levels. Whether these concepts underlie why autism is diagnosed at higher rates in males versus females is unclear; however, they do indicate a sex-biased impact of altered MeCP2 levels on social development.

To further clarify the importance of MeCP2 during brain development in organizing lasting changes in male juvenile social behavior, it was recently examined what socially relevant genes are impacted by transient alterations in MeCP2 levels within the developing amygdala. Vasopressin (AVP) is known to be linked to a number of social behaviors (Winslow et al. 1993; Bielsky et al. 2005; Bielsky and Young 2004), including juvenile social play behavior (Veenema and Neumann 2009), and MeCP2 appears to be important for regulating AVP expression within the hypothalamus (Murgatroyd et al. 2009). While AVP within the hypothalamus is important for numerous homeostatic responses, AVP within the amygdala is important for modulating a variety of social behaviors (Caldwell et al. 2008). Furthermore, AVP levels within the amygdala are higher in males contrasted to females (De Vries and Simerly 2002), suggesting a potential target of MeCP2 in regulating male social behavior. Indeed, transiently reducing MeCP2 levels within the developing amygdala results in a lasting decrease in AVP levels in males (Forbes-Lorman et al. 2012). Interestingly, while reductions in MeCP2 expression did impact other sexually dimorphic genes within the developing amygdala, these reductions were transient and did not last into adulthood. Therefore, MeCP2 may be important for organizing certain neuronal phenotypes (Fig. 3.3). These data suggest that reductions in MeCP2 may impact male juvenile social behavior by disrupting the organization of AVP within the developing male amygdala. The consequences of a lasting decrease in AVP expression within males on other social and cognitive behaviors remain to be fully explored. It is interesting to note that the programming of AVP expression was not altered in females, suggesting again a greater resistance to neonatal disruptions of MeCP2 expression in females.

3.3.4 Histone Modifications and Brain Sex Differences

Sex differences in epigenetic mechanisms are also observed upon histone modifications, which can include acetylation, phosphorylation, methylation,

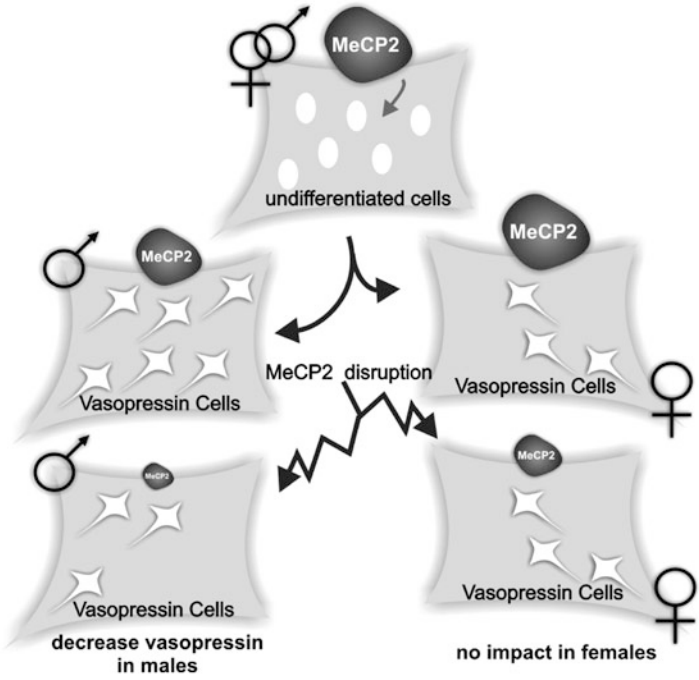

Fig. 3.3 During brain development, vasopressin cells become differentiated such that males express more cells contrasted to females. Sex differences in MeCP2 have also been reported with males having lower levels during the early neonatal period. Brief reductions in MeCP2 expression result in a lasting decrease in the number of vasopressin cells in males but not females, suggesting that the male brain may be less adept at compensating for MeCP2 loss of function

ubiquitination, sumoylation, citrullination, and ADP-ribosylation, that influence transcriptional activation and silencing. Recently, it was demonstrated that a sex difference exists in acetylated histone H3 and methylated H3 that appear to be modulated over development (Tsai et al. 2009). In the late embryonic period, male mice have higher levels of H3 acetylation contrasted to females, and this difference disappears early in postnatal development in combined cortex/hippocampus samples. Males also have higher levels of H3 trimethylation beginning at day 0 and extending to postnatal day 6 within the same brain area. The acetylation of H3, but not H3 trimethylation, is under hormonal influence, as testosterone exposure late in gestation increased H3 acetylation in females to male-like levels. Interestingly, the sex difference in H3 acetylation and methylation is only observed in cortex/hippocampus samples and not in areas typically considered important for sexual differentiation of the brain; however, these data may point toward a mechanism of early programming of learning and memory systems in the brain. Therefore, the sensitivity of this area to alterations in epigenetic profiles may underlie the basis for sex differences in disorders of learning and memory in adulthood.

Further evidence for the notion that histone modifications are instrumental in creating sex differences comes from the disruption of HDAC function in the developing mouse brain. The principal nucleus of the bed nucleus of the stria terminalis is sexually dimorphic, with males having a larger volume and more cells compared to females. It was recently reported that the treatment of male mice on the day of birth with the HDAC inhibitor, valproic acid, disrupts the sex difference within the bed nucleus of the stria terminalis (Murray et al. 2009). Additional support of the notion that histone modifications are important for sexual differentiation of the brain comes from recent data indicating that embryonic acetylation of the ER-α and aromatase promoters is higher in males compared to females around the day of birth. This sex difference is reversed by postnatal day 3. Furthermore, when the acetylation of histones H3 and H4 within the ER-alpha and aromatase promoter regions is blocked in the preoptic area, components of male sex behavior are also disrupted (Matsuda et al. 2011). These data suggest that some chromatin remodeling through acetylation of histones is necessary for appropriate sexual differentiation of the male brain and that these acetylation profiles can be region specific.

3.3.5 Corepressor Proteins and Brain Sex Differences

Methyl-CpG-binding proteins that bind to methylated DNA can initiate the recruitment of nuclear corepressors and histone deacetylase to form large corepressor complexes (Tetel et al. 2009; Auger and Jessen 2009). These repressor complexes can inhibit gene expression by removing acetyl groups from histones leading to chromatin condensation and gene repression. A large variety of corepressors can be recruited by methyl-binding proteins to form rather unique combinations, such as Sin3, NuRD, CoREST, and the NCoR/SMRT repressor complexes. It is likely that different combinations of corepressors allow for increased diversification of gene regulation. Of particular interest for sexual differentiation of the brain is nuclear corepressor (NCoR). NCoR was one of the first corepressors to be identified and appears to interact with a variety of receptors, such as thyroid hormone receptors, androgen receptors, estrogen receptors, and progestin receptors (Yoon et al. 2005; Chen and Evans 1995; Horlein et al. 1995). NCoR has been shown to interact directly, as well as indirectly, with methyl-binding proteins, such as Kaiso and MeCP2 (Yoon et al. 2003; Cukier et al. 2008). While the function of corepressor complexes within the developing brain is still being elucidated, recent data indicate that there is a sex difference in NCoR levels within the developing brain (Jessen et al. 2010). Specifically, females express higher levels of NCoR within the neonatal amygdala contrasted to males. These differences appear partly due to differences in hormone exposure, as treatment with estradiol can decrease the expression of NCoR in females to male-like levels. As NCoR levels can be altered in response to hormonal changes, it is likely that other endogenous or exogenous perturbations, such as early-life adversity, may alter NCoR levels within the

developing amygdala and impact chromatin remodeling. Importantly, transient disruptions of NCoR within the developing amygdala can have lasting consequences on both juvenile social play and anxiety-like behaviors. Reducing NCoR levels within the neonatal amygdala produced lasting alterations in juvenile social play behavior in males only. In contrast, the transient reduction of NCoR levels increased anxiety-like behavior in both juvenile males and females. These data indicate that transient disruptions in some corepressor complexes within the developing brain can have sexually dimorphic consequences on juvenile social behavior. This is consistent with the idea that targeting some epigenetic molecules may have a greater impact within the male versus female brain, at least when considering juvenile social interactions. It is important to note that lowering NCoR expression levels within the developing amygdala further *increased* juvenile play behavior in males. In contrast, lowering MeCP2 levels within the developing amygdala *decreased* juvenile social play in males. This suggests that the organization of juvenile social behavior in the male brain is under the tight control of these epigenetic molecules.

3.3.6 Coactivator Proteins and Brain Sex Differences

In contrast to the function of corepressors, coactivators increase the acetylation of histones leading to increased gene expression. Within the brain, ligand-bound steroid receptors interact with a variety of coactivator proteins, which can include steroid receptor coactivator-1 (SRC-1) and the cAMP response element-binding protein-binding protein (CPB), upon DNA (Tetel et al. 2009). These coactivator complexes function to alter the acetylation of histones either through their own histone acetyltransferase (HAT) activity or through their association with other proteins having HAT activity. Acetylation of histones relaxes chromatin structure to allow for more efficient binding of transcription factors and thereby gene transcription. As coactivators are important for steroid receptor activity, it is not surprising that SRC-1 and CBP are critical for sexual differentiation of the brain. Specifically, reducing the expression of SRC-1 within the developing hypothalamus during the first few days of neonatal life blocks sexual differentiation of adult male sexual behavior (Auger et al. 2000). Similarly, transient reductions of CBP within the developing hypothalamus during the first few days of neonatal life also disrupt the differentiation of adult male sexual behavior. Taken together, these data indicate that steroid receptor action within the developing brain requires interactions with epigenetic molecules that function to increase histone acetylation and relax chromatin structure (Auger et al. 2002). This idea is consistent with the recent data indicating that males have higher levels of acetylated histone H3 within the neonatal brain contrasted to females (Tsai et al. 2009). Therefore, subtle chromosomal alterations that are induced by steroid hormone exposure during brain development are essential for organizing sex differences in the brain. These

data further support that male brain development may be more sensitive to alterations in epigenetic molecules during the early neonatal period.

3.4 Epigenetic Programming and Its Reversibility in the Brain

Conrad Waddington is often cited as the first to define epigenetics as the branch of biology that investigates how genes and their products produce a phenotype (Waddington 1942). Later, David Nanney refined the term epigenetics to explain how cells with the same genotype can have different phenotypes (Nanney 1958). Over the years, epigenetics has been conceptualized as a relatively stable phenomenon that persists throughout the lifespan of an individual and can even be passed on to future generations. While the notion that the conversion of cytosine into 5-methylcytosine is relatively stable and is maintained through DNA replication was suggested over 30 years ago, it was also suggested that there may be a "shuffling" of DNA methylation patterns during cellular differentiation (Razin and Riggs 1980). A recent finding has indicated that the methylation of cytosine can be rather dynamic in vitro and that the methylation patterns of a particular gene can undergo cyclical changes within minutes to hours (Metivier et al. 2008). These findings suggest the possibility that some methylation patterns are not as stable as previously thought, but rather could be reversible in the brain. In fact, a recent report indicated that some DNA methylation patterns need to be *maintained* by an exogenous signal within the adult brain and that removal of the signal allows for a reversal of DNA methylation patterns (Auger et al. 2011). In particular, adult castration results in a near elimination of AVP expression and an increase in ER expression within the bed nucleus of the stria terminalis of the male brain. These castration-induced changes in mRNA levels are coupled with an *increase* in DNA methylation of a specific region on the AVP gene promoter as well as a *decrease* in methylation of the ER-α promoter. Interestingly, a CpG site within the ER-α promoter region that is altered by maternal touch during development is also modified by circulating steroid hormone levels in the adult brain (Edelmann and Auger 2011; Auger et al. 2011). This suggests that steroid hormones may be needed to maintain the methylated or demethylated states of some genes in order to sustain appropriate expression levels (Fig. 3.4). The concept that some DNA methylation patterns being laid down during early brain development are reversible later in life is consistent with the data concerning MeCP2 re-expression in knockout mice and the reversal of Rett symptoms observed in these animals (Guy et al. 2007).

The molecular pathways by which a gene can cycle from an unmethylated state to a methylated state are currently being elucidated. Understanding the mechanisms involved in methylation or demethylation of DNA will be important for removing aberrant methylation patterns that may underlie disease. Emerging data indicate that there is an active mechanism involved in the demethylation of DNA. These data have identified the occurrence of multiple forms of DNA methylation or modifications to cytosine residues that potentially progress from

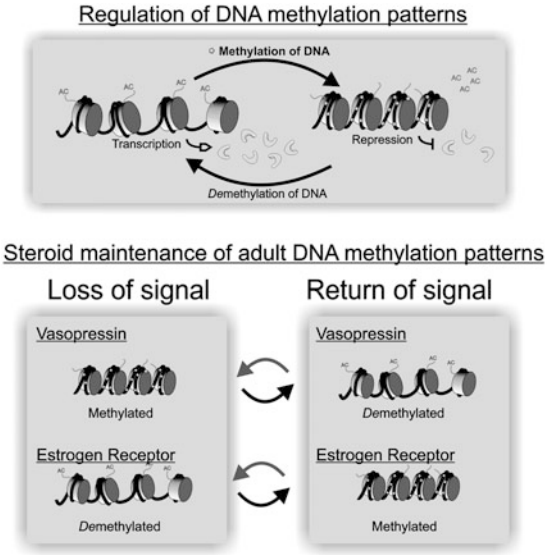

Fig. 3.4 Emerging data indicate that DNA methylation patterns may be more plastic within the adult brain than originally thought. While some DNA methylation patterns are stable and can be transmitted transgenerationally, other DNA methylation patterns may cycle between methylated and demethylated states. Within the adult male brain, circulating testosterone maintains DNA methylation patterns of both vasopressin and estrogen receptor promoter regions. Loss of testosterone levels results in increased methylation of the vasopressin promoter region and demethylation of the estrogen receptor promoter region. Restoration of testosterone levels returns methylation patterns and transcription levels back to baseline. Thus, a signal is needed to actively maintain some DNA methylation patterns in the adult brain

5-hyrdoxymethylation (5hmC) to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC). It is currently believed that the TET (ten eleven translocation) family of proteins is instrumental in mediating the progression through much of this epigenetic pathway, at least the conversion of 5mC to 5hmC, as well as from 5hmC to 5fC and 5caC (Ito et al. 2011). The decarboxylation of 5acC appears to be accomplished by thymine-DNA glycosylase (He et al. 2011). These data outline a potential pathway by which demethylation of DNA might be regulated. It remains to be determined how perturbations during development impact this pathway and to what extent disturbances in this pathway induce pathology.

The observed reversibility of DNA methylation in adulthood suggests that the adult brain may be more plastic than neonatal brain in terms of epigenetic reprogramming of DNA methylation patterns. This idea is consistent with the hypothesis that there are gradual shifts in human DNA methylation patterns during aging. Sometimes these shifts can produce aberrant methylation patterns that result in neurologic diseases later in life (van Vliet et al. 2007) or age-related cognitive decline (Penner et al. 2010). In human genetic twin studies, DNA methylation patterns can show a striking similarity during early child development but show

dramatic differences later in adulthood (Fraga et al. 2005). These alterations in DNA methylation taking place over longer periods of time are likely indicative of more transient drifts in DNA methylation. As a result of aging, these drifts in DNA methylation patterns later in life may result from the loss of signals (e.g., declining steroid hormone levels) needed to maintain methylated states. Whether there are different pathways regulating active changes in methylation versus these more graduate drifts in methylation and how these pathways relate to pathology remain to be determined.

3.5 Conclusions

Sexual differentiation of the brain is a powerful model to study how epigenetic mechanisms impact brain development, as brief exposure to steroid hormones can reorganize the brain and have lasting consequences on behavior. Studying sex differences within the male and female brain also provide a natural model for investigating the mechanisms involved in the risk or resilience to some mental health disorders. For example, autism spectrum disorders, Rett syndrome, attention deficit hyperactivity disorder, and schizophrenia all have sex differences in prevalence, time of onset, and/or severity. As these disorders are believed to have an epigenetic component, it is conceivable that sex differences in epigenetic processes underlie the risk or resilience to develop a particular disorder. Indeed, the data discussed above suggest that males are at greater risk for developing impairments in social behavior as a result of perturbations to MeCP2 expression during brain development. Males also seem to be more sensitive to alterations to other epigenetic molecules, such as coactivators and corepressors, during early brain development, suggesting a male-biased vulnerability to disturbances in epigenetic mechanisms important for brain development. Therefore, elucidating the epigenetic mechanisms governing sexual differentiation of the brain will provide valuable information for discerning why some individuals are at greater risk or are more resilient to developing mental health disorders.

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Part II
Epigenetics and Neurological Disorders

Chapter 4
Phenotypic Plasticity, Pleiotropy, and the Growth-First Theory of Imprinting

Jon F. Wilkins

Abstract The kinship theory of genomic imprinting suggests that parent-of-origin-dependent gene expression is the consequence of asymmetric selection on maternally and paternally inherited alleles. The theory has been most thoroughly developed in the context of early growth and development but applies, in principle, to any trait. The taxonomic and functional distribution of imprinted genes suggests that intragenomic conflict over maternal resources may play a particularly important role in the evolution of genomic imprinting in mammals, with those traits being the primary drivers of the acquisition of imprinted gene expression. In this scenario, imprinted gene effects on other traits (e.g., certain aspects of cognition and behavior) emerge initially as an epiphenomenon. However, once the gene is imprinted, more subtle selection asymmetries can have substantial consequences for the evolution of these traits. In this chapter, I motivate and formalize this "growth-first" theory of imprinting and lay out predictions that could be tested by future experiments.

Keywords Cost of imprinting • Epigenetics • Genetic conflict • Genomic imprinting • Kinship theory

Abbreviations

ICE Imprinting Control Element
ICR Imprinting Control Region

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4.1 Introduction

The term "genomic imprinting" refers to the phenomenon where the pattern of gene expression at a locus depends on whether the allele was maternally or paternally inherited (Wilkins 2008). In the simplest cases, an imprinted gene is expressed from one of the two alleles, while the other is transcriptionally silent. However, many imprinted loci exhibit complex patterns of expression, with different isoforms being maternally, paternally, or biallelically expressed, often in a tissue-specific manner (Peters et al. 1999; Blagitko et al. 2000; Hayward et al. 2001; Peters and Williamson 2008; Monk et al. 2009; Garfield et al. 2011).

In this chapter, I use the term *imprinted* to refer to any locus where alleles have evolved to two different expression patterns, conditional on the allele's parent of origin. Equivalently, we could say that an allele at an imprinted locus encodes two distinct, conditional strategies, whereas alleles at an unimprinted locus are subject to the constraint that they encode a single, unconditional strategy that does not depend on the allele's parental origin.

The kinship theory of genomic imprinting, in its fully developed form, proposes that imprinted gene expression is the result of natural selection favoring alleles that encode two conditional expression strategies (Haig 1997, 2000b; Wilkins and Haig 2003b), although the theory is most often described in the language of an evolutionary conflict between maternally and paternally derived alleles. In the standard models, this conflict results from the fact that the inclusive fitness effects associated with an allele depend on the patterns of relatedness for that allele, which depend in turn on the allele's parental origin.

For example, mammalian imprinting has been most thoroughly researched in the context of genes that are expressed in the fetus and/or placenta during pregnancy and influence the distribution of maternal resources. In this context, alleles favor placing a greater demand on the maternal resources when paternally inherited, but lower demand when maternally inherited (Haig and Graham 1991; Moore and Haig 1991; Mochizuki et al. 1996; Wilkins and Haig 2001). While the direct benefit of increased maternal resources is the same for an allele whether it is maternally or paternally inherited, the indirect, inclusive fitness effects will be different in the two contexts.

In general, an allele present in an offspring will be more closely related to its mother's other offspring when it is maternally inherited than when it is paternally inherited. All of the mother's offspring share the same mother, by definition, whereas they may or may not share the same father, where that probability depends on the mating structure of the species. Note that this simple verbal argument assumes that sibs and maternal half-sibs are the only individuals competing for resources and that those resources come exclusively from the mother. More complex models permit a wider range of results and predictions (Brandvain 2010; Úbeda and Gardner 2010a, b; Van Cleve et al. 2010; Brandvain et al. 2011).

However, the prenatal distribution of maternal resources among her offspring is by no means the only context in which an expression strategy that is conditional on

parent of origin might outcompete an unconditional (unimprinted) strategy. Numerous behavioral and cognitive phenotypes have been associated with imprinted genes (Davies et al. 2008; Goos and Ragsdale 2008), and models have been developed in an effort to understand those effects (Haig 2000a; Wilkins and Haig 2003a; Crespi and Badcock 2008; Úbeda 2008; Wild and West 2009; Brandvain 2010; Úbeda and Gardner 2010a, b; Van Cleve et al. 2010).

In fact, for *any* phenotype, the best possible conditional strategy will always be at least as successful as the best possible unconditional strategy. Put another way, it is vanishingly unlikely that natural selection acts *identically* on maternally and paternally inherited alleles for any gene in any species. Viewed from this perspective, the question becomes why are there any *unimprinted* genes? In the vast majority of cases, the selective asymmetry between maternally and paternally inherited alleles is extremely small. Even if the magnitude of the asymmetry is never *exactly* zero, it is typically small enough that it is *effectively* zero, in that selection will be too weak to drive the transition from unimprinted to imprinted expression.

This point becomes more obvious once we recognize that genomic imprinting represents a particular form of phenotypic plasticity. For example, heat-shock proteins are normally expressed at low levels, but expression increases under conditions of stress. In the context of environmental variation, when do we expect to find evolved phenotypic plasticity in the form of two (or more) conditional strategies? Intuitively, we expect two requirements. First, the environments must be experienced sufficiently often. Second, natural selection must be sufficiently different in the environments. Otherwise, we expect to find a single, unconditional strategy that is selected for its average properties across the environments.

In the imprinting case, the two "environments" are "maternally derived" and "paternally derived." Because an autosomal allele occupies each of these environments half of the time, this case seems to fulfill our first requirement. However, the selective asymmetry in most models derives from indirect, inclusive fitness effects and is likely small relative to the direct fitness effects for most genes and traits.

Thus, perhaps the thing that is special about eutherian pregnancy, and the "placental habit" more broadly (Haig and Westoby 1991), is not that it generates asymmetric selection on maternally and paternally inherited alleles. Rather, pregnancy is special because it creates a context in which the selective asymmetry surpasses some critical threshold required to overcome the barriers to parent-of-origin-specific gene expression. In the following two sections, I will discuss what some of those barriers might be, and the implications for functional properties of imprinted genes.

4.2 What Limits the Evolution of Genomic Imprinting?

4.2.1 Chance, Time, and Entropy

What are the features of genomic imprinting that lead to the persistence of unimprinted expression at most loci, despite the fact that there will almost always be an asymmetry, however slight, in the way that natural selection acts on maternally and paternally inherited alleles? The first set of features is one that has already been alluded to in the previous section. Namely, we expect that for most genes and most traits, the selective advantage for an allele that encodes two different expression strategies is likely to be small.

The rule of thumb from population genetics is that the efficacy of selection depends on the product of the effective population size (N_e) and the selective advantage of the favored allele (s). When $N_e s << 1$, traits are effectively neutral, meaning that the likelihood that a favored allele becomes fixed in the population is not substantially different from that for a truly neutral variant. So, for any effective population size, there will be some minimum asymmetry required to escape this domain of effectively neutral variants.

In those cases where $N_e s > 1$, chance will still play a role. Even a selectively favored allele is likely to be lost from the population due to drift. In the limit of large population size, the probability of fixation for a newly introduced allele with selective advantage s is approximately $2s$. Thus, for an allelic variant with a 1 % selective advantage (likely at the high end of what we might expect for the differential inclusive fitness effects of maternally and paternally inherited alleles), we should expect that the variant would have to arise by mutation on the order of 50 separate times before one of these variants becomes fixed.

Does the time required for a favored allele to arise by mutation and fix matter? That depends on the frequency with which the relevant mutations arise. It seems likely that mutations that actually give rise to imprinting (mutations that enable the allele to encode two different expression strategies) might be quite rare. If so, we should expect to find many unimprinted genes where imprinting would be selectively favored in principle.

If the evolution of imprinting is mutation limited, this could help to explain the fact that many imprinted genes are clustered in the genome. In many of these clusters, there is a single region that is subject to differential epigenetic marking in the germ line, often termed an Imprinting Control Element (ICE) or Imprinting Control Region (ICR) (Lewis and Reik 2006; Peters and Williamson 2008). The epigenetic marks that regulate imprinted expression of individual genes in the cluster are often the result of epigenetic spreading in *cis*. It is possible that the evolution of imprinted gene expression at a locus close to a gene that is already imprinted is mutationally easier than the problem of generating imprinted expression in an otherwise unimprinted region of chromosome.

Finally, note that imprinted expression is more complex than unimprinted expression. The establishment, propagation, and reprogramming of the epigenetic

modifications associated with imprinted genes require the existence and targeted deployment of a complicated set of molecular machinery. Thus, imprinted gene expression entails a type of complexity cost: one that we might think of as a type of entropy. The strength of selection favoring conditional regulation must be sufficiently strong to overcome this "complexity" or "entropy" barrier.

The additional complexity comes in two forms. The first is the molecular machinery of imprinting, and the capacity for differential epigenetic regulation in the male and female germ lines. This first form represents a barrier to the existence of genomic imprinting, in that the burden of developing these additional features will affect the evolution of the first imprinted gene in a species. Once these features have been established, they can be exploited at little additional cost in the establishment of imprinting at other loci.

The cost associated with this first form of complexity may be relatively small, however, as most of the molecular machinery involved in regulation of imprinting is also used for other epigenetic phenomena, such as retrotransposon silencing and tissue differentiation (Lane et al. 2003; Bourc'his and Bestor 2004; Suzuki et al. 2007; Kaneko-Ishino and Ishino 2010). In fact, there seem to be only a small number of gene products that are truly specific to imprinting in mammals, including Dnmt3L (Hata et al. 2002; Bourc'his and Bestor 2004; Arima et al. 2006; Yokomine et al. 2006) and Dnmt1o (Howell et al. 2001; Ding et al. 2003; Cirio et al. 2008). Similarly, sex differences in the patterns of gene expression at loci that are involved in epigenetic modification in the germ line may predate the evolution of imprinted gene expression.

The second complexity is the targeting of the imprinting machinery to individual loci. This is closely related to the idea that the frequency of mutations giving rise to imprinted expression may be limiting. This barrier will apply to each new imprinted locus, but the difficulty in overcoming that barrier may vary significantly among loci. The establishment of imprinting at loci that are physically close to existing imprinted genes may be less complex than the establishment of imprinting at an isolated locus. This would be the case, for example, if the *cis*-acting sequence elements that would permit propagation of epigenetic information along the chromosome were less complex than the *trans*-acting elements that are differentially targeted in the male and female germ lines.

4.2.2 Costs of Imprinting

Discussions of the evolution of imprinted gene expression often refer to a "cost of imprinting" that is different from the entropic or mutational "cost" described above. The idea is that there is a fitness cost directly associated with monoallelic gene expression. The most commonly cited cost of imprinting is the exposure of the phenotypic effects of deleterious mutations that would normally be recessive at a standard, biallelically expressed locus.

However, it is not clear that the unmasking of deleterious, recessive mutations represents a significant selective pressure on imprinted gene expression (Spencer 1997). First, while most deleterious mutations at most loci are recessive, the loci that are most likely to evolve imprinted expression likely violate this trend. For a loss-of-function mutation to be recessive, either the phenotype must be relatively insensitive to dosage effects at that locus (so that a ~50 % reduction in expression does not impose a significant selective cost), or there must be feedback mechanisms that result in a compensatory upregulation of expression from the other allele. Either way, recessiveness implies that a dramatic change in the level of expression from one of the two alleles has a negligible selective effect.

In contrast, we expect imprinting to evolve when the phenotype is most sensitive to changes in the expression or activity level of one of the two alleles. So, the loci where intragenomic conflict most strongly favors imprinting are exactly those loci where deleterious mutations are least likely to be recessive. In these cases, the existence of deleterious mutations in the population may actually *favor* monoallelic expression (Van Cleve and Feldman 2007).

4.2.3 Multidimensional Conflict and Molecular Mechanisms

Many simple models of imprinting consider only the inclusive fitness of alleles at the imprinted locus. An implicit assumption underlying such models is that the expression level of an allele is entirely under the control of *cis*-acting regulatory elements. In fact, the full life cycle of an allele at an imprinted locus involves the erasure of previously established epigenetic marks, establishment of new marks, and propagation, reprogramming, and interpretation of those marks. Each of these steps involves interaction between the *cis*-acting elements at the imprinted locus with *trans*-acting factors such as methyltransferases.

In general, the selective pressures acting on these *trans*-acting factors are not identical to those acting on alleles at an imprinted locus (Wilkins 2005). In the male and female germ lines, the factors that erase the previous generation's epigenetic modifications and establish new ones consist of gene products derived from both of the mother's (or father's) alleles. In the developing embryo, the genomic origin of the *trans*-acting factors depends on the stage of development. In the earliest rounds of cell division (the preimplantation embryo, although the exact timing varies from species to species), the zygotic genome is transcriptionally inactive. At this stage, nearly all of the potential *trans*-acting factors are maternal-store proteins, which represent premeiotic transcripts from the mother. Following the activation of zygotic transcription, most *trans*-acting factors will represent the product of biallelic expression from unimprinted loci.

Many *trans*-acting factors interact with a very large number of targets, including imprinted and unimprinted loci. These *trans*-acting factors may be sufficiently constrained by functional demands that they can be treated as effectively fixed. In such cases, it is probably reasonable to restrict ourselves to modeling the evolution

of *cis*-acting elements against this fixed background. However, some *trans*-acting factors participate specifically or predominantly in the regulation of imprinted genes. These factors may be less functionally constrained and may undergo evolutionary change at a rate comparable to the *cis*-acting elements, generating coevolutionary dynamics that undermine the conclusions of the simple models.

Evolutionary conflicts between *cis*-acting and *trans*-acting factors can also interfere with the creation and persistence of imprinted gene expression (Wilkins 2005, 2006b). For example, in the female germ line, if selection on cis-acting elements favors silencing, silencing will also be favored by *trans*-acting. In the male germ line, however, it is possible for the *cis*-acting elements at a locus to favor silencing, while the *trans*-acting factors do not (Burt and Trivers 1998; Wilkins 2005).

In the preimplantation embryo, conflict is primarily expected between paternally inherited *cis*-acting elements and maternal-store proteins, which represent premeiotic transcripts from the mother. In this context, *cis*-acting elements at both maternally and paternally inherited alleles will favor the maintenance and propagation of epigenetic marks established in their respective germ lines. The *trans*-acting maternal-store proteins will favor the maintenance of any maternally inherited epigenetic marks but may favor the elimination of paternally inherited marks. This conflict may explain the genome-wide demethylation of the paternal pronucleus following fertilization in mammals (Mayer et al. 2000; Oswald et al. 2000) and may give rise to complex molecular (Howell et al. 2001; Wilkins 2006b) and evolutionary (Wilkins 2005) dynamics.

In both the male germ line and the preimplantation embryo, then, there is an asymmetry in the evolutionary pressures acting on maternally and paternally inherited alleles. In each context, we expect paternally modified loci to be subject to a greater degree of evolutionary conflict than maternally modified loci. This asymmetry suggests that it may be easier to establish epigenetic modifications on the maternally inherited allele and that those modifications may be more evolutionarily stable than paternally inherited modifications (Wilkins and Haig 2002). This prediction is consistent with the observation that maternal silencing is often accomplished through simple methylation, while paternal silencing often involves more baroque, highly evolved molecular mechanisms (Reik and Walter 2001). Also consistent is the observation that the majority of loci that have undergone loss of imprinting in the human lineage are paternally silenced in the mouse (Isles 2009).

4.3 The Pleiotropic Origins of Imprinted Gene Effects

The evolutionary barriers to imprinting may help to explain the taxonomic and phenotypic distribution of mammalian imprinting effects. Current knowledge suggests that mammalian imprinting first arose along with the origin of placentation, in the common ancestor of eutherian mammals and marsupials. This origin, along with the fact that most imprinted genes affect early growth and development,

supports the idea that conflict over the distribution of maternal resources is the primary driver of imprinted gene expression.

How should we understand the origins of other phenotypes, though? Mammals may represent a special case in terms of how their offspring acquire resources. However, as a group, mammals do not differ qualitatively from other species in ways that lead to a straightforward explanation of the other phenotypes that are subject to imprinted gene effects. In the next section, I will discuss this issue specifically in the context imprinted gene effects on cognition and behavior.

4.3.1 Behavioral Effects and Pleiotropy

Recent work has shown that imprinted genes play a major role in the central nervous system. A pair of recent studies identified approximately 1,300 transcripts (including more than 800 known genes) exhibiting imprinted expression in the mouse brain (Gregg et al. 2010a, b). Individual imprinted genes have been associated with a variety of cognitive and behavioral effects, including maternal care for offspring (Lefebvre et al. 1998; Li et al. 1999), reactivity to novel environments (Plagge et al. 2005), social dominance (Garfield et al. 2011), and memory consolidation (Chen et al. 2011).

No clear pattern has yet emerged for these behavioral effects, either in terms of the set of phenotypes involved or in terms of which allele is silenced. Individual behavioral effects can be (and have been) rationalized in terms of assumed effects on the fitness of related individuals, but formal tests of these proposed explanations will require the characterization of additional imprinted genes, as well as the extension of these studies to a wider range of species (Brandvain et al. 2011). It is therefore too early to say exactly how and exactly how well these effects can be accommodated by conflict-based models of imprinting.

One possibility, of course, is that imprinting effects on behavior are simply epiphenomena. This view is supported by the fact that most of the imprinted genes with known behavioral effects also have significant growth effects. Furthermore, while mammalian pregnancy is particularly susceptible to conflicts over resource distribution, there is no obvious reason why any behavior should be more particularly subject to intragenomic conflict in mammals as opposed to other taxa. Yet, our current knowledge of the taxonomic distribution of imprinting suggests that birds, reptiles, and monotremes do not have imprinted genes.

In fact, I want to suggest that mammalian imprinting in the brain likely did first arise as an epiphenomenon, a side effect of the intragenomic conflict over early growth. However, the behavioral effects of the imprinted gene may still be comprehensible in terms of its matrilineal or patrilineal inclusive fitness. Once an allele has become monoallelically expressed, the action of natural selection on other properties of the allele change. Consider an allele that is expressed only when it is paternally inherited (as a result of its growth-enhancing effect of the gene product). Mutations that affect other properties of the allele, such as the level,

location, or timing of expression, or the biochemical properties of the gene product are under selection only when that allele is paternally inherited. Thus, the evolution of these properties is controlled entirely by the patrilineal inclusive fitness function.

At this point, conflicts that were too weak to drive the evolution of imprinting (due to the "costs" or "barriers" described previously) may begin to have a real effect. This will be particularly true once there are multiple imprinted genes exerting opposing effects on the phenotype. Even if the difference between the matrilineal and patrilineal optimal phenotypes is small, paternally expressed and maternally expressed imprinted genes will preferentially fix mutations that enhance the opposing phenotypic effects of those genes. Thus, cognitive and behavioral effects of imprinted genes originate as epiphenomena but become amplified by natural selection once imprinted expression is established.

How this amplification plays out will depend on the relationship between the growth and behavioral effects of alleles at the locus. Qualitatively speaking, there are three possible relationships, which I will call positive alignment, null alignment, and negative alignment. Positive alignment refers to the case where increased expression is favored by the same allele in both the growth and behavioral contexts. For example, at a growth-enhancing locus, natural selection favors higher expression from the paternally inherited allele. If increased expression or activity in the brain were also favored by the paternally inherited allele, this would be a case of positive alignment. Negative alignment would be the opposite (in this example, maternally inherited alleles favoring higher expression in the brain). Null alignment would be the case where maternally and paternally inherited alleles favored the same expression or activity level in the brain.

For many genes the nature of this alignment may vary from cell type to cell type, as the effects of increasing the activity level of the same gene in different cells may have very different consequences for cognition and behavior. The scenarios for the three possible alignments will be described here in terms of a single phenotypic effect, corresponding to a single cell type or class of cell types. The conclusions will apply to more complex situations to the extent that the regulation and activity of the locus is capable of evolving independently in the different cell types. To the extent that these are not independent, more complex outcomes should be expected.

In describing these scenarios, I will refer to the "behavioral phenotype," which is represented by a high-dimensional phenotype space. The patrilineal and matrilineal optimal phenotypes exist as points in this space, as in previous work (Wilkins 2009). In the arguments constructed below, I assume that there are many loci that affect the behavioral phenotype and that the majority of these loci are unimprinted, so that the realized behavioral phenotype lies between the matrilineal and patrilineal optima.

4.3.2 Positive Alignment

In this first case, selection will favor an increase in the level of gene expression from the locus. This prediction follows from the assumption that the realized phenotype is most likely to lie somewhere between the matrilineal and patrilineal optima. Increased expression shifts the phenotype towards the patrilineal optimum.

If this were the only locus capable of influencing the phenotype, we would expect the phenotype to evolve to the patrilineal optimum and then for directional selection on the expression level to stop. However, if there are other (unimprinted or maternally expressed) loci that affect the same trait, those loci will be under selection to shift the phenotype back away from the patrilineal optimum. Thus, there will be continual selection on this paternally expressed locus to increase its expression level. Over time, this could result in a large increase in expression level. At that point, a paternally inherited loss-of-function mutation at the locus will result in a large phenotypic perturbation, shifting the phenotype roughly in the direction of the matrilineal optimum, but most likely far overshooting that optimum.

4.3.3 Null Alignment

If the newly imprinted locus does not influence the behavioral phenotype, there will not be immediate selection on the level of expression from the locus. However, it may be possible for mutations to give rise to novel functions (and phenotypic consequences) for the gene product(s) from the locus. For example, a novel function could arise from the addition or modification of a binding site on a protein product of the gene, resulting in a novel biochemical interaction. A novel function could also arise from a mutation to a regulatory element that caused the gene to be expressed in a cell type, or at a developmental stage, where the locus had previously been silent.

In this case, selection on the novel function will be determined, in part, by the effect that the functional change has on the behavioral phenotype. Using the example of a growth-enhancing (paternally expressed) locus, that novel function will be selectively favored if it shifts the phenotype towards the patrilineal optimum. The expectation, then, is that the locus will gradually accumulate additional functions and that those additional functions will shift the behavioral phenotype towards the patrilineal optimum. If and when these additional functions arise, the situation then becomes similar to the case of positive alignment, and selection will increase expression from the gene.

The substantial difference between the null alignment and positive alignment cases will be one of time scale. Whereas mutations that quantitatively change the level of expression of an allele may be quite common, mutations that generate novel functions may be orders of magnitude less so. On the other hand, these functional mutations may be substantially more common than mutations that give rise to

imprinted gene expression. Thus, after a locus becomes imprinted, it may linger for a substantial time before acquiring novel functional properties, but once a novel function has evolved, the locus may be driven relatively rapidly to higher expression.

4.3.4 Negative Alignment

As in the case of positive alignment, if selection is negatively aligned, it can then act on the level of gene expression. However, in this case, selection will tend to *reduce* expression from the active allele. Assuming again that the phenotypic effects of selection on this imprinted locus are being compensated by selection acting at other loci, the directional selection on the expression level will persist, even as the level of expression changes. Taking the example of a paternally expressed imprinted locus, selection would favor reducing the level of expression from the paternally inherited allele. This also implies that selection would, in principle, favor increased expression from the maternally inherited allele.

This suggests two possible outcomes of the evolution following in the wake of the establishment of imprinted expression. One possibility is that expression of the paternally inherited allele could be driven to low levels, possibly even to zero in certain cell types. An alternative is that imprinting may be lost within a particular cell lineage (Wilkins 2006a), leading to tissue-specific patterns of imprinting of the type that have been observed for a large number of loci. If the equilibrium phenotype is determined predominantly by other, unimprinted loci, that equilibrium will lie close to the unimprinted optimum. Once imprinting is lost, directional selection on the expression level will cease.

4.3.5 Alternative Transcripts

The behavioral effects of imprinted genes are difficult to study in part because these genes typically also have growth effects. Even if a gene has a substantial cognitive or behavioral effect, the phenotype produced using standard gene knockout technology may be difficult to interpret if it accompanies a large-scale growth perturbation. In the long run, a full understanding of the pattern of cognitive and behavioral effects of imprinted genes is going to require the use of more sophisticated techniques that permit the manipulation of genes in specific cell lineages.

There are only two transcripts for which knockouts have been constructed that cleanly separate the cognitive and behavioral effects from early growth effects: the *NESP* transcript from the complex *GNAS* locus (Plagge et al. 2005) and the paternally expressed transcripts from the *GRB10* locus (Garfield et al. 2011). Both the *GNAS* and *GRB10* loci produce multiple transcripts, each of which has

its own promoter region and its own first exon. These different first exons are coupled to a shared set of downstream exons.

How do these transcripts, which affect behavior, but not growth, fit into the growth-first model? One possibility is that these transcripts did not exist at the time(s) when the two loci first acquired imprinting. Transcription at complex imprinted loci such as *GNAS* and *GRB10* is highly co-regulated, such that a single mutation or epigenetic change may alter the expression of multiple transcripts. Similarly, if a novel promoter should arise within this complex locus, it seems possible, if not likely, that the new transcript will exhibit imprinted gene expression.

Thus one possibility is that imprinting at the *GNAS* and *GRB10* loci was driven by the growth effects of their gene products. Novel transcripts at the loci first arose in an imprinted state and were subject to selection on the basis of the parent-of-origin-specific fitness functions (matrilineal for *NESP*, patrilineal for the *GRB10* transcripts). These transcripts, then, are analogous to the novel gene functions described in the null-alignment scenario above. We may be able to understand the cognitive and behavioral effects of these transcripts using the logic of intragenomic conflict, even if that conflict would have been too subtle to drive the evolution of imprinting at a preexisting unimprinted locus with the same phenotypic effect.

4.3.6 Testing the Growth-First Theory

In this chapter, I have outlined an argument in support of a growth-first theory of imprinting. Briefly, the argument is that growth effects drive the acquisition of imprinting in mammals, since this is the only context in which there is a selection asymmetry large enough to overcome the barriers to imprinting. Systematic patterns in the other phenotypic effects of these genes, such as those on cognition and behavior, are acquired and/or elaborated later. Testing this proposal is difficult at the moment but is possible in principle. In the hope of making the ideas amenable to future empirical examination, I attempt to describe some specific, testable predictions here.

The general prediction is that if we look at the homologs of mammalian imprinted genes in other species, we will find that knockouts have a less pronounced effect on the cognitive and behavioral phenotype. This reduced effect could come in different forms, depending on the locus. For example, at some loci, the qualitative behavioral effect of the knockout will be similar but will be less pronounced. This might coincide with the gene having lower expression in non-mammalian species. At other loci, we might expect to find that the cognitive or behavioral effects associated with the gene do not exist in species where the locus is unimprinted.

For example, consider the *Peg1(Mest)* and *Peg3* loci in the mouse. Both are paternally expressed, and both have the expected positive growth effect, meaning that knockouts result in undergrowth. Both loci have also been associated with

maternal care effects: both are expressed in the brains of adult females, and loss of expression produces deficits in certain maternal behaviors, including placentophagy, pup retrieval, and nest building (Lefebvre et al. 1998; Li et al. 1999). The evolutionary conflict underlying the effect on maternal care is not obvious but could be related to inbreeding effects and the allocation of maternal resources between present and future litters (Wilkins and Haig 2003a).

If we were to look at the homologs of *Peg1* and *Peg3* in species without imprinting (likely birds, reptiles, and monotremes), the growth-first theory would predict either that these loci would not have an effect on maternal care in those species or that the effect would be less pronounced. Similarly, it would predict that the expression level from these genes would be higher in mice than in species without imprinting.

The growth-first theory also suggests that the brain-specific transcripts at complex imprinted loci such as *GNAS* and *GRB10* might only have come into existence only after those loci acquired imprinting. Alternatively, the gene products generated from these transcripts might have undergone substantial changes in function and/or expression pattern following the acquisition of imprinting. Again, this hypothesis could be tested by comparing the existence and behavior of these transcripts in species with and without imprinting at the loci.

4.4 Conclusions

Studies focusing on humans and model organisms like the mouse are powerful tools for revealing the molecular mechanisms of imprinting and for understanding the medical implications of mutations and epimutations at imprinted loci. However, in order to understand the evolutionary factors that have given rise to the patterns of imprinted gene expression that we observe today, we need to expand the breadth of our knowledge to additional taxa, including species where imprinting is known, or thought, to be absent. This will involve additional work in non-eutherian mammals (marsupials and monotremes), as well as reptiles and birds.

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Chapter 5
The Imprinted Brain: How Genes Set the Balance Between Autism and Psychosis

Christopher Badcock

Abstract The imprinted brain theory proposes that autism spectrum disorder (ASD) represents a paternal bias in the expression of imprinted genes. This is reflected in a preference for mechanistic cognition and in the corresponding mentalistic deficits symptomatic of ASD. Psychotic spectrum disorder (PSD) would correspondingly result from an imbalance in favor of maternal and/or X-chromosome gene expression. If differences in imprinted gene expression were reflected locally in the human brain, as mouse models and other evidence suggests they are, ASD would represent not so much an "extreme male brain" as an extreme paternal one, with PSD correspondingly representing an extreme maternal brain. To the extent that copy number variation resembles imprinting and aneuploidy in nullifying or multiplying the expression of particular genes, it has been found to conform to the diametric model of mental illness peculiar to the imprinted brain theory. The fact that non-genetic factors like nutrition in pregnancy can mimic and/or interact with imprinted gene expression suggests that the theory might even be able to explain the notable effect of maternal starvation on risk of PSD—not to mention a part of the "autism epidemic" of modern affluent societies. Finally, the theory suggests that normality represents balanced cognition and that genius is an extraordinary extension of cognitive configuration in both mentalistic and mechanistic directions. Were it to prove correct, the imprinted brain theory would represent one of the biggest single advances in our understanding of the mind and of mental illness that has ever taken place and would revolutionize psychiatric diagnosis, prevention, and treatment—not to mention our understanding of epigenetics.

Keywords Imprinting • Autism • Imprinted brain theory • Schizophrenia

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Abbreviations

ADCYAP1R1	PACAP receptor
AG	Androgenetic
AS	Angelman syndrome
ASD	Autism spectrum disorder
BPD	Borderline personality disorder
BWS	Beckwith-Wiedemann syndrome
CMV	*Cytomegalovirus*
CNV	Copy number variation
MDD	Major depressive disorder
PACAP	Pituitary adenylate cyclase-activating polypeptide
PG	Parthenogenetic
PSD	Psychotic spectrum disorder
PTSD	Posttraumatic stress disorder
PWS	Prader-Willi syndrome
RORA	Retinoic acid-related orphan receptor-alpha
SRS	Silver-Russell syndrome
TPJ	Temporoparietal junction

5.1 Introduction

The discovery of epigenetics has effectively given a new, third dimension to depth of the nature/nurture issue. Once, nature/nurture was two-dimensional to the extent that, for example, where identical twins raised apart differed significantly, it was assumed that the differences had to be the result of nurture and the similarities seemingly the result of their shared nature. But now we know this is not so and that monozygotic twins can differ epigenetically. A recent study of DNA methylation profiles in monozygotic and dizygotic twins pointed out that "molecular mechanisms of heritability may not be limited to DNA sequence differences." Indeed, the authors speculate that because identical twins reared together or apart are generally quite similar on measures such as brain imaging, IQ, and other psychometrics, epigenetic differences between identical twins "are much more important than environment" (Kaminsky et al. 2009).

Another finding is that if one of two identical twins has a mental disorder such as autism or schizophrenia, there is a much-higher-than-average probability that the other will too. Autism spectrum disorder (ASD) is sometimes associated with genetic syndromes, such as Rett, Down, and Turner's, phenylketonuria, tuberous sclerosis, and fragile X syndrome, where between a quarter and a half of all cases are diagnosed autistic (Aitken 2008). But neither autism nor schizophrenia obeys

classical Mendelian laws of inheritance, suggesting that genetics cannot be the sole cause.

Is nurture also a factor? There is certainly good evidence for social, environmental causes of mental illnesses. Studies of the Dutch wartime famine and of the Chinese famine of 1959–1961 reported increased incidence of schizophrenia among children born just after the events (St Clair et al. 2005; Susser et al. 2008). Again, a study of two million Swedish children born between 1963 and 1983 revealed a significant link between schizophrenia and poverty in childhood. Those with four out of five measured indicators of hardship had an almost threefold greater risk of schizophrenia than those with none (Wicks et al. 2005).

At first sight, it would seem that no single theory could explain these seemingly contradictory facts—and certainly not an evolutionary or genetic one—but an attempt is under way to do exactly that. According to the so-called imprinted brain theory, the paradoxes can be explained in terms of the expression of genes and not simply their inheritance. Furthermore, it proposes that the pattern of expression in question is part of a more general phenomenon rooted in evolution and explained by conflicts over parental investment (Badcock 2008, 2009; Badcock and Crespi 2006, 2008; Crespi and Badcock 2008; Crespi et al. 2009a; Crespi 2008). The implication is that this new, third dimension of depth which epigenetics adds to the nature/nurture dichotomy is an evolved, natural one centered on nurture, understood principally as parental investment and secondarily as environmental factors which mimic, reinforce, or interact with genetics. The imprinted brain theory poses a provocative challenge to neo-Lamarckian interpretations of epigenetics (Jablonka and Lamb 1995) and as such is a crucial test case of the so-called selfish-gene neo-Darwinism (Badcock 1995).

5.2 Genomic Imprinting: The Epigenetics of Nurture

One way in which our understanding of nurture has been transformed and placed on a secure scientific and quantitative footing is via its definition as *parental investment* in terms of *additions to an offspring's survival and/or reproductive success at a cost to the remainder of its parent's survival and/or reproductive success*. The sexes can also be defined in terms of their contribution to offspring's reproductive success, with an almost universal anisogamy evident in the contrast between the microscopic and highly mobile male gamete (sperm or pollen cell) and the relatively massive and immobile female one (ovum or ovule). Mammals in particular are characterized by an extreme asymmetry between the sexes in this respect, thanks to gestation and lactation being exclusive to females and male parental investment being minor or negligible in many species. As a result, the vast majority of mammalian species are polygynous, with males investing primarily in mating effort and females primarily in parental investment (Trivers 1972).

Such striking asymmetry between the sexes where reproductive success is concerned may explain the finding that some genes in mammals are only expressed

from one allele depending on its parent of origin (Haig 2002). More surprisingly still, many of the genes in question are strategic, controlling ones like *IGF2,* and most are found to be expressed in critical organs—specifically the placenta and brain (Barlow 1995; De Chiara et al. 1991; Reik and Surani 1997). However, conflicts between maternal and paternal genetic self-interest might explain why, for example, *IGF2* is often paternally active and maternally imprinted or silenced. Since growth of the offspring benefits both parents but only the mother pays the costs involved in gestation and lactation, this growth-factor gene is predominantly expressed only from the father's copy. Indeed, in mice a maternally expressed but paternally imprinted gene, *Igf2r* effectively contradicts murine *Igf2* by creating receptors which act as non-growth-inducing sinks for the hormone (Haig and Graham 1991). (For a review of the evidence, see Haig (2004).)

Where humans are concerned, researchers have argued that Silver-Russell syndrome (SRS) and Beckwith-Wiedemann syndrome (BWS) "may now be regarded as two diseases caused by opposite (epi)genetic disturbances of the same chromosomal region displaying opposite clinical pictures" (Eggermann et al. 2005). The region to which they refer includes *IGF2*, and symptoms associated with SRS feature pre- and postnatal growth retardation arguably associated with non-expression of *IGF2,* while BWS symptoms include generalized pre- and postnatal overgrowth, arguably accounted for by biparental expression of *IGF2* (Holm et al. 1993).

Nor is this an isolated case: similar diametrically opposite symptoms are associated with reversed imprinting of the same genes on chromosome 15 in Angelman syndrome (AS) and Prader-Willi syndrome (PWS). AS is associated with increased expression of paternal and/or reduced expression of maternal genes at 15q11–13 and PWS with the opposite pattern of expression. In some variants of PWS, the entire maternal chromosome 15 is duplicated. In other cases, duplication of the paternal chromosome 15 occurs (Nicholls et al. 1998). Significantly in view of the conflict theory of imprinting, AS children are notably demanding: hyperactive, sleepless, effusive, and prolonged sucklers with a low pleasure threshold ("paroxysms of laughter" is one of the diagnostic criteria of what is otherwise known as "happy puppet syndrome") and an unusual readiness to smile. Indeed, Brown and Consedine (Brown and Consedine 2004) suggested that smiling produced by infants with Angelman syndrome may reflect a patri-gene adaptation to extract resources from the mother, as smiling positively correlates with night feeding and gaining positive social attention from caregivers (for an independent anticipation of the same insight, see Badcock (1999)). By contrast, PWS infants—those with the maternal bias—are the opposite: inactive, sleepy, and poor sucklers with a high pain threshold (such that they often develop severe skin lesions as a result of picking at scabs normal children would leave alone) (Angelman 1965; Nicholls et al. 1998).

Although some studies link PWS with autism (Milner et al. 2005; Veltman et al. 2004), all cases of maternal uniparental disomy chromosome 15 PWS known to the Cambridge Prader-Willi study were diagnosed with a psychotic spectrum disorder (PSD) in adulthood. As the researchers who demonstrated it point out, this is the

only example of such a direct and apparently absolute relationship between a specific genetic abnormality and psychotic illness known at the present time (Whittington and Holland 2004). AS children, by contrast, tend to be diagnosed with ASD (Bonati et al. 2007; Steffenburg et al. 1996) (although there are contrary assertions (Veltman et al. 2005)), and in one sample of 87 BWS cases (double expression of paternally active *IGF2*), 6.8 % of the sample had been diagnosed autistic (Kent et al. 2008)—in other words approximately seven times the highest estimate of prevalence of ASD in the general population, which is about 1 % (Baird et al. 2006).

Further examples of such genomic sister syndromes associated with duplication versus deletion of the same genomic regions have recently been described by Crespi, Summers, and Dorus. Velocardiofacial syndrome deletion 22q11 carries the second highest known risk of PSD after maternal uniparental disomy PWS, with about 30 % being diagnosed schizophrenic. Duplication of the same region, however, has been linked with ASD. Williams syndrome cases with deletion at 7q11 have visuospatial deficits, but are hyper-social and highly verbal to the point of being described as possessing "cocktail party" skills and also show heightened levels of anxiety and phobias. By contrast, duplication of the same region is associated with spared visuospatial skills but severe language impairment, ASD, and seizures (which are commonly associated with ASD). Similarly, Smith-Magenis syndrome, which features deletions at 17p11, shows evidence of good verbal skills, high sociability, and a tendency to PSD, while duplication of the same region in Potocki-Lupski syndrome is associated with high risk of ASD and seizures (Crespi et al. 2009b).

X-chromosome genes resemble maternally active autosomal ones for the simple reason that female mammals have two X chromosomes to the male's one and that all mothers are female. This means that selection for female-benefiting traits acts twice as often on X genes as it does for male-benefiting X-chromosome traits. As a result, X-chromosome aneuploidies which increase the representation of X-chromosome genes resemble maternally active autosomal ones and shift the balance of gene expression in favor of the mother and against the father (Haig 2006).

Crespi, Summers, and Dorus also point out that the short arm of the X chromosome has a high concentration of genes involved in psychosis and micro-deletions on the long-arm link to autism. There is a three times greater incidence of schizophrenia in Turner's cases with mosaic karyotypes mixing XO, XX, and XXX, and true X trisomy is linked to schizophrenia, with X trisomics having brain imaging similar to that of schizophrenics. In Klinefelter's syndrome (XXY) there is a four to ten times heightened risk of psychosis, with more positive female-typical symptoms (auditory hallucinations and paranoia) and a female-typical age of onset along with neuroanatomy similar to that seen in schizophrenia. As Crespi, Summers, and Dorus note, these examples "suggest that diametric copy-number alterations can, like diametric alterations to imprinted genes, generate contrasting phenotypes associated with autistic-spectrum and psychotic-spectrum conditions" (Crespi et al. 2009b).

5.3 The Imprinted Brain and the Epigenetics of Mental Conflict

Genes build brains to provide real-time responses to environmental challenges in motile organisms which cannot be predicted or directly encoded in DNA (Badcock 2000, pp. 69–71). However, sexual reproduction implies that the organism's genes originate in two parents of the opposite sex with conflicting or contradictory genetic self-interests—for example, as a result of the asymmetries in parental investment and mating effort mentioned above. The consequence is that such conflicts and contradictions are likely to be built into the brain before birth and fought out throughout life—and nowhere more so than in mammals with large brains and evolved minds such as human beings (Hamilton 1996, pp. 133–5).

A precedent for this expectation can be found in mice. Chimeric mice can be engineered to express a preponderance of one parent's genes as opposed to the other, androgenetic (AG) chimeras expressing the father's or parthenogenetic (PG) chimeras expressing the mother's, and staining can be incorporated to show where these genes are expressed in the developing embryo (Allen et al. 1995). As the conflict theory of imprinting would lead you to expect, the resulting AG embryos are larger than normal (excepting the brain) and have massive placentas. By contrast, PG embryos are small overall (except for the brain, where, interestingly, *Igf2* is maternally active and paternally imprinted (Gregg et al. 2010)) and have little or no placenta (Keverne et al. 1996). Naturally occurring triploid human fetuses with a double set of the mother's genes and one of the father's are small except for the head, show a retardation of growth, and have small placentas. Those with a double set of their father's genes and a single set of the mother's are well grown except for the head and have a large placenta (Hannah et al. 2002; Newton 2001).

Such experiments also show that while genes from both parents are equally expressed in the brain stem of mice as Mendelian norms might suggest, PG cells are found in large numbers in the cerebral cortex (and the underlying striatum) and in the forebrain, but very few are found in the so-called limbic system (MacLean 1996)—especially in the hypothalamus. This is true of mature, fully grown mice but even more so of fetuses where there is a complete absence of PG cells in the hypothalamus. In both cases, PG cells are found to be particularly clustered in the frontal lobes of the cortex. AG cells, by contrast, are the exact opposite: these are found in the hypothalamus and limbic system, but not in the cerebral cortex. The few that are found in the forebrain tissue of embryos do not proliferate and are subsequently eliminated (Allen et al. 1995).

More recent research on normal, fully developed mouse brains found imprinting at 1,300 different places in the murine genome. Three hundred and forty-seven non-sex chromosome genes were found to have sex-specific imprints: in other words, these genes not only were limited to being expressed from one parent but were further limited by the sex of the offspring in which they found themselves. For example, *Il18*, a gene linked to multiple sclerosis (a disease which predominates in women and runs down the maternal line of descent) was found to be preferentially

expressed from the mother in the female brain but not in the male. In the hypothalamus, sex-specific imprinted genes were found in females, suggesting, as the authors point out, "parental influence over the hypothalamic function of daughters" (Gregg et al. 2010).

In the same study it was found that the mother's genes made a greater contribution during development, but that the father's contributed more in adulthood. Furthermore, 40–50 % more neurons expressed the mother's X chromosome as compared to the father's in the prefrontal and other parts of the cortex. By contrast, there was no difference in X-chromosome expression in the hypothalamus. As the authors point out, there are many genes involved with brain function on the X in both mouse and man, and as we have just seen, theory suggests that maternally biased inheritance of X-chromosome genes serves maternal genetic self-interest. As the authors of the latest research on the mouse brain conclude, "parental expression bias emerges as a major mode of epigenetic regulation in the brain" (Gregg et al. 2010).

Hints of a similar pattern of maternal expression in the frontal cortex in humans are found in a study by Goos and Silverman. Intra-familial correlations on cognitive tests involving occipital, temporal, parietal, and frontal lobe functions in 65 families found that abilities mediated by frontal, parietal, and temporal lobes—but not occipital lobes—were more closely correlated between children and mothers than between children and their fathers (Goos and Silverman 2006). The prefrontal cortex tends to be larger in women, while elements of the limbic system such as the amygdala and hippocampus tend to be larger in men and larger still in autism but smaller in schizophrenia (Gur et al. 2004; Mendrek 2007).

The difference in the average volume of the orbitofrontal cortex between men and women accounts for about half of the variance in antisocial behavior between the sexes (Jones 2008), and reduced frontal volume is associated with antisocial behavior and psychopathy (Gur et al. 2002). If maternal genes are predominantly expressed in the prefrontal cortex, we might be justified in thinking of it as a critical part of the *maternal brain*. If so, then we could see the hypothalamus, amygdala, and other parts of the lower or limbic brain as *paternal* for parallel reasons: paternal genes are mainly expressed there, and these regions are also proportionately larger in men (Goldstein et al. 2001). The limbic system is sometimes called "the emotional brain" (LeDoux 1996) and certainly contains centers concerned with gratification of basic drives, appetite, and gut reactions such as fear, pleasure, and disgust. Bearing this in mind, we can now begin to see that the relation between the paternal and maternal brains is reminiscent of that between paternally active and maternally active genes, such as *Igf2* and *Igf2r*. (Indeed, in man *IGF2R* is linked to high intelligence (Chorney et al. 1998), although it is not imprinted (Killian et al. 2001).) The paternal brain could be seen as serving the father's genetic interest in the offspring's growth and consumption of resources, but as we can also now see, the maternal brain—and the prefrontal cortex in particular—could equally be seen as serving maternal genetic self-interest to the extent that it is able to inhibit, control, and contain the paternal brain.

5.4 The Battle of the Sexes in the Brain and the Epigenetics of Mental Illness

According to the so-called extreme male brain theory of autism (Baron-Cohen 2002), ASD represents a pathological hypertrophy of typical male cognitive tendencies, variously described as *systemizing* (Baron-Cohen et al. 2009) or *mechanistic* (Badcock 2004) in contrast to typical female ones described correspondingly as *empathizing* (Baron-Cohen 2005) or *mentalistic* (Badcock 2004). High-functioning male autistics certainly outnumber female ones by at least four to one, but paradoxically for the extreme male brain theory, the sex ratio is much closer to unity where severe ASD is concerned (Baron-Cohen et al. 2005). If autism were indeed an extreme male brain condition, you would expect the exact opposite: more males with severe symptoms and more females with mild ones. This anomaly—not to mention the paradox of how females could be said to have "extreme male" brains—is readily resolved by the imprinted brain theory. According to this way of looking at things, ASD is caused not by an extreme *male* brain but by a bias in gene expression in the brain in favor of *paternally* expressed imprinted genes.

Because the mother's genes are equally present in all her offspring, her genetic self-interest is best served by cooperation and family unity. Any net benefit from social behavior among her offspring is also a benefit to the ultimate reproductive success of her genes invested equally in all of them (Trivers 1974). Thanks to gestation and lactation, the mother is biologically the prime nurturer, and so it serves her interests to be able to nurture, educate, and instruct her children—for example, to teach them their "mother tongue" and then use it to program their thinking in ways she approves. By these means the mother can indoctrinate, condition, and socialize her offspring in behavior that is likely to benefit her equitable genetic investment in all of them.

The father, on the other hand, need make no obligatory biological contribution to his offspring beyond a single sperm, and other children of the same mother need not share his genes: *Mother's baby—father's? Maybe!* As a result, we have seen that the father's genes build parts of the brain that tend to motivate self-interested, instinctual, and nonsocial behavior: the limbic system. The father's genetic self-interest is not necessarily served by his child seeing things its mother's way—for example, in making sacrifices for siblings to which its paternal genes may not be related in any way whatsoever. The verbal deficits of autism would be explained by the fact that the paternal brain—alias the limbic system—"eludes the grasp of the intellect because its animalist, primitive structure makes it impossible to communicate in verbal terms" (MacLean 1996, p. 455).

As Crespi has pointed out (Crespi 2007), "The origin of speech and language is arguably the most important transition in the evolution of modern humans." He adds that there is now good evidence that one gene in particular—*FOXP2*—is critical both to the mirror neuron system in humans and to articulate speech. Mirror neurons fire when a person sees someone else performing an act which larger-scale firing of motor neurons in the same region would produce in the observer. As such,

they have been seen as part of the neural basis of empathy, and there are averagely more of them in women than in men and fewest of all in autistics, whose deficits in empathy have been claimed to epitomize the disorder (Baron-Cohen 2005).

Empathy is important in language—at the very least you need to understand what other people are trying to say. But Crespi goes on to note that evidence that *FOXP2* is imprinted and predominantly expressed from the paternal chromosome reminds us that language is also much concerned with self-assertion—and in the case of young children, with demands on the parents. "By this hypothesis, articulate human speech evolved as it develops, predominantly in the context of mother-offspring interactions, which are permeated by a complex mix of cooperation and conflict" (Crespi 2007).

Recently, Brown (2011) has added a new twist in his contribution to what he calls "The parental antagonism theory of language evolution." Like Crespi, he notes that imprinted patri-genes will select for language skills related to extracting resources from the mother early in development, but adds that matri-genes will select for language skills related to cooperation with the mother and kin later in development. And of course, the mother is much more likely to be the primary caregiver than the father throughout childhood. Brown suggests that this may explain why although only about 2 % of human genes overall are imprinted, about 35 % involved with language are subject to such differential expression by parent of origin. Brown notes that loss of *FOXP2* is linked to expressive but not receptive verbal dyspraxia, just as the parental antagonism theory would predict. Moreover, he also points out that Turner's syndrome cases with a paternal X chromosome are more likely to have hearing impairments which may filter out maternal speech. Again, and as predicted by its maternal bias, he notes that the X chromosome has more language-related loci (29 %) than expected by chance and that maternal genes of humans and chimps are more distant than paternal, suggesting recent maternally mediated selection for social learning.

So-called *mands* (verbal demands and requests) result from aversion and deprivation and should be paternal in origin if they followed the precedent set by resource-demanding genes like *IGF2*. Interestingly in this respect, children diagnosed with AS do indeed show high levels of mands, and the condition is indeed caused by a paternal bias on chromosome 15. Brown adds that in Williams syndrome you see hyper-social behavior with high levels of receptivity and cooperativeness—just as its associated maternal bias on chromosome 7 would lead you to expect (Brown in press).

According to such a view of the matter (Badcock and Crespi 2006), autism could be the consequence of the failure of the maternal brain in this respect and the notable impulsiveness, compulsiveness, and contrariness of autistics the inevitable result of the paternal brain's corresponding success. The striking social deficits seen in autism would seem to fit the idea that paternal genetic self-interest underlies the disorder because autistic children seem perversely committed to doing things their own way, in their own time, and for their own selves. If they can learn at all, they usually refuse to do so in the way adults think they should and inevitably pose a severe challenge to any caregiver (who in our evolutionary past would

predominantly have been the mother and her relatives). Certainly, the reduced empathy, uncooperativeness, and insistence of routine seen in autism hardly contribute to easy parenting. Indeed, there is evidence that in experimental animals, failure to cope with change is a central characteristic of paternal brain lesions, and a persuasive case can be made for the limbic system being centrally involved in the problems associated with autism (Lathe 2006).

The same reasoning would certainly explain why the brain systems that malfunction in autism seem to be critical to a child's social interaction with its mother (Maestro et al. 2002; Zwaigenbaum et al. 2005). Indeed, when paternal brain centers such as the hypothalamus and amygdala are active in dreams, aggressive impulses on the part of the dreamer emerge. However, when what I am calling maternal brain centers are activated in dreaming (the forebrain and neocortex), aggressive impulses are inhibited and cooperative and pro-social ones expressed (McNamara et al. 2005). As you would predict if autism was indeed caused by enhanced expression of the father's genes, proliferative overgrowth of the placenta is found at three times the normal rate in autism (Anderson et al. 2007), and studies suggest that autistics—and males in particular—are heavier than normal at birth and have elevated levels of growth hormones such as *IGF2* (Mills et al. 2007), confirming that they are indeed predisposed to consume more than usual of the mother's resources (Sugie et al. 2005). Again, there is evidence that autistics show early brain growth during gestation at the expense of the mother and have a 100-fold greater risk of neurofibromatosis (in other words, pathological overgrowth in the form of benign nerve-tissue tumors) along with genetic alterations relating to the PI3K (phosphatidylinositol 3-kinase) pathway resulting in greater vulnerability to cancer—despite the fact that autistics smoke less (Crespi and Badcock 2008).

These facts are all the more telling when contrasted with the corresponding situation in PSD: there, in accordance with the growth-limiting effect of maternal genes, you find intrauterine growth restriction, placental undergrowth, and higher incidence of fetal hypoxia; low birth weight, low levels of brain growth factors and smaller brain size, thinner cortex, and smaller hippocampus and amygdala; decreased risk of cancer among schizophrenics (despite increased smoking) and their first-order relatives; and reduced expression of growth factors and decreased stem cell proliferation, reduced thresholds for apoptosis (programmed cell death, e.g., if a cell becomes precancerous), and evidence of increased expression of tumor-suppressor genes in schizophrenics (Crespi and Badcock 2008) (Fig. 5.1).

The fact that all fathers are male explains why you could mistake autism for an extreme male brain disorder. But because males and females have both paternal and maternal brains, as we are calling them, you can easily account for the fact that females as well as males can suffer from autism. More high-functioning autistics might be expected to be male if only their paternal brain were affected—perhaps driven to an extreme early in development by male sex hormones (Ingudomnukul et al. 2007; Knickmeyer et al. 2004). The intact intelligence and verbal abilities of high-functioning autistics seen in Asperger's syndrome would therefore be the result of predominantly normal maternal brain development, while the occasional

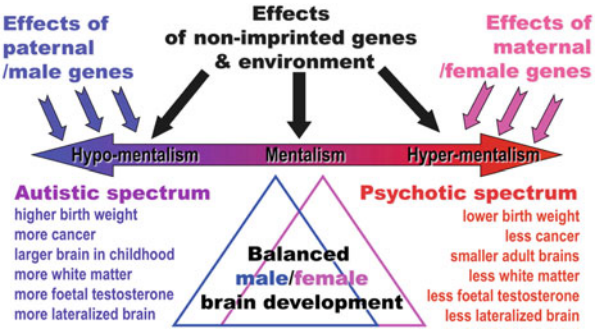

Fig. 5.1 The imprinted brain theory (Redrawn and modified from Crespi and Badcock (2008))

appearance of savant skills could be explained by an enhancement of characteristically male cognitive skills associated with an extreme paternal brain.

Finally, the obvious implication of all this is that if ASD is an extreme paternal brain disorder, PSD must be an extreme *maternal* one. Simon Baron-Cohen, who developed the extreme male brain theory of autism in recent times, explicitly rejected the proposition that psychosis might be the extreme female/paternal brain counterpart of autism. However, recent research revealed findings consistent with the imprinted brain theory, particularly those linking measures of hyper-mentalism to paranoia and positive symptoms of PSD in 70 healthy female students (Brosnan et al. 2010).

5.5 The Diametric Model of the Mind and Mental Illness

Mental disorders can be located along a dimension of mentalism (otherwise known as "theory of mind," "folk psychology," or "people skills") defined as our evolved ability to comprehend others' actions and behavior in purely mental terms (such as intention, belief, desire, emotion). Autistics, notoriously, are poor where mentalistic skills like inferring intention or understanding false belief are concerned. ASDs therefore belong on the *hypo-mentalistic* side of the continuum. However, PSD can be typified as *hyper-mentalistic:* paranoid schizophrenics, for example, symptomatically over-interpret intention either positively in erotomania (delusions that others are in love with you) or negatively in delusions of persecution. They also entertain bizarre false beliefs about themselves and others, and generally exhibit excessive mentalism, often enshrined in quasi-religious or mystical delusions (Badcock 2004). Indeed, the symptoms and signs of autism and psychoses like paranoid schizophrenia exhibit a remarkable pattern of antithesis similar to that seen in the sister syndromes mentioned above, such as BWS/SRS and AS/PWS (Table 5.1).

The concepts of hypo- and hyper-mentalism readily explain the last item in Table 5.1: age of onset. Typically, this is early childhood for autism but late adolescence or adulthood for schizophrenia: a difference which up until now has

Table 5.1 Diametrically different symptoms of ASD and PSD

Autism spectrum disorder	Psychotic spectrum disorder
Gaze-monitoring deficits	Delusions of being watched/spied on
Apparent deafness/insensitivity to voices	Hallucination of and hypersensitivity to voices
Intentionality deficits	Erotomania/delusions of persecution
Shared-attention deficits	Delusions of conspiracy
Theory of mind deficits	Magical ideation/delusions of reference
Deficits in sense of personal agency	Megalomania/delusions of grandeur
Literalness/inability to deceive	Delusional self-deception
Pathological single-mindedness	Pathological ambivalence
Early onset	Adult onset

lacked an obvious explanation. But the fact that you need years to develop normal mentalistic skills before you can overdevelop them to the point of psychosis readily explains why the mentalistic deficits of autism are apparent in childhood and why the hyper-mentalism of psychosis can normally only become fully apparent much later.

Recent brain imaging studies have found evidence of hyper-mentalism in the brain. The studies presented normal and paranoid schizophrenic subjects with cartoon-based tests depicting various forms of intentions while scanning their brain activity. It confirmed earlier findings (Walter et al. 2009) suggesting that the medial prefrontal cortex is peculiarly involved in the social dimension of mentalizing but not active when it is a question of purely private intentions. Private prior intentions activated only the right temporoparietal junction (TPJ) and the precuneus (a deeply buried part of the parietal cortex involved with episodic memory, visuospatial processing, and self-awareness). The left TPJ became active when there was a social dimension to communicating intention, but only if it related to the present. In contrast to normal subjects, the schizophrenics' intentional thinking was found to be permanently active, even when unwarranted and inappropriate: for example, in relation to inanimate objects. The authors cite the diametric model (Crespi and Badcock 2008) and then add that "Adopting a similar approach, we claim that the impairments in understanding others' intentions exhibited by paranoid patients and autistic patients, respectively, can be considered as the two extremes of a continuum" (Bara et al. 2011)—just as the imprinted brain theory proposes.

One of the most counterintuitive implications of the concept of hyper-mentalism and its relation to psychosis is that if ASD is symptomatically deficient in mentalistic skills, PSD should reveal better than normal mentalistic abilities in certain respects. Psychiatrists since Bleuler have been commenting on the uncanny ability of some PSD patients where tuning into other people's minds and emotions is concerned. Recent experiments with borderline personality disorder (BPD) and schizotypical patients in particular have revealed better than normal skills in reading expression and detecting other's state of mind. BPD patients get more correct responses than healthy volunteers on the Reading the Mind in the Eyes Test. But as the concept of hyper-mentalism might suggest, they do particularly well on

neutral emotional expressions but tend to over-personalize their responses (Fertuck et al. 2009). When playing trust games with and without relevant facial cues, BPD players equaled normal controls in recognizing emotions and assessing fairness but were superior to normal controls when emotional cues were present and were more objective in assessing fairness (Franzen et al. 2010). Indeed, in the first laboratory study to explicitly test the diametric model, 318 students were assessed for autistic versus schizotypical (i.e., mildly psychotic) tendencies and tested for their ability on an Embedded Figures Test. The authors report that their results offer support for the claim made by the diametric model "that autistic and positive schizophrenia traits are diametrically opposed with regard to their effect on local versus global processing" (Russell-Smith et al. 2010).

Copy number variation (CNV) is the recently discovered and very surprising finding that individuals vary in the number of copies of particular genes they carry by up to 12 % of the total. CNV can result from duplication or deletion of genes and to this extent resembles imprinted gene expression, which can produce nil or double expression of normally singly expressed genes. Crespi, Stead, and Elliot used CNV, single-gene associations, growth-signaling pathways, and brain-growth outcomes to evaluate the diametric model. They found that CNV findings support the diametric model, which holds that autism and schizophrenia stand in opposition to one another: at four places in the genome, deletions predispose to one, while duplications predispose to the other. They also found that single-gene associations are inconsistent with the model which sees ASD and PSD as separate entities because schizophrenia and autism frequently share associated genes. Where brain growth was concerned, they found that autism goes with enhanced brain growth, whereas schizophrenia is characterized by reduced brain size—just as the diametric model predicts (Crespi et al. 2009a). Indeed, Shinawi et al. report independently that autism and macrocephaly observed with deletion and microcephaly seen in duplication of a site on chromosome 16 support the diametric model (Shinawi et al. 2010).

5.6 The Genes that Made Us Human and the Epigenesis of Culture

Schizophrenia, like autism, poses a striking paradox because, along with this high degree of heritability, it manifestly damages individuals' survival and reproductive success. So how could the genes responsible evolve? Nevertheless—and again like autism—it persists at a prevalence of about 1 % across all human cultures (Tamminga and Holcomb 2005). A proposed solution is that genetic liability to schizophrenia has evolved as a consequence of selection for human traits involving social cognition, creativity, and language (Crow 1997; Horrobin 1998)—or what you could call mentalism (Badcock 2004). According to this hypothesis, genes that increase risk of schizophrenia have been subject to positive selection in the

evolution of human beings, thanks to their key role in mentalistic cognition. Recently, Crespi, Summers, and Dorus evaluated this hypothesis by screening human and primate genes for evidence of positive selection (Crespi et al. 2007). They found statistically significant evidence for positive selection on 26 of 80 genes mediating liability to schizophrenia, including some which exhibit some of the best-supported functional and genetic links to this disorder. Previous studies indicated that recent positive selection in humans has driven the evolution of a suite of additional genes linked with schizophrenia risk, and variants of three genes associated with schizophrenia have recently been linked with measures of creativity. Taken together, the authors conclude that these findings provide evolutionary and genetic support for the hypothesis that schizophrenia represents "the illness that made us human" (Horrobin 1998)—or at least, *half* of what makes us human.

If mentalism gave us our mental culture, then there is good evidence for a contrasting form of cognition—what you might call mechanistic cognition. This is the mode of cognition that we have evolved to interact with the physical, nonhuman, natural environment, and it stands in contrast to mentalistic cognition, which evolved to facilitate social contact and cognition in relation to other people (Badcock 2004).

Significantly, autistics sometimes show remarkable compensations for their mentalistic deficits in mechanistic cognitive skills—something otherwise known as *autistic savantism*. Among the most common such skills are calendar calculation (such as knowing the date of Easter in any year you care to name), rote memorization, and math skills (Happé and Frith 2009). But as the diametric model of the mind might also suggest, psychotics show the contrary cognitive configuration: despite the mentalistic gifts mentioned above, they also reveal deficits in mechanistic skills (Toulopoulou et al. 2006). According to one authority, "Intellectual asymmetry with a relative superiority of verbal skills to spatial skills represents a putative endophenotype of schizophrenia" (Kravariti et al. 2006). Indeed, a recent finding confirms that visuospatial ability (and especially mental rotation) is impaired in schizophrenia patients when compared with healthy controls and implicates one particular gene (*S100B*) in accounting for this deficit (Zhai et al. 2011).

According to a survey of 919 families of children with ASD which listed occupations of parents, fathers of children with ASD were twice as often employed in engineering as were fathers in any of four control groups of children with Tourette's or Down syndrome. This was also true of grandfathers: among the fathers of children with autism, the ratio of those working in engineering to those working in other fields was 6:1, whereas in the Tourette's and Down cases, it was less than 3:1. The authors conclude that there seems to be a small but statistically significant link between autism and engineering and that their result might also help to explain why a condition like autism persists in the gene pool. They speculate that the very same genes that lead an individual to have a child with autism can lead to superior functioning in folk physics and observe that engineering and related folk physics skills have transformed the way in which our species lives. Indeed, they

conclude that "without such skills, Homo sapiens would still be pre-industrial" (Baron-Cohen et al. 1997).

Part of the paradox of why severe mental illnesses like autism and schizophrenia have genetic causes may therefore lie in the fact that the very same genes that can produce these pathological conditions also underpin the twin cognitive systems on which human preeminence as a species relies: mentalistic and mechanistic cognition. One gave us our society, culture, language, and ability to empathize and interact with other people's minds. The other gave us science, technology, and all the manual, mechanical, and technical skills on which our civilization depends. If this view is correct, autism and psychoses like schizophrenia are the price we pay for these critical cognitive adaptations (Badcock 2004).

5.7 Nurture via Nature: Environmental Epigenetics

A final and equally challenging and controversial implication of the imprinted brain theory is that it may be able to explain the seemingly non-genetic, environmental, and social factors in the incidence of mental illnesses like autism and schizophrenia mentioned at the beginning.

A possible explanation for the findings relating maternal starvation to schizophrenia mentioned earlier is that maternal starvation has the same effect as maternally active genes in restricting growth and, according to the hypothesis advanced here, also predisposes towards the risk of psychosis in later life: nurture—or the lack of it—via nature, so to speak. Furthermore, a study of *IGF2* expression in children born during the Dutch wartime famine provided the first evidence that transient environmental conditions early in human gestation can affect the expression of such imprinted genes (Heijmansa et al. 2008). Although this effect was found among those with normal birth weight who were exposed to famine early in gestation but was not found among those with low birth weight unrelated to *IGF2* expression exposed to famine late in gestation, the finding suggests that more direct effects cannot be ruled out in principle. On the contrary, it establishes a strong precedent for thinking that environmental factors could directly or indirectly affect gene expression in accordance with the theory set out here.

The suggestion that severe deficits in nutrition like those associated with maternal starvation during pregnancy might have pathological consequences where development is concerned is hardly surprising. But if that were true, the theory proposed here would have the contrary, very counterintuitive implication. This is that environmental influences which enhanced growth might predispose towards ASD, perhaps by way of increasing the expression of genes like *IGF2* or at the very least by mimicking their effects. This in itself might explain quite a lot of the so-called autism epidemic of recent years. Growth enhancement, thanks to higher standards of living in developed countries, could be predicted to predispose towards milder forms of ASD such as Asperger's syndrome. Indeed, birth weights of newborn babies in Vienna rose an "unprecedented amount" (from a mean of 3 kg

to a peak of 3.3 kg) during the 1920s (Ward 1993, p. 56), and perhaps this partly explains why Asperger was to discover the syndrome named after him during the next couple of decades.

Again, critics of Kanner, autism's other independent discoverer, have pointed out that he portrayed it as an "upper class" disorder, but that later research, particularly in Sweden (the Gothenburg studies) contradicted this and found no clear link to social class (Gillberg 1992). However, it might simply be that during the 1940s the heavier-birth-weight effect was mainly seen among better-off people in the USA, but that by the 1980s it had spread to just about everyone in welfare-state Sweden—and today to most people in modern Western societies, where obesity, rather than undernourishment, has become the primary health problem related to food intake.

A controversial and counterintuitive prediction of the theory for which there is already much evidence is that if ASD has increased in modern societies with higher standards of living as it so spectacularly has done, then PSD should be falling. Interestingly in this respect, rates of admission for PSDs like schizophrenia have decreased by between 10 % and 57 % in England and Wales, Scotland, Denmark, Australia, and New Zealand. Indeed, even Bleuler, who coined the term schizophrenia, noticed a secular decline in his own lifetime (Der et al. 1990), and a recent Canadian study showed a 42 % decrease in the number of first-admission schizophrenia cases over 20 years. It found that annual inpatient prevalence rates decreased by 52 % between 1986 and 1996, with no corresponding change in outpatient rates, regardless of sex (Woogh 2001).

The exception is major depressive disorder (MDD); however, there are now reasons for thinking that MDD may be an immunoregulatory disorder with major psychiatric symptoms: in other words, the outcome of an immune system dysregulated by modern hygienic living conditions, rather than a dysregulated brain (Raison et al. 2010). Again, where schizophrenia is concerned, there are suspicions that the protozoan parasite *Toxoplasma gondii* may sometimes be a contributing factor to the development of the illness (Webster et al. 2006). But as I have argued elsewhere, *T. gondii* is known to attack the amygdala, a key component of what I have been calling the paternal brain, perhaps explaining its link with PSD by way of disturbing the balance of brain function in a maternal direction, as predicted by the theory (Badcock 2009). Indeed, given its known affinity for the limbic system, much the same might be said for the other suspected infectious cause of schizophrenia: *Cytomegalovirus* (CMV) (Yolken and Torrey 2008).

5.8 Summary and Conclusion

The imprinted brain theory proposes that development can be pushed to either mental extreme by any factors that affect gene expression either before or after birth (Fig. 5.1). Valproic acid is known to do this, as is thalidomide and other

environmental causes of autism (Rodier 2000). Where purely genetic factors are concerned, we have seen that the theory proposes that increased expression of paternal genes like *IGF2* will predispose to autism—and expression of that gene is now known to be enhanced in individuals with ASD. And because all fathers are male, the new theory can also be reconciled with the extreme male brain theory of autism, which persuasively argues that ASD can often be linked to increased testosterone exposure in utero, and to the more lateralized brain characteristic of males. But because all mothers are female, enhanced expression of maternal genes also goes with reduced fetal testosterone and the less lateralized brain typical of women. Moreover, in cases where an extra X chromosome is present, it results in brain features similar to those found in schizophrenia, along with a notably increased vulnerability to psychosis, just as the theory would predict.

Two recent discoveries both illustrate and corroborate the imprinted brain theory. *Retinoic acid-related orphan receptor-alpha (RORA)* is the first candidate gene for autism that has been found to be responsive to both male and female sex hormones. In research on twins, the expression of *RORA* and other candidate autism genes was shown to be affected by a key epigenetic factor, DNA methylation, which in turn might explain how one of a pair of monozygotic twins could be affected by ASD, but the other not (Nguyen et al. 2010). *RORA* is involved in several key processes implicated in autism, including Purkinje cell differentiation, muscle tone and development of the cerebellum, protection of neurons against oxidative stress, suppression of inflammation, and regulation of circadian rhythm. Testosterone acts on androgen receptors to reduce *RORA*, while estrogen acts to increase it. Aromatase (an enzyme responsible for the conversion of testosterone to estrogen) is also a target for *RORA*, and both *RORA* and aromatase are strongly correlated in brain tissue and relatively reduced in the frontal cortex of ASD subjects—a region of the brain known to be critically involved in mentalistic cognition (Sarachana et al. 2011). As such, this is a finding that beautifully endorses the imprinted brain theory because it demonstrates how male genetic influence predisposes to ASD and how the presence of female sex hormones such as estrogen is protective.

A second recent discovery (Ressler et al. 2011) illustrates epigenetic effects working as predicted by the theory in the opposite direction. Over a lifetime, posttraumatic stress disorder (PTSD) is diagnosed in as many as 40 % of individuals exposed to traumatic events. The classic triad of symptoms is as follows: waking or dreaming flashbacks or recurrent involuntary reactions to the trauma or things recalling it; avoidances, fears, and phobias associated with the trauma; and finally, hyperarousal, hypervigilance, and exaggerated startle response. As such, these symptoms clearly mark PTSD out as a hyper-mentalizing disorder on the psychotic side of the spectrum according to the diametric model of mental illness peculiar to the imprinted brain theory. Indeed, in severe or chronic cases, classic psychotic symptoms such as paranoid delusions and auditory hallucinations may be present. And as the imprinted brain theory would predict, females may be at twice the risk of PTSD compared to males (Breslau 2001).

A study of 1,200 highly traumatized subjects with and without PTSD matched for age, sex, race, and trauma history found that levels of pituitary adenylate cyclase-activating polypeptide (PACAP) correlated strongly with PTSD symptoms and diagnosis in female, but not in male subjects (Ressler et al. 2011). This hormone is involved in activation and growth of neurons and their connections, and in rodents an-order-of-magnitude higher concentration of it is found in a part of the brain involved with conditioned fear reactions (the amygdala and associated regions). A separate experiment on startle reflexes in 16 male and 11 female subjects revealed that only female participants with the high PACAP levels showed correlated conditioned fear responses. A presumed estrogen receptor for PACAP seems to explain the striking limitation of the effect to women just as in the previous case we saw that *RORA's* links with androgens and androgen receptors might explain some of the male-biased incidence of autism. Furthermore, it is worth pointing out that the PACAP receptor gene is subject to differential methylation. This is the same epigenetic mechanism found in imprinted genes, in X-chromosome gene inactivation, and in RORA, and it is the methylation of the critical part of the PACAP receptor gene (*ADCYAP1R1*) which correlates with PTSD symptoms (Ressler et al. 2011). Such epigenetic mechanisms are known to be affected by environmental factors and insults during development, perhaps suggesting a further explanation for constitutional variations in susceptibility to PTSD.

In other words, PTSD and its associated receptor gene now seems to fit in as aptly on the psychotic side of the spectrum as *RORA* does on the autistic side. Both discoveries vindicate the twin predictions of the imprinted brain theory: namely, that mental illness is caused by epigenetic mechanisms affecting gene expression as well as by inheritance and that enhanced expression on the male/paternal side results in autistic spectrum disorders (as with RORA), while biased expression on the female/maternal side results in psychotic spectrum disorders—such as PTSD. Together, these two very recent discoveries suggest that as the genetic basis of mental disorders is discovered, more and more of them will be found to fit into the new paradigm proposed by the imprinted brain theory and its associated diametric model of the mind and of mental illness.

A further conclusion is that normality represents a more or less balanced expression of genes and environmental developmental influences; however, the sexes are likely to be slightly offset. This would fit with the finding that ASD afflicts more males than females and that men typically do worse on tests of mentalistic competence than do women. Women, on the other hand, would be symmetrically offset to the more mentalistic side of the spectrum, and this might explain why BPD is three times more common in women than in men and why rates of incidence of schizophrenia among family members of women with the disorder are higher than those among family members of men with schizophrenia. And although there is a slightly higher incidence of schizophrenia overall in men, erotomania appears to be a predominantly female pathology, with women suffering more paranoid delusions and hallucinations than men, particularly in late-onset cases.

The model appears to rule out anyone suffering from an ASD and a PSD simultaneously, and such comorbidity does appear to be rare—but is not unknown.

There are cases of individuals diagnosed with bipolar disorder who also show unmistakable signs of ASD during their non-manic phases. Indeed, in my book I quote one who suffers from severe gaze-aversion, autistic deficits in a sense of self and social anxiety most of the time, but who becomes comfortable with other people during manic episodes when his sense of self hypertrophies into megalomania with the feeling that he is the returned Jesus Christ (Badcock 2009, pp. 96–7)! Furthermore, there is evidence of both ASD and PSD in Newton and Beethoven and also so in the Nobel Prize-winning mathematician John Nash. Here the theory predicts that the ASD must come first (typically in childhood) and leave a permanent savant-like basis later built on by hyper-mentalistic tendencies to produce an unusually broadened and dynamically balanced cognitive configuration: that of true genius.

5.9 Future Prospects: The Epigenetic Revolution

Such a speculative theory as this can be expected to be controversial, and much remains to be done to work out its details. But the hypothesis does have one outstanding merit: it makes clear and counterintuitive predictions about which genes are likely to be involved, about how they should be expressed, and about what effects they should be found to have in the brain and on cognition. Rapid progress now being made in genomics and neuroscience should be able to disprove the basic concept quickly if it is indeed as wrong as some of its critics believe. Full corroboration may take some time and in practice would probably need something of a Human Imprintome Project (i.e., one focused on differential paternal/maternal methylation or other evidence of actual imprinting—something not currently provided by the Human Epigenome Project) (Crespi, 2010, personal communication). But were the theory to be proved even partly true, it would have a number of far-reaching and indeed revolutionary implications.

First and foremost, the new theory would place human epigenetics in its proper biological and evolutionary context and, if proved, would vindicate the kinship/conflict theory of imprinting in its most crucial application: the mind and brain. The study of human evolution would be put on a secure genetic basis, with genes and their expression, rather than phenotypes, being the new focus of research and explanation in accordance with the "selfish-gene" approach of modern Darwinism (Badcock 2009; Dawkins 1989).

Secondly, the diametric model and its basis in genetic conflict outlined above would provide a completely new paradigm for psychology. The twentieth century saw the emergence of two opposed psychological paradigms: psychoanalysis, which was guilty of hyper-mentalizing to an almost paranoid extent, and behaviorism, which was hypo-mentalistic in its autism-like denial of the mind (Badcock 2004). The new theory's dualistic mentalistic/mechanistic model of cognition does full justice to the mind without going to either extreme and roots cognition in a sound biological foundation (Badcock 2009).

Finally, success for the theory would revolutionize psychiatry and psychotherapy as a whole. Mental disorders could be classified in terms of their position on the mentalistic continuum and diagnosis confirmed by epigenetic testing for the patterns of gene expression predicted by the theory. Prevention and therapy would also be revolutionized by such objective measures, along with new drugs and other interventions tailored to the individual's epigenome. Indeed, individual epigenetic profiling might open up a completely new window on personal development, especially if psychological changes over a person's lifetime could be linked to variations in the pattern of gene expression as they conceivably might (Abu-Akel, 2011, private communication). Psychotherapy could certainly be founded on a secure basis in epigenetics, and the diametric model would become the basis of a completely new rationale for intervention. Mentalistic skills training has already been shown to benefit autistics, but only the diametric model explains why mechanistic skills training appears to benefit psychotics (e.g., pitch discrimination, which is often perfect in autistics (Fisher et al. 2009)).

At the very least, the imprinted brain theory poses a challenging new perspective for thinking about epigenetics and its relationship to the brain, the mind, and mental illness. The next few years will certainly reveal how successful it is going to be in that respect.

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Chapter 6
Epigenetics at the Interface of Genetics and Environmental Factors in Autism

Janine M. LaSalle, Roxanne O. Vallero, and Michelle M. Mitchell

Abstract Autism spectrum disorders have a complex etiology, suggesting that multiple genetic and environmental factors play a role in their etiology. The apparent rise in the prevalence of autism spectrum disorders in recent decades is currently an unresolved problem. Epigenetic mechanisms, such as DNA methylation, act at the interface of genes and environment and are of critical importance for human brain development. This chapter reviews the known epigenetic pathways with known genetic bases in autism spectrum disorders and some recent studies showing epigenetic changes in human autism samples. In addition, environmental factors are discussed for their roles in impacting DNA methylation, with a focus on the persistent organic pollutants because of their bioaccumulation in brain. Dietary factors are also discussed, particularly folic acid supplementation during pregnancy because of the known contributions to DNA methylation and the protection for neural tube defects and, more recently, autism. Further investigation into epigenetic factors at the gene/environment interface will likely be important for making progress in preventing and treating autism in the future.

Keywords Autism • DNA methylation

Abbreviations

CNV	Copy-number variation
DNMT	DNA methyltransferase
ICF	Immunodeficiency, centromeric region instability, and facial anomalies

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MECP2	Methyl-CpG-binding protein 2
RTT	Rett syndrome
IAP	Intracisternal A particle
POP	Persistent organic pollutants
PBDE	Polybrominated diphenyl ether
PCB	Polychlorinated biphenyl
CHARGE	Childhood Autism Risks from Genetics and the Environment
SAM	S-adenosylmethionine
SNP	Single-nucleotide polymorphism

6.1 Introduction

Autism is a neurodevelopmental disorder characterized by severe impairments in social interaction and communication, combined with restrictive and repetitive interests and behaviors. The term "autism spectrum disorders" is also used to reflect that autism is not a single disorder with a single identifiable cause or clinical end point but reflects a wide spectrum of clinical diagnoses defined by impairments in these three domains: communication, social behavior, and restrictive/repetitive behaviors. The onset of symptoms for autism spectrum disorders generally occurs around 1–3 years of age and is characterized by stereotyped mannerisms, abnormal preoccupations, lack of pragmatic language and imaginative play, impaired eye gaze, and impaired joint attention (Volkmar and Pauls 2003). Males with autism outnumber females by around 4–1, and the frequency in the population has increased dramatically in recent decades to around 1 in 110 children, representing a significant public health burden. However, shifts in the interpretation of diagnostic criteria could not completely explain the increased prevalence, thereby suggesting that environment may affect the rate of autism (Hertz-Picciotto and Delwiche 2009). While the understanding of environmental risk factors in autism is a field still in its infancy, epidemiological studies are emerging. One such study showed that maternal residence near agricultural fields in Central Valley of California has suggested a potential risk factor of organochlorides used as agricultural pesticides (Roberts et al. 2007). In addition, air pollution was found to be a potential risk factor in the development of autism (Windham et al. 2006). Additional reproductive risk factors for autism include advanced maternal and paternal age (Hultman et al. 2011; Shelton et al. 2010), maternal early age of menarche and obesity at age 18 (Lyall et al. 2011), and decreased spacing between subsequent pregnancies (Cheslack-Postava et al. 2011).

6.2 Autism Is a Complex Genetic Disorder

Historically, twin studies have provided compelling evidence for a genetic origin of autism, showing a concordance rate of 70–90 % for monozygotic and 0–10 % for dizygotic twins (Zoghbi 2003). A recent publication, which looked at 277 twin pairs having at least one affected twin with autism, found monozygotic twins to have an 88 % concordance rate versus a 31 % concordance rate for dizygotic twins, supporting earlier publications that had only looked at less than 50 twin pairs (Rosenberg et al. 2009) but an additional study of twins suggested that shared in utero environment was more critical than genetic similarities (Hallmayer et al. 2011). Despite the high heritability in monozygotic twins, autism has a heterogeneous etiology, with multiple genes and chromosomal regions suspected to be involved (Veenstra-Vanderweele et al. 2003). Direct approaches to investigating the genetic etiology of autism have involved cytogenetic studies, linkage analyses, genome-wide association studies, and, more recently, the analysis of copy-number variations (CNVs). Cytogenetic methods have historically detected the fragile site caused by CGG-repeat expansion at *FMR1* responsible for fragile X syndrome, as well as the ~12-MB deletions of 15q11–q13 that cause Prader-Willi, Angelman, or 15q duplication syndromes. The 15q11–q13 rearrangements and *FMR1* CGG expansions each account for about 1 % of autism etiology by recent estimates (Geschwind 2008). Homozygosity mapping of families with shared ancestry and autism revealed homozygous deletions of *DIA1* upstream of *NHE9* and a region upstream of *PCDH10*, all of which are involved in neural activity, suggesting that defective regulation of gene expression after neural activity may be shared between seemingly diverse autism mutations (Morrow et al. 2008). Genome-wide association studies (GWAS) in autism have been somewhat conflicting, but large studies have shown significance associated with a cadherin gene cluster on chromosome 5p14 (Wang et al. 2009) and the MACROD2 gene on chromosome 20p12.1 (Anney et al. 2010). Complex genetic diseases are characterized by both polygenic inheritance (multiple genes interacting in the susceptibility) and locus heterogeneity (mutations in different genes cause the same phenotype). The investigation of autism genetics, like that of other neuropsychiatric disorders, has therefore moved from a prediction of "common disease, common variant" to a "common disease, rare variant" prediction that suggests that many alternative rare variants may disrupt overlapping pathways (Cook and Scherer 2008; Geschwind 2008).

Rapid developments in microarray technologies have spawned the discovery and greater appreciation of extensive copy-number variations (CNVs) in the human genome, which are gains or losses of DNA segments ranging from several kb to several Mb (Feuk et al. 2006; Iafrate et al. 2004; Sebat et al. 2004). While CNVs are polymorphic and often inherited in humans, a higher frequency of de novo rare CNVs are found in patients with autism and schizophrenia compared to unaffected controls (International Schizophrenia Consortium 2008; Sebat et al. 2007; Stefansson et al. 2008). CNVs mapping to 15q11–q13.5 are among the most frequently observed in both disorders (International Schizophrenia Consortium

2008; Christian et al. 1999; Miller et al. 2008; Stefansson et al. 2008). In addition to the higher burden of CNV observed in autism, there was a significant enrichment of CNVs disrupting function gene sets in autism compared to controls (Pinto et al. 2010). While progress in CNV detection and frequent occurrences in autism spectrum disorders is an exciting recent development, challenges to understanding causality of specific genes within specific CNVs remain (Cook and Scherer 2008). Gains and losses of the same locus often leads to overlapping phenotypes, as is observed in 22q11.2, 7q11.23, 17p11.2, and 15q11–q13 deletion and duplication syndromes with comorbid autism (Cook and Scherer 2008; Ramocki and Zoghbi 2008). Complicating CNV genotype-phenotype studies even further are issues of variable penetrance or variable expressivity of the phenotype. For instance, while maternal duplication of 15q11–q13 is associated with autism in 85 % of cases (Hogart et al. 2010), paternal 15q11–q13 has been observed in healthy unaffected individuals (Cook et al. 1997) as well as cases with autism or language and social defects (Depienne et al. 2009; Mao et al. 2000; Milner et al. 2005; Mohandas et al. 1999). Within the subset of patients with maternally derived idic(15), there is much clinical heterogeneity even within patients with similar duplication breakpoints, including a subset of patients with Prader-Willi-like features (Depienne et al. 2009). Therefore, other genetic liabilities and nongenetic factors are considered to be important but uncharacterized determinants in determining the pathogenicity and expressivity of CNVs in autism spectrum disorders (Cook and Scherer 2008).

6.2.1 Epigenetics

Epigenetics is defined as reversible and heritable modifications to nucleotides or chromosomes that can change gene expression and chromatin structure without altering the primary genetic sequence. Epigenetic modifications include DNA methylation, histone acetylation or methylation, and chromatin architecture. Epigenetic characteristics can differ in individuals even with identical nucleotide sequences. Methylation, histone modifications, and chromatin architecture can all influence gene regulation and gene expression, leading to susceptibility or protective effects for disease pathogenesis. Because DNA methylation is the epigenetic mark most commonly examined and correlated with environmental factors, it will be the focus of this chapter.

Determining how epigenetics plays a role in human health and disease is a major question. Because the nature of epigenetic marks is that they do not alter the nucleotide sequence, the challenges for epigenetic research are greater than those for genetic investigators. Although epigenetic marks can be heritable through the germ line as parental imprints, most are erased and reestablished during gametogenesis and early embryogenesis. Some epigenetic changes may be caused throughout human lifespan directly by changes in environment, such as diet and early experiences. However, complicating the analyses of direct environmental changes on epigenetic changes is that most epigenetic changes are stochastic or randomly derived.

6.2.2 DNA Methylation

The first "layer" of epigenetic modification is DNA methylation, the addition of a methyl group ($-CH_3$) to nucleotides within DNA, which occurs in a number of different organisms. In humans, the methylation site can occur on 5' of cytosine when followed by a guanine (CpG). CpG dinucleotides are distributed less frequently in the mammalian genome than statistical expectations and they cluster in CpG islands, typically overlapping gene promoters (Bird et al. 1985). CpGs outside of CpG islands in the genome are generally methylated, whereas CpG islands do not have dense methylation (Bird 2002). Methylation of cytosines renders them sensitive to deamination C > T mutations, thus explaining the nonrandom occurrence of unmethylated CpGs clustered in islands in the mammalian genome (Gonzalgo and Jones 1997; Vairapandi and Duker 1994). In normal cells, DNA methylation of CpG sites is predominant (75–90 %) in repetitive genomic regions including satellite DNA and parasitic elements (e.g., LINEs, SINEs, endogenous retroviruses, and segmental duplications) (Yoder et al. 1997).

Although DNA is highly methylated in all cells of organisms during early embryonic development, the methylation patterns inherited from the parental gametes are erased and reestablished in every individual around the stage of implantation (Shemer et al. 1996). Previous studies suggested that a combination of concerted epigenetic marks including histone acetylation or methylation directs the local methylation status at CpG islands (Meissner et al. 2008). Strasseman et al. showed that the CpG islands at the 5' ends of housekeeping genes are largely unmethylated, suggesting that the primary effect of DNA methylation acts outside of gene promoters (Straussman et al. 2009). Using a genome-wide assay with a single-base resolution, Lister et al. recently showed that almost one-quarter of methylation marks in embryonic stem cells were in a non-CpG context (Lister et al. 2009). Their data also suggests that within gene bodies, there is an increased non-CpG methylation.

DNA methyltransferases (DNMTs) are essential enzymes that establish and maintain the DNA methyl marks. One study using embryonic stem cells elucidated their importance using a cell line deficient for DNMTs which are viable but, however, expire after differentiation (Panning and Jaenisch 1996). DNMT1, the maintenance methyltransferase which catalyzes the addition of methyl groups to newly synthesized DNA, is highly expressed in the adult central nervous system (CNS) in postmitotic neurons, although theoretically it is not needed to maintain postmitotic DNA methylation patterns (Inano et al. 2000). However, one study showed DNMT1 may possess some de novo methylation activity as well. A brain-specific knockout mouse mosaic for *Dnmt1*$^{-/-}$ cells revealed loss of mutant neurons and glia, while the full knockout was early postnatal-lethal (Fan et al. 2001). A double-mutant mouse model of both *Dnmt1* and *Dnmt3a* showed deficits in learning and memory (Feng et al. 2010). Inhibitors of DNA methylation administered in adult animals also inhibited memory formation (Miller and Sweatt 2007).

While most of the focus of DNA methylation studies has been on individual genes, it is important to note that repetitive DNA makes up approximately 50 % of the human genome and a majority of DNA methylation occurs in repetitive regions, predominately *Alu* and *LINE1* elements (Yoder et al. 1997). *Alu* elements are heavily methylated in normal tissue including the brain (Ladd-Acosta et al. 2007). Xie et al. used high-throughput sequencing methods to show that *AluY*/*S* elements may be enriched at the interface between hypermethylated and hypomethylated regions (Xie et al. 2009). Their results also showed that some *Alu* elements may be tissue-specifically methylated and hypothesized that *Alu* elements may serve as sensors for DNA methylation and signal changes in methylation status.

6.3 DNA Methylation and Disease

DNA methylation has been implicated in disease states for several decades. The link between DNA methylation and cancer was first demonstrated in 1983 when it was shown that genomes of cancer cells are hypomethylated relative to their normal counterparts (Feinberg and Tycko 2004). Disruption to any of the interacting epigenetic systems can lead to inappropriate expression or silencing of genes, resulting in "epigenetic diseases." One such example of an epigenetic disease is ICF (immunodeficiency, centromeric region instability, and facial anomalies) syndrome, a rare recessive autosomal disorder and one of the first genetic diseases to be associated with a constitutional methylation defect that affects mostly heterochromatin (Jeanpierre et al. 1993; Miniou et al. 1994; Smeets et al. 1994). ICF is caused by mutations in the DNMT3b gene on the long arm chromosome 20 (Hansen et al. 1999; Okano et al. 1999; Xu et al. 1999). DNMT3b is the essential enzyme for establishment of methylation patterns.

In 1999, mutations in the gene *MECP2*, encoding methyl-CpG-binding protein 2, were found to be the major cause of Rett syndrome (RTT) (Amir et al. 1999). This finding of an involvement of MECP2 in RTT has provided some of the strongest evidence to date for a major role for epigenetics in neurodevelopmental disorders. Several reports have linked epigenetics to disease phenotypes in humans. An example includes the incidence of discordant MZ twins for the X-linked disorder Rett syndrome (Bruc et al. 1991; Migeon et al. 1995). The discordance in severity of RTT in monozygotic twins is presumed to be due to differences in X chromosome inactivation and is a female-specific epigenetic mechanism, although other explanations are also possible. A previous study has also demonstrated that multiple epigenetic differences between monozygotic twins increase with age, including X chromosome inactivation and alterations in methylation at several specific gene promoters and *Alu* repeats (Fraga et al. 2005).

Methylation aberrations have been implicated in other neurodevelopmental and psychiatric disorders. Increased methylation of the *MECP2* promoter correlated with decreased expression in autism compared to control brain samples (Nagarajan

et al. 2006). DNA methylation abnormalities have also been observed in autism blood samples, including hypermethylation of the oxytocin receptor gene (*OXTR*) (Gregory et al. 2009) and the circadian regulatory gene *RORA* (Nguyen et al. 2010). In schizophrenia brain, hypermethylation of the reelin (*RLN*) promoter CpG island has been observed (Grayson et al. 2005).

6.4 Epigenetics at the Interface of Gene and Environmental Interactions

Although it has yet to be understood exactly how environmental factors alter DNA to influence gene expression throughout the lifetime of an organism, epigenetics seems to be at the interface of gene x environment interactions that can ultimately alter gene expression. The epigenetic profile of an individual is influenced by both stochastic and environmental factors. Although monozygotic twins are genetically identical, the methylation patterns become more variable between MZ twins throughout their lifetime (Fraga et al. 2005).

The *Agouti* gene which encodes coat color in mice provides an excellent mutation model of how environmental factors bring about both random and directed changes within the epigenome. An intracisternal A particle (IAP) retroviral element spontaneously inserted into the *Agouti* gene, giving rise to several agouti viable yellow alleles (A^{vy}) (Millar et al. 1995). Mice that inherit the A^{vy} alleles have one of the three phenotypes: yellow, mottled, or wild-type agouti. Methylation status of the IAP element was found to correlate with the variation in phenotype. When the A^{vy} allele was unmethylated, mice were yellow and obese. In contrast, when the A^{vy} allele was methylated, mice exhibited a normal phenotype. In a separate study, agouti female mice which are yellow, fat, and prone to certain disease were fed a methyl donor-rich diet during pregnancy. As a result, the offspring of these mice had virtually no mutant phenotype; they were mottled in color, were smaller, and did not develop any of the diseases to which their mothers were predisposed (Dolinoy et al. 2006). Further study of this model showed that an environmental pollutant, perinatal exposure to bisphenol A, was a risk factor for hypomethylation of the A^{vy} allele and the yellow, obese phenotype, while a diet rich in methyl donor protected against this phenotype (Dolinoy et al. 2007). These findings demonstrate that environmental factors can have both harmful and protective effects on DNA methylation that may be important in human neurodevelopmental disorders.

6.5 Environmental Pollutants and Neurodevelopment

A variety of environmental chemicals of concern for human health have been investigated for their potential effects on DNA methylation and other epigenetic effects (reviewed in Baccarelli and Bollati 2009). Most studies have shown that exposures correlated negatively with global methylation but positively with promoters of specific genes. Arsenic, cadmium, benzene, and air pollutants are all chemicals linked to global hypomethylation of human tissues (Baccarelli et al. 2009; Bollati et al. 2007; Tarantini et al. 2009; Wright et al. 2010; Zhao et al. 1997). In mouse models, methylmercury exposure resulted in hypermethylation of brain-derived neurotropic factor (*Bdnf*) in hippocampus (Onishchenko et al. 2008) and diethylstilbestrol exposure reduced global methylation in uterus (Sato et al. 2009). In a human Greenlandic Inuit population with high persistent organic pollutant (POP) levels, reduced global DNA methylation was observed with increased POP levels (Rusiecki et al. 2008). Furthermore, prenatal exposure of a rat model with organochloride pesticides, methylmercury, POPs, or a mixture of all three chemical classes showed that POPs and the mixture reduced DNA methylation levels significant in liver. Lastly, the POP PCB-95 was observed to be significantly higher and correlated with reduced global DNA methylation in postmortem brain samples of autistic individuals with duplication of chromosome 15q11-q13 compared to than controls matched for year of birth (Mitchell et al. 2012).

POPs are of particular concern for a causal role in neurodevelopmental disorders because of their widespread use, bioaccumulation, and characterized developmental neurotoxicity. Polybrominated diphenyl ethers (PBDEs) are a specific family of POPs that are used as flame retardants in various items such as furniture, electronics, and carpeting. The structure of PBDE is similar to polychlorinated biphenyls (PCBs), another class of POPs that were used in various consumer goods but banned in the 1977 due to their neurotoxicity (Steenland et al. 2006). Despite the ban, levels of PCBs still persist in the environment and in organisms because of their bioaccumulation in lipid-rich tissues (Hopf et al. 2009). PBDEs are not covalently bound to the articles or items to which they have been added, and as a result they easily leach into the environment (Alaee 2003). Flame retardant DE-71 is a penta-PBDE mixture containing 23 PBDE congeners with a 47 % BDE-47 content. BDE-47 is the most abundant congener in the environment and has the greatest burden on Western populations (North 2004). The bioaccumulation of PBDE in the environment has raised concerns with regard to the potential effects on human health, and several studies have found adverse effects of PBDEs on the environment and on the health of human and wildlife populations (Dufault et al. 2005; Stoker et al. 2005).

Concentrations of PBDEs found in the environment have doubled every 4–6 years since the 1970s, and levels in North America are approximately 20 times greater than in Europe (Hites et al. 2004). Women in California have strikingly higher tissue levels compared to individuals in Europe (North 2004; Petreas et al. 2003; Rose et al. 2010). The extensive use of brominated flame retardants in

consumer products in California is mostly due to the high flammability standards the state has set for upholstered furniture (Zota et al. 2008). The Childhood Autism Risk from Genes and Environment (CHARGE) study at the University of California at Davis M.I.N.D. Institute found that levels of PBDEs in the individuals sampled in CA are higher than those reported for any other population worldwide (Rose et al. 2010).

The structural similarity between PBDEs and the banned PCBs has raised concerns that they may have adverse developmental effects. A number of studies report altered thyroid hormone homeostasis by PBDEs (Ellis-Hutchings et al. 2006; Fernie et al. 2005). Studies in rats and mice have shown the negative impact BDE-47 has on the endocrine system, especially with regard to the thyroid (Darnerud et al. 2007; Hallgren and Darnerud 2002; Hallgren et al. 2001). Of note, both the hydroxylated forms of PBDEs and PCBs exhibit structural similarity with thyroxine, the major thyroid hormone important for proper neurodevelopment (Penner et al. 2001). Interestingly, PBDEs were only able to compete with thyroxine binding after complete metabolic conversion, indicating the importance of hydroxylation in the mechanism of action (Meerts et al. 2000).

The studies described thus far look at the mechanism by which PBDE may be affecting development. Interestingly, a recent paper reported on a mechanism for PBDEs uptake by the liver. The study reports that a class of organic anion transporting polypeptides (OATPs), which are responsible for the hepatic uptake of a variety of larger amphipathic compounds, mediate PBDE uptake by the liver and contribute to their bioaccumulation in the body (Pacyniak et al. 2010). An *ex vivo* study showed that PBDEs can be transported through human placenta and that BDE-47 is transported more rapidly and extensively than another PBDE, BDE-99 (Frederiksen et al. 2010). Recently, a perinatal exposure of BDE-47 was performed in a mouse model with genetic and epigenetic susceptibility to social behavioral deficits that showed adverse reproductive success, reduced female postnatal growth, reduced sociability, and a compounding interaction with Mecp2 mutation on spatial learning (Woods et al. 2012). Reduced global DNA methylation was observed in the brains of female offspring in this experiment, corresponding to reduced sociability.

Because humans are exposed to a variety of PBDE congeners, it will be important to understand the neurodevelopmental effects of combined exposures. A recent study reports that the exposure to the combination of BDE-47 and BDE-99 induced a synergistic neurotoxic effect in human neuroblastoma cells mediated by oxidative stress (Tagliaferri et al. 2010). PBDEs are known to accumulate in adipose tissue, and as such, the importance of establishing how BDE-47 perinatal exposure affects infant neurodevelopment becomes evident because breast milk produced from the mother's fat stores is the main diet of many newborns. A Swedish study confirmed the presence of PBDEs in women's breast milk (Lind et al. 2003). Infants' exposure to PBDEs is higher than that of adults; exposure for a breast-fed infant is estimated at 306 ng/kg/day compared to 1 ng/kg/day for adults (Schecter et al. 2006).

6.6 Dietary Influences in Neurodevelopment and Autism

Folic acid is a natural B vitamin obtained from the diet from leafy green vegetables, beans, peas, and liver. Folic acid is the synthetic form of folate found in prenatal vitamins, supplements, and fortified foods. As part of one-carbon metabolic cycle, folate acts as a donor and acceptor of one-carbon units required for biosynthesis of nucleic acids, proteins, and methyl groups for DNA, RNA, and protein methylation. Vitamin B_{12} is another vitamin linked to the one-carbon cycle, as a required cofactor for homocysteine to methionine conversion. Methionine is a substrate for S-adenosylmethionine (SAM), a key enzyme for DNA methylation reactions. Choline is another dietary nutrient found in lipid-rich foods such as eggs and liver and is known to be in high demand during pregnancy. Betaine is an important methyl donor and metabolite of choline that participates in SAM synthesis.

Folic acid has a strong protective role in the prevention of neural tube defects from two randomized clinical trials (MRC Vitamin Study Research Group 1991; Czeizel and Dudas 1992). Based on these findings, the Center for Disease Control recommended that women of childbearing age consume at least 400 mg of folic acid per day. The United States Food and Drug Administration mandated the addition of folic acid to enriched breads, cereals, flours, and other grain products in 1998 (MRC Vitamin Study Research Group 1991; Czeizel and Dudas 1992). Because of the increased prevalence of autism over the last decade, some individuals have hypothesized that folic acid fortifications may be responsible (Currenti 2009; Leeming and Lucock 2009). However, a recent case-controlled study from the UC Davis M.I.N.D. Institute has shown a significant protective role for the use of prenatal vitamins in autism risk, where mothers of children with autism were less likely than those of typically developing children to have taken prenatal vitamin during the 3 months before and/or first month after pregnancy (Schmidt et al. 2011). Significant gene x environment interaction effects were observed for maternal and child genotypes of known methylation risk alleles. These findings suggest that the protective nature of prenatal vitamin consumption in early pregnancy in autism may relate to DNA methylation pathways that perhaps serves to counteract the global hypomethylation observed from multiple environmental toxins.

6.7 Conclusions

Autism and autism spectrum disorders are complex human disorders, affected by multiple genes and environmental factors. Figure 6.1 diagrams some of the known risk factors acting on the developing brain that are implicated in autism etiology, as well as reflecting the unknown factors yet to be discovered. While specific genetic mutations, CNVs, and environmental exposures appear to strong causal factors, they are only in rare cases. The more common cases of autism spectrum disorders

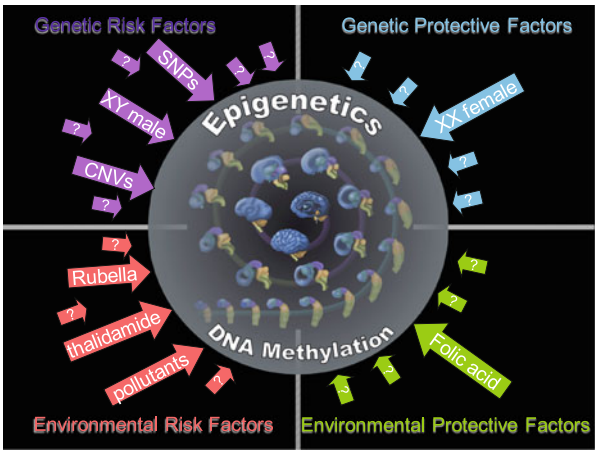

Fig. 6.1 The epigenetic interface of genetic and environmental risk and protective factors on the developing brain. The development of the human brain (*center*) and outcome on human behavior is impacted by a variety of genetic and environmental factors, only some of which are represented as individual arrows. Epigenetic mechanisms such as DNA methylation are critical for the developing brain and are impacted by both genes and environment. Protective factors are less well understood but are likely to be important for future prevention of autism spectrum disorders

are likely to involve combinations of multiple genetic and environmental risk factors. In addition to the obvious focus on genetic and environmental risk factors relevant to autism, research into protective factors may likely be as important for future prevention and treatment of autism spectrum disorders. Epigenetic pathways act at the interface genetic and environmental factors and are of critical importance for the developing brain. Furthermore, therapeutic strategies targeted to epigenetic mechanisms have promise in that defects in epigenetic pathways may be reversible. Therefore, further understanding of epigenetic pathways and how they are impacted by specific genetic and environmental factors will be instrumental to future progress in preventing and treating autism.

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Chapter 7
Epigenomic and Noncoding RNA Regulation in Addictive Processes

John S. Satterlee

Abstract The phenotypic effects of drugs of abuse are partially mediated by transcriptional and epigenetic regulatory mechanisms. This chapter will provide a brief overview of substance abuse and then focus on the roles of three epigenetic regulatory mechanisms in addictive processes: histone modifications, DNA modifications, and noncoding RNAs. This chapter will conclude with a focus on three other important areas: (1) the potential for long-lasting epigenetic effects due to drugs of abuse, (2) obstacles and opportunities in this scientific area as they pertain to addiction biology, and (3) the potential for translating epigenomic and noncoding RNA discoveries into improvements in human health and the treatment of substance use disorders.

Keywords Addiction • Chromatin regulation • DNA modifications • Drug abuse • Epigenetic regulation • Gene expression • Histone modifications • MeCP2 • Methylation

Abbreviations

ADAR	Adenosine deaminase
Ago2	Argonaute 2
AML	Acute myeloid leukemia
BDNF	Brain-derived neurotrophic factor
BRD4	Bromodomain-containing protein 4
Brg1	Brahma-related Gene 1
caC	5-carboxylcytosine
CBP	CREB-binding protein

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Cdk5	Cyclin-dependent kinase 5
CHD	Chromodomain helicase DNA-binding protein
ChIA-PET	Chromatin interaction analyses – paired end tags
ChIP-seq	Chromatin immunoprecipitation-sequencing assay
CPP assay	Conditioned place preference assay
CREB	cAMP-response element-binding protein
D1R	Dopamine 1 receptor
DARPP-32	Dopamine- and cyclic-AMP-regulated phosphoprotein 32
DAT	Dopamine transporter
DNMT	DNA methyltransferase
DOHaD	Developmental origins of health and disease
EMX2OS	EMX2 opposite strand
ENCODE	Encyclopedia of DNA elements
eRNA	Enhancer RNA
EWAS	Epigenome-wide association studies
fC	5-formylcytosine
GABA	Gamma-aminobutyric acid
GAD67	Glutamic acid decarboxylase 67
GFP	Green fluorescent protein
GluR2	Glutamate receptor 2
GWAS	Genome-wide association studies
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
Hi-C	Chromatin conformation capture assay
Histone modifications	Example H3K4me3 = histone H3 with trimethylated lysine-4
hmC	5-hydroxymethylcytosine
HOTAIR	HOX antisense intergenic RNA
HOTTIP	HOXA transcript at the distal tip
IGFBP-3	Insulin-like growth factor binding protein 3
JARID1C	Jumonji AT-rich interactive domain 1C protein
lincRNA	Large intervening noncoding RNA
LINE	Long interspersed nuclear element
MBD	Methyl-CpG-binding domain protein
mC	5-methylcytosine
MeCP2	Methyl CpG-binding protein 2
MEG3	Maternally expressed Gene 3 ncRNA
methylC-seq	5-methylcytosine sequencing assay
mGluR5	Metabotropic glutamate receptor 5
MGMT	Methyl guanine DNA methyltransferase
MIAT	Myocardial infarction associated transcript
miRNA	MicroRNA
MOR	Mu opioid receptor
mPFC	Medial prefrontal cortex

MSK1	Mitogen and stress-activated protein kinase 1
NAc	Nucleus accumbens
nAChR	Nicotinic acetylcholine receptor
ncRNA	Noncoding RNA
NEAT1	Nuclear paraspeckle assembly transcript 1 ncRNA
NEAT2	Nuclear paraspeckle assembly transcript 2 ncRNA
PET	Positron emission tomography
PFC	Prefrontal cortex
PHD	Pleckstrin homology domain
piRNA	Piwi-interacting RNA
PRC2	Polycomb repressive complex 2
RIP-seq	RNA immunoprecipitation sequencing assay
SAHA	Suberoylanilide hydroxamic acid
SINE	Short interspersed nuclear element
siRNA	Small interfering RNA
SIRT1	Sirtuin 1
SNP	Single-nucleotide polymorphism
SPRED1	Sprouty-related EVH1 domain containing 1 protein
SUD	Substance abuse disorder
TORC	Transducers of regulated CREB
TPH2	Tryptophan hydroxylase-2
Uhrf1	Ubiquitin-like containing PHD and RING finger domains 1 protein
VTA	Ventral tegmental area
WD40 domain	WD dipeptide-containing domain

7.1 Introduction

7.1.1 The Environment and Epigenomic Regulation

With certain exceptions, an individual's genome is believed to be more or less identical in every cell. However, the epigenomes of different cell types within an individual appear to differ significantly from one another (Hawkins et al. 2010). This is consistent with the distinct phenotypes, functions, and gene expression profiles of particular cell types. There have been a number of reviews indicating that our epigenomes may be sensitive to "environmental" influences which can be broadly defined to include diet, toxins, stressors, and psychosocial influences (Jirtle and Skinner 2007; Zhang and Meaney 2010; Caldji et al. 2011). It has been hypothesized that environmental exposures may lead to changes in signaling in specific cell or tissue types. These changes may in turn impact epigenetic regulation

of gene expression, ultimately leading to transient or long-lasting changes in gene expression and cellular or organismal phenotypes. In some cases, these changes appear to be passed on through mitosis or even to subsequent generations (Youngson and Whitelaw 2008).

7.1.2 Epigenetic Regulation in the Nervous System

Epigenetic regulation has been shown to be important in neurogenesis, neural fate specification, neuronal development, behavioral plasticity, synaptic plasticity, circadian regulation, and learning and memory (Day and Sweatt 2011; Haggarty and Tsai 2011; Ma et al. 2010; Zocchi and Sassone-Corsi 2010; Bellet and Sassone-Corsi 2010; Maze and Nestler 2011; Nelson and Monteggia 2011; Ma et al. 2010; Dulac 2010; Namihira et al. 2008). Additionally, misregulation of epigenetic processes has been implicated in a number of human disorders including neurodevelopmental disorders (e.g., Rett and Prader-Willi syndromes) and psychiatric disorders (e.g., schizophrenia, depression) (Horsthemke and Wagstaff 2008; Graff and Mansuy 2009; Moretti and Zoghbi 2006; Tsankova et al. 2007; Pidsley and Mill 2011; Renthal and Nestler 2009a). Epigenetic regulation has also been implicated in response to early childhood abuse associated with suicide completion (McGowan et al. 2009). For the remainder of this chapter, I will focus on what is known about the role of epigenetic regulation in substance use and abuse.

7.2 Substance Abuse

7.2.1 Substance Use and Abuse

Our brains are inherently plastic, possessing connections and signaling processes that can change in response to distinct environmental exposures. Brains exposed to drugs of abuse on a continuing basis develop changes in particular neuronal regions, including those involved in the reward system. In addicted individuals, these brain changes can lead the individual to seek out drugs of abuse despite serious negative consequences. The definition of drug addiction has evolved over the years. According to Drs. Koob and Volkow: “Drug addiction is a chronically relapsing disorder that has been characterized by (1) compulsion to seek and take the drug, (2) loss of control in limiting intake, and (3) emergence of a negative emotional state (e.g. dysphoria, anxiety, irritability) reflecting a motivational withdrawal syndrome when access to drug is prevented” (Koob and Volkow 2010; American Psychiatric Association 2000; Koob and Le 1997). Common addictive substances include nicotine, alcohol, caffeine, cocaine, methamphetamine, opioids, certain prescription medications, inhalants, and cannabis. Food and sex are sometimes

referred to as "natural rewards" and can exhibit effects similar to those caused by drugs of abuse (Olsen 2011; Avena et al. 2008). There are also compulsive "behavioral addictions" such as gambling or internet addiction that have some of the hallmarks of drug addiction (Grant et al. 2010; Ambermoon et al. 2011).

In broad strokes, exposure to addictive substances, such as alcohol, opiates, cannabinoids, and nicotine, leads to increased levels of dopamine within the mesolimbic dopamine system (Sulzer 2011; Justinova et al. 2009). For example, cocaine inhibits dopamine reuptake such that more dopamine remains at the synapse (Newman and Kulkarni 2002; Fleckenstein et al. 2007). Continued drug exposure ultimately leads to adaptations in the strength of circuit connections between different brain regions including the nucleus accumbens (NAc). These changes in circuit strength are mediated in part by alterations in signaling by the neurotransmitters dopamine and glutamate in specific types of neurons with concomitant gene expression changes (Luscher and Malenka 2011; Kalivas et al. 2009; Gardner 2011). A strengthening of the reward connections leads to changes such that an individual craves the drug of abuse more, even if substance use leads to adverse consequences. A weakening of the inhibitory influence of the prefrontal cortex (PFC) on the reward circuitry can also decrease the ability of the individual to resist substance use (Van den Oever et al. 2010).

Not all individuals exposed to drugs of abuse become addicted. Based on heritability measurements, this complex disease appears to have an important genetic component with some individuals particularly susceptible to addiction, while others are resistant to it (Johnson et al. 1996; Kendler et al. 1999; Uhl et al. 2008; Buckland 2008). Individuals that are particularly impulsive or have an enhanced propensity for risk taking are more likely to explore the use of drugs, and this impulsivity phenotype may have a genetic component (Perry and Carroll 2008; Dalley et al. 2011). Environmental influences are important in the development of substance abuse disorder (SUD); access to drugs of abuse, early life adversity, poverty, or exposure to drugs during critical periods such as adolescence can all influence the potential of individuals to develop substance abuse disorder (Caspi et al. 2005; Buka et al. 2003; Hill et al. 2005).

The overall economic cost of substance abuse in the USA has been estimated to be greater than $600 billion per year (Table 7.1). For illicit drugs alone, the cost is estimated to be $193 billion, while for alcohol and tobacco, the costs are estimated to be $235 billion and $193 billion, respectively. In addition to the serious economic cost to society from SUD, the consequences of addiction are extraordinarily destructive to the addicted individuals and their families. Behavioral therapies can be used to improve outcomes in substance abusers, and in some cases, therapy in concert with medication can improve outcomes (Carroll and Onken 2005). Despite ongoing efforts to develop safe and effective medications for the treatment of SUDs, only limited success has been achieved. Currently, approved medications exist to aid in smoking cessation as well as to treat opiate and alcohol dependence, including therapies for the initiation of and maintenance of abstinence (e.g., nicotine replacement therapy, buprenorphine, varenicline, naltrexone), to alleviate symptoms of withdrawal (e.g., varenicline, diazepam), and to prevent relapse

Table 7.1 The estimated yearly economic cost of licit and illicit abused substances

Substance	Estimated economic cost per year	Reference
Illicit drugs	$193 billion	(National Drug Intelligence Center [NDIC] 2010)
Alcohol	$235 billion	(Rehm et al. 2009)
Tobacco	$193 billion	(Centers for Disease Control and Prevention [CDC] 2007)
All	>$600 billion	

(e.g., naltrexone, bupropion). However, with that said, no approved medications exist for the treatment cocaine, methamphetamine, or cannabis addiction even though efforts to develop these are ongoing (Montoya and Vocci 2008; McCann 2008). Unfortunately in the absence of good treatment options for the various SUD-related indications, addicted individuals will continue to struggle with this devastating disease.

In order to advance the development of effective medications and therapies to treat SUDs, it is critical that we understand the molecular and neurobiological mechanisms that lead to the development of addiction. Since exposure to drugs of abuse leads to long-lasting brain changes, it is not surprising that molecular studies exploring changes in gene expression in the nervous system have been very illuminating. For the rest of this chapter, I will focus on the role of transcriptional regulation in substance abuse with a focus on epigenomic regulation.

7.2.2 Transcriptional Regulation and Substance Abuse

Because exposure to drugs of abuse can lead to long-lasting brain changes including changes in neurotransmission in specific brain circuits, it has been proposed that alterations in gene expression via transcriptional regulation play a significant role (Nestler et al. 2001). The role of transcriptional changes in response to drugs of abuse has been well studied and reviewed (Nestler 2008; Nestler and Malenka 2004). Several transcription factors have been shown to play a role in addictive processes. For example, the cAMP-response element-binding protein (CREB), which has a well-established role in learning and memory, can alter drug abuse behaviors (Briand and Blendy 2010; Carlezon et al. 2005). In many ways addiction is an example of learning and memory gone seriously awry, so the identification of CREB is perhaps not so surprising.

Probably the most well-characterized transcription factor involved in drug addiction is the delta-FosB protein. Delta-FosB can be induced in the nucleus accumbens by opiates, cocaine, nicotine, other drugs of abuse, and natural rewards (such as sucrose and sex), and the targets of delta-FosB (e.g., GluR2, dynorphin, Cdk5) are congruent with the signaling molecules previously implicated in addictive processes (Nestler 2008). It has been proposed that significant delta-FosB

induction can lead to "excessive sensitization of the nucleus accumbens circuitry" and ultimately lead to compulsive drug taking (Nestler 2008).

Transcription factors bind to and can alter the properties of chromatin, and conversely chromatin state may impact transcription factor binding and function (Birney 2011; Adrian et al. 2010; Koche et al. 2011). Although the role of transcription factors in mediating long-term changes to gene expression in the brain has been fairly well studied, the role of epigenetic regulation in addictive processes had not been investigated to a significant extent until recently.

7.2.3 Epigenetic Regulatory Mechanisms in Substance Abuse

Of the major epigenetic regulatory mechanisms, histone posttranslational modifications including histone acetylation, histone methylation, and histone phosphorylation have been the best studied in the area of substance abuse. For DNA modifications, DNA methylation has been recently investigated, while the role of the recently discovered DNA hydroxymethylation has not been investigated to date. The roles of ATP-dependent chromatin remodeling and nucleosome position have not been well characterized in substance abuse as yet. Noncoding RNAs can be important regulators of gene expression. While microRNAs have been investigated with respect to substance abuse, other noncoding RNA types including lincRNAs (long intergenic noncoding RNAs) have not been well characterized to date.

7.3 Histone Modifications and Addictive Processes

More than 100 distinct posttranslational histone modifications have been identified and more are likely to be discovered in the future. While some modifications are associated with active chromatin and others are associated with silenced chromatin, the function of the majority of these modifications is currently unknown (Campos and Reinberg 2009). It is also unclear whether histone modifications at particular chromatin regions cause chromatin structural changes or are simply a consequence of these changes (Henikoff and Shilatifard 2011). The enzymes that are responsible for the deposition and removal of posttranslational histone modifications, such as histone acetyltransferases (HATs), histone deacetylases (HDACs), histone methylases, and histone demethylases, are sometimes referred to as the "writers" and "erasers" of the histone code. Additionally, there are proteins that bind to histone posttranslational modifications, which are important for functions relevant to the modification. These molecules are sometimes referred to as "readers" of the histone code. Some of these "readers" contain protein domains such as PHD, Tudor, or WD40 that bind to methylated lysines or arginines, bromodomains that can bind to acetylated lysines, or 14-3-3 domains which can bind to phosphorylated residues (Gardner et al. 2011; Musselman and Kutateladze 2009; Sanchez and Zhou 2011; Kim et al. 2006;

Bonasio et al. 2010). There has been limited work studying histone modification "readers" in the nervous system, although the little information that exists is tantalizing. For example, the JARID1C protein is involved in X-linked mental retardation and in addition to being an H3K4 demethylase (a "writer"); it also appears to bind to H3K9me3 residues (Jensen et al. 2005; Iwase et al. 2007).

One of the most informative assays for detecting histone modifications is chromatin immunoprecipitation (ChIP) in which an antibody specific for a particular histone modification is used to immunoprecipitate cross-linked chromatin. The DNA regions associated with the histone modification can then be analyzed using either microarray analysis (ChIP-chip) or high-throughput sequence analysis (ChIP-seq) (Park 2009). Many genome-wide histone modification datasets for a diversity of cell or tissue types have been generated, enabling one to look at similarities and differences in histone modifications across cell types (Bernstein et al. 2010; Myers et al. 2011; Ernst et al. 2011). These datasets are accessible through a variety of web links including http://www.roadmapepigenomics.org/data and http://www.ncbi.nlm.nih.gov/epigenomics. In addition, a few cell types have been mapped genome-wide for up to 24 different histone modifications to reveal the extent to which histone posttranslational modifications co-occur with one another and with other genomic features as well as to identify chromatin states associated with particular sets of modifications (Heintzman et al. 2009; Hawkins et al. 2010; Ernst and Kellis 2010). Unfortunately, ChIP-quality affinity reagents do not exist for many histone modifications, and so their genomic pattern and function remain mysterious.

Histone acetylation has been the most well-studied histone modification in the nervous system. Histone acetyl marks are covalently attached to histone tails via HATs which include a variety of structurally distinct enzymes including the well-characterized CREB-binding protein (CBP) which has important functions in the nervous system (Dekker and Haisma 2009; Hallam and Bourtchouladze 2006). The HDAC enzymes that can remove these modifications comprise three classes: Class I, Class II, and Class III (sirtuins) (Thiagalingam et al. 2003). Histone acetylation tends to be associated with actively expressed genes, while deacetylated regions tend to be associated with gene silencing (Thiagalingam et al. 2003).

In the nervous system, histone modifications are known to have important functions (Miller 2011; Akbarian and Huang 2009; Tsankova et al. 2007; Bredy et al. 2010; Morris et al. 2010; Haggarty and Tsai 2011). For example, disruption of the HAT enzyme CBP leads to memory defects (Korzus et al. 2004). Mutations in CBP are associated with Rubinstein-Taybi syndrome, which has an intellectual disability phenotype (Petrij et al. 1995). Histone deacetylases have been implicated in depression (HDAC5), regulation of dendritic spine density and memory formation (HDAC2), negative regulation of long-term memory formation (HDAC3), cognition (the Class III HDAC SIRT1), and synaptic transmission (Tsankova et al. 2006, 2007; Guan et al. 2009; McQuown et al. 2011; Gao et al. 2010; Morris et al. 2010).

Chromatin modifications, such as histone acetylation, mediate some of the neuronal and behavioral changes induced by cocaine. As can be seen in Table 7.2,

Table 7.2 Selected epigenetic changes associated with exposure to drugs of abuse

Drug class	Drug of abuse	Epigenetic modification	Enzyme/molecule involved	Tissue/brain region	Reference
Stimulants					
	Cocaine	Histone acetylation (H3,H4)	HDAC/HAT?	Striatum	(Kumar et al. 2005)
	Cocaine	Histone acetylation (H3)	HDAC/HAT?	Unknown	(Malvaez et al. 2010)
	Cocaine	Histone acetylation	HDAC Class I/II	Striatum and ventral midbrain	(Schroeder et al. 2008)
	Cocaine	Histone acetylation	HDAC5	Nucleus accumbens	(Renthal et al. 2007)
	Cocaine	Histone acetylation (H3)	HDAC/HAT?	Medial prefrontal cortex	(Sadri-Vakili et al. 2010)
	Cocaine	Histone acetylation	HDAC/HAT	Nucleus accumbens	(Host et al. 2011)
	Cocaine	Histone acetylation?	HDAC/HAT?	Unknown	(Romieu et al. 2008)
	Cocaine	Histone acetylation?	HDAC/HAT?	Unknown	(Sun et al. 2008b)
	Cocaine	Histone acetylation (H3K5ac)	HDAC/HAT?	Dorsal striatum	(Brami-Cherrier et al. 2005)
	Cocaine	Histone acetylation (H4)	HAT (CBP)	Striatum	(Levine et al. 2005)
	Cocaine	Histone acetylation	Sirt1, Sirt2	Nucleus accumbens	(Renthal et al. 2009b)
	Cocaine	Histone methylation (H3K9me2)	Histone methyltransferase G9a	Nucleus accumbens	(Maze et al. 2010)
	Cocaine	Histone methylation (H3K9me3)	H3K9methyltransferase/ demethylase?	Nucleus accumbens	(Maze et al. 2011)
	Cocaine	Histone methylation (H3K4me3)	H3K4methyltransferase/ demethylase?	Hippocampus	
	Cocaine	Histone phosphorylation (H3S10)	Protein phosphatase-1	Striatum	(Stipanovich et al. 2008)
	Cocaine	Histone phosphorylation (H3S10)	MSK1	Dorsal striatum	(Brami-Cherrier et al. 2005)
	Cocaine	DNA methylation	DNMT3a	Nucleus accumbens	(Laplant et al. 2010)
	Cocaine	DNA methylation	DNMTs	Hippocampus	(Novikova et al. 2008)
	Cocaine	DNA methylation	DNMT3A, DNMT3B, MeCP2	Nucleus accumbens	(Anier et al. 2010)

(continued)

Table 7.2 (continued)

Drug class	Drug of abuse	Epigenetic modification	Enzyme/molecule involved	Tissue/brain region	Reference
	Cocaine	DNA methylation?	DNMT1, DNMT3a	Seminiferous tubules	(He et al. 2006)
	Cocaine	DNA methylation?	MeCP2 and microRNA-212	Dorsal striatum	(Im et al. 2010)
	Cocaine	Noncoding RNAs	microRNA-212	Dorsal striatum	(Hollander et al. 2010)
	Cocaine	Noncoding RNAs	miRNAs and Argonaute2	Striatum	(Schaefer et al. 2010)
	Cocaine	Noncoding RNAs	microRNA-124, miR-181a, let-7d	Nucleus accumbens	(Chandrasekar and Dreyer 2011)
	Cocaine	Noncoding RNAs	microRNA-8 family	Nucleus accumbens and striatum	(Eipper-Mains et al. 2011)
	Amphetamine	Histone acetylation?	HDAC/HAT?	Unknown	(Kalda et al. 2007)
	Amphetamine	Histone methylation (H3K9me2)	KMT1A histone methyltransferase	Striatum	(Renthal et al. 2008)
	Amphetamine	DNA methylation?	MeCP2	Nucleus accumbens	(Deng et al. 2010)
	Methamphetamine	Histone methylation (H3K4me3)	H3K4 methyltransferase?	Nucleus accumbens	(Ikegami et al. 2010)
	Methamphetamine	DNA methylation?	DNMT2	Hippocampus	(Numachi et al. 2004)
	Caffeine	Histone methylation (H3K9me2)	Histone methyltransferase G9a/GLP	Striatum	(Schaefer et al. 2009)
	Nicotine	Histone acetylation (H3K9ac)	HDAC2?	Striatum, prefrontal cortex	(Pastor et al. 2011a)
	Nicotine	DNA methylation	DNMT1	Frontal cortex	(Satta et al. 2008b)
	Nicotine	DNA methylation of MAOA	DNMT?	Lymphoblasts, whole blood	(Philibert et al. 2008); (Philibert et al. 2010)
	Nicotine?	Noncoding RNAs	microRNA-504	Cultured cells	(Huang and Li 2009)
	Smoking	DNA methylation	DNMT?	Blood cells	(Launay et al. 2009)
Inhalants					
	Solvents	Histone acetylation	HDAC/HAT?	Fly heads	(Wang et al. 2007b)

Cannabinoids					
	THC	?	?	?	
Opiates					
	Morphine	Histone acetylation (H3)	HDAC/HAT?	Unknown	(Jing et al. 2011)
	Morphine	Histone acetylation?	HDAC	Unknown	(Wang et al. 2010b)
	Morphine	Histone acetylation?	HDAC/HAT	Striatum?	(Sanchis-Segura et al. 2009)
	Morphine?	Histone acetylation	HDAC/HAT?	Neuronal cell culture	(Hwang et al. 2010)
	Morphine?	Histone methylation	Methyltransferase/ demethylases	Neuronal cell culture	(Hwang et al. 2010)
	Morphine	Unknown	Unknown	Not applicable	(Byrnes 2005)
	Heroin/methadone	DNA methylation	DNMT?	Lymphocytes	(Nielsen et al. 2009)
	Heroin	Noncoding RNAs	Long noncoding RNAs (lincRNAs)	Nucleus accumbens	(Michelhaugh et al. 2011)
Depressants					
	Alcohol	Histone acetylation (H3 and H4)	HDAC/HAT?	Amygdala	(Pandey et al. 2008)
	Alcohol	Histone methylation (H3K4me3)	H3K4methyltransferase/ demethylase?	Hippocampus	(Zhou et al. 2011b)
	Alcohol	DNA methylation	DNMT?	Prefrontal cortex	(Taqi et al. 2011)
	Alcohol	DNA methylation	DNMT?	Cultured neurons	(Marutha Ravindran and Ticku 2004)
	Alcohol	DNA methylation of MAOA	DNMT?	Lymphoblasts	(Philibert et al. 2008)
	Alcohol	DNA methylation	DNMT?	Blood	(Muschler et al. 2010)
	Alcohol	DNA methylation	DNMT?	Blood	(Bonsch et al. 2004)
	Alcohol	Noncoding RNAs	microRNA-9	Cultured neurons	(Pietrzykowski et al. 2008)

This table provides an overview of selected studies in the realm of epigenetic regulation and addictive processes. When known, the epigenetic modification, enzymes, or other associated molecules are indicated. Also indicated are the tissue types or brain regions investigated when known

histone acetylation has been the best studied class of histone modifications for addictive processes, although no doubt much more remains to be learned about its functions. Histone acetylation is particularly interesting from a translational point of view since certain medications that inhibit HDAC activity are clinically approved for treating seizure disorders and particular types of cancer (Sharma et al. 2010). The effects of these inhibitors on nervous system function and drug-taking behaviors are discussed in more detail in the Therapeutics section.

The role of histone acetylation and deacetylation in addictive processes in rodents has been recently reviewed; therefore, I will focus on a few of the key studies related to cocaine responses (Renthal and Nestler 2009b; McQuown and Wood 2010; Laplant and Nestler 2011; Wong et al. 2011). This will be followed by descriptions of some recent work on the role of the Class III HDACs (sirtuins) and then studies on tolerance to benzyl alcohol in a *Drosophila* model of inhalant exposure which helps delineate some of the detailed molecular events that may be occurring. I will then touch on several other histone modifications of particular interest including histone dimethylation, histone trimethylation, and histone phosphorylation.

7.3.1 Histone Acetylation and Cocaine Responses

Acute, but not chronic, cocaine exposure is known to induce expression of the mRNA that encodes the cFOS transcription factor. Using ChIP Dr. Eric Nestler and colleagues found that acute, but not chronic, cocaine exposure led to increased acetylation of histone H4 at the cFOS gene promoter, but had no significant effect on histone H3 acetylation (Kumar et al. 2005). Conversely chronic, but not acute, cocaine exposure induces the BDNF and Cdk5 genes. Chronic cocaine exposure led to increased acetylation of histone H3 but had no significant effect on histone H4 acetylation. These data suggest that a chromatin state (acetylation of histones H4 or H3 near the promoters of particular genes) may in part indicate which genes are modulated in response to acute or chronic cocaine administration. Taking these experiments one step further, the investigators showed that administration of the HDAC inhibitor trichostatin A to rodents prior to cocaine administration enhanced the reward response to cocaine, while overexpression of the HDAC4 gene in the striatum using herpes simplex virus decreased the reward response to cocaine.

In a second paper further exploring the roles of HDACs in drug responses, several HDACs were found to be expressed in the NAc, with HDAC3 and HDAC5 having the highest expression levels (Renthal et al. 2007). Viral overexpression of HDAC5 in the NAc led to a reduction in the rewarding properties of cocaine using a conditioned place preference assay, while HDAC5 knockout animals were found to have the converse phenotype, with increased preference for the cocaine-paired chamber. HDAC5 was required specifically in the NAc to regulate this behavioral response, since viral expression of HDAC5 in the NAc of HDAC5 knockout animals reduced the preference for the cocaine-paired chamber.

Interestingly, the investigators also looked at the role of HDAC5 in chronic stress using a social defeat behavioral assay. While acute stress had no effect on HDAC5 levels in the NAc, chronic stress was associated with reduced levels of the HDAC5 mRNA, and HDAC5 knockouts exhibited hypersensitivity to chronic stress. HDAC5 modulation of behavioral responses to cocaine reward and chronic stress responses is quite significant given the important role of stress in drug abuse relapse.

7.3.2 Histone Acetylation, Sirtuins, and Cocaine Responses

In experiments described in the previous section, Dr. Renthal and coworkers characterized genome-wide levels of histone acetylation from the nucleus accumbens (NAc) brain region of rodents treated chronically with cocaine. These studies revealed that many genes previously known to be upregulated by cocaine exposure also have increased acetylation of histone H3 and H4. The investigators then looked genome-wide to identify the binding sites of the cocaine-induced transcription factors delta-FosB and CREB in the NAc of cocaine-treated animals (Renthal et al. 2009a). Cross comparison of these datasets identified many genes that had not previously been implicated in response to cocaine, including the Sirtuin genes (Sirt1 and Sirt2) which function as NAD-dependent histone deacetylases (Vaquero et al. 2007). While these genes function in many biological processes, including circadian and metabolic regulation and aging, their role in the nervous system is not well understood (Haigis and Sinclair 2010; Herranz and Serrano 2010). The investigators used pharmacological activators and inhibitors of sirtuins to look at their function in cocaine responses. Interestingly, systemic pharmacological activation of sirtuins dramatically enhanced the rewarding effect of cocaine, while inhibition of sirtuins had the opposite effect. Pharmacological modulation of sirtuin function may be a fruitful future avenue to explore in the development of therapeutic agents to treat cocaine addiction.

7.3.3 Histone Modifications and Inhalant Exposure in Drosophila

Dr. Nigel Atkinson and coworkers exploited the genetically powerful fruit fly model system to investigate the molecular basis of inhalant tolerance. It has been observed that adult flies become tolerant to sedation by organic solvents (which sometimes are abused as inhalants) and this reduced sensitivity to inhalant requires increased expression of the *slowpoke* potassium channel gene which in turn alters neuronal function (Wang et al. 2007a).

In order to investigate the molecular mechanism behind this observation, Dr. Atkinson and coworkers found that a single exposure to inhalant led to epigenetic changes in regulatory regions of the *slowpoke* gene leading to altered expression of the *slowpoke* gene and reduced sensitivity (tolerance) to additional inhalant exposure. Specifically, they observed that the pattern of acetylation of histone H4 was altered across the *slowpoke* gene, which likely led to a more open localized chromatin structure and subsequent increased expression of the *slowpoke* gene. Exposure of the animals to a pharmacological inhibitor of histone deacetylases, the class of enzymes responsible for the histone H4 acetylation, also led to similar epigenetic and gene expression change as well as tolerance of the animals to inhalant.

Interestingly, Dr. Atkinson and colleagues found DNA elements within the *slowpoke* promoter that could be bound by the CREB transcription factor. A number of labs have shown that the CREB transcription factor is important in the responses of organisms to illicit substances, as well as in other neuroplastic processes such as learning and memory (Han et al. 2007). Using a genetic manipulation to "turn off" CREB, the researchers found that the epigenetic and expression changes to *slowpoke* gene and the development of behavioral tolerance no longer occurred. These results indicated that the CREB transcription factor is required for these processes.

Dr. Atkinson and coworkers also found that sedation with benzyl alcohol leads to increased expression of positively acting CREB isoforms and reduced expression of negatively acting CREB isoforms (including dCREB2). Specifically the dCREB2 isoform shows increased occupancy at the *slowpoke* promoter immediately after benzyl alcohol sedation in a chromatin immunoprecipitation assay. Animals with a knockout in dCREB2 no longer have increased benzyl alcohol-induced *slowpoke* gene expression and also no longer develop tolerance to this organic solvent.

Overall, this work clearly shows that exposure to an organic solvent can alter future sensitivity to the solvent via epigenetic regulatory mechanisms. It also provides insight into the precise mechanisms by which exposure to an inhalant can lead to epigenetic and expression level changes for a single gene, resulting in altered neuronal function and altered behavioral responses of an animal to future inhalant exposure. Although this work was performed using model inhalant, similar mechanisms may well be utilized for responses to other drugs of abuse.

7.3.4 Histone Dimethylation (H3K9me2) and Cocaine Responses

Histone dimethylation (H3K9me2) is normally associated with gene silencing (Wen et al. 2009; Barski et al. 2007). In an article published in *Science*, Dr. Maze and colleagues observed that histone methylation levels are reduced in the nucleus

accumbens (NAc) of rodents (Maze et al. 2010). To further explore this phenomenon, the researchers investigated the gene expression levels of the histone dimethyltransferases and demethylases that regulate this chromatin modification and found that levels of the G9a and GLP histone dimethyltransferases are downregulated upon cocaine administration. The investigators then looked at the effect of G9a manipulation in the NAc on the behavioral effects of cocaine and found that overexpression of G9a decreased the rewarding properties of cocaine, while knockdown of G9a increased the rewarding properties of cocaine. The researchers showed that these behavioral changes were correlated with concomitant changes in G9a levels and global histone dimethylation. Looking upstream of G9a, repeated cocaine exposure increased the levels of the transcription factor delta-FosB, leading to decreased G9a levels. Looking downstream, many of the genomic targets of histone dimethylation are known to play roles in the regulation of dendritic plasticity, and in fact dendritic spine density was shown to be altered by G9a levels. Overall Dr. Nestler and colleagues have elucidated an elegant multistep molecular pathway in which repeated cocaine exposure leads to delta-FosB activation, downregulation of G9a, and reduction in global histone dimethylation levels. Histone dimethylation is normally associated with gene silencing, so decreased histone dimethylation likely leads to increased expression of genes that regulate dendritic plasticity. This change in gene expression leads to increased dendritic spine density and ultimately increased behavioral preference for cocaine. Thus, small molecules that target the activities of histone demethylases or histone dimethyltransferases could have potential efficacy as therapeutic agents for treating cocaine addiction.

7.3.5 Histone Trimethylation (H3K9me3) and Cocaine Effects on Heterochromatin

Histone H3 Lysine 9 trimethylation (H3K9me3) is associated with silencing of heterochromatic regions of the genome (Schotta et al. 2004; Yamada et al. 2005). Work by Dr. Maze and colleagues found that cocaine exposure results in changes in H3K9me3 levels in the NAc but not in brain regions such as the caudate putamen or medial prefrontal cortex (Maze et al. 2011). When ChIP-seq assays were performed on the NAc from cocaine-treated animals, thousands of repetitive elements (e.g., LINEs, SINEs) were associated with increased H3K9me3 binding, while thousands of other sites had decreased binding. Overall, "repeated cocaine decreases H3K9me3 binding and un-silences several specific retrotransposons (e.g. LINE-1)" in the NAc (Maze et al. 2011).

7.3.6 Histone Trimethylation (H3K4me3) Changes Associated with Cocaine and Alcohol Exposure

Histone trimethylation (H3K4me3) tends to be associated with gene promoters (Guenther et al. 2007; Bernstein et al. 2005). Work by Drs. Goldman and Mash and coworkers have shown that histone trimethylation is important in chronic exposure to cocaine and to alcohol (Zhou et al. 2011a). Genome-wide ChIP-seq for H3K4me3 was performed on postmortem hippocampal samples from chronic cocaine addicts, alcoholics, and controls. The investigators found that chronic cocaine use was associated with H3K4me3 changes at >1,100 gene promoters, while chronic alcohol use was associated with changes at >700 promoters. The authors indicate that "there was significant overlap of the changes between the cocaine and alcohol exposure groups" (Zhou et al. 2011a). Interestingly, these H3K4me3 changes did not correlate well with gene expression changes measured in parallel, suggesting that perhaps additional chromatin or transcriptional regulation is important in mediating gene expression changes in response to cocaine and alcohol exposure.

7.3.7 Histone H3S10 Phosphorylation and Cocaine Responses

Phosphorylation of H3S10 has previously been shown to be important in chromatin condensation and transcriptional activation (Nowak and Corces 2004; Crosio et al. 2003). The DARPP-32 protein (dopamine-regulated and cyclic-AMP-regulated phosphoprotein) has been a well-characterized role in responses to cocaine and other drugs of abuse (Svenningsson et al. 2005). Interestingly, Stipanovich and colleagues have identified a regulatory cascade whereby DARPP-32 leads to altered Histone H3 phosphorylation (Stipanovich et al. 2008). Exposure to drugs of abuse via dopamine 1 receptor (D1R) regulation leads to the accumulation of DARPP-32 in the nuclei of D1R-expressing striatal neurons. This nuclear accumulation appears to be regulated by phosphorylation of Ser-97 of DARPP-32, such that when Ser-97 is unphosphorylated, DARPP-32 is primarily nuclear. The dephosphorylation of Ser-97 is mediated by protein phosphatase 2A. The researchers then looked at the effects on histone phosphorylation and found that cocaine exposure led to increased levels of H3S10 phosphorylation. This pathway reveals a mechanism by which drugs of abuse, via dopamine signaling, can influence chromatin and presumably impact gene expression.

7.4 DNA Modifications and Addictive Processes

Methylation of cytosine nucleotides (mC) has long been thought to be the only covalent mammalian DNA modification and is often referred to as the "fifth base" (Lister and Ecker 2009; Miranda and Jones 2007). DNA methyltransferases (DNMTs) are the enzymes that methylate DNA; this methylation frequently occurs at cytosines that occur as dinucleotides followed by guanine (CpG) (Turek-Plewa and Jagodzinski 2005). DNA methylation appears to be a dynamic rather than a static process, especially in the nervous system (Guo et al. 2011; Ma et al. 2009). The enzymes responsible for active DNA demethylation have been difficult to pin down despite significant efforts, and a number of candidate enzymes and mechanisms have emerged (Wu and Zhang 2010; Ooi and Bestor 2008). There have been recent discoveries addressing DNA methylation reversal mechanisms which involve conversion of mC to an intermediate molecular form which can then be excised by thymine DNA-glycosylase (He et al. 2011; Cortellino et al. 2011). DNA methylation has been traditionally associated with gene silencing, but the context of DNA methylation (methylation in CpG islands, CpG island shores, in promoters, or in gene bodies) appears to be important in mediating the functional effects of mC (Ndlovu et al. 2011). Interestingly, genome-wide single-base resolution DNA methylation maps reveal that human embryonic stem cells and reprogrammed induced pluripotent stem cells contain high levels of DNA methylation in a non-CpG context, although the function of non-CpG methylation is not clear (Lister et al. 2009, 2011). As more whole methylome datasets are generated for different cell/tissue types and this information is compared to histone modification, gene expression, and other data, the precise role of mC and the cross talk between DNA methylation and other regulatory mechanisms will become more clear.

Additional covalent DNA modifications were known to occur in other organisms such as plants and bacteriophage (Vanyushin 2006; Fleischman et al. 1976). However, in 2009 a "sixth base" was discovered in mammalian cells: hydromethylcytosine (hmC) (Kriaucionis and Heintz 2009; Tahiliani et al. 2009). Hydroxymethylcytosine was discovered in Purkinje cells in the cerebellum, and an independent paper showed that the TET1 enzyme can convert mC to hmC. Since then, researchers have been working to discover putative functions for hmC and have identified likely roles for hmC in transcriptional regulation and regulation of pluripotency (Pastor et al. 2011b; Ndlovu et al. 2011; Ficz et al. 2011; Wu and Zhang 2011; Stroud et al. 2011; Wossidlo et al. 2011; Szulwach et al. 2011). A very exciting publication indicates that TET proteins can catalyze the in vitro formation of 5-carboxylcytosine (caC) and 5-formylcytosine (fC) from mC (Ito et al. 2011). These new DNA modifications may be intermediates in a TET-mediated DNA demethylation pathway or perhaps could have unexpected regulatory properties of their own. Only time will tell whether these or other novel DNA modifications will be discovered in the genomes of nervous system cells.

As described earlier, specific proteins can bind to different histone modifications and may play an important role in mediating their effects. Similarly for DNA modifications, proteins in the MBD and Kaiso families have been shown to bind to mC, while a recent report indicates that the Uhrf1 protein can bind to hmC (Bogdanovic and Veenstra 2009; Frauer et al. 2011). Methyl CpG-binding protein-2 (MeCP2), a member of the MBD family discussed below, can bind to mC and presumably plays a role in mediating the effects of DNA methylation, perhaps by recruiting additional proteins or protein complexes to a particular region of chromatin.

There are a number of useful assays to determine DNA methylation status including MethylC-seq, which provides genome-wide DNA methylation information at single-base resolution (Lister et al. 2009; Harris et al. 2010). Unfortunately, bisulfite-based sequencing strategies do not distinguish between mC and hmC, but the development of new assays to detect and distinguish between hmC and mC should help us to elucidate the function of hmC in the nervous system (Pastor et al. 2011b). In fact a recently developed Tet-based bisulfite sequencing protocol (TAB-Seq) in combination with traditional bisulfite sequencing enables base resolution mapping of hmC and confirms widespread distribution of 5hmC in embryonic stem cells (Yu et al. 2012).

There is now a substantial body of work showing that DNA methylation has multiple roles in the nervous system, including a significant role in memory formation (Day and Sweatt 2010; Feng and Fan 2009). Mutations in the DNA methyltransferase DNMT1 lead to neurodegeneration (Klein et al. 2011), while the GABAergic neurons of some human schizophrenics show increased DNMT levels (Klein et al. 2011; Zhubi et al. 2009; Mill et al. 2008). In addition, the methyl CpG-binding protein-2 (MeCP2) is a transcriptional regulator originally identified as a protein that can bind to mC (Lewis et al. 1992). Work by Dr. Huda Zogbhi and colleagues have indicated that defects in MeCP2 function are associated with the neurodevelopmental disorder Rett syndrome (Amir et al. 1999).

As can be seen in Table 7.2, there have been a number of studies investigating the effects of drugs of abuse on DNA methylation. I will discuss the role of DNA methylation with respect to cocaine and nicotine exposure as well as the role of the mC-binding protein MeCP2 in addictive processes.

7.4.1 DNA Methylation and Cocaine Responses

Work by Dr. Laplant and coworkers in the Nestler group shows that both chronic cocaine exposure and chronic social defeat stress can lead to changes in the expression of the DNMT3a DNA methyltransferase gene. DNA methylation was found to be required for the cocaine-induced formation of thin dendritic spines in the NAc. To functionally test the role of DNMT3a in cocaine reward, DNMT3a was conditionally knocked down in the NAc, and the treated animals preferred the

cocaine-paired chamber in a CPP assay. In the converse experiment, DNMT3a was overexpressed in the NAc using a herpes simplex virus vector, and the preference of the animal for the cocaine-paired chamber was reduced. NAc injection of the DNA methylation inhibitor RG108 led to increased cocaine CPP, while administration of methionine, which promotes DNA methylation, led to a decrease in cocaine CPP. The authors conclude from these pharmacological and genetic manipulations of DNMT3a that "increased Dnmt3a expression in NAc negatively regulates cocaine reward, whereas decreased Dnmt3a enhances cocaine reward" (Laplant et al. 2010).

7.4.2 DNA Methylation and Nicotine Responses

DNA methylation appears to also play a role in mediating responses to nicotine. Mice injected with nicotine had decreased levels of DNMT1 in the frontal cortex and the hippocampus, but had normal DNMT1 levels in the GABAergic neurons of the striatum (Satta et al. 2008a). Pharmacological experiments were used to show that nicotinic acetylcholine receptor (nAChR) function was required to achieve the change in DNMT levels in the frontal cortex. Further experiments revealed that nicotine exposure led to upregulation of GAD67 (glutamic acid decarboxylase 67) protein in the frontal cortex, but not in the striatum, and that this upregulation was associated decreased CpG methylation in the GAD67 promoter. Overall this study identifies a potential mechanism of action by which nicotine could mediate neuronal gene expression changes via DNA methylation. This study also suggests that therapeutic agents that modulate DNA methylation changes in the appropriate brain regions could be of potential use in treating nicotine addiction or perhaps schizophrenia.

7.4.3 A Role for MeCP2, a Methyl-C-Binding Protein, in Substance Abuse

MeCP2 is known to play a role in the neurodevelopmental disorder Rett syndrome; however, the neurobiological functions of MeCP2 are not completely understood and are under active investigation. MeCP2 can bind to methylated cytosine residues and presumably can recruit additional proteins or protein complexes to a particular region of chromatin to regulate transcription or other molecular processes. Some studies have suggested that MeCP2 regulates alternative splicing, can bind promoters that are not methylated, or may regulate neuronal genome function through histone acetylation in a more global fashion (Yasui et al. 2007; Young et al. 2005; Skene et al. 2010). Neuronal activity is known to stimulate CaMKII phosphorylation of MeCP2 Serine 421 to modulate "dendritic patterning, spine morphogenesis, and the activity-dependent induction of *Bdnf* transcription"

(Zhou et al. 2006; Chen et al. 2003). Functional work indicates that disruption of MeCP2 specifically in GABA-releasing neurons leads to behavioral phenotypes, including compulsive grooming, reminiscent of phenotypes characteristic of Rett syndrome patients (Chao et al. 2010). MeCP2 is not just required during a specific developmental window but is required in adult animals for proper brain function (McGraw et al. 2011). Recent studies suggest that MeCP2 loss stimulates L1 transposition (Muotri et al. 2010).

Several publications point to a role for MeCP2 in regulation of responses to drugs of abuse. Work by Dr. West and colleagues has shown that MeCP2 knockdown in the NAc leads to increased preference of mice for amphetamine using a CPP assay, while animals no longer had preference for the amphetamine-paired chamber when MeCP2 was overexpressed in the NAc (Deng et al. 2010). Interestingly, in a strain of MeCP2 mutant mice, the authors found an almost twofold increase in GABAergic synapses in the NAc as compared to control animals, indicating that MeCP2 is required for the developmental wiring of this brain structure. Furthermore, MeCP2 mutant mice did not show normal amphetamine-induced changes in dendritic spine density of medium spiny neurons, nor did they show normal amphetamine-induced changes in immediate early gene expression in the striatum, both of which correlate with impaired amphetamine-induced behavioral changes in this strain.

In related work Dr. Kenny and colleagues showed that lentiviral knockdown of MeCP2 in the striatum leads to decreased cocaine consumption (Im et al. 2010). Interestingly, MeCP2 was found to repress expression of the miR-212 microRNA involved in regulation of cocaine-taking (discussed in more detail in the microRNA section of this chapter) (Im et al. 2010; Hollander et al. 2010). Furthermore, miR-212 can repress MeCP2 expression, while cross talk between miR-212 and MeCP2 regulates the impact of cocaine on brain-derived neurotrophic factor (*Bdnf*) levels in the striatum. Interestingly, Dr. Sadri-Vakili and colleagues found that cocaine self-administration was associated with decreased MeCP2 binding to brain-derived neurotrophic factor (*Bdnf*) promoter IV in the medial prefrontal cortex (mPFC) (Sadri-Vakili et al. 2010). The medial prefrontal cortex (mPFC) is one of several brain regions that has dopaminergic inputs from the ventral tegmental area (Le et al. 2005). Based on this study and the two publications above, it appears that MeCP2 has an important role in addictive processes and may have special functions in distinct brain regions.

Work by Teresa Reyes and colleagues has shown that in an animal model of diet-induced obesity, animals on a chronic high-fat diet were found to have epigenetic changes in the mu opioid receptor (MOR) promoter in reward areas of the brain (VTA, NAc, PFC). These epigenetic changes included increased H3K9me2, decreased H3ac, increased DNA methylation, and increased MeCP2 binding to the MOR promoter (Vucetic et al. 2011). Related work revealed that high-fat diet influenced dopamine reuptake transporter (DAT) gene expression in the VTA, NAc, and PFC (Vucetic et al. 2010). DNA methylation changes were also observed in DAT and other reward-related genes in the hypothalamus, NAc, and PFC. These molecular changes associated with exposure to high-fat diet suggest that epigenetic

regulation in response to diet and to drugs of abuse could have significant overlap and raises the possibility of whether or not specific dietary regimens, in combination with other therapeutic interventions, could influence addictive processes.

7.4.4 MeCP2 and Epigenomic Regulation of Genomic Structure

As mentioned earlier, MeCP2 was found to play a role in regulation of transposition of L1 retrotransposons (Muotri et al. 2010). Dr. Muotri and colleagues found that there is increased L1 retrotransposition in neural precursor cells derived from patients with MeCP2 mutations. Additionally in a mouse strain designed to detect L1 transposition events using a fluorescent reporter, there were higher numbers of GFP-positive cells in brain tissue from MeCP2 knockout animals as compared to wild type. The results from this paper suggest a possible scenario in which L1 retrotransposons may be methylated and bound by MeCP2, inhibiting retrotransposition. In the absence of MeCP2, retrotransposition becomes more frequent. This study raises the intriguing possibility that our somatic genomes may be much more diverse than we previously expected and that epigenomic regulation may play an important role in regulating somatic genomic diversity.

Could drugs of abuse impact somatic genomic structure via epigenomic regulation? Work by Dr. Maze and colleagues described earlier showed that cocaine exposure leads to changes in Histone H3 Lysine 9 trimethylation (H3K9me3) in the NAc but not two other brain regions (Maze et al. 2011). H3K9me3 is associated with heterochromatin silencing (Schotta et al. 2004; Yamada et al. 2005). The authors indicate that overall "repeated cocaine decreases H3K9me3 binding and unsilences several specific retrotransposons (e.g. LINE-1)" in the NAc (Maze et al. 2011).

LINE-1 retrotransposition during neurodevelopment and neurogenesis could contribute to genomic diversity within the somatic genomes of neurons (Singer et al. 2010). Taken together, the effects of MeCP2 mutation on LINE-1 retrotransposition and the H3K9me3 work suggest the testable hypothesis that repeated cocaine administration could lead to unsilencing and retrotransposition of LINE-1 elements in the NAc leading, in some cases, to permanent gene disruption or long-lasting alterations in gene expression (Muotri et al. 2010). Obviously animal and postmortem brain studies of cocaine-exposed individuals would be needed to assess whether cocaine exposure induces LINE-1 retrotransposition in the NAc and, if so, what the functional consequences of retrotransposition might be.

7.5 Noncoding RNAs and Addictive Processes

Many large and small noncoding RNAs (ncRNAs) have been identified in recent years, and some of these have significant regulatory functions. In particular, small ncRNAs (approximately 20–30 nucleotides) play key roles in gene transcription and translation. For example, Piwi-interacting RNAs (piRNAs) are involved in transposon silencing, and small interfering RNAs (siRNAs) are involved in regulating mRNA levels and chromatin formation (in plants and yeast) and in antiviral responses (in animals), while microRNAs (miRNAs) can simultaneously regulate the mRNA levels and translational efficacy for tens to hundreds of genes (Kaikkonen et al. 2011; Hannon et al. 2006; Czech and Hannon 2011; Marques and Carthew 2007). In the nervous system, a recent paper shows that piRNAs and the piRNA-associated protein MIWI are found in the hippocampus and may play a role in the morphogenesis of spines (Lee et al. 2011). miRNAs have been shown to play diverse roles in processes including neural development, survival, and degeneration, synaptic plasticity, dendritic spine morphology, and memory formation (Olde Loohuis et al. 2011; Saba and Schratt 2010; Davis et al. 2008; Schratt 2009; Schaefer et al. 2007; Schratt et al. 2006; St. Laurent et al. 2009; pp. 81–88; Lin et al. 2011). In mammalian cells miRNAs have important cytoplasmic functions, although as yet they have not been shown as yet to have epigenetic regulatory function (Khraiwesh et al. 2010). Some miRNAs have a profound effect on addictive behaviors and for this reason have been included in this chapter.

In addition to small RNAs, some of the longer ncRNA species include enhancer RNAs (eRNAs) and large intergenic noncoding RNAs (lincRNAs). eRNAs are associated with enhancer regions of the genome that likely play a role in transcriptional regulation (Wang et al. 2011). Recent work utilizing a specific chromatin signature identified more than 1,000 mammalian lincRNAs, and some lincRNAs have been shown to be involved in regulation of cellular differentiation (Guttman et al. 2009; Guttman et al. 2011). The lincRNA HOTAIR seems to be able to impact chromatin remodeling by binding to multiple enzymes which are able to modify histones, while the lincRNA HOTTIP seems to be able to "transmit information from higher order chromosomal looping into chromatin modifications" in order to regulate gene expression (Tsai et al. 2010; Wang et al. 2011). Some lincRNAs form complexes with proteins such as polycomb repressive complex 2 (PRC2), and the development of assays such as RIP-seq has allowed the identification of RNAs associated with PRC2 (Zhao et al. 2010). Assays enabling the identification of genomic regions associated with lincRNAs will be an important future tool needed to help uncover regulatory cross talk that may occur between lincRNAs and other epigenetic regulatory mechanisms. Some lincRNAs are expressed in brain and may play a role in the specification of glial and neuronal fates (Mercer et al. 2008, 2010). For example, the RCNR2 RNA has been show to play a role in specification of retinal cell fate (Rapicavoli et al. 2010). Future research is likely to uncover additional roles for lincRNAs in the nervous system.

As shown in Table 7.2, investigations into the role of noncoding RNA regulation in addictive processes have been quite limited to date, and only one study has investigated any role for lincRNAs. This section of this chapter will focus on an emerging role for lincRNAs in heroin abuse followed by a more complete story on microRNA regulation in cocaine-taking behavior.

7.5.1 lincRNAs and Heroin Use

Drs. Bannon and Lipovich and colleagues found that 23 lincRNAs were represented on microarrays being used to characterize gene expression changes in postmortem NAc tissue from heroin and non-heroin users (Michelhaugh et al. 2011). These investigators found that five of these lincRNAs (MIAT, MEG3, NEAT1, NEAT2, and EMX2OS) were expressed in the NAc and further that all five were upregulated in the heroin users as compared to non-heroin users. Thus, a potential lincRNA function may be to regulate widespread gene expression, and this regulation may be disrupted in the NAc of drug-abusing individuals. Further exploration of the role of lincRNAs in neuroplastic and addictive processes is an important area of investigation for the future.

7.5.2 miRNAs and Cocaine

The role of miRNAs in addictive processes has recently been reviewed (Li and van der Vaart 2011; Pietrzykowski 2010). As indicated in Table 7.2, roles have been described for microRNA regulation of the dopamine 1 receptor involved in nicotine dependence and for microRNA regulation of response to alcohol exposure (Huang and Li 2009; Pietrzykowski et al. 2008). There have been several projects that have successfully identified miRNAs involved in cocaine responses (Eipper-Mains et al. 2011; Chandrasekar and Dreyer 2011). Another study shows that rodents with an Argonaute 2 (Ago2) protein deficiency in the dopamine receptor 2 expressing neurons have altered intravenous cocaine self-administration in mice (Schaefer et al. 2010). Ago2 is known to play an important role in miRNA biogenesis and function, supporting a role for miRNAs in cocaine reinforcement (O'Carroll et al. 2007).

A major discovery in this area has been made regarding miRNA regulation of cocaine-taking behavior. Dr. Paul Kenny and coworkers have identified a 21-nucleotide microRNA, miR-212, that is found at higher levels in the dorsal striatal brain region of animals that self-administer cocaine (Hollander et al. 2010). In rats with extended access to cocaine, reduction of miR-212 levels in the striatum leads to increased cocaine intake, while overexpression of miR-212 leads to decreased cocaine intake. Further molecular experiments revealed that miR-212 achieves its effects via simultaneous reduction in expression of several mRNAs encoding

regulatory proteins that impinge upon the Raf1 protein kinase signaling pathway (including the SPRED1 repressor of Raf1). Overall these gene expression changes lead to increased levels of Raf1 protein kinase activity, increased expression of the CREB regulatory protein TORC, and ultimately increased activity of the transcription factor CREB. In a separate publication, miR-212 was found to regulate and be regulated by MeCP2 (see MeCP2 section of this chapter for details) (Im et al. 2010).

The identification of novel miRNA regulatory pathways that control cocaine intake, as well as responses to other drugs of abuse, could reveal new and unexpected targets for the development of potential therapeutic agents to treat addiction. Two key areas for future research in the miRNA arena are the identification and characterization of the target mRNAs regulated by these miRNAs as well as studies investigating whether or not these and/or other miRNAs are associated with addictive behaviors in human populations.

7.6 The Perdurance of Epigenomic Changes

While some chromatin changes are transient and occur as a normal part of transcriptional regulation, others are more long lasting and could be particularly important in the case of terminally differentiated post-mitotic neurons (Miller et al. 2010). There is also evidence for mitotically heritable chromatin changes that may impact progeny cells (Ng and Gurdon 2008). In some cases, epigenomic changes may even be meiotically heritable and affect the next generation (Youngson and Whitelaw 2008). The occurrence and perdurance of some types of epigenetic changes is likely to be influenced by factors including the nature of any environmental exposures involved, the cell type involved, and whether or not that cell type is exposed during a particular developmental window. There is increasing evidence that certain environmental exposures during critical developmental periods such as prenatal development, adolescence, or periods of germline maturation are associated with disease consequences. This concept has been captured in the developmental origins of health and disease (DOHaD) hypothesis which "proposes that during critical periods of prenatal and postnatal mammalian development, nutrition and other environmental stimuli influence developmental pathways and thereby induce permanent changes in metabolism and chronic disease susceptibility" (Waterland and Michels 2007). In the following two sections, we will discuss the limited literature concerning the stability of epigenomic changes associated with exposure to drugs of abuse during particular developmental periods.

7.6.1 Somatic Effects of Drugs of Abuse

The stability of epigenomic changes is a particularly difficult problem to investigate in the brain. For animal studies, individuals need to be sacrificed, and thus, only a

single time point can be generated per animal. Surprisingly few studies have been performed looking at how long epigenomic changes can last within the nervous system. Work by Dr. Courtney Miller and colleagues indicates that fear conditioning-associated DNA methylation changes in the *Calcineurin* gene can last for at least 30 days in the dorsal medial PFC (Miller et al. 2010). Researchers in the Nestler laboratory found H3K9me2 changes in the nucleus accumbens that last for at least 28 days using a social defeat behavioral paradigm (Wilkinson et al. 2009). Investigations into the stability and dynamics of somatic epigenomic changes in response to drugs of abuse and potential drug abuse therapeutics will be important for the future.

7.6.2 *Multigenerational and Transgenerational Effects of Drugs of Abuse*

There is evidence that exposure to certain chemical toxins, social environments, or nutrient levels can, in some cases, lead to specific phenotypes in subsequent generations. These phenotypes can be transmitted without an apparent DNA change through multiple generations even when these generations have not been exposed to the inducing factor (Youngson and Whitelaw 2008; Skinner et al. 2010; Champagne 2008). The phenotypic consequences can in part be dependent upon when the exposure occurred. When an individual encounters an environmental exposure such as a drug of abuse, the exposure could potentially impact the individual, any fetuses present, and any germ cells or gametes present. Effects on progeny derived from the exposed parent can be referred to as multigenerational phenotypic effects, while transgenerational effects usually refer to phenotypes observed in progeny that were not exposed in utero or derived from exposed germ cells (Skinner et al. 2010).

For example, there is evidence that caloric restriction can lead to impaired glucose tolerance in subsequent generations (Zambrano et al. 2005). Exposure to certain chemical toxins or social environments can also impact phenotypes across generations (Skinner et al. 2008; Champagne and Meaney 2007). Several groups of researchers have shown that early life stress or adversity can lead to a number of important phenotypic effects including altered transcription factor binding to and histone acetylation of the glucocorticoid receptor (which plays a critical role in stress responses), altered DNA methylation of the BDNF gene in the adult prefrontal cortex, altered serotonin signaling in the dorsal raphe nucleus, depressive-like phenotypes, and changes to energy metabolism and feeding behavior (Weaver et al. 2004; Roth et al. 2009; Franklin et al. 2010; Dietz et al. 2011; Pankevich et al. 2009; Weiss et al. 2011). The mechanism for phenotypic transmission in all of these cases has not been fully elucidated, although the potential involvement of epigenetic processes is an important avenue of investigation.

There are several lines of evidence suggesting that it would be worthwhile to explore whether or not exposure to drugs of abuse could lead to multigenerational

or transgenerational effects. Human epidemiological studies by Dr. Marcus Pembrey and colleagues indicate that preadolescent paternal smoking is associated with greater body mass indices in sons (at 9 years of age), with no significant effects observed in daughters (Pembrey et al. 2006). There is also preliminary evidence that paternal cocaine exposure in mice could lead to phenotypic consequences in the progeny. In this study, mice were exposed to cocaine via inhalation in an animal model of crack cocaine use (He et al. 2006). Cocaine-exposed males were mated to unexposed females and the progeny characterized morphologically and behaviorally. The progeny had reduced head diameters, perhaps reflecting reduced cerebral volume, and also had impaired spatial working memory and attention. Interestingly, the investigators did not observe significant DNA breaks in the sperm of cocaine-exposed fathers, but did observe "reduced levels of expression of Dnmt1, together with increased levels of expression of Dnmt-3a, in the germ cell-rich seminiferous tubular tissue" of cocaine-exposed males which may mean that cocaine exposure could impact progeny phenotypes through DNA methylation changes in the sperm (He et al. 2006). Related work indicates that cocaine exposure of pregnant female mice can lead to altered DNA methylation patterns in the hippocampus of progeny (Novikova et al. 2008).

In 1972, an article published in Science indicated that maternal morphine exposure prior to fertilization was associated with a decrease in body weight in the progeny (Friedler and Cochin 1972; Friedler 1978). Further studies indicated that male adolescent morphine exposure could impact endocrine responses in offspring (Cicero et al. 1991). More recent work by Dr. Elizabeth Byrnes and colleagues indicates that maternal morphine exposure prior to conception can lead to phenotypic effects on the progeny (Byrnes et al. 2011; Byrnes 2005; Johnson et al. 2011). Female rats were exposed to multiple doses of morphine during adolescent development, drug exposure was halted for at least 20 days, and the animals were mated to males not exposed to morphine (Byrnes et al. 2011). The adult female, but not male, progeny from morphine-exposed mothers had decreased anxiety as measured by the open-field assay. However, adult male, but not female, offspring of morphine-exposed mothers had increased sensitivity to morphine. The potential phenotypic consequences of adolescent or young adult morphine exposure on the next generation are particularly significant given the recent sharp increase in prescription opioid use among adolescents (Sung et al. 2005).

Genomic imprinting is an "epigenetically regulated process that causes genes to be expressed in a parental-origin-specific manner rather than from both chromosome homologues" (Ferguson-Smith 2011). In at least one case, the parental origin of a single genetic variant has been associated with disease protection and disease risk, depending upon whether the variant came from the mother or father (Kong et al. 2009). Imprinting could play a very interesting role in nervous system function and is known to be important in certain neurodevelopmental disorders such as Prader-Willi syndrome (Allen et al. 1995; Gregg et al. 2010; Gurrieri and Accadia 2009). There has been some work suggesting a role for imprinting in alcohol dependence, and it has been reported that the imprinting control region H19-IGF2 may play a role in specification of dopaminergic precursor neurons

(Strauch and Baur 2005; Freed et al. 2008). However, overall, little is known about the role of parental imprinting in drug abuse resilience or susceptibility.

It is clear from this section that additional research, including extremely well-controlled studies, need to be carried out in order to prove unequivocally whether or not any drugs of abuse have authentic transgenerational effects. If substance use/abuse were conclusively shown to lead to deleterious transgenerational phenotypic effects, this new knowledge would have significant public health implications and would likely influence the development of future drug prevention programs. If exposure to drugs of abuse were shown to lead to transgenerational phenotypes, then the mechanism for such transmission would need to be elucidated. There have been a number of mechanisms proposed for transmission of transgenerational effects including (1) viral, microbiome, or prion transmission, (2) neurobehavioral or societal transmission, and (3) altered epigenomic states of germ cells or gametes via altered parental imprinting, or other epigenetic effects (Youngson and Whitelaw 2008).

7.7 Challenges and Opportunities in Epigenomics and Addiction Research

As research proceeds in the area of transcriptional and epigenomic regulation in human disease, there are a number of scientific challenges and opportunities that present themselves. These include investigations into less well-studied chromatin features, renewable affinity reagents, addressing cell-type heterogeneity in the nervous system, epigenomic maps of brain tissues or cell types, epigenome-wide association studies (EWAS), and manipulation of epigenetic changes to understand function and mechanism. These challenges and opportunities are discussed in the following section with an emphasis on their impact on neuroscience and addiction research.

7.7.1 Underexplored Areas: From Novel Modifications to Higher Order Chromatin Structure

There are a number of research areas that have the potential to be quite exciting but have received very limited attention with respect to neuroplasticity and drugs of abuse. Several of these are discussed below including new DNA, histone, and RNA modifications; histone variants; RNA editing; ATP-dependent chromatin remodeling; and higher order chromatin structure.

Histone posttranslational modifications and DNA modifications have both been shown to be important in regulation of gene function, and it is likely that our catalog of these modifications is not complete. Histone variants are known to be important

in a number of biological processes including regulation of transcription; however, little is known about whether they have any special roles in post-mitotic neurons (Talbert and Henikoff 2010). RNAs have a surprising number of posttranscriptional modifications (e.g., 6-methyladenine, 1-methyladenine), but the functional roles of these modifications have not been carefully explored in the nervous system (Cantara et al. 2011; He 2010).

Some mRNAs are modified through the process of A-I RNA editing in which an adenosine in the RNA is converted to inosine by adenosine deaminases (ADARs). The inosine can be translated as a guanosine by the ribosome which can result in the presence of an amino acid in the protein product that was not encoded by the original DNA sequence (Mattick and Mehler 2008). RNA editing may serve to increase the diversity of proteins that can be produced, but it could also enable neurons to modify their properties in response to particular environmental changes. The extent to which RNA editing occurs in noncoding RNAs is poorly characterized but could impact their regulatory functions. mRNAs that have been shown to be edited include the serotonin biosynthetic enzyme TPH2 and the serotonin 2C receptor mRNA (Grohmann et al. 2010; Iwamoto et al. 2009; Dracheva et al. 2008). Little work has been done looking at the role of RNA editing in addictive processes, although Dr. Stella Dracheva and colleagues have found higher serotonin 2C editing in the prefrontal cortex associated with rats that exhibit high locomotor response to novelty (Dracheva et al. 2009).

The ATP-dependent chromatin remodeling proteins, such as members of the SNF2, ISWI, or CHD families, are able to "disrupt nucleosome DNA contacts, move nucleosomes along DNA, and remove or exchange nucleosomes" (Hargreaves and Crabtree 2011; Gkikopoulos et al. 2011). These nucleosome changes regulate access to genomic DNA which can have consequences in terms of gene expression. Some of these chromatin remodeling proteins have been shown to function in neural development and differentiation (Yoo and Crabtree 2009; Brown et al. 2007; Pirotte et al. 2010). When neurons exit from mitosis, there is a switch in the subunit composition of the BAF chromatin remodeling complexes that appears to be important in regulating dendritic morphogenesis, and this switch is regulated by the microRNAs miR-9* and miR-124 (Yoo et al. 2009). For neuroplasticity and substance abuse, investigations into chromatin remodeling has been limited; however, it has been reported that increased levels of the ATP-dependent chromatin remodeling protein Brg1 are found at the Cdk5 promoter in response to chronic cocaine exposure (Kumar et al. 2005).

Higher order chromatin structure within the nucleus may play an extremely important role in regulation of gene expression and in mediating other cellular functions (Eskiw et al. 2010; Li and Reinberg 2011). Technologies have recently been developed that enable the characterization of higher order chromatin (e.g., Hi-C, ChIA-PET) (Lieberman-Aiden et al. 2009; Fullwood et al. 2009; Espinoza and Ren 2011). Studies investigating the role of higher order chromatin structure in the nervous system, or in response to neuroplastic changes or drugs of abuse, are a very interesting area for future investigation.

7.7.2 *Renewable Affinity Reagents for Epigenomic Research*

Chromatin immunoprecipitation (ChIP) assays can provide extremely valuable information concerning the chromatin landscape of particular cells and tissues. These assays have only become possible due to the development of very low-cost and very high-throughput sequencing (Zhang and Pugh 2011; Park 2009). The continued reduction in the cost of sequencing DNA will improve the ability of researchers to apply this technique to important biological processes and disease studies. However, one of the key needs for successful ChIP assays is a high-quality antibody or other affinity reagent that binds specifically to the target of interest enabling chromatin enrichment of DNA regions in the vicinity of that particular epitope. Unfortunately some commercially available antibodies do not have appropriate specificity or are not useful for ChIP assays (Egelhofer et al. 2011). Efforts have been made to begin validating commercially available antibodies through Western blot, dot blot, or ChIP-seq analyses (http://compbio.med.harvard.edu/antibodies/). Even if an antibody is found to be useful for ChIP assays, the supplies are finite unless the antibody is monoclonal. Thus, there is a great need to develop a renewable resource of ChIP-grade antibodies (such as monoclonal antibodies) or affinity reagents (using recombinant affinity technologies) so that the scientific community has an unlimited supply of these reagents. A ChIP affinity reagent resource would allow researchers to compare ChIP experiments performed in different labs using identical antibodies, which is not always possible when the ChIP assays are performed with polyclonal antibodies. The development of a panel of one or more renewable ChIP-grade affinity reagents for each posttranslational histone modification, DNA modification, and ultimately each transcriptional regulatory protein would be an extremely valuable resource for the scientific community as a whole.

7.7.3 *Addressing Cell-Type Heterogeneity in the Nervous System*

One of the major barriers impeding epigenetic studies in the nervous system, as well as other organ systems, is cellular heterogeneity. The mixture of neurons, glia, microglia, and cardiovascular tissue in different brain regions may mask or confound epigenomic changes that may be taking place. One strategy to address this problem includes the sorting of labeled nuclei from specific brain regions or other clinically relevant tissue to enrich for cell types of interest while preserving the relevant epigenomic information (Cheung et al. 2010). Genetically tractable systems have been used to label ribosomes from specific cell types for purification and molecular identification of cell-specific mRNAs (Heiman et al. 2008; Doyle et al. 2008). In recent years, our ability to epigenomically characterize smaller and smaller numbers of cells has improved significantly, and in the future it might even

be possible to assay the epigenomes of single cells (Gu et al. 2010; Goren et al. 2010; Geng et al. 2011; Cipriany et al. 2010).

7.7.4 Epigenomic Maps of Brain Cell/Tissue Types

Scientific consortia such as the NIH Roadmap Epigenomics program are generating comprehensive maps of chromatin from a wide variety of "normal" human cell and tissue types (Bernstein et al. 2010). These maps typically include DNA methylation information, ChIP assays for several highly informative histone modifications (H3K4me1, H3K4me3, H3K9me3, H3K27me3, H3K36me3, H3K9ac, or H3K27ac), chromatin accessibility information using the DNAse1 hypersensitivity assay, and gene expression information. At the moment there are 65 epigenomic maps of cells that have all of these data types, while partial datasets are available for around 180 additional cell or tissue types. For brain researchers there are currently partial datasets for fetal brain from six time points between 85 and 142 days, as well as postmortem adult brain from anterior caudate, cingulate gyrus, hippocampus, inferior temporal lobe, midfrontal lobe, and substantia nigra (http://www.roadmapepigenomics.org/). Other epigenomic datasets for human and model organisms cells/tissues can be found at the NCBI Epigenomics gateway (http://www.ncbi.nlm.nih.gov/epigenomics) or produced by the ENCODE consortium (http://www.genome.gov/10005107).

One goal for the future would be to develop a comprehensive atlas of chromatin maps for a wider variety of brain regions and brain-specific cell types for both human and mouse. It will be important to link these epigenomic features with other molecular phenotypes such as mRNA and ncRNA expression, transcription factor binding sites, and higher order chromatin structure information. It will also be important to link molecular phenotypes to other cellular phenotypes such as morphology, connections with other neuronal or support cells, and the electrophysiological properties of the cells. These maps would be an important aid to researchers studying neuropsychiatric, neurodevelopmental, and cognitive disorders and may also yield neuronal cell type-specific targets for developing small molecular probes and therapeutic compounds.

For drug abuse researchers, the systematic generation of an "addiction epigenomics" data resource cataloging molecular phenotypes for drug abuse relevant brain regions with and without exposure to different drugs of abuse would provide an invaluable resource. Researchers would be able to mine this data to identify novel candidate loci to test for their involvement in addictive processes. They would also be able to compare profiles of molecular phenotypes responses for different drugs of abuse to begin to identify loci and networks common to addictive processes in general as well as those that might be unique for a particular drug of abuse.

Genome-wide datasets for DNA methylation, histone modifications, chromatin accessibility, ncRNAs, and transcription factor binding sites can be harnessed to

interpret data from genome-wide association studies (GWAS) for various diseases. Of particular interest, recent investigations reveal that disease SNPs identified by GWAS frequently fall in regions of accessible chromatin or in enhancer elements of cell types relevant to the disease (Maurano et al. 2012; Pennisi 2011; Ernst et al. 2011). Overall these studies "can facilitate the interpretation of GWAS data sets by predicting specific cell types and regulators related to specific diseases and phenotypes" (Ernst et al. 2011). Application of this strategy to polymorphisms associated with addictive behaviors could help to shed light on the function of some of these SNPs, particularly those in noncoding genomic regions, help flesh out the gene networks involved, as well as to confirm or generate new hypotheses concerning the brain regions and cell types involved in addiction to particular drugs of abuse.

7.7.5 *Epigenome-Wide Association Studies (EWAS) for Drug Abuse Research*

Although GWAS have been valuable in identifying unexpected genes and loci involved in particular human diseases, some diseases have a significant environmental component. If certain epigenomic states are indeed influenced by environmental exposures, then EWAS, which look at the epigenomic states of disease-relevant tissues in a case/control design, could be of great value in identifying loci involved in particular environmental exposures (Rakyan et al. 2011). Identification of genes and loci using EWAS approach could point the way to new therapeutic targets to treat disease. As scientists begin to perform EWAS using readily accessible human tissues, it will be interesting to see how the genes and loci identified compare and contrast with those identified in GWAS. At this time, EWAS for an addiction phenotype would be difficult to implement since this would necessitate the ability to monitor brain epigenomic regulation either through in vivo imaging techniques or through the use of an accessible surrogate tissue type that accurately reflects epigenomic changes that occur within the relevant brain regions.

7.7.6 *Pharmacological and Molecular Manipulation of Epigenetic Changes to Understand Function*

As correlative hypotheses are generated, it becomes essential to determine the functional role of a particular epigenetic or ncRNA regulatory pathway on a phenotype. Small-molecule probes that activate or inhibit specific epigenetic regulators provide an invaluable resource for testing the function of specific regulation for substance abuse phenotypes. As interest in epigenomic and ncRNA regulation unfolds, more small-molecule modulators are being made available to

the scientific community. The NIH Molecular Libraries Program has several projects to identify epigenetic modulators underway (http://mli.nih.gov/mli/) as does the Structural Genomics Consortium (http://www.thesgc.org/chemical_probes/epigenetics/) (Austin et al. 2004). These small-molecule modulators could be used as probes to confirm whether or not a given pathway is functionally important and should be further investigated. These small molecules could also serve as the foundation for developing therapeutic agents. As small-molecule reagents become more readily available, it will become easier to determine which epigenetic regulatory pathways have the most impact on drug abuse phenotypes and might be useful to target therapeutically.

Molecular genetic approaches can also be useful for investigating the function of epigenomic and noncoding RNA regulation. Unfortunately most small molecules and molecular genetic manipulations impact epigenetic function on a global level. Efforts have been made to manipulate chromatin states in a locus-specific manner, typically by using fusion proteins to target epigenetic modifying enzymes to particular DNA loci (Hansen et al. 2008). These techniques will need further development to enable robust locus-specific manipulation of chromatin states in the future.

7.8 Translating Epigenomic Discoveries into Improvements in Human Health

Although a deeper understanding of the biological mechanisms of drug abuse is of great significance, this understanding has the potential to be translated into improvements in human health. Most substance abuse studies to date have investigated epigenomic regulation in the brain regions of rodents since the level of drug exposure can be readily quantitated and tissues from the exposed brains are accessible to the investigator. However, it will be important to pursue epigenomic studies on postmortem brain samples from substance users and abusers to begin to determine the extent to which the elegant discoveries in rodents are recapitulated in humans. In addition to these types of studies, some of the fundamental discoveries that have been made in addiction epigenomics could have future impact on addiction diagnosis, prevention, and therapy.

7.8.1 Future Substance Use Disorder Diagnostics?

Epigenetic changes have been identified that could serve as potentially useful cancer biomarkers or diagnostics. For example, promoter methylation of a panel of genes may be useful for early detection of colorectal cancer, a DNA methylation phenotype has been used to identify a glioma subgroup, DNA methylation of the

promoter of the MGMT DNA repair enzyme can be used as a biomarker to predict glioblastoma chemotherapy outcome, and promoter methylation of the IGFBP-3 growth factor binding protein may predict cisplatin chemotherapy outcome in non-small lung cancer (Lind et al. 2011; Noushmehr et al. 2010; Weller et al. 2010; Ibanez et al. 2010). In the realm of brain disorders, DNA methylation information can be useful for predicting the efficacy of treatment of Fragile X using an mGluR inhibitor, suggesting the potential for epigenomically informed personalized medicine (Jacquemont et al. 2011).

A significant barrier to developing diagnostic tools for substance abuse based on epigenomic changes is our current inability to assess the epigenomic state of tissue types within the human brain. Unlike genomic studies which can readily be carried out using blood samples, different cell types within the brain express different suites of genes and are thus expected to have epigenomes that differ from one another (Doyle et al. 2008). Thus, to study epigenomic dysregulation in disease, one would ideally investigate the cell or tissue type of most relevant to the disease. In the case of substance abuse, epigenomic studies would thus focus on postmortem human or animal brain tissue. There has been speculation that epigenomic changes in human samples such as specific blood cell types, olfactory neurons, or other more accessible tissues could serve as a surrogate for epigenomic changes in particular brain regions, but to date there has been little compelling evidence that surrogate tissues are of significant utility in studying epigenomic processes in psychiatric diseases.

The ability to image epigenomic processes or changes within the nervous system in a noninvasive manner would be a major technological advance that could help bring epigenomic studies of substance abuse and other psychiatric diseases into living humans. One could imagine using this technology in future clinical settings for diagnosis, monitoring of disease progression, or monitoring of therapeutic efficacy. To date, only limited efforts have been made to image epigenomic processes in vivo. As a good first step, Dr. Joanna Fowler and colleagues have generated reagents to visualize the levels of histone deacetylases in vivo using positron emission tomography (PET) (Hooker et al. 2010; Reid et al. 2009). These and related strategies could eventually be used to image gross changes in the levels and/or activity of epigenetic modifying enzymes relevant to substance abuse and other diseases in vivo.

Measurement of epigenomic regulatory changes in brain using in vivo imaging techniques, or perhaps through assay of more accessible tissues that serve as a surrogate for the brain, might one day be used to help predict susceptibility to substance use disorder, to diagnose disease progression, or perhaps to provide biomarkers that accurately reflect levels and duration of chronic drug use. Future development of substance use disorder diagnostics will require us to more fully understand what epigenomic changes truly mean with respect to addictive processes as well as how long these changes persist.

7.8.2 Preventing Substance Use Disorder?

As mentioned earlier, there is evidence that exposure to certain chemical toxins, social environments, or nutrient levels can occasionally lead to organismal phenotypes in subsequent generations. Whether or not this phenomenon is also true for any drugs of abuse remains unclear. However, if particular drugs of abuse were shown to have phenotypic effects on subsequent generations, then this scientific information could be used to strengthen public health messages documenting the known adverse health consequences of drug abuse for public dissemination to facilitate scientifically informed choices on the use of licit and illicit drugs.

7.8.3 New Therapeutics for Substance Use Disorder?

Epigenetic changes are fundamentally more plastic than genetic changes and thus appear to be more amenable to therapeutic intervention (Haberland et al. 2009). Epigenetic therapeutics have shown great potential in cancer and other diseases. For example, DNA methyltransferase inhibitors have been approved by the FDA to treat myelodysplastic syndromes and may be useful for treating certain leukemias (Sharma et al. 2010). There are also HDAC inhibitors such as SAHA that have been approved to treat T-cell lymphoma (Sharma et al. 2010). Other HDAC inhibitors have been previously approved to treat urea cycle disorders, while the HDAC inhibitor valproic acid has been used to treat seizures, migraines, and bipolar disorder (Mack 2006; Bialer and Yagen 2007). HDAC inhibitors have also shown very promising effects in certain animal models of neurodegeneration, depression, and cognitive disorders (Fischer et al. 2010).

There is interest in testing FDA approved compounds for efficacy in treatment of a wider variety of diseases. As one example, clinicians have been investigating whether HDAC inhibitors can be used to activate latent HIV within the genome making the cells susceptible to antiretroviral therapy (Margolis 2011). If successful for all the tissue reservoirs that contain the latent virus, this strategy could point the way to a possible cure for HIV/AIDS. In another very exciting example, the HDAC inhibitor SAHA was used to successfully treat a patient with seizure disorder likely due to a genetic mutation, suggesting that in some cases epigenetic therapies may have the potential to treat genetic diseases (Almeida et al. 2007).

Scientists are also developing new compounds that impact epigenetic targets other than HDACs and DNMTs, such as histone methyltransferases and histone demethylases (Grant 2009; Hamada et al. 2010; Fiskus et al. 2009). There have even been efforts to target proteins that bind to histone modifications. For example, a molecule that can inhibit the BRD4 protein, which can bind to acetylated lysines, has potential for treating acute myeloid leukemia (AML) (Zuber et al. 2011).

The effects of small-molecule modulators of HDACs and other epigenetic regulatory enzymes suggest an important role for histone posttranslational

regulation in the nervous system (Haggarty and Tsai 2011). For example, Class I HDAC inhibitors have been shown to ameliorate cognitive defects in an Alzheimer's rodent model system, while environmental enrichment and HDAC inhibitors have been shown to enable "the recovery of impaired learning and lost long-term memories after animals had developed severe neurodegeneration and synaptic loss" (Kilgore et al. 2010; Fischer et al. 2007).

Histone acetylation is particularly interesting from a translational point of view since, as described above, certain medications based on inhibition of HDAC activity are clinically approved for treating seizure disorders and particular types of cancer (Sharma et al. 2010). Administration of nonspecific inhibitors of HDACs has yielded mixed results with respect to responses to drugs of abuse (Table 7.2). In some cases these inhibitors led to an increase in the rewarding properties of cocaine or an increase in cocaine intake (Kumar et al. 2005; Renthal et al. 2007; Sun et al. 2008a; Wang et al. 2010a). In other cases, these compounds have led to decreased cocaine intake (Romieu et al. 2008). The precise timing of HDAC inhibitor administration may play a crucial role in determining the effects of the compound. For example, in work by Dr. Marcelo Wood and colleagues, the HDAC inhibitor sodium butyrate was found to "facilitate extinction and prevent reinstatement of drug-induced behavioral changes" (Malvaez et al. 2010). In aggregate, these studies suggest that epigenetic therapies should be further explored as a potential treatment for addictive disorders.

As described earlier, despite ongoing efforts to develop safe and effective medications for the treatment of substance use disorders (SUDs), only limited success has been achieved, and no approved medications exist for the treatment of cocaine, methamphetamine, or cannabis addiction even though efforts are ongoing. It is possible that future epigenetic therapies could serve to complement current gaps in the treatment of individuals who are addicted to these drugs of abuse. In this chapter, several possible new avenues of inquiry for possible therapeutic intervention are indicated, including the development of isoform-specific HDAC inhibitors, sirtuin modulators, H3K9me2 demethylase inhibitors, DNA methylation inhibitors, and MeCP2 modulators. In addition, targeting of miRNAs or components of the pathways they regulate (Raf1 protein kinase, SPRED1, TORC, and CREB) could be of therapeutic value.

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Chapter 8
Epigenetic Therapies in Neurological Diseases

Hsien-Sung Huang, Benjamin D. Philpot, and Yong-hui Jiang

Abstract The potential to reverse epigenetic abnormalities makes them suitable targets for therapeutic intervention. In addition to directly targeting an epigenetic defect, therapeutic strategies can also be used to indirectly ameliorate disease pathophysiology by modifying the broader epigenome. Towards these goals, a host of epigenetic modifiers, such as DNA methylation and histone deacetylation inhibitors, have been developed and tested in human clinical trials. Several of these compounds have been approved by the US Food and Drug Administration for clinical use. In addition to treating peripheral disorders, there is a growing appreciation of the potential for epigenetic therapies to treat central nervous system disorders, including neurological and psychiatric disorders. In this chapter, we will review the current understanding of epigenetic disorders in the central nervous system and the progress and challenges in developing therapies for these disorders.

Keywords DNA methyltransferase inhibitor • Epigenetic mechanisms • Epigenetic therapy • Histone deacetylase inhibitor • Neuroepigenetic disorders • Topoisomerase inhibitor

Abbreviations

APP	Amyloid beta (Aβ) precursor protein
ATRX	Alpha thalassemia/mental retardation syndrome X-linked

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BDNF	Brain-derived neurotrophic factor
CREBBP	CREB-binding protein
EHMT1	Euchromatic histone-lysine N-methyltransferase 1
GAD1	Glutamic acid decarboxylase 1
H3	Histone 3
H3K4me3	Trimethylated forms of histone H3 at lysine 4
H4	Histone 4
HDACi	Histone deacetylase inhibitor
ICF syndrome	Immunodeficiency, centromeric region instability, and facial anomalies syndrome
JARID1C	Jumonji/ARID domain-containing protein 1C
MECP2	Methyl-CpG-binding protein 2
MLL1	Mixed-lineage leukemia 1
NSD1	Nuclear receptor binding SET domain protein 1
RELN	Reelin
RSK2	Ribosomal protein S6 kinase 90 kDa, polypeptide 3
SETDB1	SET domain, bifurcated 1
SMCX	Selected mouse cDNA on the X
SNORD116	Small nucleolar RNA C/D box 116 cluster
UBE3A	Ubiquitin protein ligase 3A
UPD	Uni-parental dissomy
ASD	Autism Spectrum Disorder
AD	Alzheimer's disease
TSA	Trichostatin A

8.1 Introduction

For more than a decade, studies of epigenetic therapies have focused on cancers and related conditions (Baylin and Jones 2011; Egger et al. 2004). Consistent with this focus, almost all clinical trials and FDA-approved epigenetic therapies to date have targeted cancers (Kaminskas et al. 2005; Sharma et al. 2010). There is an increasing understanding that a host of neurological disorders could also be ameliorated by epigenetic interventions, either because these disorders have an epigenetic basis that could be treated directly or because these disorders might be overcome indirectly through modifying the epigenome. This appreciation has led to recent explorations of epigenetic therapies in brain disorders (Bird et al. 2011; Huang et al. 2012; Peters et al. 2010; Roth and Sweatt 2009). In this chapter, we will introduce the roles of epigenetic factors during brain development and function (Sect. 8.2), review how epigenetic factors contribute to neurological and psychiatric disorders (Sect. 8.3), and discuss how novel therapies, targeting epigenetic mechanisms, could be developed for the treatment of neurological and psychiatric disorders (Sect. 8.4).

8.2 The Roles of Epigenetic Factors on Brain Development and Function

Our brains undergo remodeling throughout life, and epigenetic mechanisms contribute to much of this plasticity. These plastic processes include, but are not limited to, cell differentiation, adult neurogenesis, and experience-driven gene regulation. Thus, proper brain development and function requires coordinated transcriptional regulation in distinct cell lineages at different developmental epochs. Epigenetic modifiers such as DNA methylation/demethylation, histone modifications, and microRNAs play an important role in regulating gene expression (Dulac 2010; Fagiolini et al. 2009; West and Greenberg 2011). Our knowledge of how the epigenetic landscape contributes to brain functions is in its infancy, and new advances in this open frontier are being made at a rapid pace. For example, new research has demonstrated that epigenomic localization of the DNA base 5-hydroxymethylcytosine is dynamically regulated across postnatal brain development in a manner that is influenced by the dosage of genes such as the disease-associated *MECP2* gene (Szulwach et al. 2011; Tahiliani et al. 2009). However, how 5-hydroxymethylcytosine-mediated epigenetic modifications contribute to neuronal gene regulation and brain function remains to be investigated. Although it is clear that there are many unanswered questions, we are beginning to better appreciate how the epigenetic landscape contributes to normal brain functions. Below we will discuss how epigenetic machinery and modifications play a role in synaptic plasticity, memory, and cognition.

8.2.1 The Role of Epigenetic Machinery in Synaptic Plasticity, Memory, and Cognition

The importance of transcription in forming long-lasting synaptic modifications and memory has been appreciated for more than 40 years, since the initial experiment showing that transcription is needed for long-term memory in goldfish (Agranoff et al. 1967). However, the epigenetic contribution to synaptic plasticity and memory is just beginning to be explored (Dulac 2010; Roth and Sweatt 2009). The importance of epigenetic contributions to plasticity and cognitive function is underscored by evidence that mutations in epigenetic machinery such as histone acetyltransferases and DNA methyltransferases cause cognitive impairments. For example, monogenetic disorders such as Rubinstein-Taybi syndrome and immunodeficiency-centromeric instability-facial anomalies syndrome (ICF) are caused by dysfunction in a histone acetyltransferase (CREB-binding protein; CREBBP) and a DNA methyltransferase (DNMT3B), respectively (Ehrlich 2003; Roelfsema and Peters 2007). Mouse models of these disorders, with genetically modified mutations of the affected epigenetic machinery (e.g., deletion/mutation of CREBBP or DNMT3B), exhibit impaired synaptic plasticity and memory

formation (Alarcon et al. 2004; Ueda et al. 2006). Other epigenetic-associated proteins, such as the histone deacetylases HDAC2 and HDAC3, are critical negative regulators of long-term memory formation and synaptic plasticity in animal models (Guan et al. 2009; McQuown et al. 2011). HDAC2 serves as an epigenetic blocker of cognitive functions in the aging brain (Gräff et al. 2012). These human genetic disorders and accompanying mouse models demonstrate that intact epigenetic machinery is necessary for synaptic plasticity, memory, and cognition.

8.2.2 The Role of Epigenetic Modifications in Synaptic Plasticity, Memory, and Cognition

Consistent with the genetic evidence described above, rodent studies also indicate a role for epigenetic modifications such as histone and DNA modifications in synaptic plasticity, learning, and memory. For example, the consolidation and reconsolidation of fear memory and synaptic plasticity in the lateral amygdala require epigenetic modification such as histone H3 acetylation (Maddox and Schafe 2011; Monsey et al. 2011). Not surprisingly, pharmacological manipulations that disrupt normal epigenetic modifications impair synaptic plasticity and learning. This is exemplified by the fact that inactivation of DNA methyltransferases impairs synaptic transmission, reduces the late phase of long-term potentiation, and impairs hippocampal-dependent memory formation (Feng et al. 2010; Nelson et al. 2008). Moreover, inactivation of histone acetyltransferase (HAT) genes and HDAC inhibitors both impair synaptic plasticity and memory formation (Guan et al. 2009; Haggarty and Tsai 2011). Epigenetic modifications may also contribute to age-related cognitive decline as altered histone acetylation is associated with age-dependent memory impairment in mice (Peleg et al. 2010; Sweatt 2010). This idea is further supported by findings showing that histone acetylation changes with age and that this change is associated with reduced BDNF expression and synaptic plasticity in the aging brain (Zeng et al. 2011). It has also been shown that the DNA methylation status and expression of *Arc*, an important immediately early gene that contributes to synaptic strength and plasticity (Korb and Finkbeiner 2011), is altered in the aged hippocampus (Penner et al. 2011). These alterations in *Arc* could contribute to impaired memory storage and retrieval during aging (Penner et al. 2011).

The above observations support a role for epigenetic modifications in age-related memory loss and in the pathogenesis of age-related neurological and psychiatric disorders. These observations also suggest that epigenetic modifiers might present new therapeutic opportunities for treating these disorders. While it was previously believed that some epigenetic modifications, such as DNA methylation, are immutable, increasing data indicate that DNA methylation states can be dynamic and reversible in postnatal brains (Kangaspeska et al. 2008; Kim et al. 2009; Miller and Sweatt 2007). Although the exact molecular mechanism of how

disrupting the epigenetic machinery alters synaptic plasticity and memory is not fully known, the cumulative data support a critical role for epigenetic modifications in cognition and provide a rationale to explore epigenetic therapies to treat memory disorders (Guan et al. 2009; Haggarty and Tsai 2011).

8.3 Neuroepigenetic Disorders

Accumulating evidence supports a causative role of epigenetic dysregulation in human diseases (Jiang et al. 2004a; Santos-Reboucas and Pimentel 2007), including complex neuropsychiatric disorders like autism and schizophrenia (Bell and Beck 2010; Hatchwell and Greally 2007; Petronis 2010; Ptak and Petronis 2008; van Vliet et al. 2007). We provide a list of epigenetic defects associated with CNS-related disorders in Table 8.1 and discuss some examples in more detail below.

8.3.1 Definition of Neuroepigenetic Disorders

Epigenetic mechanisms are believed to exert long-lasting influences on brain function by controlling gene expression without altering the underlying genetic code (Dulac 2010; Fagiolini et al. 2009; Graff et al. 2011; West and Greenberg 2011). Dysregulation of these epigenetic mechanisms are associated with a myriad of neurological diseases related to neurodevelopmental, neuropsychiatric, and neurodegenerative processes in the brain (Jiang et al. 2004a; Petronis 2010). We define "neuroepigenetic disorders" as those caused directly or secondarily by alterations of the epigenetic machinery or code. Because of the potential reversibility of epigenetic codes, researchers over the last decade have begun to search for therapeutic agents targeting neuroepigenetic disorders. The results of these studies will help elucidate the molecular mechanisms regulating the epigenome and offer a promise for treating epigenetic disorders.

8.3.2 Neuroepigenetic Disorders with Mutations in Genes Encoding Epigenetic Machinery

Mutations in genes encoding the epigenetic machinery have been well documented in many genetic diseases with Mendelian inheritance (Jiang et al. 2004a). Mutations in DNA methyltransferase 3B (*DNMT3B*) cause ICF syndrome, while mutations in methyl-CpG-binding protein (*MeCP2*) and the *CREBBP* gene that encodes a histone acetyltransferase (HAT) cause Rett syndrome (Amir et al. 1999; Ehrlich 2003) and Rubinstein-Taybi syndrome, respectively (Petrij et al. 1995).

Table 8.1 List of candidate neuroepigenetic disorders

Epigenetic code	Diseases	Molecular target	References
Histone modifications	AD	Increased histone phosphorylation	(Myung et al. 2008)
	ASD	Excess spreading of H3K4me3	(Shulha et al. 2012)
		Mutation in *JARID1C*/*SMCX*	(Adegbola et al. 2008)
	Huntington's disease	Increased SETDB1 and H3K9me3	(Ryu et al. 2006)
	Rubinstein-Taybi syndrome	Deletion or mutation of *CREBBP*	(Roelfsema and Peters 2007)
	Schizophrenia	Decreased H3K4me3 by MLL1	(Huang et al. 2007)
		Increased H3K9me2	(Gavin et al. 2009)
	Sotos syndrome	Mutation in *NSD1*	(Kurotaki et al. 2002)
	X-linked mental retardation	Mutation in *JARID1C*/*SMCX*	(Iwase et al. 2007)
		Mutation in *RSK2*	(Merienne et al. 1999)
		Mutation in *ATRX*	(Guerrini et al. 2000)
		Mutation in *MECP2*	(Meloni et al. 2000)
	9q34 subtelomeric deletion syndrome	Mutation in *EHMT1*	(Kleefstra et al. 2006)
DNA methylation or methylation-related defect	AD	Hypomethylation at *APP*	(West et al. 1995)
	ASD	Dysregulation	(Melnyk et al. 2012; Nagarajan et al. 2008)
	Bipolar disorders	Dysregulation	(Mill et al. 2008)
	Fragile X syndrome	Dysregulation	(Sutcliffe et al. 1992)
	Rett syndrome	Mutation in *MECP2*	(Amir et al. 1999)
	Schizophrenia	Dysregulation	(Abdolmaleky et al. 2005, 2006; Grayson et al. 2005; Huang and Akbarian 2007; Mill et al. 2008)
Noncoding RNA regulation	Alzheimer's disease	Dysregulation	(Satoh 2010)
	Bipolar	Dysregulation	(Moreau et al. 2011)
	Schizophrenia	Dysregulation	(Beveridge et al. 2010; Perkins et al. 2007)
Genomic imprinting	Angelman syndrome	Loss of *UBE3A*	(Kishino et al. 1997; Matsuura et al. 1997)
	Prader-Willi syndrome	Loss of *SNORD116*	(de Smith et al. 2009; Sahoo et al. 2008)
	UPD14	Chromosome 14	(Papenhausen et al. 1995)

Additionally, mutations within the genes encoding the H3K4-specific histone demethylase (*JARID1C*/*SMCX*), the H3K9-specific methyltransferase (*EHMT1*), and the chromatin remodeling protein (*ATRX*) have been linked to genetic disorders with significant intellectual disabilities (Guerrini et al. 2000; Iwase et al. 2007; Jacquot et al. 1998; Kleefstra et al. 2006). Although these types of disorders are not considered primary epigenetic disorders because they follow Mendelian inheritance, these disorders currently provide the most direct evidence supporting epigenetic modifications as a basic molecular mechanism underlying certain neurological pathologies.

With continued advances in genetic sequencing and other genome-wide profiling techniques, many more epigenetic disorders are likely to be identified. For instance, molecular defects in MBD5, a member of methyl-CpG-binding protein, were recently identified in autism spectrum disorder by genome-wide copy number variant analysis, followed by sequencing of the candidate gene (Talkowski et al. 2011). This type of genetic analysis was not readily possible a decade ago.

8.3.3 Neuroepigenetic Disorders with Aberrant Epigenetic Modifications

Disruption of epigenetic modifications may contribute to any human disease with a genetic etiology. The list of diseases with aberrant epigenetic modifications including DNA methylation or histone modification continues to grow (Table 8.1). For example, a link between aberrant DNA methylation and Alzheimer's disease (AD) has been observed in a wide range of studies in human brains (Mill 2011). Hypomethylation of the promoter of gene encoding amyloid precursor protein (APP) has been reported in the parietal cortex of AD patients (Tohgi et al. 1999), and more recently a global reduction of DNA methylation has also been identified in the cerebral cortex of AD patients (Mastroeni et al. 2010). Similarly, psychiatric disorders such as schizophrenia and bipolar disorder may also have an epigenetic basis (Abdolmaleky et al. 2011; Akbarian and Huang 2006; Veldic et al. 2005). Aberrant DNA methylation of the Rett syndrome gene *MECP2* and the Angelman syndrome gene *UBE3A* has also been reported in brain tissues from patients with autism spectrum disorders (Jiang et al. 2004b; Nagarajan et al. 2008). The list of diseases associated with aberrant epigenetic modifications is expected to increase significantly with increases in (1) the ability to profile the epigenome and (2) human brain tissue repositories that are permissive to relating epigenetic modifications to diseases. However, establishing a cause-effect relationship between aberrant epigenetic modifications and disease pathophysiology in human or animal models is still a major hurdle to be overcome.

8.3.4 Neuroepigenetic Disorders with Defects in Genomic Imprinting

Genomic imprinting is an epigenetic process through which certain genes are monoallelically expressed in a parent-of-origin manner. Disorders can arise if these genes are mutated, deleted, or abnormally imprinted (Butler 2009). These imprinting disorders can be associated with significant intellectual disabilities and other behavioral manifestations (Table 8.1). For example, Angelman and Prader-Willi syndromes are imprinting disorders caused by genetic disruptions 15q11–q13 region of the maternal and paternal chromosomal copy, respectively (Jiang et al. 1998; Mabb et al. 2011). Notably, maternal, but not paternal, duplication of proximal chromosome 15q is strongly associated with autism (Cook et al. 1997) and other broad-spectrum neuropsychiatric disorders (Ingason et al. 2011; Stewart et al. 2011).

Parent-of-origin effects have also been reported in other disorders such as schizophrenia (Crow et al. 1989; Pun et al. 2011), bipolar disorder (McMahon et al. 1995), attention-deficit hyperactivity disorder (Goos et al. 2007), seizure (Ottman et al. 1988), Tourette syndrome (Lichter et al. 1995), and multiple sclerosis (Hoppenbrouwers et al. 2008). However, it remains to be determined whether disruptions in genomic imprinting are causal to the pathogenesis of these diseases.

How genomic imprinting is established, maintained, and erased is still poorly understood. Because imprinted genes may have a tissue/cell type and developmentally specific pattern, the number of disorders with an imprinting component may be underestimated due to our current technical inability to identify these genes. A recent report suggests that more than 5% of murine genes may have parent-of-origin expression in the brain (Gregg et al. 2010). This observation, if confirmed in humans, would have a significant impact on the estimated number of genomic imprinting disorders in humans.

8.3.5 Relationship Between Genetic and Epigenetic Defects in Neuroepigenetic Disorders

Genetic and epigenetic defects can be clinically indistinguishable. This relationship is well illustrated in genomic imprinting disorders like Angelman syndrome (Jiang et al. 1999; Mabb et al. 2011). DNA point mutations in the *UBE3A* gene and chromosome deletion of 15q11–q13, which includes the *UBE3A* gene, are known *genetic* causes of Angelman syndrome. Angelman syndrome (AS) can also be caused epigenetically by paternal uniparental disomy of chromosome 15 and by rare cases of imprinting defects without detectable imprinting center mutations. The clinical features of AS caused by either genetic or epigenetic defects are indistinguishable. This paradigm holds true for Prader-Willi and Beckwith-Wiedemann syndromes as well (Cassidy and Driscoll 2009; Engel et al. 2000). Therefore, the

possibility of epigenetic dysregulation should be considered for genetic diseases even when a corresponding genetic etiology has been identified. While relatively few diseases have been associated with epigenetic dysregulations, this may be due to a dearth of epigenetic profiling, a lack of high quality of brain tissues for study, and difficulties associated with establishing a definitive cause and effect relationship.

8.4 Current and Potential Epigenetic Therapy

Epigenetic modifications can be reversible, hence presenting a therapeutic opportunity. Histone acetylation and DNA methylation have been major targets for possible epigenetic cancer therapies over the last decade (Baylin and Jones 2011), and there are now ongoing clinical trials evaluating epigenetic therapies for certain types of cancer (Table 8.2). Epigenetic therapies for nonneoplastic conditions are also beginning to emerge (Gray 2011; Mikaelsson and Miller 2011). One of the major challenges to developing such therapies is that they must be sufficiently specific to minimize potentially damaging off-target effects. However, it is possible that a number of the available therapeutic agents may lack significant toxicities despite having relatively nonspecific or genome-wide side effects. The development of epigenetic therapies is clearly challenging, but promising. Below we will briefly summarize challenges associated with developing epigenetic therapies as well as insights gained from current epigenetic strategies.

8.4.1 Lessons Learned from the Epigenetic Therapy of Cancer

A number of DNA methyltransferase and histone deacetylase (HDAC) inhibitors have been developed, and some of these have been tested as epigenetic therapies for various types of cancer (Kelly et al. 2010; Peedicayil 2006; Yoo and Jones 2006). 5-azacytidine (5-AZA, Vidaza) and 5-aza-2′-deoxycytodine (5-aza-dC, decitabine) are the two best studied DNA methyltransferase inhibitors of the last decade (Table 8.2) (Kaminskas et al. 2005). In 2006, clinical trials of Vidaza and decitabine were approved by the FDA for the treatment of hematopoietic malignancies. Contrary to the prediction that the DNA methylation inhibitors would have significant toxicity due to genome-wide or off-target effects, these two drugs appear to be relatively well tolerated in humans (Issa et al. 2004; Samlowski et al. 2005). Although the long-term toxicity of these treatments remains to be investigated, available data suggest that genome-wide effects or off-target effects may not be as significant as predicted.

Several HDAC inhibitors have also gained approval by the FDA to treat certain neoplastic diseases (Dokmanovic et al. 2007). Vorinostat and romidepsin were the first two HDAC inhibitors approved by the FDA for the treatment of cutaneous

Table 8.2 Candidate epigenetic drugs and their potential clinical uses

Target	Drug	Clinical use or trials	References
DNA methylation			
Nucleoside analogue	5-azacytidine (Vidaza®, Celgene)	Hematologic malignancy	
	Decitabine (Dacogen®, Astex)	Hematologic malignancy	
	FCdR	Phase I (advanced cancer)	
	Zebularine	Preclinical	(Chen et al. 2012)
Non-nucleoside analogue	DNMT1 ASO (MG98)	Phase I (advanced solid tumors)	
	EGCG	Preclinical	(Lin et al. 2012)
	Hydralazine (Apresoline®)	Hypertension; phase II (cervical cancer, refractory solid tumors)	
	MG98	Phase I (advanced solid tumor)	
	RG108	Preclinical	(Brueckner et al. 2005)
	Procainamide (Pronestyl®)	Anti-arrhythmic, preclinical	(Gao et al. 2009)
	Procaine	Local anesthetic, preclinical	(Tada et al. 2007)
	Psammaplin A	Preclinical	(Ahn et al. 2008)
Histone deacetylase			
Short-chain fatty acids	Phenylbutyrate (Buphenyl®)	Urea cycle disorders, phase I (hematologic malignancy)	
	Valproic acid (Depakene®)	Epilepsy, mood disorders, phase I (advanced cancer)	
Hydroxamic acid	CBHA	Preclinical	(Takai et al. 2006)
	LBH589	Phase I (advanced solid tumor)	
	NVP-LAQ824	Preclinical	(Weisberg et al. 2004)
	Oxamflatin	Preclinical	(Sonoda et al. 1996)
	PDX 101	Phase I (solid tumor)	
	Pyroxamide	Phase I (advanced tumor)	
	Scriptaid	Preclinical SAHA	(Giacinti et al. 2012)
	SAHA (Vorinostat®)	Cutaneous T-cell lymphoma	
	TSA	Preclinical	(Vigushin et al. 2001)
Cyclic tetrapeptides	Apicidin	Preclinical	(Ahn et al. 2011)
	CHAP	Preclinical	(Komatsu et al. 2001)
	Depsipeptide (romidepsin)	Phase II (cutaneous T-cell lymphoma)	

(continued)

Table 8.2 (continued)

Target	Drug	Clinical use or trials	References
	Trapoxin	Preclinical	(Komatsu et al. 2001)
Benzamides	CI-994	Phase III (lung cancer)	
	MS-275	Phase II (melanoma)	
Others	α-ketoamides	Preclinical	(Wada et al. 2003)
	Trifluoromethyl ketones	Preclinical	(Frey et al. 2002)

More detail information is in the following webpage and references: (http://clinicaltrials.gov) (Egger et al. 2004; Peedicayil 2006; Robak 2011; Yoo and Jones 2006)
ASO antisense oligonucleotides, *CBHA* m-carboxy cinnamic acid bishydroxamic acid, *CHAP* TPX-HA analogue, *EGCG* epigallocatechin gallate, *FCdR* 5-fluoro-2′- deoxycytidine, *SAHA* suberoylanilide hydroxamic acid, *TSA* trichostatin A

T-cell lymphoma. More than a dozen other HDAC inhibitors are currently in phase I or phase II clinical trials (Baylin and Jones 2011). The use of HDAC inhibitors in neurological and psychiatric disorders has been long documented (Abel and Zukin 2008). For example, valproic acid, a known HDAC inhibitor, is commonly used to treat epilepsy and mood disorders. However, the development of new HDAC inhibitors for CNS-related disorders is lagging behind those for the treatment of cancer. The specificity for many of these HDAC inhibitors remains to be elucidated. In addition, trials combining HDAC and DNA methylation inhibitors, as well as other types of chemotherapeutic agents, are being conducted to determine if combinations of these agents will have synergistic effects and increase their therapeutic potential (Ai et al. 2012; Rius and Lyko 2012).

8.4.2 Epigenetic Therapy in Animal Models of CNS-Related Disorders

Although there are a few reports of epigenetic therapies being used to treat neurological and psychiatric disorders in humans, many more studies have evaluated the effectiveness of epigenetic pharmaceuticals in animal models of these disorders. Similar to human studies, the majority of epigenetic studies in animal models have targeted DNA methyltransferases and HDACs (Kazantsev and Thompson 2008; Peedicayil 2006). Combined therapies (DNA methylation with histone deacetylase inhibitors) (Belinsky et al. 2003) and RNAi-based therapeutics are also currently under investigation (Aouadi et al. 2009; Czech et al. 2011).

The power of epigenetic modifications to influence brain development has long been known, as folic acid supplements are given to pregnant women to reduce the chance for neural tube defects (Pitkin 2007). The idea that epigenetic codes such as DNA methylation can be modified in vivo by drugs or dietary supplementation has

been well documented in animal models (Choi et al. 2005; Waterland 2006; Wolff et al. 1998). While the epigenetic effects of these treatments on the brain can be difficult to parse, the epigenetic effects on the periphery are sometimes clear. For example, the supplementation of methyl donors *prenatally* in mice changes the methylation of intracisternal A particle (IAP) and the A^{vy} allele in Agouti mice, and this epigenetic change manifests as a change in the coat color of the mice (Waterland and Jirtle 2003; Wolff et al. 1998). Likewise, maternal methyl donor supplements can prevent bisphenol A-induced DNA hypomethylation and shift coat color in Agouti mouse offspring (Dolinoy et al. 2007).

Excitingly, some disorders associated with epigenetic modifications have a remarkable capacity to be reversed *postnatally* (Silva and Ehninger 2009). For example, genetic reintroduction of normal MECP2 protein in adulthood can reverse neurological impairments normally observed in the Rett syndrome mouse model (Gadalla et al. 2011; Guy et al. 2007). Similarly, postnatal treatments with the HDAC inhibitor, trichostatin A, can rescue defective long-term memory in a mouse model of Rubinstein-Taybi syndrome (Alarcon et al. 2004). HDAC inhibitors have been studied in other animal models of neurological and psychiatric disorders (discussed below), but it is unclear if these are acting directly or indirectly. Also unknown is whether the promising results of epigenetic therapies in mouse models will translate to treatment of human neurological disorders. Several examples of recent advances in epigenetic therapeutic approaches are discussed below.

8.4.2.1 Alzheimer's Disease (AD)

Alzheimer's disease is a common form of dementia that has a strong genetic etiology. The involvement of epigenetics in the molecular pathogenesis of AD has been suggested in multiple studies looking at human postmortem brain tissues and in mouse models of AD. Elevated levels of histone phosphorylation have been observed in the hippocampus and cortex of postmortem brain tissues from AD patients (Myung et al. 2008). Hypomethylation of the promoter of the *APP* gene was also observed in the parietal cortex of AD patients (West et al. 1995). Consistent with an epigenetic contribution to some forms of AD, the HDAC inhibitor sodium 4-phenybutyratre has been shown to ameliorate spatial learning and memory deficits in the Tg2576 mouse model of AD (Ricobaraza et al. 2009, 2011). Similar results using different HDAC inhibitors have been observed in other AD mouse models (Kilgore et al. 2010). It is interesting to note that AD is not the only disorder with an age-dependent onset. Other age-dependent epigenetic changes linked to neurological disturbances have been documented in both animal models (Peleg et al. 2010) and humans (Salpea et al. 2012; Siegmund et al. 2007). This observation may suggest epigenetic modifications are a possible general mechanism underlying age-dependent decline in cognitive ability.

8.4.2.2 Depression

The pathophysiology and molecular basis of depression are poorly understood. Given this, it is perhaps not surprising that there are few effective treatments for depression. It may be possible to develop better therapies by gaining a better understanding of the epigenetic contributions to depression (Schroeder et al. 2010). Changes in genome-wide DNA methylation have been observed in patients suffering from major depressive disorder (Sabunciyan et al. 2012), raising the possibility that epigenetic therapies may exist. Consistent with this idea, several studies have shown that epigenetic therapies hold promise for treating depression. One specific epigenetic modification linked to depression is hypermethylation in the promoter region of the P11 (S100A10) gene. Expression of the P11 gene is reduced in individuals with depression (Anisman et al. 2008), and loss of P11 in mice models depression (Svenningsson et al. 2006). The loss of P11 may contribute causally to depression, because antidepressants such as escitalopram (a selective serotonin reuptake inhibitor, SSRI) have been shown to reduce DNA methylation at the P11 promoter and increase P11 levels (Melas et al. 2012).

Other epigenetic therapies have also been suggested for treating depression. For example, sodium butyrate, a HDAC inhibitor, shows antidepressant-like effects in mice running anxiety and behavioral despair tests (forced swim test) (Schroeder et al. 2007). Sodium butyrate also enhances the antidepressant-like effects of fluoxetine (a selective serotonin reuptake inhibitor) in mice running behavioral despair tests (Schroeder et al. 2007). Likewise, the HDAC inhibitor MS-275 reverses the effect of chronic defeat stress through regulation of gene expression in the nucleus accumbens (Covington et al. 2009). Defeat stress can also downregulate *Bdnf* transcripts and increase repressive histone methylation at *Bdnf* promoters. Decreased BDNF has been observed in the serum of depressed patients and in postmortem brains of suicide victims (Karege et al. 2005a, b; Pandey et al. 2010). Chronic imipramine (a tricyclic antidepressant) administration reverses this downregulation of *Bdnf* transcripts and increases histone acetylation at *Bdnf* promoters through a selective downregulation of HDAC5 (Tsankova et al. 2006). Zebularine, a DNA methylation inhibitor, acts by restoring BDNF expression by decreasing *Bdnf* promoter methylation in early defeated adult rats (Yoo et al. 2004). Collectively these experiments suggest a critical role of chromatin remodeling in the pathophysiology and treatment of depression. These findings also raise the hope that certain DNA methyltransferase inhibitors or HDAC inhibitors may help treat depression.

8.4.2.3 Schizophrenia

Schizophrenia is a common psychiatric disorder that affects about 1% of the population worldwide. Similar to other psychiatric disorders, the pathophysiology of schizophrenia remains poorly understood. Currently, most drug treatments for

schizophrenia act to modulate dopaminergic pathways in the brain. Several studies have suggested an epigenetic basis in the pathogenesis of schizophrenia (Akbarian 2010; Gavin and Akbarian 2012), which would present additional therapeutic opportunities. One putative contributor to schizophrenia is the epigenetic regulation of glutamate decarboxylase 1 (GAD1), the key enzyme of GABA biogenesis. Dysfunction of GABAergic neurons has been linked to schizophrenia, and this dysfunction might arise in part because *GAD1* mRNA and protein is reduced in the prefrontal cortex and other brain regions (Akbarian and Huang 2006; Akbarian et al. 1995; Guidotti et al. 2000; Volk et al. 2000). Decreased *GAD1* mRNA has been correlated with decreased levels of histone H3 lysine 4 trimethylation (H3K4me3), a chromatin marker associated with active transcription, at the *GAD1* promoter in the prefrontal cortex of schizophrenic subjects (Huang et al. 2007). Interestingly, the antipsychotic medication clozapine increases levels of H3K4me3 at the *Gad1* promoter by recruiting mixed-lineage leukemia 1 (Mll1), a histone H3 lysine 4 methyltransferase (Huang et al. 2007). These findings suggest that MLL1-mediated histone-lysine methylation at the *GAD 1* promoter could be involved in the pathophysiology of schizophrenia.

Schizophrenia might be caused by epigenetic loss of *GAD1* in conjunction with other genes, such as reelin (*RELN).* Reelin is a large, extracellular, matrix protein and is important for regulating neuronal migration and positioning in the developing brain (Ikeda and Terashima 1997). In the adult brain, reelin can be synthesized and secreted from GABAergic interneurons (Pesold et al. 1998). Decreased *RELN* mRNA and protein has been repeatedly observed in the prefrontal cortex and other brain regions of schizophrenic patients (Eastwood and Harrison 2003, 2006; Fatemi et al. 2000; Guidotti et al. 2000; Impagnatiello et al. 1998). Additionally, aberrant DNA methylation of the *GAD1* and *RELN* promoters has been observed in the cortex of schizophrenic subjects (Grayson et al. 2006; Huang and Akbarian 2007). These observations raise the possibility that the reduction of *GAD1* and *RELN* in schizophrenia subjects could be regulated by epigenetic mechanisms such as DNA methylation and histone modifications. In support of an epigenetic mechanism for regulating *Reln* and *Gad1* expression, downregulation of DNA methyltransferase 1 (DNMT1) induced *Reln* and *Gad1* expression in NT-2 neuronal precursor cells (Kundakovic et al. 2007). Valproate, an HDAC inhibitor, can decrease the hypermethylation of *Reln* promoter by reducing the binding of the methyl-CpG-binding domain protein to the *Reln* promoter in an epigenetically methionine-induced mouse model of schizophrenia (Dong et al. 2005). These observations provide a rationale to explore epigenetic targets for treating schizophrenia.

8.4.2.4 Friedreich Ataxia (FRDA)

Friedreich ataxia is an inherited neurodegenerative disease characterized by progressive ataxia and caused by mutation in frataxin (*FXN*) gene. The mutation mechanism involves a GAA repeat expansion in the first intron of *FXN* gene. The GAA expansion results in epigenetic silencing of the *FXN* gene (Al-Mahdawi et al.

2008). Treatment with pimelic o-aminobenzamide, an HDAC inhibitor, ameliorates the disease state of an FRDA in cell and mouse models (Herman et al. 2006; Sandi et al. 2011). Additionally, two new pimelic diphenylamide-related HDAC inhibitors induce upregulation of *FXN* in cultured cells from FRDA patients and in a mouse model (Rai et al. 2010). At this time, no clinical trial data has been reported for these compounds to assess their effectiveness in human FRDA patients.

8.4.2.5 Spinal Muscular Atrophy (SMA)

Spinal muscular atrophy (SMA) is a severe and progressive neuromuscular disorder caused by the deficiency of the *SMN1* gene. *SMN2*, an *SMN1* homologue, differs by five nucleotides but encodes the same protein as *SMN1*. The increase of *SMN2* copy number correlates to the mild phenotypes in SMA caused by mutation in *SMN1* gene. For this reason, upregulating the *SMN2* gene by epigenetic modification such as HDAC inhibitors in cell and animal models has been tested extensively. The HDAC inhibitors, SAHA and TSA (trichostatin A), ameliorate phenotypes in two mouse models for SMA (Avila et al. 2007; Riessland et al. 2010). Similar results have also been reported in humans using different types of HDAC inhibitors, although a formal clinical trial is needed to fully evaluate the potential of these treatments (Brichta et al. 2003; Garbes et al. 2009).

8.4.3 Novel Approach to Discover New Epigenetic Drugs for Neuroepigenetic Disorders

Genomic imprinting disorders provide a unique opportunity to explore novel epigenetic therapies, as their molecular and epigenetic basis is relatively well understood. These disorders are usually caused because a monoallelically expressed gene is mutated or deleted. A potential therapy, therefore, is to activate the dormant but intact allele to replace lost expression from the mutated/deleted allele. Such an approach has recently been attempted for the imprinting disorder Angelman syndrome (Huang et al. 2012), which is caused by loss of maternal *UBE3A* expression. No effective therapy currently exists for Angelman syndrome. The structurally intact, but functionally silent, paternal *UBE3A* allele in the brain of affected individuals provides a potential target for therapeutic treatment of Angelman syndrome. Using an unbiased and high-content drug screen, it was found that topoisomerase inhibitors can unsilence paternal *Ube3a* in cultured cortical neurons and Angelman syndrome model mice (Fig. 8.1). Four of the identified effective topoisomerase inhibitors are FDA-approved drugs. This discovery provides a novel therapeutic strategy for reactivating the paternal *UBE3A* allele in Angelman syndrome patients. This study also shows that topoisomerase

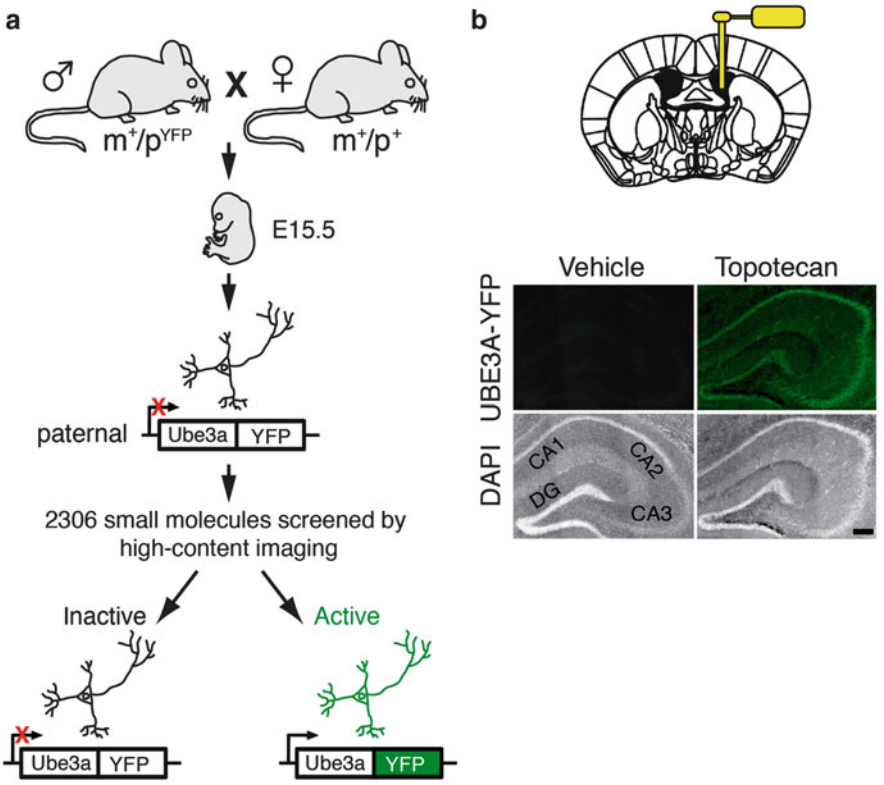

Fig. 8.1 Novel approach for identifying epigenetic drugs for potential treatment of Angelman syndrome. (**a**) High-content screen flowchart. E15.5 cortical neurons with a paternally inherited *Ube3a-YFP* allele were cultured in 384-well plates and treated with small molecules from days 7 to 10 in vitro. Active compounds that unsilence the paternal *Ube3a-YFP* allele were detected by antibody-enhanced fluorescence and high-content imaging. (**b**) Topotecan unsilences paternal *Ube3a* in the brain. *Top*, schematic showing unilateral delivery of topotecan (i.c.v.) using a mini-osmotic pump into the lateral ventricle of $Ube3a^{m+/pYFP}$ mice in vivo for 2 weeks. *Bottom*, brain sections showing unsilencing of paternal *Ube3a* (*green*) in the hippocampus of topotecan—but not vehicle-treated mice. Scale bar = 200 μm (Huang et al. 2012)

inhibitors unsilence paternal *Ube3a* by reducing the antisense transcript of *Ube3a* (*Ube3a-ATS*). Many studies have suggested that downregulation of *Ube3a-ATS* could unsilence paternal *Ube3a* (Chamberlain and Brannan 2001; Chamberlain and Lalande 2010; Rougeulle et al. 1998; Yamasaki et al. 2003). The correlation between a reduction in *Ube3a-ATS* and an increase in paternal *Ube3a* expression was supported by a more direct study demonstrating that breakdown of *Ube3a-ATS* in mice activates the silent paternal *Ube3a* allele (Meng et al. 2012). Identifying drugs that can break down *Ube3a-ATS* or otherwise reduce *Ube3a-ATS* expression presents a promising therapeutic strategy for Angelman syndrome. Similar strategies could be used to identify potential therapies for other genomic imprinting

disorders such as Prader-Willi syndrome. An important caveat of all these studies is that identified compounds that can activate a dormant allele must be tested for off-target effects and carefully vetted for safety.

8.4.4 Challenges of Epigenetic Therapy

Given the significant progress in using epigenetic therapies to treat certain cancers, it is becoming a reasonable and popular therapeutic approach to treat neurological patients with epigenetic modifiers. However, progress has been slow for the epigenetic therapy of CNS-related disorders. This is due in part to difficulties in identifying an epigenetic locus of neurological disorders, although advanced high-throughput approaches are rapidly overcoming this obstacle. Other difficulties include proving the cause-effect relationship between epigenetic modifications and disease states. Finally, the difficulty of performing drug screens in neurons limits the speed at which epigenetic therapies can be identified.

Many questions remain before epigenetic therapies can be more effectively implemented. For example, what is the cellular permeability for epigenetic drugs? Can epigenetic drugs cross the blood-brain barrier? When should epigenetic drugs be administered during development to achieve a therapeutic effect? What are the cytotoxicity and potential mutagenicity of current epigenetic drugs? These are very real obstacles. Indeed, many potentially effective HDAC and DNA methyltransferase inhibitors are known to be cytotoxic. Despite the significant challenges, epigenetic therapies present a new and exciting opportunity for CNS drug development.

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Part III
Epigenetics, Nutrition, Diabetes, and Obesity

Chapter 9
Nutrition, Histone Epigenetic Marks, and Disease

Janos Zempleni, Dandan Liu, and Jing Xue

Abstract The dietary intake of essential nutrients and bioactive food compounds is a process that occurs on a daily basis for the entire life span. Therefore, your diet has a great potential to cause changes in the epigenome. Known histone modifications include acetylation, methylation, biotinylation, poly(ADP-ribosylation), ubiquitination, and sumoylation. Some of these modifications depend directly on dietary nutrients. For other modifications, bioactive dietary compounds may alter the activities of enzymes that establish or remove histone marks, thereby altering the epigenome. This chapter provides an overview of diet-dependent epigenomic marks in histones and their links with human health.

Keywords Bioactive food compounds • Diet • Epigenome • Minerals • Nutrition • Vitamins

Abbreviations

A^{vy}	Viable yellow agouti
CoA	Coenzyme A
DNMT1	DNA methyltransferase 1
FAD	Flavin adenine dinucleotide
H3K4me3	K4-trimethylated histone H3
H3K9ac	K9-acetylated histone H3
H3K9me2	K9-dimethylated histone H3
H3K9me3	K9-trimethylated histone H3
H4K5ac	K5-acetylated histone H4

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H4K8ac	K8-acetylated histone K12
H4K12ac	K12-acetylated histone H4
H4K12bio	K12-biotinylated histone
H4H4K16ac	K16-acetylated histone H4
H4K20me3	K20-trimethylated histone H4
HAT	Histone acetyl transferase
HDAC	Histone deacetylase
HLCS	Holocarboxylase synthetase
HMT	Histone methyl transferase
JmjC	Jumonji C
K	Lysine
LSD1	Lysine-specific demethylase 1
SET	Suppressor of variegation enhancer of zeste trithorax
Sir2	Silent information regulator 2

9.1 Introduction

The human epigenome is constantly exposed to a variety of environmental factors that may alter chromatin structure, gene regulation, genome stability, and an individual's predisposition to disease. Frequently, individuals are exposed to these factors in a controlled environment over a short period of time in subsets of the general population. The treatment of cancer patients with the histone deacetylase inhibitor, belinostat (Ma et al. 2010), and lifestyle choices such as smoking are examples (Launay et al. 2009). This is not the case for human nutrition.

The dietary intake of essential nutrients and bioactive food compounds is a process that occurs on a daily basis for the entire life span. Therefore, it has great potential for causing changes in both the epigenome and disease risk. A well-documented example is the feeding of defined diets to agouti mice, where dietary supplementation with methyl donors and genistein resulted in increased DNA methylation of a long terminal repeat in the viable yellow agouti (A^{vy}) locus and an alteration in offspring coat color and disease susceptibility in adulthood (Cooney et al. 2002; Dolinoy et al. 2006; Waterland and Jirtle 2003; Wolff et al. 1998). Epidemiologic studies also indicate that adult disease risk is associated with poor nutritional status early in development. Individuals exposed to the famine during the Dutch Hunger Winter of 1944 and 1945 is the most widely recognized example (Heijmans et al. 2008). Put simply, dietary choices during pregnancy can alter the disease risk of the unborn child later in life. It remains to be seen whether such scenarios hold true under conditions that are more moderate than the Dutch Hunger Winter.

A large number of covalent modifications have been identified in the N-termini of histones, while some modifications also exist in the hinge regions and the C-termini. Known modifications include acetylation, methylation, biotinylation, poly (ADP-ribosylation), ubiquitination, and sumoylation (Boulikas 1988; Boulikas et al. 1990; Camporeale et al. 2004; Chambon et al. 1966; Kouzarides and Berger 2007; Shiio and Eisenman 2003; Stanley et al. 2001; Wolffe 1998). For many of these modifications, unambiguous links have been established with human disease and nutrition.

One might consider classifying diet-dependent histone modifications according to the following scheme. In one group of histone marks, nutrient supply is a limiting factor only under exceptional circumstances, but bioactive compounds and essential nutrients in food may modulate the abundance of the modification by altering enzyme activities. This group of histone marks would include iron-, calcium-, and riboflavin-dependent demethylation of histones and the inhibition of histone deacetylases by sulforaphane. In a second group of histone marks, essential nutrients or functional groups thereof are attached to histones, and dietary nutrient availability can be a limiting factor for creating these marks. Histone methylation, biotinylation, and poly(ADP-ribosylation) belong in this group. In a third group of histone marks, specific nutrients are attached to histones, but the availability of these nutrients is not a limiting factor under normal circumstances. Sumoylation and ubiquitination belong in this group.

9.2 Acetylation of Histones

Histone acetylation marks are associated with transcriptionally active chromatin (Kouzarides and Berger 2007). Lysine (K)-9 acetylated histone H3 (H3K9ac), H4K5ac, H4K8ac, H4K12ac, and H4K16ac are the most frequently studied markers for acetylation-mediated gene activation. There are two dietary compounds directly involved in acetylation events. The first is acetate, which can be derived from the metabolism of glucose, amino acids, fatty acids, and other compounds in intermediary metabolism (Garrett and Grisham 1995). Except for circumstances of prolonged total starvation, it is nearly impossible to deplete the body pool of acetate. The second is coenzyme A (CoA), which is required for generation of the energy-rich acetyl-CoA for subsequent acetylation reactions. The vitamin pantothenic acid is an integral part of the CoA molecule (Garrett and Grisham 1995). In the most recent edition of the Dietary Reference Intakes, the Food and Nutrition Board acknowledges that human pantothenic acid requirements are unknown (National 1998). Thus, only recommendations for Adequate Intake are available for pantothenic acid. These recommendations are based solely on the intake of pantothenic acid in the general, apparently healthy, population (National 1998). There is currently no compelling evidence to suggest that the prevalence of pantothenic acid deficiency is quantitatively meaningful in Western societies; the

Table 9.1 Histone deacetylases

Class	Member
I	HDAC1, HDAC2, HDAC3, HDAC8
II	HDAC4, HDAC5, HDAC6, HDAC7A, HDAC9, HDAC10
III	SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6, SIRT7
IV	HDAC11

prevalence of deficiency in developing countries or in unique, small subgroups within the general population is unknown.

Acetylation of histones is catalyzed by histone acetyl transferases (HATs) such as PCAF, STAGA, and TFTC, which are grouped on the basis of their catalytic domains such as GCN5 and PCAF {see (Lee and Workman 2007) for an excellent review}. Little is known about dietary modulation of HAT activity; however, the dietary polyphenol curcumin is an inhibitor of the acetyl transferase p300 (Morimoto et al. 2008).

Histone deacetylases (HDACs) catalyze the enzymatic removal of acetyl mark from the ε-amino group of lysines in histones during gene repression (Minucci and Pelicci 2006). The family of HDACs comprises 18 isoforms, which are categorized into four classes, depending on sequence identity and domain organization (Table 9.1) (Dokmanovic et al. 2007). Histone deacetylation by class III HDACs (sirtuins) is coupled to NAD+ hydrolysis, which is a niacin-dependent event; the dietary supply of niacin and niacin precursors is discussed below. Sir2 (silent information regulator 2) in simple eukaryotes and its mammalian ortholog SIRT1 play important roles in life span extension in response to caloric restriction (Boily et al. 2008). The efficacy of SIRT1 in enhancing the life span in mammals appears to depend on salvaging NAD in the nicotinamide phosphoribosyl transferase pathway (Ho et al. 2009a). The phenolic compound resveratrol activates sirtuins and increases the life span in *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, *Drosophila melanogaster*, and the fish *Nothobranchius furzeri*, but its efficacy in mammals is uncertain (Evason et al. 2005; Howitz et al. 2003; Kang et al. 2002; Valenzano et al. 2006). Resveratrol is present in grapes and red wine at relatively high concentration (Celotti et al. 1996).

Hypoacetylation of histones is a hallmark of human cancer. HDAC inhibitors may induce cell cycle arrest, differentiation, and apoptosis in cancer cells. Thus, HDAC inhibitors are considered promising tools in the prevention and treatment of the disease (Liu et al. 2006), and synthetic inhibitors such as belinostat are already being tested in clinical trials (Ma et al. 2010). Importantly, HDAC activity may be modulated by a number of dietary compounds. Sodium butyrate is an HDAC inhibitor that can be produced by gastrointestinal fermentation of fermentable fiber, particularly resistant starch (Cummings et al. 2001; Davie 2003). Nicotinamide is a competitive inhibitor of the class III sirtuins, and it may restore some of the cognitive deficits in Alzheimer's disease (Green et al. 2008). Sulforaphane is an isothiocyanate found in relatively high concentrations in cruciferous vegetables such as broccoli and brussels sprouts. Although it inhibits HDACs

(Ho et al. 2009b; Myzak et al. 2006), it is uncertain whether pharmacologically effective concentrations can be achieved through a normal diet. Synthetic selenium-containing analogs of suberoyl hydroxamic acid also show promise as HDAC inhibitors (Desai et al. 2010). The extent to which dietary selenium compounds affect HDAC activity is unknown.

Diseases other than cancer are also linked with acetylation events in the epigenome, although unambiguous cause-and-effect relationships still remain to be demonstrated for many of these diseases. A link to diabetes is proposed because of the physical interactions of the HATs p300, CBP, and PCAF with hepatocyte nuclear factor and glucokinase (Gray and De Meyts 2005). These interactions do not constitute a classical epigenetic mechanism but rather a change in transcription factor and enzyme activity. The abundance of histone H4 acetylation marks is abnormally increased in the promoters of NFκB-dependent proinflammatory genes in lung diseases such as asthma and cystic fibrosis (Selvi and Kundu 2009). Nevertheless, the roles of HATs and HDACs and the possible modulation by dietary intervention in these diseases are far from being understood. Roles for histone acetylation are also proposed for neurodegenerative disorders, cardiac hypertrophy, and malaria; however, the underlying mechanisms and environmental perturbations remain to be elucidated (Freitas-Junior et al. 2005; McKinsey and Olson 2004; Saha and Pahan 2006).

It is difficult to establish causal links between changes in acetylation marks in the epigenome and roles for HDACs in human health because HDACs also target nonhistone proteins such as transcription factors (Drummond et al. 2005; Selvi and Kundu 2009). It also appears that both drugs and dietary compounds that affect HDACs elicit off-target effects by modifying the transcriptional activity of genes not related to disease. For example, evidence suggests that HDAC inhibitors increase the acetylation of histones in transposable elements. This is associated with their transcriptional activation (Brunmeir et al. 2010a, b; Montoya-Durango et al. 2009), and transcriptional activation of transposable elements impairs genome stability (Fan 2007; Gasior et al. 2006). More studies are needed to investigate aberrant gene regulation caused by these off-target effects.

9.3 Methylation of Histones

Methylation events in the epigenome depend on the dietary supply of methyl donors and other essential cofactors. Both cytosines in DNA and histones are targets for methylation (Kouzarides and Berger 2007; Li and Bird 2007). This chapter focuses exclusively on histone epigenetic marks, whereas cytosine methylation is covered in other chapters in this book. The reader should note, however, that the histone methyl transferase (HMT) G9a is known to interact with DNA methyltransferase 1 (DNMT1) (Esteve et al. 2006). When DNMT1 is knocked down by using siRNA, both cytosine and H3K9 methylation on chromatin are impaired, confirming DNMT1 as the primary loading factor. Consistent with this notion, aberrant

cytosine methylation impairs both histone methylation and histone biotinylation (Chew et al. 2008).

Both arginine and lysine residues in histones are potential targets for methylation (Bedford and Clarke 2009; Martin and Zhang 2005). HMT activity toward lysine and arginine residues is found in a family of enzymes with a conserved catalytic domain called SET (suppressor of variegation, enhancer of zeste, trithorax) (Albert and Helin 2010). The human genome encodes 48 SET domain-containing proteins and DOT1L, which does not contain a SET domain but has lysine methyltransferase activity. The domain structures, phylogenetic tree, and histone targets of HMTs are covered in depth in an excellent recent review (Albert and Helin 2010).

Histone methylation is a dynamic and reversible process. As of today, three classes of histone demethylases have been identified (Klose et al. 2006). The largest class of demethylase enzymes contains a Jumonji C (JmjC) domain. The JmjC domain-containing enzymes can demethylate mono-, di-, and trimethylated histones by an oxidative mechanism that requires Fe(II) and alpha-ketoglutarate as cofactors (Tsukada et al. 2006). The following subgroups in the general population are at risk for developing iron deficiency: infants (particularly premature and low-birth-weight babies), young children, menstruating and pregnant women, vegetarians, and people who have internal bleeding or who get kidney dialysis treatment (National 2011). Nevertheless, it is unclear to what extent iron deficiency affects histone demethylation events. Lysine-specific demethylase 1 (LSD1) is the founding member of the second class of histone demethylases. Enzymes in this class demethylate only mono- or dimethylated H3K4me and H3K9me and depend on flavin adenine dinucleotide (FAD) as a coenzyme for oxidative demethylation. FAD is one of the two coenzyme forms of the vitamin riboflavin (Rivlin 2007). Dietary riboflavin deficiency is uncommon in developed countries, but deficiency may be precipitated by adrenal and thyroid hormone deficiency, psychotropic agents such as chlorpromazine, antidepressants such as imipramine and amitriptyline, chemotherapeutic drugs such as Adriamycin, and antimalarial agents such as quinacrine (Rivlin 2007). It is unknown whether riboflavin deficiency affects histone demethylation to a meaningful extent. Peptidylarginine deiminase 4 is the third class of histone demethylases, and it was the first to be identified. It converts methylated arginine to citrulline (as opposed to producing unmethylated arginine) in a Ca^{2+}-dependent reaction (Wang et al. 2004). It is unknown whether calcium deficiency affects histone demethylation to a meaningful extent. Please note that vitamin D and biotin play important roles in calcium homeostasis and cellular compartmentalization, respectively (Griffin et al. 2006; Norman and Henry 2007).

Histone methylation depends on a sufficient supply of S-adenosylmethionine (SAM) as a methyl donor (Fig. 9.1). In this reaction, a methyl group is transferred from SAM to histones (or other methyl acceptors) and SAM is converted to S-adenosylhomocysteine (SAH). In addition to SAM, numerous other nutrients and metabolites also play key roles in one-carbon metabolism and, hence, methylation reactions {see (Bailey 2007) for an elaborate review}. Briefly, L-homocysteine can be remethylated to produce L-methionine in a reaction that depends on both 5-methyltetrahydrofolate and vitamin B_{12}. Deficiency of vitamin B_{12} is frequently

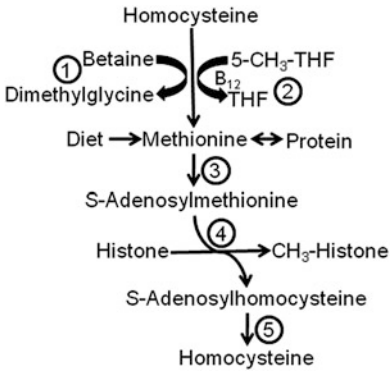

Fig. 9.1 One-carbon flux. Abbreviations: CH_3 methyl, *THF* tetrahydrofolate. *1* betaine: homocystein methyltransferase, *2* methionine synthase, *3* methionine adenosyltransferase, *4* histone methyltransferase(s), *5* S-adenosylhomocysteine hydrolase

seen in vegans, the elderly, and after surgical removal of the ileum. Furthermore, it leads to folate depletion by trapping the latter as 5-methyltetrahydrofolate (Green and Miller 2007). Dietary compounds such as betaine and choline also play important roles in replenishing the one-carbon pool (Bailey 2007). Finally, zinc is a cofactor for DNMT1 (Wolff et al. 1998); impaired methylation of cytosines may precipitate aberrant patterns of histones marks, as described above.

Folate deficiency used to be relatively common in the general population and was blamed for being a factor contributing toward birth defects, particularly neural tube defects and congenital heart defects (Bailey 2007). Many countries have adopted a policy mandating folate fortification of staple foods. These policies have proven effective with regard to decreasing the incidence of neural tube defects and, perhaps, congenital heart defects (Botto et al. 2003; Honein et al. 2001). Most studies of folate and birth defects have focused on abnormalities in cytosine methylation and impaired thymidine synthesis, with the latter leading to uracil misincorporation into DNA.

It is difficult to unambiguously attribute adverse effects of methyl deficiency to distinct patterns of histone methylation for the following reasons. Firstly, the great number of histone methyltransferases (48 SET domain-containing proteins and DOT1L) requires a combination of sound enzyme kinetics data with regard to substrate affinity and fairly advanced computer algorithms to predict effects of altered methyl supply on the biological activity of individual enzymes. This information is not yet available. It is also important to consider possible effects of single nucleotide polymorphisms on substrate affinity and enzyme activity. Secondly, it is currently unclear to what extent betaine and choline can substitute for folate in one-carbon metabolism, although some mathematic models are emerging (Reed et al. 2006). Thirdly, histone methylation marks can have opposing effects in gene regulation. Classical examples include H3K4me3 and H3K9me, which are transcriptional activation and repression marks, respectively (Kouzarides and Berger 2007). Thus, methyl deficiency might affect both gene activation and repression. Finally, the flow of information among marks in the epigenome is immense and goes far beyond a cross talk among methylation marks. Epigenomic synergies have been documented for H3K4acme3 and H3K9ac in gene activation

(Kouzarides and Berger 2007) and for cytosine methylation, H3K9me2, and H4K12bio in the repression of retrotransposons (Chew et al. 2008). Despite these limitations, the following themes have emerged in the field of diet-dependent changes in the histone methylome and their consequences for human health.

Loss of H4K16ac and H4K20me3 is a hallmark of human cancer (Fraga et al. 2005), and there is now consensus that aberrant methylation marks in the epigenome play a significant role in cancer development and progression (Poke et al. 2010). Evidence suggests that HATs, HDACs, and HMTs can act as tumor suppressor genes or oncogenes. Occasionally, these effects also depend on chromosomal rearrangements and the expression of fusion proteins (Albert and Helin 2010; Fraga and Esteller 2005; Huang 2002). Clearly, the abundance of histone methylation marks depends on the dietary methyl supply. For example, when mice are fed a choline- and methionine-deficient diet, the enrichment of the repression mark H3K9me2 decreases in the promoters of imprinted genes *Igf2* and *H19* in the prostate; however, the changes in H3K9me2 enrichment do not affect the imprinting of these two genes (Dobosy et al. 2008). In contrast, feeding a methyl-deficient diet increases the abundance of H3K9me3 and the expression of the Suv39h1 methyltransferase in preneoplastic nodules and liver tumors in rats compared with controls on a normal diet (Pogribny et al. 2006). It is possible that these apparently contradictory observations are caused by locus-specific effects, by epigenomic synergies with other epigenetic marks, or by a combination of both mechanisms.

Distinct H3K9, H3K27, and H4K20 methylation marks are enriched across tandem repeats (e.g., major and minor satellites), DNA transposons, retrotransposons, long interspersed nucleotide elements, and short interspersed nucleotide elements in the mouse genome (Martens et al. 2005). Evidence that epigenetic mechanisms are important in retroelement control and cancer prevention are as follows (Chen et al. 1997; Chen and Townes 2000; Chew et al. 2008; Jahner et al. 1982; Slotkin and Martienssen 2007; Walsh et al. 1998; Yoder et al. 1997): Firstly, the human genome contains at least 54 transcriptionally active LTRs (Buzdin et al. 2006). Retrotransposons and any integrating virus produces DNA breaks during integration (Gasior et al. 2006) that may directly or indirectly lead to tumorigenesis (Fan 2007). Secondly, retrotransposition events account for ~10 % of known spontaneous mutations in mice (Kazazian and Moran 1998; Smit 1999). Thirdly, retroelements are associated with break-prone segmental duplications in tumors (Darai-Ramqvist et al. 2008). Finally, chromosomal instability caused by aberrant epigenetic marks and the insertion of retrotransposons lead to oncogene activation, tumor suppressor gene inactivation, and the disruption of essential genes (Check 2003; Eden et al. 2003; Feinberg and Tycko 2004).

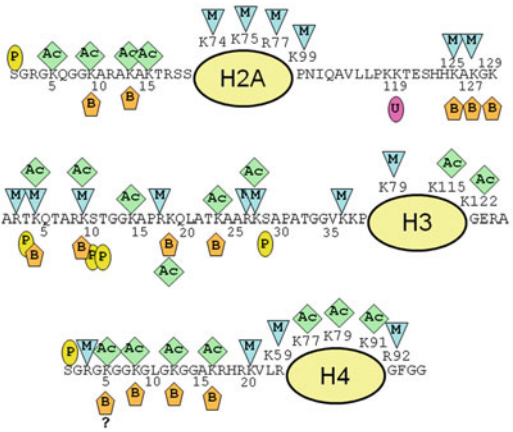

Fig. 9.2 Modifications in histones H2A, H3, and H4. Biotinylation sites in histone H2B are unknown. Abbreviations: *Ac* acetate, *B* biotin, *M* methyl, *P* phosphate, *U* ubiquitin. "?" = awaits confirmation

9.4 Biotinylation of Histones

Biotinylation of histones is a novel epigenetic mark that was discovered in the Zempleni laboratory (Bao et al. 2011a; Camporeale et al. 2004; Chew et al. 2006; Kobza et al. 2005; Kobza et al. 2008; Stanley et al. 2001) and subsequently confirmed by two independent laboratories (Bailey et al. 2008; Ghosh 2009). To date, 12 distinct biotinylation sites have been identified in histones (Fig. 9.2), and all known species of biotinylated histones are gene repression marks (Camporeale et al. 2007a; Chew et al. 2008; Gralla et al. 2008; Pestinger et al. 2011). Biotinylation marks appear to be more abundant in histones H3 and H4 than in other histones. Evidence suggests that nucleosomal condensation increases in response to biotinylation of K12 and possibly other target sites in histone H4 (Filenko et al. 2011).

Holocarboxylase synthetase (HLCS) plays a pivotal role in covalently linking biotin to histones (Bao et al. 2011a; Camporeale et al. 2006, 2007; Kobza et al. 2008). Consistent with the important roles of HLCS in epigenomics, no living HLCS null individual has ever been reported, indicating this condition may cause embryonic lethality. HLCS knockdown studies (~30 % residual activity) produced phenotypes such as decreased life span and heat resistance in *Drosophila melanogaster* (Camporeale et al. 2006), and aberrant gene regulation in human cell lines (Chew et al. 2008; Gralla et al. 2008; Pestinger et al. 2011). Mutations have been identified and characterized in the human *HLCS* gene. These mutations cause a substantial decrease in HLCS activity and metabolic abnormalities (National 2008; Suzuki et al. 2005). Unless diagnosed and treated early, HLCS deficiency appears to be uniformly fatal (Thuy et al. 1999).

HLCS is present in both nuclear and extranuclear structures (Chew et al. 2006; Narang et al. 2004). Nuclear HLCS is a chromatin protein (Camporeale et al. 2006); its binding to chromatin is mediated by physical interactions with histones H3 and H4 (Bao et al. 2011a). Our knowledge of HLCS regulation consists of the

following: (a) Both the abundance of HLCS mRNA and the nuclear translocation of HLCS depend on biotin (Gralla et al. 2008). (b) The human HLCS promoter has been tentatively identified (Warnatz et al. 2010) but not yet characterized in great detail. (c) The expression of HLCS is repressed by miR-539 (Bao et al. 2010). (d) HLCS-dependent histone biotinylation cross talks with cytosine methylation in gene regulation. When cytosine methylation marks are erased by treatment with 5-aza-2'-deoxycytidine, the expression of HLCS decreases compared with untreated controls. The effects of 5-aza-2'-deoxycytidine on HLCS expression are partly mediated by cytosine demethylation of the promoters in the two human *miR-153* genes, leading to high levels of miR-153 and, subsequently, miR-153-dependent degradation of HLCS mRNA (Bao et al. 2011b).

The binding of biotin to histones is a reversible process, but the identity of the histone debiotinylase is uncertain. Circumstantial evidence indicates that biotinidase has histone debiotinidase activity (Ballard et al. 2002; Chew et al. 2007). Biotinidase has histone biotinyl transferase activity in vitro (Camporeale et al. 2004; Hymes et al. 1995), but HLCS appears to be more important than biotinidase for catalyzing histone biotinylation in vivo (Camporeale et al. 2006). Presumably, the histone transferase activity of biotinidase is an artifact caused by artificially high concentrations of the biotin donor biotin-ε-lysine in vitro, thereby shifting the reaction equilibrium toward biotinylation of histones.

Histone biotinylation is a comparably rare event in human tissues (i.e., <0.001 % of histones are biotinylated) (Bailey et al. 2008; Kuroishi et al. 2011; Stanley et al. 2001); however, the abundance of an epigenetic mark is not an indicator of biological importance. For example, serine-14 phosphorylation in histone H2B and histone poly(ADP-ribosylation) are detectable only after induction of apoptosis and major DNA damage, respectively, but the role of these epigenetic marks in cell death is unambiguous (Boulikas 1988, 1989; Cheung et al. 2003). The abundance of histone biotinylation marks is much greater in confined genomic loci compared with bulk histones. For example, about one out of three molecules of histone H4 is biotinylated at K12 (Wijeratne et al. 2010). Please note that about 50 % of the histones are biotinylated in *Candida albicans* chromatin (Ghosh 2009).

While the abundance of biotinylated proteins depends on biotin supply in adults (Chew et al. 2008; Stratton et al. 2006) and human cell cultures (Camporeale et al. 2007a; Chew et al. 2008; Gralla et al. 2008; Pestinger et al. 2011), human biotin requirements are still unknown (National 1998). Depletion of histone biotinylation causes deregulation of genes (Camporeale et al. 2007b; Gralla et al. 2008; Pestinger et al. 2011). The production of viral particles, the frequency of retrotransposition events, and the number of chromosomal abnormalities increase when long terminal repeats are derepressed by biotin depletion or HLCS knockdown in cell cultures, humans, and *Drosophila* (Chew et al. 2008). Retrotransposition events can also cause cancer (Check 2003; Darai-Ramqvist et al. 2008; Eden et al. 2003; Fan 2007; Feinberg and Tycko 2004; Kazazian and Moran 1998; Smit 1999). Thus, biotin deficiency may be a risk factor for cancer formation. Recently, we have proposed an alternative model to explain the roles of biotin in epigenetic mechanisms of gene regulation (Kuroishi et al. 2011). According to that model, biotin regulates the

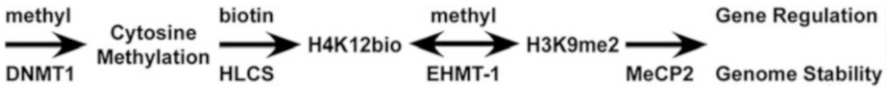

Fig. 9.3 Epigenomic synergies between diet-dependent methylation and biotinylation events. Abbreviations: *DNMT1* DNA methyltransferase 1, *EHMT-1* euchromatic histone methyltransferase, *H3K9me2* K9-dimethylated histone H3, *H4K12bio* K12-biotinylated histone H4, *HLCS* holocarboxylase synthetase, *MeCP2* methyl-CpG-binding domain protein 2

assembly of a HLCS-containing multiprotein gene repression complex. This protein complex mediates gene repression through histone methylation and histone deacetylation events, whereas histone biotinylation is a mere side product created by this complex with no meaningful biological functions.

It is now widely appreciated that nutrients may have synergistic effects in gene regulation by epigenomic mechanisms. For example, while folate deficiency alone is typically insufficient to impair gene regulation, a combined deficiency of folate and other methyl donors can have detrimental effects for health (Christman 2003; Cooney 2008, 2009; Kirkland et al. 2007; Wolff et al. 1998). Evidence also demonstrates the existence of cross talk among biotinylation and methylation marks in maintaining genome stability (Camporeale et al. 2007a; Chew et al. 2008; Gralla et al. 2008; Pestinger et al. 2011). Specifically, we reported that histone biotinylation is substantially impaired when cytosine methylation marks are erased by treating cells with 5-aza-2'-deoxycytidine but that the depletion of histone biotinylation marks does not affect cytosine methylation (Chew et al. 2008). These previous studies suggest that biotinylation of histones depends on prior methylation of cytosines and that H3K9me2 marks cross talk with K12-biotinylated histone H4 (H4K12bio) in the repression of long terminal repeats (Fig. 9.3).

Derepression of retroelements by biotin depletion and HLCS deficiency unambiguously links biotin status with cancer risk; however, the causal links between histone biotinylation and the teratogenic effects of biotin deficiency remain to be demonstrated (Mock 2009; Watanabe 1983). The human requirement for biotin is unknown (National 1998). Thus, only recommendations for Adequate Intake are available for biotin. These recommendations are based solely on the intake of biotin in the general, apparently healthy, population (National 1998). This approach is flawed in the case of biotin where dietary intake data are only crude estimates. Currently, no studies are available that quantified biotin in foods by using chemically specific assays (Zempleni and Mock 2000), and it is unclear whether intake estimates exceed or underestimate the true biotin intake. Also, the "normal state" is defined by using biotin-dependent carboxylases or urinary metabolites as markers while ignoring the apparently subtle changes occurring at the chromatin level.

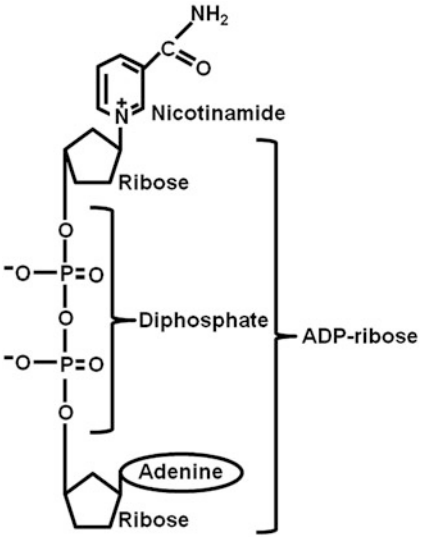

Fig. 9.4 Chemical structure of nicotinamide adenine dinucleotide

9.5 Poly(ADP-ribosylation) of Histones

All five major classes of histones are targets for poly(ADP-ribosylation), but the mark is more abundant in histones H1 and H2B than in other classes (Boulikas et al. 1990; Kim et al. 2005). Lysine residues in histones are among the prime poly(ADP-ribosylation) sites (Messner et al. 2010). Poly(ADP-ribosylation) of histones is catalyzed by poly(ADP-ribose) polymerase and depends on nicotinamide adenine dinucleotide (NAD+) as ADP-ribose donor; nicotinamide is released in this reaction (Fig. 9.4). Poly(ADP-ribosylation) is characterized by the binding of multiple subunits of ADP-ribose. Branching of the poly(ADP-ribose) chains may occur every 30–50 residues and involves ribose-ribose 1" → 2' bonds (Kim et al. 2005; Miwa et al. 1981). The abundance of poly(ADP-ribosylation) marks is low in normal cells but can increase substantially in response to treatment with mitogens and DNA damaging agents (Boulikas 1988; Boulikas et al. 1990). It has been suggested that poly(ADP-ribosylated) histones are intermediates in nuclear processes that involve DNA strand breaks including repair, replication, and recombination (Boulikas 1990; Boulikas et al. 1990; Kim et al. 2005; Malanga and Althaus 2005). It is speculated that poly(ADP-ribose) can induce free DNA domains by removing histones from specific nucleosomes whose DNA has been damaged (Boulikas 1993). The massive negative charge introduced by poly(ADP-ribosylation) might play a role in histone removal. Poly(ADP-ribosylation) of histones is a reversible process; ADP-ribose residues are removed by poly(ADP-ribose) glycohydrolase (Miwa et al. 1981).

The water-soluble vitamin niacin (as nicotinamide) is an essential building block of NAD+, and poly(ADP-ribosylation) of histones depends on niacin supply. Please note that NAD+ can also be derived from dietary sources other than niacin. Humans

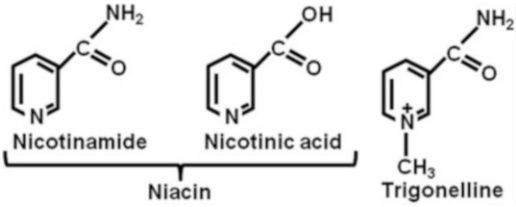

Fig. 9.5 Chemical structures of nicotinamide, nicotinic acid, and trigonelline

can convert the essential amino acid L-tryptophan to niacin (National 1998), and trigonelline can be demethylated to produce nicotinic acid, which is a bioactive niacin derivative (Fig. 9.5). Coffee is a good source of trigonelline, and it can contribute meaningful quantities of niacin to the daily intake (Casal et al. 2000). Niacin deficiency is rare in Western societies, but might be observed in societies where most of the dietary niacin comes from plant-based foods. In plants, most of the niacin is present as nicotinic acid esters with various macromolecules, which have a low bioavailability (Mason et al. 1973). Nicotinic acid can be released by alkaline treatment during cooking such as during preparation of corn-based tortillas. There are no published reports linking dietary niacin with the cellular response of DNA strand breaks in humans.

9.6 Ubiquitination and Sumoylation of Histones

Ubiquitin is a 76-amino acid protein that is covalently attached to K119 in histone H2A and K120 in histone H2B via an isopeptide bond with G76 in ubiquitin (Zhang 2003). About 5–15 % and 1–2 % of histones H2A and H2B, respectively, are ubiquitinated (Robzyk et al. 2000; West and Bonner 1980). Histones H3 and H1 are also targets for ubiquitination, but the abundance of ubiquitination marks in these histones is low (Chen et al. 1998; Pham and Sauer 2000). Ubiquitination of histone H2B is catalyzed by Rad6 (or its mammalian orthologs HR6A and HR6B) and Bre1 (or its human orthologs) (Hwang et al. 2003; Koken et al. 1991; Pickart 2001). The enzymes responsible for the ubiquitination of histone H2A have not yet been identified. Apparently, $TAF_{II}250$ plays a role in the ubiquitination of histone H1 (Pham and Sauer 2000). At least four lysine residues in ubiquitin (i.e., K11, K29, K48, and K63) can serve as attachment sites for additional ubiquitin molecules, thereby creating polyubiquitin chains (Pickart 2001). The majority of ubiquitinated histone H2A is monoubiquitinated, but polyubiquitinated H2A has also been detected (Nickel et al. 1989). In contrast, no polyubiquitinated histone H2B has been reported to date. Deubiquitination of histones is catalyzed by isopeptidases such as Ubp8 (Henry et al. 2003; Wilkinson 2000); at least 19 histone deubiquitinases have been identified in yeast (DAndrea and Pellman 1998). Sequential ubiquitination and deubiquitination are both involved in transcriptional activation in a process that is mediated by methylation of K4, K36, and K79 in histone H3 (Briggs et al. 2002).

Histones are also modified by covalent binding of small ubiquitin-like modifier (SUMO) proteins. SUMO shares 18 % identity with ubiquitin and has a similar 3D structure (Melchior 2000). Sumoylation of lysine residues in histones is mediated by the ubiquitin-like protein SUMO-1 conjugating enzyme, UBC9, and it plays a role in gene repression. Sumoylation marks can be removed enzymatically by ULP-related proteases (Nathan et al. 2003).

As of today, there are no published reports linking nutrient intake to aberrant patterns of histone ubiquitination and sumoylation. Theoretically, one can envision scenarios where impaired protein synthesis in cells might affect ubiquitination and sumoylation, but this is pure speculation.

9.7 Conclusions

While the link between diet and epigenetic mechanisms is most apparent for dietary methyl donors (e.g., folate, choline, and betaine) (Kouzarides and Berger 2007; Li and Bird 2007), it is now widely appreciated that other dietary molecules also modify the epigenome. Examples include the biotin-dependent assembly of gene repression complexes (Kuroishi et al. 2011), the pantothenic acid-dependent generation of acetyl-CoA (Garrett and Grisham 1995) as a substrate for acetylation of histones, the curcumin-dependent inhibition of histone acetyl transferases (Morimoto et al. 2008), the niacin-dependent deacetylation of histones by class III histone deacetylases (HDACs) (Boily et al. 2008; Dokmanovic et al. 2007), the butyrate- and sulforaphane-dependent inhibition of HDACs (Cummings et al. 2001; Davie 2003; Ho et al. 2009; Myzak et al. 2006), the iron-, riboflavin-, and calcium-dependent demethylation of histones (Tsukada et al. 2006; Wang et al. 2004, 2009), and the niacin-dependent poly(ADP phosphorylation) of histones (Boulikas et al. 1990; Kim et al. 2005; Messner et al. 2010). Nutrient-dependent modification of the epigenome is an exciting field of research, because diet is the one environmental factor that the entire population is exposed to on a daily basis during all stages of life, and where exposure can be modified by lifestyle choices.

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Chapter 10
Chromatin Switching and Gene Dynamics Associated with Type 2 Diabetes

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Abstract Type 2 diabetes (T2DM) is a chronic disease with a rapidly increasing global burden. An early event in the disease is deregulation of glycaemic control resulting in periods of hyperglycaemia. Large-scale clinical studies have shown that complications resulting from this hyperglycaemia can be manifest long after glycaemic control has been restored (UKPDS, Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33) UK Prospective Diabetes Study (UKPDS) Group. Lancet 352:837–852, 1998; Chalmers J, Cooper ME, UKPDS and the legacy effect. N Engl J Med 359:1618–1620, 2008), a phenomenon known as the "legacy effect" (Holman RR et al., 10-year follow-up of intensive glucose control in type 2 diabetes. N Engl J Med 359:1577–1589, 2008). Such continued development of cardiovascular complications, which result from prior exposure to hyperglycaemia, has led to the proposal of a "metabolic memory" (Cooper ME, Metabolic memory: implications for diabetic vascular complications. Pediatr Diabetes 10:343–346, 2009). Such a hypothesis suggests that a transient exposure to hyperglycaemia results in persistent changes in gene expression that are not reversed merely by restoring glycaemic control. Support for early, persistent changes came from the Diabetes Control and Complications Trial (DCCT) which revealed that early glycaemic control in diabetic patients led to sustained benefits and better outcomes (Cooper ME, Metabolic memory: implications for diabetic vascular complications. Pediatr Diabetes 10:343–346, 2009), and it has recently been proposed that minimising early exposure to hyperglycaemia is paramount (Aizawa T, Funase Y, Intervention at the very early stage of type 2 diabetes. Diabetologia 54:703–704; author reply 707–708, 2011). Currently, the most attractive potential mechanism responsible for the "legacy effect" is epigenetic, manifested by changes in DNA methylation and/or posttranslational modifications

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on histones. Over the last decade, numerous studies have identified correlations of specific epigenetic marks with type 2 diabetes, and more recently the mechanisms by which these changes lead to persistent alterations in gene expression levels have been interrogated.

Keywords Crosstalk Disease • Diabetes • DNA methylation • Histone acetylation • Histone methylation • Hyperglycaemia • Metabolic memory • Vascular

Abbreviations

CBP	CREB-binding protein
COMPASS	Complex proteins associated with Set1
CREB	cAMP response element binding protein
DCCT	Diabetes Control and Complications Trial
DNMT	DNA methyltransferase
H3K4me1	Monomethylated Histone H3 lysine 4
H3K4me2	Dimethylated histone H3 lysine 4
H3K4me3	Trimethyl histone H3 lysine 4
H3K9me2	Dimethyl histone H3 lysine 9
HDAC	Histone deacetylase
HAT	Histone acetyltransferase
HMT	Histone methyltransferase
HUVECS	Human umbilical vein endothelial cells
IL	Interleukin
JMJD2	Jumonji domain 2
LSD1	Lysine-specific demethylase 1
NFAT	Nuclear factor of activated T cell
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator-1 alpha
SAM	S-adenosylmethionine
shRNA	Short hairpin RNA
T2DM	Type 2 diabetes mellitus
TGF	Transforming growth factor
TNF	Tumour necrosis factor
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cell

10.1 Introduction

The proposal that epigenetic mechanisms contribute to the progression and complications associated with type 2 diabetes has been around for many years. In 1990, Roy et al. reported a persistent change in the levels of fibronectin mRNA in tissues from diabetic rats and cultured human endothelial cells after a short period of exposure to high levels of glucose (50 mmol/l) and suggested heritable changes in chromatin structure as a potential mechanism underlying their observations (Roy et al. 1990). More recently, such a suggestion has been gaining support with further studies that provide evidence supporting the notion that changes in chromatin are induced during diabetes and by exposure to hyperglycaemia (Maier and Olek 2002; Ling and Groop 2009; Reddy and Natarajan 2011; Villeneuve et al. 2011). Though there is still relatively limited understanding of the complete molecular details, changes in posttranslational modifications on histones have been associated with the gene expression changes seen in diabetes (Pirola et al. 2010). Alterations in several types of posttranslational modification including DNA methylation, histone acetylation and histone methylation have been observed in animal and cell models of diabetes and associated with changes in gene expression (Pirola et al. 2010).

10.2 DNA Methylation

Methylation of DNA involves the addition of a methyl group to specific cytosine residues of DNA by DNA methyltransferase enzymes (DNMTs). DNA methylation was the first described epigenetic mark and methylation of the DNA for a specific gene generally leads to silencing of expression of that gene. DNA methylation has long been known to function as a cellular memory, and the mechanisms by which DNA methylation can be inherited over many cell generations are well understood (Jaenisch and Bird 2003). There is some good correlative data which, collectively, points an accusing finger at DNA methylation for a role in metabolic memory and diabetes, reviewed in Maier and Olek 2002. DNMTs use the donor S-adenosylmethionine (SAM) for the methyl group, and perturbations in the level of available SAM, which is influenced by diet, can have some profound effects on the extent of DNA methylation. Low levels of SAM are associated with global hypomethylation and may contribute to formation of cancers (Singh et al. 2003). Individuals with diabetes show reduced concentrations of SAM in their blood suggesting a potential hypomethylation of genes in these individuals and potential for perturbed gene expression levels (Poirier et al. 2001). In addition to global effects on methylation resulting from fluctuations in the levels of SAM, DNA methylation is also influenced by many cell signalling pathways. Methylation of cytosines within specific genes is controlled by the recruitment of DNMTs by regulatory cofactors in response to local environmental cues (Szyf and Detich

2001; Turek-Plewa and Jagodzinski 2005). DNA methylation was originally thought to be an essentially irreversible modification that could only be removed through the process of cell division and DNA replication. However, there is now compelling evidence supporting a mechanism of active demethylation, though a precise molecular explanation for this process is still the subject of some conjecture (Wu and Zhang 2010). Given that DNA methylation can be regulated by the local cellular environment and, once deposited, methyl marks have the potential to be stably maintained over many cell generations, it is an excellent candidate for a mechanism responsible for cellular memory in type 2 diabetes. Consistent with such notion, a role for DNA methylation has recently been identified as a contributory mechanism to learning and memory in the brain, and memories can be maintained many years after the event that initiated them (Day and Sweatt 2010). The discovery of active DNA demethylation does provide some optimism that any cellular memory encoded by DNA methylation could eventually be reset with the use of an appropriate intervention.

Though there is no definitive experimental data that has unequivocally demonstrated that DNA methylation is responsible for metabolic memory in type 2 diabetes, many studies have reported associations between altered methylation in models of diabetes and as a result of hyperglycaemia in culture. In the Zucker (type 2) diabetic fatty rat, increased levels of global DNA methylation were observed at 12 and 21 weeks in the liver although not in the kidney or heart (Williams and Schalinske 2011). The authors also observed an increase in expression of DNMT1 in the liver at 21 weeks, but not at 12 weeks. The significance of this increase is not entirely clear, as it follows the increase in DNA methylation; it cannot be the driver for it though it may be involved in maintaining high methylation levels. Cultures of the human hepatocellular carcinoma cell line, HepG2, also show an increase in DNMT activity and global DNA methylation in response to a 72-h exposure to high (16.7 mmol/l) glucose (Chiang et al. 2009).

The db/db mouse contains a natural mutation in the leptin receptor gene and is a well-established model of diabetes, providing an in vivo model to study the progression of and mechanisms underlying diabetes (Lee et al. 1996). In these mice, the gene encoding the insulin-like growth factor 1 receptor (Igfr1) shows enhanced levels of DNA methylation and reduced levels of mRNA expression in skeletal muscle compared to wild-type mice (Nikoshkov et al. 2011). Human patients with type 2 diabetes also show increased methylation within the gene for peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α) within skeletal muscle, and the levels of methylation showed inverse correlation with mRNA levels (Barrès et al. 2009). In pancreatic islet cells from patients with type 2 diabetes, the insulin gene shows increased levels of DNA methylation and decreased levels of mRNA compared to nondiabetic individuals (Yang et al. 2011). Consistent with a role for hyperglycaemia in this response is the observation that rat pancreatic beta cells showed similar changes when cultured in high glucose (16.7 mmol/l) for 72 h (Yang et al. 2011). These recent data provide some suggestions that perturbation in DNA methylation resulting directly or indirectly

from hyperglycaemia results in changes of gene expression relevant to type 2 diabetes in different tissues.

Technological advances such as microarray analysis and high-throughput sequencing techniques over recent years now allow researchers to interrogate the entire genome and uncover novel target genes whose involvement was previously unrecognised. One recent study used a high-throughput sequencing approach to examine the epigenetic landscape of cultured human aortic endothelial cells exposed to hyperglycaemia (30 mmol/l glucose). The analysis revealed an increase in DNA methylation across many sites within the genome (Pirola et al. 2011). Such methylation would be predicted to result in reduced gene expression, and although this was not directly tested, it was shown that the regions of increased DNA methylation did not overlap with regions of high levels of histone acetylation which were associated with gene expression (Pirola et al. 2011).

These data suggest an involvement of DNA methylation in defining the pathophysiological phenotype of cells from individuals with type 2 diabetes. Such changes in DNA methylation may be the direct result of a short period of hyperglycaemia, and the hypothesis that such changes are responsible for, or at least contribute to, the observed metabolic memory is attractive. To date much of the data collected on DNA methylation and type 2 diabetes is correlative, and it is not clearly understood if DNA methylation changes cause or are the result of complications associated with type 2 diabetes. Hopefully such questions will be addressed in the future.

10.3 Histone Methylation

Like DNA, histones are also a substrate for methylation. Both lysine and arginine residues on histones can be methylated and multiple methyl groups can be added to each. Lysine can be mono-, di- or trimethylated, while arginine can be mono-, symmetrically di- or asymmetrically dimethylated (Fig. 10.1). The consequence of the methylation is dependent on the exact nature of methylation. For example, trimethylation of histone H3 lysine 4 (H3K4me3) is associated with active gene promoters, while methylation of H3K9 is associated with gene repression. Histones are methylated by a series of enzymes known as histone methyltransferases (HMTs) and histone methylation was originally thought to be irreversible. However, over the last few years, several enzymes have been identified that that can actively remove methyl groups from histones, and it is now appreciated that histone methylation is very dynamic and contributes to many physiological responses (Shi et al. 2004; Tsukada et al. 2006). Unlike DNA methylation, the process by which posttranslational modifications such as histone methylation can be preserved across multiple cell divisions remains unclear. However, some data suggest that during DNA replication, the histones are partitioned between the old and newly synthesised DNA and that newly inserted histones are modified to match their neighbours (Xu et al. 2010). Histone methylation impacts on chromatin function by

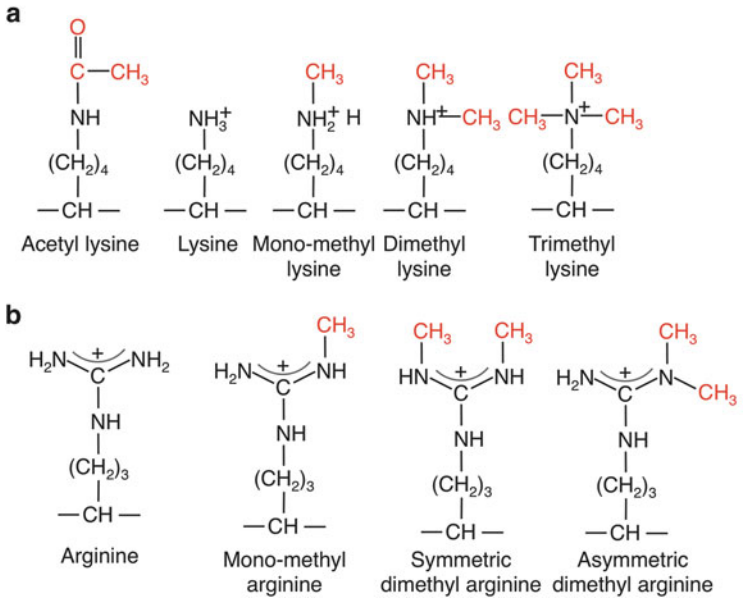

Fig. 10.1 Posttranslational modification of lysine and arginine residues in histones. (**a**) Illustration shows the results of posttranslational modifications on the structure of lysine. Acetyltransferases can add an acetyl moiety onto the lysine to give acetyl lysine (*left*) which not only provides a structural change but also removes the positive charge carried by the native lysine. Methyltransferases can add one, two or three methyl groups onto the lysine to produce three forms of methylated lysine (*right*). Unlike acetylation, the addition of methyl groups does not affect the positive charge carried by the lysine. (**b**) Illustration showing the results of posttranslational modifications on the structure of arginine

influencing the set of proteins that are recruited to a specific gene. Adding or removing methyl groups on specific histone residues creates, or destroys, binding sites for specific proteins via their chromodomains, PHD fingers or Tudor domains (Chen et al. 2011; Sanchez and Zhou 2011; Yap and Zhou 2011). Ultimately the proteins recruited induce changes in the expression of the underlying genes, either directly by influencing the activity of the RNA polymerase or its associated subunits or indirectly by initiating further chromatin modifications or changes to the overall structure.

To uncover the consequences of metabolic disturbance on histone methylation, Miao et al. profiled the levels of dimethylated histone H3 lysine 4 (H3K4me, which is preferentially, but not exclusively, associated with active genes) and dimethylated histone H3 lysine 9 (H3K9me2, which is associated with repressed genes) of monocytes cultured in normal or high (25 mmol/l) glucose (Miao et al. 2007). This study identified 9 and 26 genes that showed decreased and increased H3K4me2 levels in high glucose conditions, respectively. One of the genes showing increased levels of H3K4me2 was the histone K9 demethylase JMJD2A. Increased expression of JMJD2A could reduce epigenetic silencing at other loci by

removing H3K9 methylation, inhibiting the H3K9me dependant repression at these genes and thus providing a feed forward mechanism. It is not clear that JMJD2A expression is actually increased though as no measurements of this were reported and overall the changes in H3K4me2 levels at genes did not correlate well with changes in expression of the associated mRNA. Thus, although the H3K4me levels were increased, there is not really a strong argument that the levels of JMJD2A would necessarily go up as a result. The levels of H3K9me2, which is associated with gene repression, were increased in 39 genes and decreased in 11 genes. Unlike H3K4me2 and consistent with many other studies, the changes in the levels of H3K9me2 did correlate very well with changes in gene expression (Miao et al. 2007).

Increased inflammatory gene expression has long been associated with type 2 diabetes and insulin resistance (Dandona et al. 2004), and many groups focus on the mechanisms responsible for such changes. A major driver of inflammatory gene expression is the transcription factor NF-κB (though it also has other roles too) which is regulated both by expression levels and through a cytoplasmic sequestration mechanism. In bovine endothelial cells, a transient (16 h) exposure to hyperglycaemia (30 mmol/l glucose) increased levels of NF-κB mRNA which were sustained over a 6-day period (Brasacchio et al. 2009). Increased NF-κB levels were associated with monomethylation of H3 lysine 4 (H3K4me1) most likely brought about as a result of recruitment of the histone methyltransferase SET7 to the NF-κβ promoter (Brasacchio et al. 2009). Indeed knockdown of SET7 abolished the increase in NF-κB in response to hyperglycaemia. As well as the addition of an activating histone modification, hyperglycaemia produced a reduction of the repressive H3K9 methylation mark by the histone demethylase, LSD1 (El-Osta et al. 2008; Brasacchio et al. 2009). Induction of NF-κB activity may also be important in modulating the VSMC phenotype during diabetes, and cultured porcine VSMCs show increased NF-κB function when cultured in high (25 mmol/l) glucose (Yerneni et al. 1999). NF-κB activity drives expression of many inflammatory genes such as tumour necrosis factor (TNF)-α and interleukin (IL)-1β and plays a contributory role to vascular complications such as atherosclerosis. Hyperglycaemia also increases expression of a second transcription factor, NFAT, associated with proliferation and migration of VSMC as well as neointima formation. Mouse cerebral arteries cultured in 20 mmol/l glucose and blood glucose levels of 17.6 mmol/l in mice resulted in an increase of nuclear NFAT activity and potentially contribute to vascular dysfunction in patients with type 2 diabetes (Nilsson et al. 2006; Nilsson-Berglund et al. 2010).

Consistent with increased function of transcription factors that regulate inflammatory associated genes, VSMC from type 2 diabetic mice (db/db) show persistent changes in inflammatory gene expression even after isolation and culturing in vitro (Li et al. 2006). Correlating with this increased gene expression are reduced levels of the repressive histone mark H3K9me3 at the promoters of inflammatory genes (Villeneuve et al. 2008). The reduced levels of H3K9me3 are associated with reduced expression and a reduced recruitment of the histone methyltransferase Suv39h1 (Fig. 10.2a), which is the key methyltransferase responsible for the

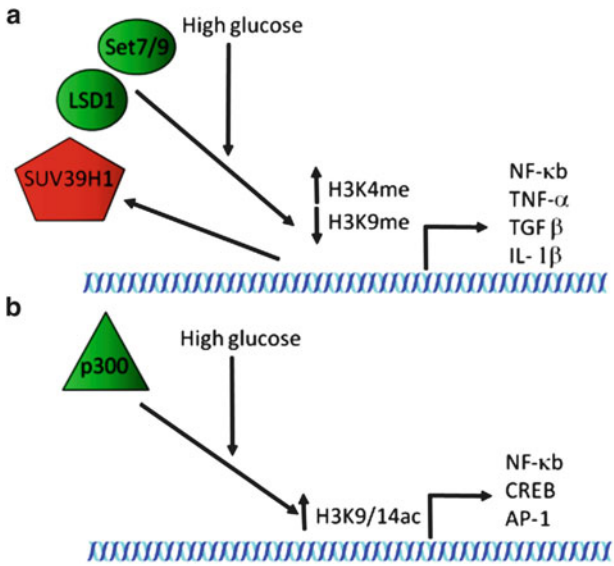

Fig. 10.2 Changes in chromatin modifications in response to hyperglycaemia. Exposure of endothelial cells to high concentrations of glucose leads to changes in the recruitment of different chromatin-modifying enzymes to specific gene promoters and a subsequent change in the expression levels of those genes. (**a**) In the presence of high glucose, the histone methyltransferase Set7/9 and the histone demethylase LSD1 both show increased recruitment to chromatin in endothelial cells, while recruitment of the histone methyltransferase SUV39H1 is reduced. Changes in the recruitment of these enzymes result in an increase of H3K4me and decrease in H3K9me and increased expression of genes including NF-κb, TNF-α, TGF-β and IL-β. (**b**) In the presence of high glucose, the histone acetyltransferase p300 shows enhanced recruitment to some genes and leads to increased levels of H3K9 and H3K14 acetylation levels and expression of genes such as NF-κb, CREB and AP-1. The precise molecular details connecting high glucose levels to the recruitment of these chromatin-modifying enzymes are yet to be uncovered

deposition of this mark, to the promoters of inflammatory genes. Ectopic expression of Suv39h1 in VSMC from db/db mice could reverse the inflammatory phenotype and led to the decrease in inflammatory gene expression. When cultured for a 2-week period in high glucose (25 mmol/l), human VSMC also showed a decrease in the levels of H3K9me3 at inflammatory gene promoters, and consistent with the mouse model, SUV39H1 is implicated in this response. In this case, knockdown of SUV39H1 by shRNA potentiated inflammatory gene expression, while ectopic expression of SUV39H1 reduced the induction of inflammatory gene expression by TNF-α (Villeneuve et al. 2008).

Mesangial cells are smooth muscle cells which regulate capillary blood flow in the kidney and show a loss of contractility in diabetes. When exposed to high glucose (30 mmol/l) for 48 h, cultured mesangial cells showed an increased expression of several extracellular matrix-encoding genes. This increased gene expression could be inhibited by an antibody to TGF-β1 (a secreted peptide known to play a role in type 1 diabetes) suggesting the increase involves TGF-β1

activity (Sun et al. 2010). The increases in gene expression correlated with a decrease in the repressive marks H3K9me2 and H3K9me3 and an increase in the active marks H3K4me1, 2 and 3 across those genes. The changes seen in H3K4 methylation appear to involve the histone methyltransferase Set7/9 as recruitment of Set7/9 is enhanced in response to high glucose and knockdown of Set7/9 inhibits the increase in expression (Sun et al. 2010). Clearly other chromatin-modifying factors must also be involved as Set7/9 is only able to monomethylate H3K4 and has no direct effect on the levels of H3K9 methylation.

These data identify some changes in histone methylation levels at specific genes associated with type 2 diabetes. Short periods of hyperglycaemia have been suggested as the priming event for long-term progression of complications in type 2 diabetes, and it is clear that increasing the glucose levels to which cells are exposed is sufficient to trigger changes in histone methylation in some cell types. The identification of specific histone methyltransferases and demethylases that respond to hyperglycaemia should facilitate future investigations into the nature of the signalling pathways involved.

10.4 Histone Acetylation

Lysine residues on histones are subject to the addition of an acetyl group (Fig. 10.1a) by enzymes known as histone acetyltransferases (HATs), and this acetylation can be reversed by a group of enzymes known as histone deacetylases (HDACs). Inappropriate regulation of acetylation is associated with a host of human disease states; mutations within the histone acetyltransferase and CREB-binding protein (CBP) result in Rubinstein-Taybi syndrome, while HDAC inhibitors are used as anticancer agents (Kalkhoven et al. 2003; Witt et al. 2009). The addition of the acetyl group to the lysine residue neutralises the positive charge of the amino group of the lysine residue and is thought to promote a more open chromatin structure (Hansen et al. 1998). Such a change allows increased accessibility to transcription factors and RNA polymerase and thus enhances levels of gene transcription. Acetylated lysine residues are also recognised by bromodomain-containing proteins, and thus, a second mechanism by which acetylation regulates gene expression is by the recruitment of proteins and/or complexes that regulate gene expression (Zeng and Zhou 2002).

As with histone methylation, there are a selection of studies that implicate a role for histone acetylation changes in regulating the phenotype of cells in individuals with type 2 diabetes. Rats fed a high-fat diet to induce hyperglycaemia showed increased levels of histone acetylation of the fibrillin 1 gene in both the heart and the kidney. The increased levels of acetylation were associated with increased levels of fibrillin mRNA (Gaikwad et al. 2010). Monocytes from patients with diabetes also show enhanced levels of histone acetylation (Miao et al. 2004) and increased histone acetylation of some genes, e.g. Mll3 correlates with increased mRNA levels (Miao et al. 2007). In a genome-wide study using human aortic endothelial cells,

Pirola et al. identified a number of genes that were associated with increased levels of H3K9 and H3K14 acetylation subsequent to high glucose (30 mmol/l) exposure as well as a number of genes that were associated with reduced levels of acetylation (Pirola et al. 2011). Analysis of the cohort of genes that showed hyperacetylation identified regulation of apoptosis and the NF-κβ pathways as common pathways that are influenced by these gene sets. The authors however could not uncover any clear association with the hypoacetylated gene set and a specific cellular pathway. Incubation of the endothelial cells with the histone deacetylase inhibitor, suberoylanilide hydroxamic acid (SAHA), mimicked at least some of the hyperacetylation seen with hyperglycaemia. How much overlap there is between hyperglycaemia-induced hyperacetylation and that induced as a result of histone deacetylase inhibition is not known; presumably the latter would be more widespread but it may be important to interrogate the response in the future given the clinical use of histone deacetylase inhibitors to treat diseases such as cancer. In general, histone acetylation of H3K9 and H3K14 is associated with gene expression and hyperacetylation of genes in endothelial cells correlated well with an increase in mRNA levels (Pirola et al. 2011). A mechanism leading to hyperacetylated histones is suggested by experiments with human umbilical vein endothelial cells (HUVECS). Incubation of cultured HUVECS in high glucose (25 mmol/l) resulted in the increase of mRNA and protein level of the histone acetyltransferase p300 and enhanced recruitment of p300 at the NF-κB and fibronectin genes (Chen et al. 2010). Several transcription factors, including NF-κB, CREB and AP-1, were all induced by high glucose, and this induction was inhibited by knockdown of p300 while ectopic expression of p300 resulted in the increased expression of fibronectin, endothelin 1 and VEGF mRNA levels under normal levels of glucose (Chen et al. 2010).

Hyperglycaemia induces the expression of some genes in VSMC though whether VSMC shows increases in histone acetylation in type 2 diabetes similar to other cell types is not clear. However, it is likely and changes in acetylation do play an important role in VSMC proliferation and neointima formation. Inhibition of HDAC enzymes (which should presumably result in hyperacetylation) with the inhibitor Scriptaid reduced the proliferation of cultured rat VSMC by causing them to arrest at the G1 phase of the cell cycle. Additionally, in vivo administration of Scriptaid reduced the level of neointima in a mouse blood vessel injury model (Findeisen et al. 2011). Such evidence is apparently contradictory, and taken at face value, it would suggest that hyperacetylation in VSMC would be beneficial. Of course actively promoting acetylation is not quite the same as inhibiting deacetylation and these two approaches may not target the exact same set of genes.

Since it was first uncovered, regulation of histone acetylation has proven to be an important mechanism by which expression levels of genes are regulated in all tissues and in response to many pathophysiological stimuli. Clinically approved HDAC inhibitors are already available to treat some cancers so a more detailed understanding of acetylation in the progression of type 2 diabetes may uncover some potential opportunities for alternative uses of them.

10.5 Combinatorial Interactions

Histone proteins can be modified by acetylation, methylation, ubiquitination, phosphorylation and sumoylation. Many enzymes exist within the cell that can add or remove specific marks, usually only at a subset of the potential amino acids within a single histone. The availability of such an array of modifications led to the proposal of a histone code (Turner 1993; Strahl and Allis 2000) – histone modifications are read, either individually or in combination, by other proteins to bring about distinct functional outcomes. Since then, researchers have come a long way in understanding how single modifications are deposited and removed; however, it is clear that knowledge of a single epigenetic mark is not sufficient to predict functional changes in gene activity. Such observations suggest that combinations of histone modifications must interact, though our understanding of such interactions, or "crosstalk," is still in its infancy. It is known that some modifications on histones are dependent on or enhanced by the presence of others. An initial study identified a requirement for the ubiquitination of histone H2B for the COMPASS complex to methylate histone H3 in yeast (Fig. 10.3) (Dover et al. 2002). Enhanced acetylation of histones can promote the methylation of H3K4 in cardiac myocytes (Fig. 10.3) (Bingham et al. 2007), while histone deacetylation is required for demethylation of H3K4 by LSD1 (Lee et al. 2005; Shi et al. 2005).

In type 2 diabetes, no specific interaction between histone modifications or association with combinatorial marks has yet been uncovered though it is clear that knowledge of a single mark is not sufficiently predictive of functional outcomes. For example, the mRNA levels of Mll3 are increased in monocytes from patients with type 2 diabetes, yet the gene is associated with an increase in the repressive mark H3K9me2. Similarly, histone acetylation levels of the SBF1 gene are also increased in monocytes which are exposed to high glucose, but the mRNA levels are decreased (Miao et al. 2007). Clearly our understanding of the precise relationship with specific epigenetic marks and gene activity is still incomplete, and we need to discover how individual marks interact with each other to produce a specific outcome. Advances in technology are enabling researchers to study a greater number of histone marks across entire genomes (Barski et al. 2007) as well as identifying epigenetic marks that coexist on the same nucleosome (Eberl et al. 2011), while interrogation of the vast amounts of data being generated is uncovering functional associations between marks and uncovering sets of chromatin marks or chromatin signatures (Hon et al. 2008, 2009).

10.6 Conclusions

Periods of transient hyperglycaemia in patients with type 2 diabetes appear to induce persistent changes that impact on an individual's health after many years. There is compelling evidence for an epigenetic mechanism involving changes in the

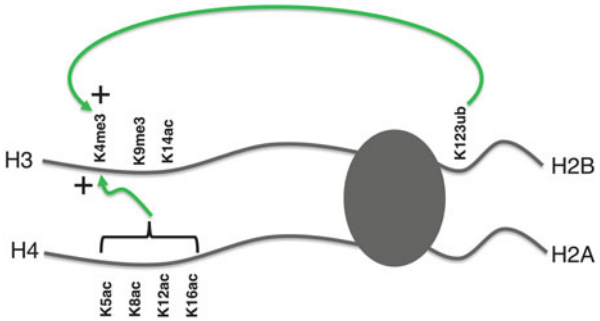

Fig. 10.3 Combinatorial interactions between chromatin modifications. Shown are two examples of the presence of one chromatin modification influencing the deposition of another. Ubiquitination of H2BK123 is required for methylation of H3K4 by the COMPASS complex, while acetylation of histone H4 promotes the methylation of H3K4. Other interactions are known, and for a more in-depth discussion, see Wood (2011)

posttranslational modifications of DNA and histones around specific sets of genes which alter their long-term expression levels. Understanding which genes are affected and which specific epigenetic marks are involved should open up new avenues for treatment and/or management of complications associated with diabetes. Gaining an appreciation of the timeline of chromatin changes (and in which cells these are important) and how these relate to changes in gene expression levels and disease progression may also provide new opportunities for more accurate diagnoses that do not rely on quantifying the current metabolic state of an individual. Such data may provide a better predictive model regarding future potential complications for an individual who has suffered a period of metabolic disturbance.

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Chapter 11
Developmental Epigenetic Programming in Diabetes and Obesity

Caroline L. Relton, George Davey-Smith, and Susan E. Ozanne

Abstract It is well established from animal and human studies that suboptimal environmental exposures during fetal and early postnatal life can have long-term effects on metabolic health, including the risk of developing type 2 diabetes and obesity. Nevertheless, the mechanisms by which an event in early life can have phenotypic effects many years later, following multiple rounds of cell division are poorly defined. Alterations in epigenetic modifications are emerging as a plausible mechanism underlying such developmental programming, not least because they are normally retained following mitotic cell division. There is good evidence showing that epigenetic patterns are altered by early environmental factors known to be associated with obesity or type 2 diabetes. Since these changes in epigenotype are present in early postnatal life, they could represent biomarkers of disease risk. If a causal relationship between changes in the epigenotype and long-term health causality can be established, this raises the possibility of also using epigenetic changes as therapeutic targets for intervention and prevention.

Keywords Biomarkers • Diabetes • Growth patterns • Maternal diet • Nutrition • Programming

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Abbreviations

DOHaD	Developmental origins of health and disease
FTO	Fat mass and obesity associated gene
HNF4A	Hepatic nuclear factor 4 alpha gene
IGF1	Insulin-like growth factor 1
IGT	Impaired glucose tolerance
IL13RA2	Interleukin 13 receptor alpha 2
INS	Insulin
IUGR	Intrauterine growth restriction
KCNQ1	Potassium voltage-gated channel KQT-like subfamily member 1
LINE-1	Long interspersed element 1
MC4R	Melanocortin 4 receptor
NPAS2	Neuronal PAS domain protein 2
PEPCK	Phosphoenolpyruvate carboxykinase
POMC	Proopiomelanocortin
PPARGC1A	Peroxisome proliferator-activated receptor gamma co-activator 1 alpha
TFAM	Transcription factor A mitochondrial

11.1 Introduction

It is well established that events in very early life can impact on our long-term health and, in particular, our risk of developing adult-onset diseases such as type 2 diabetes and obesity. Despite extensive research efforts, the fundamental molecular mechanisms underlying this process still remain relatively poorly defined. Over the last decade, epigenetics has emerged as the leading candidate to explain this developmental programming phenomenon (Waterland and Jirtle 2003; Jirtle and Skinner 2007).

In this chapter, we first summarize the results of studies in both humans and animals that demonstrate early environmental exposures, particularly early nutrition, can influence an individual's risk of developing type 2 diabetes and obesity as an adult (Sect. 11.2). We then introduce the concept that epigenetics may provide the molecular framework by which such developmentally programmed effects are mediated (Sect. 11.3) and describe the evidence to support this concept (Sect. 11.4). Many of the studies to date simply describe associations; hence, we go on to highlight the need to establish causality (Sect. 11.5). In the last section of this chapter, we look to the future of the field and the potential that epigenetic modifications have as biomarkers for disease risk and therapeutic targets (Sect. 11.6)

11.2 Developmental Programming: The Evidence

The process whereby an event occurring during a critical period of development results in a long-term or permanent effect on the structure and function of an organism has been termed developmental programming (Lucas 1991). Great interest in this phenomenon over the past 20 years has been prompted by the results of a series of epidemiological studies that show fetal and early neonatal growth patterns are associated with a number of diseases generally occurring in later adulthood. Initial studies linked birth weight with type 2 diabetes and cardiovascular disease, showing associations across the full birth weight spectrum (Barker et al. 1989; Hales et al. 1991). Low birth weight individuals were six times more likely to have type 2 diabetes at age 64 compared to the highest birth weight individuals (Hales et al. 1991). These initial findings are now replicated in many ethnic groups in over 40 studies worldwide (Whincup et al. 2008). Additionally, in populations with a high prevalence of obesity, there is also an increased risk of type 2 diabetes at the very high birth weight end of the spectrum. This is thought to reflect the increased risk of type 2 diabetes in the macrosomic offspring of women who develop gestational diabetes during pregnancy (Dabelea et al. 1999). In light of the growing prevalence of obesity in women of childbearing age, and the increased risk of gestational diabetes, recent research emphasis is now directed towards determining the role of excess fetal weight gain and its long-term effects on metabolic health (Poston et al. 2011).

Patterns of growth during the early postnatal period are also associated with differences in later risk of metabolic disease, especially in relationship to obesity. Accelerated postnatal growth is associated with increased risk of later obesity (Ong and Loos 2006) and cardiovascular disease (Singhal et al. 2007). In contrast, slow growth during lactation is linked to reduced risk of these diseases (reviewed in Singhal 2010). Whether these associations are causal, and if so their mechanistic basis, is not established; however, there is considerable evidence to indicate that the early environment, and in particular early nutrition, plays an important role. This has been termed the developmental origins of health and disease hypothesis (DOHaD) (Barker 2004).

11.2.1 Human Studies

The strongest evidence in support of the role of the environment in mediating the relationship between birth weight and type 2 diabetes comes from twin studies. Poulsen and colleagues investigated a cohort of twins in their 60s and revealed that in both monozygotic (identical) and dizygotic (nonidentical) twins who were discordant for type 2 diabetes, the diabetic twins have a lower average birth weight than their normoglycemic co-twins (Poulsen et al. 1997). Studies of younger twins in Italy (mean age 32) revealed similar findings (Bo et al. 2000). If it is assumed that

the monozygotic twins are genetically identical, then differences in birth weights within twin pairs are likely to be due to the fetal environment. This provides strong evidence for the importance of the fetal environment in mediating the relationship between birth weight and the later development of type 2 diabetes.

Directly assessing the impact of maternal nutrition on the health of the offspring in humans is complex. Most evidence for direct effects of maternal nutrition has therefore been gained from the studies of individuals who were in utero during conditions of famine. The earliest study to link famine during fetal life with subsequent risk of type 2 diabetes was carried out by Ravelli and colleagues on a Dutch cohort (Ravelli et al. 1998). The Dutch Hunger Winter occurred for around 5 months over the winter of 1944 in a population that before the famine was reasonably well nourished. When studied in their 50s, those individuals who were in utero during the famine were less glucose tolerant than those born either the year before the famine or the year after the famine (Ravelli et al. 1998). More recently a much larger study of individuals in utero during the Chinese famine (1959–1961) showed a similar relationship between famine during fetal life and glucose homeostasis in adulthood (Li et al. 2010). Both of these studies demonstrated that a nutritionally rich environment in later life exacerbated the detrimental effects of the famine on glucose tolerance. This suggests that there is an interaction between conditions experienced in utero and those experienced postnatally.

There is accumulating evidence in humans to suggest that nutrition during early postnatal life can also influence long-term metabolic health, especially in relationship to the risk of developing obesity. Initial observational studies demonstrated that breastfed infants were at a reduced risk of becoming obese later on in life than formula-fed infants (Arenz et al. 2004; Harder et al. 2005). Comparisons of cohort studies where confounding structures are different (Brion et al. 2011) and randomized controlled trial data, however, did not support a causal relationship (Kramer et al. 2009). In contrast, recent experimental intervention studies and randomized control trials indicate that nutrition during infancy directly influences later risk of developing obesity and cardiovascular disease, with low levels of nutrient intake during the neonatal period protecting against these conditions (Singhal et al. 2007, 2010). Early observations from the Dutch Hunter Winter Cohort also indicated that low nutrient intake during early postnatal life reduced risk of obesity at age 19 (Ravelli et al. 1976).

11.2.2 Animal Models

Epidemiological studies have therefore provided much evidence for the role of the early environment in influencing the long-term risk of developing type 2 diabetes and obesity. In parallel, there is now considerable evidence from animal models in support of the DOHaD hypothesis. Animal models have the advantage of enabling controlled manipulations of the early environment and tissue sampling throughout the life course of both the parents and the offspring. The majority of studies have

used rodents; however, other animal models showing proof of principle include nonhuman primates, sheep, and pigs.

Initial studies in animals focused on the undernutrition/low birth weight end of the spectrum using models of total caloric deficiency (i.e., 70–30 % of ad libitum intake), macronutrient deficiency (i.e., restricting protein content from 20 % to 5 % of ad libitum intake), placental insufficiency through intrauterine artery ligation, and overexposure to glucocorticoids. The results of these studies show an age-dependent loss of glucose tolerance in the offspring (Lindsay et al. 1996; Garofano et al. 1998; Vickers et al. 2000; Petry et al. 2001; Simmons et al. 2001).

To reflect the growing prevalence of obesity in Westernized societies in the twenty-first century, recent students in animal models addressed the potential detrimental effects of maternal overnutrition and obesity on long-term metabolic health of the offspring. Rodents effectively regulate their food intake of high fat calorie dense food. Therefore, feeding high fat diets in general does not cause excess weight gain. To overcome this, highly palatable diets rich in simple sugars are used to override the rodent's natural satiety signals. Studies using such models provide direct evidence that maternal diet-induced obesity leads to loss of glucose tolerance in the offspring (Bayol et al. 2008; Samuelsson et al. 2008).

Animal models also indicate that the early postnatal period is a critical time window for epigenetic dysregulation. They demonstrate that increased nutrition and growth during the suckling period is associated with increased obesity later in life (Aubert et al. 1980; Faust et al. 1980; Ozanne et al. 2004). In contrast, reduced nutrition and growth during this period of development permanently reduced weight gain (Aubert et al. 1980; Faust et al. 1980; Jimenez-Chillaron et al. 2006; Cripps et al. 2009) and conferred resistance to diet-induced obesity (Ozanne et al. 2004).

11.3 Epigenetics as a Mechanism Underlying Developmental Programming

Epigenetics has captured the headlines as the most likely mechanism to explain how exposures early in life can be captured at the molecular level and perpetuated into later life by influencing gene regulation, cellular function, and whole body metabolism.

11.3.1 Rationale Underlying Epigenetics as a Candidate Mechanism

Epigenetics embraces a wide array of modifications. These include changes in chromatin organization, the modification of DNA and proteins with specific chemical moieties, and the expression of small noncoding RNAs (details can be found

elsewhere in this book). The unifying feature is the influence these alterations have in regulating gene expression.

Fuelled by advances in technologies to assess genetic variation, the methods available to measure epigenetic variation between individuals, tissues, developmental periods, and treatment regimens are now accessible, varied, and powerful. Comprehensive reviews on the current methods to interrogate the epigenome can be found elsewhere (Laird 2010; Rakyan et al. 2011). In the context of developmental programming, much of the evidence emerging recently focuses on the role of DNA methylation in mediating the influence of early life exposures on later disease risk (Gabory et al. 2011). This may not be because this will ultimately prove to be the most important epigenetic modification, but rather it is presently the most readily measurable.

Many features of epigenetic modifications make them highly relevant to the domain of developmental programming. Firstly, epigenetic markings are dynamic and change throughout the life course (Ollikainen et al. 2010) with some markings being stable while others are more variable over time (Feinberg et al. 2010). Secondly, a range of environmental exposures alter epigenetic marks (Aguilera et al. 2010; Mathers et al. 2010). Thirdly, the epigenome undergoes profound remodeling early in development at a time that coincides with the critical window of vulnerability to developmental programming (Waterland and Michels 2007). Fourthly, epigenetic markings persist through mitosis (Skinner 2011), providing a possible mechanism for the durability of an exposure-related epigenetic change throughout life. Figure 11.1 places environmental exposures that potentially impact the developmental programming of the epigenome in a temporal context with developmental epigenetic events. Although the in utero period and early postnatal life are believed to be periods of particular epigenetic plasticity (Hanson et al. 2011), it should be noted that epigenetic change is likely to occur throughout life.

Variation among individuals in epigenetic markings is now documented in cord blood DNA (Gordon et al. 2011; Kile et al. 2010). This indicates that factors acting during gestation may contribute to this variation. Some of this variation can likely be explained by genetic factors; recent estimate points predict 6–10 % of the variation in DNA methylation being attributable to genetic variation (Bell et al. 2011). The nongenetic component of interindividual variation is likely to be determined by maternal factors and exogenous exposures during pregnancy as well as by stochastic events.

The potential role of epigenetic factors in mediating the developmentally programmed phenomena reviewed in the first section of this chapter will now be considered. Discussion will be limited to those factors clearly associated with obesity and type 2 diabetes in later life in both animals and humans, such as birth weight, undernutrition, overnutrition, breastfeeding, accelerated postnatal growth, and in utero exposure to glucocorticoids.

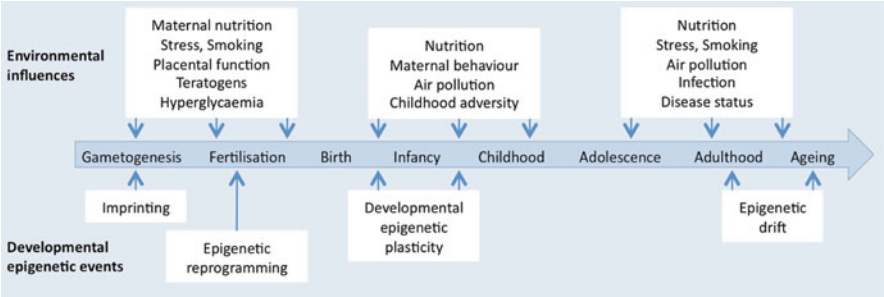

Fig. 11.1 The temporal relationship between epigenetic developmental events and the environmental influences of the developmental programming of health and disease

11.4 Evidence for Epigenetic Mechanisms Mediating the Influence of Early Life Exposures on the Risk of Obesity and Type 2 Diabetes

11.4.1 Birth Weight

Much of the literature upon which the DOHaD hypothesis is based on links between low birth weight and adverse health outcomes in later life, including type 2 diabetes. Consequently, it is pertinent to explore the association between low weight at birth and perturbed DNA methylation or other epigenetic signatures. Newborns with low or high birth weight display lower global LINE-1 DNA methylation in their cord blood compared to normal weight infants after adjusting for gestational age, sex, maternal age, and maternal smoking (Michels et al. 2011). In a small study of 12 neonates, Fryer et al. (2011) also report an inverse correlation between birth weight centile and global LINE-1 DNA methylation. In a genome-wide appraisal of DNA methylation, neonates with intrauterine growth restriction were found to contain loci with differential methylation, such as the hepatocyte nuclear factor 4 alpha gene *HNF4A*, a candidate type 2 diabetes gene (Einstein et al. 2010).

Analysis of epigenetic patterns in human placenta likewise demonstrates that DNA methylation is perturbed in small for gestational age and low birth weight infants (Guo et al. 2008; Filberto et al. 2011; Wilhelm-Bernartzi et al. 2012). Interesting work recently published by Novakovic and colleagues reports large-scale differences in DNA methylation in human placenta from the first, second, and third trimesters (Novakovic et al. 2011). The most differentially methylated regions are localized in genes involved in immune regulation, indicating an epigenetic mediation of a placental response to external stimuli. Furthermore, a gradual increase in interindividual variation in DNA methylation patterns is observed from the first through to the third trimester, supporting the postulate that environmentally induced and/or stochastic changes accrue as pregnancy proceeds.

Collectively, these observations support the role of epigenetic mechanisms in developmental programming.

Although evidence is building to link in utero events and birth weight to epigenetic perturbations, there is still a paucity of data linking this epigenetic variation to subsequent risk of obesity or type 2 diabetes. The identification of specific differentially methylated regions now opens up the opportunity to explore the role of these same loci in older cohorts in which disease is evident.

11.4.2 Undernutrition

Adverse outcomes in offspring following dietary restriction during pregnancy are widely documented (Simmons 2011). It is thought that epigenetic modifications provide a mechanism by which early life events increase disease risk in later life. Epigenetic mechanisms are implicated in adipogenesis, the development of obesity, and in the control of glucose homeostasis and insulin secretion. This is very aptly illustrated in work by Sandovici et al. (2011) who reported that poor maternal diet in a rodent model induced epigenetic silencing of *Hnf4a* gene in the offspring. This effect results in the permanent reduction in *Hnf4a* expression, a gene implicated in the etiology of type 2 diabetes. Altered offspring DNA methylation in a gene involved in gluconeogenesis (i.e., *PEPCK1*) is likewise described in a primate model of maternal nutrient restriction during gestation (Nijland et al. 2010).

In addition to the maternal low-protein diet, intrauterine growth restriction (IUGR), induced by the well-established uterine artery ligation model in rodent, is also used to explore the role of epigenetic mechanisms in undernourished fetuses. Epigenetic perturbation of the *IGF1* locus occurs in IUGR offspring using this model (Fu et al. 2009). Additionally, Carone and colleagues (2011) report a paternally induced transgenerational effect. When low-protein-fed males are crossed with control females, alterations in DNA methylation at the *Pparα* locus in offspring liver tissue are correlated with downregulation of gene.

There is less evidence in humans of in utero undernutrition and the induction of epigenetic changes. The association between exposure to famine during early gestation and epigenetic variation in offspring six decades later has been widely cited (Heijmans et al. 2008), although these findings did not demonstrate an association with phenotype.

11.4.3 Overnutrition

As with undernutrition, exposure to overnutrition during pregnancy and early postnatal life also induces adverse health consequences, potentially mediated by epigenetic mechanisms. One hypothesis to explain this phenomenon is the programming of genes involved in the regulation of food intake and body weight. In a

series of studies on DNA methylation in hypothalamic tissue from rats exposed to neonatal overfeeding, Plagemann and colleagues provide evidence for the role of epigenetic factors in this process (Plagemann et al. 2009, 2011). Hypermethylation of the main anorexigenic neurohormone proopiomelanocortin (POMC) occurs in a model of neonatal overfeeding. Increased DNA methylation of the insulin receptor promoter is also observed with concurrent elevated blood glucose levels.

In studies of lean and obese mouse strains, Widiker and colleagues (2010) reported decreased DNA methylation of the *Mc4r* gene in response to a high fat diet. Although this effect was not reported as a transgenerational phenomenon, it nevertheless highlights the potential for the appetite regulatory center of the brain to respond to overnutrition cues. Further evidence for the modulation of the appetite reward circuitry is provided by animal studies of maternal high fat consumption during pregnancy. This was associated with reduced global and gene-specific promoter methylation in the brains of offspring from dams that consumed the high fat diet (Vucetic et al. 2010). Suter et al. (2011) also reported differential fetal histone modifications in response to maternal high fat diet exposure in a gene implicated in the peripheral circadian machinery (i.e., *Npas2*).

A comparison of skeletal muscle biopsy tissue from adult human subjects of low and normal birth weight showed that a challenge of high fat overfeeding induced DNA methylation and expression changes at the *PPARGC1A* locus in this tissue (Brøns et al. 2010). Although DNA methylation did not correlate with gene expression, and methylation changes were only observed in normal birth weight individuals, a high fat feeding challenge appears to induce epigenetic changes within individuals. In a separate study, this locus also showed evidence of a positive correlation with maternal body mass index and DNA methylation in umbilical cord genomic DNA (Gemma et al. 2009). This indicates that the influence of diet on DNA methylation may indeed be transmitted from mother to fetus. Differential DNA methylation and gene expression of the leptin gene is also observed in placental tissue from women with impaired glucose tolerance (IGT) (Bouchard et al. 2010), suggesting that epigenetic mechanisms may mediate the adverse consequences of IGT on the long-term risk of obesity and type 2 diabetes in offspring.

Emerging evidence also points to a role for epigenetic programming through overnutrition via the paternal lineage. Chronic high fat feeding of male rats results in a pancreatic β-cell dysfunction in female offspring and is correlated with hypomethylation of *Il13ra2* (Ng et al. 2010).

11.4.4 Breastfeeding

Breastfeeding is implicated as a factor in determining risk of obesity and type 2 diabetes in later life; however, this relationship is difficult to disentangle from the many confounding factors associated with breastfeeding (see earlier). Our own unpublished data indicate a weak correlation between the duration of breastfeeding

and later global LINE-1 DNA methylation at age 50. In an independent cohort, we also observe an association between gene-specific methylation in women 7 years postdelivery and the duration of breastfeeding of their infants. Clearly, further research is required to interrogate the influence of breastfeeding on the epigenome.

11.4.5 Accelerated Postnatal Growth

Rapid catch-up growth is associated with later development of type 2 diabetes and obesity. Tosh et al. (2010) report decreased histone methylation and increased *Igf1* expression in the offspring of dams who were food restricted during pregnancy and fed ad libitum postnatally. In addition, recent work by Groom and colleagues (2011) describes a link between rapid postnatal growth and differential DNA methylation of the *TACSTD2* gene which is associated with childhood adiposity. Thorough interrogation of the observed associations, however, indicates that the relationships were unlikely to be causal.

11.4.6 In Utero Exposure to Glucocorticoids

Fetal overexposure to glucocorticoids induces hypertension, hyperglycemia, and increased hypothalamic-pituitary-adrenal axis activity in adulthood (Seckl et al. 2000). It is postulated that such glucocorticoid programming may be mediated by epigenetic mechanisms. Multi-generational programming effects are observed on birth weight and disease risk. Nevertheless, despite investigations of candidate loci, changes in DNA methylation have not yet been shown to underlie the transmission of biological effects (Drake et al. 2011).

11.4.7 Other Evidence Linking Epigenetics to Phenotype

The evidence presented thus far largely relates to the observed associations between early life exposures and the modulation of epigenetic events. Herein, we consider the body of evidence linking epigenetic factors to disease phenotype.

Exploration of epigenetic variation in candidate genes associated with type 2 diabetes and obesity (e.g., *FTO*, *INS,* and *KCNQ1*) highlights the small but consistent differences in DNA methylation at these loci that correlate with disease risk (Kong et al. 2009; Bell et al. 2010; Toperoff et al. 2011; Yang et al. 2011). Although these signatures may predict disease risk, further research is required to establish whether the epigenetic differences are causally related to disease or just a bystander effect. Other gene-specific analyses report the epigenetic modulation of the *PPARGC1A* locus in the pathogenesis of insulin resistance (Sookoian et al.

2010) and an inverse correlation of DNA methylation of the mitochondrial transcription factor gene *TFAM*, with features of insulin resistance (Gemma et al. 2010).

There is also evidence that epigenetic mechanisms may play a role in mediating the complications of type 2 diabetes. Hyperglycemia is linked to altered epigenetic signatures and gene expression in endothelial cells in an animal model (Siebel et al. 2010; Pirola et al. 2011). This serves to highlight the challenges in discerning cause from consequence when considering the role of epigenetic variation in type 2 diabetes and obesity, as changes can arise secondary to the disease state itself.

11.5 Establishment of Causal Relationships Between Epigenetic Programming and Phenotype

As outlined above and in other chapters, there is a sizable body of evidence that demonstrates epigenetic patterns are altered by environmental factors known to be associated with obesity or type 2 diabetes. An important question remaining to be resolved, however, is which epigenetic changes are secondary to the disease and which lie in its causal pathway? In Fig. 11.2, this conundrum is depicted by a red double-headed arrow, illustrating that reverse causation is a major issue in epigenetic studies. Furthermore, even with the observation of a temporal association between early life exposures, epigenetic change, and later disease, confounding factors remain a possibility (Fig. 11.2). Reverse causation is a particular problem in the search for epigenetic antecedents of type 2 diabetes and obesity as both of these disorders are characterized by chronic subclinical traits (e.g., inflammation and impaired glucose tolerance), which themselves can perturb epigenetic patterns.

As alluded to earlier, animal models present several advantages in epigenetic investigations and in the interrogation of programming mechanisms involved in DOHaD. They permit a life course approach involving the harvesting and analysis of numerous tissue types at multiple time points that would simply not be feasible in human studies. Establishing the temporal relationship between exposure and the events at the molecular level (e.g., epigenetic patterns and gene expression) can assist in defining a causal relationship. One caveat in relying solely on this approach is that it is not always possible to translate findings in animals to humans because the epigenomes vary markedly between species. Thus, the use of animal models should be viewed as complementary, but not sufficient, for a complete understanding of the mechanisms involved in developmental programming.

Longitudinal studies, with serial sampling, can be performed in humans to establish temporal variation in epigenetic patterns and its relationship with phenotype development. Such human studies are largely limited by the source of DNA (i.e., saliva, buccal scrapes, and peripheral blood rather than the target tissue of choice) and commonly little or no RNA. Nevertheless, there are a number of established, extensively characterized human longitudinal cohort studies that can

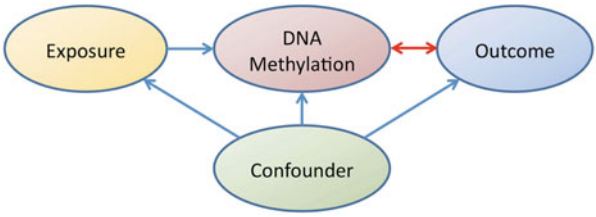

Fig. 11.2 DNA methylation as a mediator of early life exposures on disease risk during adulthood. All elements of the potentially causal pathway are vulnerable to confounding. DNA methylation is also susceptible to reverse causation (*red arrow*) where features of the disease state may act to alter the epigenome rather than vice versa

help determine the role of epigenetics in the developmental programming of obesity and type 2 diabetes. In only a minority of these studies is DNA stored for multiple time points throughout life.

In epidemiological terms, epigenetic patterns can be considered in the same way as many other intermediate phenotypes, for example, blood lipid profiles, insulin levels, and C-reactive protein. These traits are vulnerable to measurement bias, confounding, and reverse causation (Relton and Davey Smith 2012), which are problems when aiming to establish a causal relationship. Various approaches can be adopted to strengthen causal inference in human studies using epidemiological approaches.

The development and application of a Mendelian randomization approach (Davey Smith 2011) in an epigenetic context has recently been described (Relton and Davey Smith 2010). This utilizes germline genetic variation as a proxy for the exposure of interest (e.g., smoking) to establish the relationship between the exposure and DNA methylation patterns. In a second step, a separate germline genetic variant which proxies for DNA methylation itself is used to interrogate the relationship between DNA methylation and disease. This approach has been applied to establish causal relationships with regard to the role of DNA methylation in mediating the observed association between rapid postnatal growth and later childhood adiposity (Groom et al. 2011). Given the widespread and highly effective use of Mendelian randomization, this approach has enormous potential to help establish causality in complex epigenetic scenarios.

Additional methods for probing causality can also be applied, some borrowed from epidemiology, others from experimental laboratory-based science. The use of paternal controls has been advocated for epidemiological investigations when assessing maternal in utero effects (Davey Smith 2008; Macdonald-Wallis et al. 2011). For example, when assessing the relationship between maternal smoking and offspring birth weight, a comparison with paternal smoking and offspring birth weight will give some indication of likely confounding effects. Similar risk estimates from both paternal and maternal analyses would be suggestive of maternal smoking as a confounding effect if a causal biological birth weight-reducing mechanism has a larger influence than paternal smoking. This approach can be

applied to compare the relative effects of maternal (in utero) and paternal influences on epigenetic signatures at birth.

A range of experimental approaches can also be adopted to manipulate epigenetic patterns to dissect causal relationships. These might include the use of in vitro approaches utilizing demethylating agents or the more elegant targeted manipulation of specific genes through transfection of reporter gene constructs.

11.6 Developmental Programming and Epigenetics: The Future

11.6.1 Use of Epigenetic Marks as Biomarkers of Disease Susceptibility

There is presently more evidence that environmental exposures alter epigenetic patterns (Aguilera et al. 2010; Mathers et al. 2010) than support a link between epigenetic variation and disease formation (Portela and Esteller 2010). Hence, more work is required to build the evidence base linking epigenetic variation to specific phenotypes or diseases.

The importance of causality has been highlighted because without proven causality, interventions to prevent or reverse developmentally programmed type 2 diabetes, obesity, or any other disorder based upon epigenetic mechanisms will not be fruitful. Nevertheless, noncausal associations can still be informative even when not causally involved in the pathogenesis of the disease. This scenario is illustrated clearly in the case of C-reactive protein, a biomarker of atherosclerosis (Genest 2010). It is not causally related to disease pathogenesis; however, it may still remain as a useful diagnostic tool (Abd et al. 2011). Thus, defining robust prospective relationships between epigenetic patterns and type 2 diabetes or obesity may have important applications in diagnostics or in identifying high-risk individuals for non-epigenetic-based interventions (Relton and Davey Smith 2010).

In the context of developmental programming, it remains to be seen whether epigenetic signatures can truly predict later phenotype. Recent studies have reported associations between DNA methylation patterns in cord blood DNA (Relton et al. 2012) and DNA extracted from umbilical cord (Godfrey et al. 2011) and subsequent childhood adiposity. In both instances causality remains equivocal. Therefore, the predictive utility of such signatures may well lack specificity due to confounding effects by other measured or unmeasured factors. More comprehensive studies linking DNA methylation patterns at birth to disease phenotypes are required to provide compelling evidence.

11.6.2 Intervention and Prevention

Epigenetic patterns are generally considered to be a modifiable molecular target, with the potential for reversal of adverse epigenetic profiles. This makes epigenetic modifiers attractive for therapeutic intervention. Causality must be proven before interventions are used as those based upon noncausal associations will have no influence on disease phenotypes and result in wasted resources. Once epigenetic mechanisms, even if only contributory, are unequivocally implicated in disease pathogenesis, the prospect of epigenetic-based therapies becomes a realistic possibility. A wide range of pharmacological agents that target the epigenome are now used in clinical practice, mainly as anticancer treatments. The current epigenetic-based interventions are relatively crude and require more refined targeting as well as evaluation in non-cancer settings. Lifestyle and nutrition interventions are also worthy of consideration since these factors (e.g., diet, smoking, and physical activity) can modulate epigenetic patterns. These non-pharmacological strategies may also provide safer and more efficacious options if interventions are to be targeted at pregnant women in the hope of molding the epigenome of their children during pregnancy.

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Part IV
Epigenetics and Cancer

Chapter 12
Developmental Reprogramming by Environmental Estrogens: How Early Life Exposures Affect Cancer Risk in Adulthood

Cheryl L. Walker

Abstract There is an emerging consensus that development is a time of increased susceptibility to the adverse effects of environmental agents. Observations in both humans and experimental animal models have led to the "developmental origins of health and disease" or DOHaD hypothesis, which posits that environmental exposures during development reprogram the epigenome to profoundly impact susceptibility to diseases of adulthood, including cancer. Recent epigenetic data confirm that alterations in both DNA methylation and histone methyl marks are associated with developmental reprogramming and linked to environmental exposures that increase cancer susceptibility. Importantly, reprogramming of the epigenome by environmental exposures during susceptible windows of development can remain dormant until triggered by later-life events such as puberty. The identification of critical epigenetic alterations associated with developmental reprogramming holds the promise for developing biomarkers that can identify individuals at increased cancer risk as a result of early life environmental exposures. Furthermore, because epigenetic changes are reversible, it may be possible in the future to reverse the adverse effects of developmental reprogramming in affected individuals at increased risk of cancer as a result of early life environmental exposures.

Keywords Adult disease • Bisphenol A • Cancer • Developmental reprogramming • DES • DNA methylation • Epigenome • DOHaD • ER signaling • Genistein • Histone methylation • Xenoestrogens

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Abbreviations

BPA	Bisphenol A
DES	Diethylstilbestrol
DNMT	DNA (cytosine-5)-methyltransferase
DOHaD	Developmental origins of health and disease
ER	Estrogen receptor
HMD	Histone demethylase
HMT	Histone methyltransferase

12.1 Introduction

12.1.1 The Developmental Origins of Health and Disease (DOHaD) Hypothesis

Exposure of developing tissues or organs to an adverse stimulus or insult during critical periods of development can permanently reprogram normal physiological responses in such a way as to give rise to disease later in life. It is well established that this developmental reprogramming can increase risk in adulthood to several metabolic diseases, such as diabetes and cardiovascular disease, and evidence is now emerging that developmental reprogramming can increase cancer risk as well (Table 12.1) (Gillman 2005). Early seminal studies by David Barker in the 1980s were the first to link low birth weight, a proxy for an inadequate in utero environment, with increased coronary heart disease in adulthood (Barker 1994). In these and later confirmatory studies, infants having the lowest birth weights were found to have the highest rates of coronary heart disease, hypertension, and stroke as adults. Similar findings have been reported in response to starvation conditions, such as occurred during the Dutch "hunger winter" of World War II, where low birth weight as a result of maternal starvation correlated with increased risk for cardiovascular disease in adulthood and metabolic diseases such as obesity, metabolic syndrome, and diabetes (Roseboom et al. 2001). These and other epidemiologic data led to the "developmental origins of health and disease" or DOHaD hypothesis. DOHaD posits that increased risk of disease in adulthood is the result of an adverse developmental environment that reprograms cell and tissue response to normal physiological signals in a way that increases disease susceptibility.

During development, organogenesis and tissue differentiation occur via a continuous series of tightly regulated and precisely timed molecular, biochemical, and cellular events. From its earliest stages, this process is directed by epigenetic programs "installed" on the genome by epigenetic "writers" such as histone

Table 12.1 Adult diseases and disorders linked to developmental reprogramming by environmental exposures

Human diseases and neurological disorders
Asthma and allergic disorders
Cancer
Cardiovascular disease
Diabetes and metabolic syndrome
Obesity
Schizophrenia

methyltransferases (HMT) and DNA methyltransferases (DNMT) (Berdasco and Esteller 2010). In the case of histone modifications, the programs installed by these writers form an epigenetic "histone code" that are interpreted by "readers" (effector molecules that recognize methylated arginine and lysine residues) and modified by "erasers" (histone demethylases). Epigenetic methyl marks occur primarily on histones H3 and H4, are by convention denoted by specific lysine (K) or arginine (R) residues that are mono-, di-, or trimethylated, and can activate (e.g., H3K4me2) or repress (e.g., H3K27me3) gene expression. Gene-specific patterns of histone modifications can generate binding sites for histone code readers, such as proteins containing plant homeodomain (PHD), as well as direct other epigenetic "writers," such as DNA methyltransferases (Bannister et al. 2001; Lachner et al. 2001; Vire et al. 2006; Smallwood et al. 2007; Zhao et al. 2009).

It has now begun to be appreciated that developmental programming exhibits a high degree of plasticity and is modifiable by both extrinsic (e.g., environmental chemicals) and intrinsic (e.g., maternal) factors (Arici and Sozen 2003; Aguilera et al. 2010). This plasticity is thought to afford opportunities to modify epigenetic programming in response to endogenous and exogenous environmental cues and thus assist the developing organism in preparing for their adult environment. Due to the heritable nature of these epigenetic modifications, the potential exists for even a brief exposure to an environmental agent to disrupt the "installation" of epigenetic programs during development and, in so doing, reprogram the epigenome in a way that can increase disease risk in adulthood, including risk of developing cancer.

12.1.2 Evidence from Human Studies for Developmental Reprogramming of Cancer Susceptibility

To date, hormone-dependent cancers of the male and female reproductive tract provide the strongest evidence of developmental reprogramming of cancer susceptibility by early life environmental exposures. Diethylstilbestrol (DES), a synthetic stilbene estrogen, was administered to pregnant women in the 1940s–1970s to prevent complications of pregnancy. In the early 1970s, daughters of women who took DES during the first trimester (so-called DES daughters) were diagnosed at high frequency with congenital reproductive tract abnormalities ("T-shaped" uterus), dysplasia, and cervical intraepithelial neoplasia, and a significantly

increased rate of an otherwise rare type of vaginal clear cell adenocarcinoma (Herbst et al. 1971). Continued follow-up of DES daughters has now revealed they are also at increased relative risk for breast cancer (~2–3 times that of unexposed women) and uterine leiomyoma, although not all data on this point are concordant (NCI DES research update 1999; Baird and Newbold 2005; Wise et al. 2005; Palmer et al. 2006; Verloop et al. 2010). DES sons may also be at higher risk for prostate cancer; however, the final verdict on this point will require additional investigation (Schrager and Potter 2004). In males, testicular cancer has also been linked to early life environmental exposures and is one of the features of testicular dysgenesis syndrome (TDS), which includes poor semen quality, undescended testis, and hypospadia. Experimental studies from animal models and human epidemiology support a causal association between TDS and exposure to endocrine disrupting compounds (environmental estrogens such as DES and antiandrogens such as vinclozolin) during male reproductive tract development (Skakkebaek et al. 2001).

12.1.3 Evidence from Animal Models for Developmental Programming of Cancer Susceptibility

In mice, studies that simulate the human DES experience demonstrated that exposure of the developing reproductive tract to DES and other environmental estrogens imparts a permanent estrogen imprint that alters reproductive tract morphology, induces persistent expression of estrogen-responsive genes, and induces a high incidence of uterine adenocarcinoma (Newbold et al. 1990, 1997; Li et al. 2003). In Eker rats carrying a genetic defect in the tuberous sclerosis complex 2 (*Tsc2*) tumor suppressor gene, exposure to environmental estrogens during uterine development causes the tumor suppressor defect to become fully penetrant (Cook et al. 2005). These rats develop spontaneous uterine leiomyoma (Walker and Stewart 2005), sometimes referred to as "fibroids," in the smooth muscle layer of the uterus with an incidence of ~65 %. Exposure to environmental estrogens during uterine development increases incidence of these tumors to 100 %. The increased penetrance is associated with reprogramming of estrogen-responsive genes, which become hypersensitive to hormone, promoting the development of hormone-dependent uterine leiomyomas (Greathouse et al. 2008, 2012). Xenoestrogen exposure also modulates IGF signaling in the adult endometrium, decreasing negative feedback to IRS1, and increasing the incidence of endometrial hyperplasia in rats genetically predisposed to develop these preneoplastic lesions (McCampbell et al. 2008, 2010).

In rodents, the mammary gland begins developing in utero and is not fully mature until after pregnancy and lactation (Russo and Russo 2008). Inappropriate exposure during this period of development to environmental estrogens such as DES, the plasticizer bisphenol A (BPA), or the soy phytoestrogen genistein alters

susceptibility of the mammary gland to chemical carcinogenesis in adulthood. Importantly, the impact of xenoestrogen exposure differs across the life course (De Assis and Hilakivi-Clarke 2006). Xenoestrogen exposure in utero can increase risk for mammary tumorigenesis, in part by increasing the number of target cells for transformation in terminal end buds (TEBs) of the mammary gland. Conversely, xenoestrogen exposure during the postnatal period can decrease susceptibility to mammary gland tumorigenesis by inducing a program of differentiation in mammary epithelial cells that mimics the protective effects of pregnancy.

In Sprague-Dawley rats, early life exposure to environmental estrogens leads to prostatic hypertrophy and increased inflammation with age in adult animals (Prins 2008). These early life exposures also predispose to increased age-dependent prostate carcinogenesis (Ho et al. 2006). Similarly, benign tumors and adenocarcinoma of the rete testis are also frequently observed in the rodent testes in response to prenatal exposure to DES (Newbold et al. 1986, 1987).

12.1.4 Developmental Reprogramming of Gene Expression

Ample evidence exists that developmental reprogramming induces epigenetic changes that can be detected in at-risk tissues prior to tumor development (Table 12.2).

In the reproductive tract, persistent alterations in DNA methylation and developmental reprogramming occur in *Fos*, lactotransferrin (*Ltf*), *HoxA10,* phosphodiesterase type 4 variant 4 (*Pde4d4*), and high-mobility group nucleosome-binding domain 5 (*Hmgn5*; also known as *Nsbp1*) (Li et al. 1997, 2003; McLachlan et al. 2001; Ho et al. 2006; Tang et al. 2008). Early studies with DES demonstrated that this environmental estrogen induced aberrant methylation of specific CpG sites in *Ltf* (Li et al. 1997) and *Fos* (Li et al. 2003) in the mouse uterus. More recently, developmental environmental estrogen exposure was found to modulate expression and promoter methylation of *Hox* genes, which play key roles in uterine development. Exposure to DES in utero results in increased promoter methylation and decreased *HoxA10* expression (Bromer et al. 2009), whereas BPA exposure results in decreased methylation, increased estrogen receptor binding, and increased *HoxA10* expression in the adult uterus (Bromer et al. 2010). Similarly, neonatal exposure of mice to DES or genistein induced persistent hypomethylation of the *Nsbp1* promoter and aberrant overexpression of this gene in the uterus throughout life and was associated with increased risk of developing uterine tumors in adult female mice (Tang et al. 2008). Additional studies demonstrated that exposure of the developing uterus to environmental estrogens reprogrammed many estrogen-responsive genes including calbindin protein D9K (*Calb3*), glutamate receptor, ionotropic, AMPA 2 (*Gria2*), growth differentiation factor 10 (*Gdf10*), and matrix metalloproteinase 3 (*Mmp3*) causing them to become hyperresponsive to estrogen (Cook et al. 2005; Greathouse et al. 2008). In the prostate, *Pde4D4*, the gene product of which regulates intracellular levels of cAMP, is developmentally

Table 12.2 Epigenetic changes detected in at-risk tissues prior to tumor development

Gene	Tissue/ organ	Exposure	Reprogrammed phenotype	References
HOXA10	Uterus	BPA	Hypomethylation, constitutive expression	(Bromer et al. 2009)
		DES	Hypermethylation, reduced expression	(Bromer et al. 2010)
PDE4D4	Prostate	BPA	Elevated expression	(Ho et al. 2006)
LTF	Uterus	DES	Elevated expression	(Li et al. 1997, McLachlan et al. 2001)
FOS	Uterus	DES	Elevated expression	(McLachlan et al. 2001, Li et al. 2003)
HMGN5	Uterus	DES, Genistein	Elevated expression	(Tang et al. 2008)

reprogrammed in response to perinatal xenoestrogen exposure (Ho et al. 2006). In the normal adult prostate, *Pde4D4* becomes gradually hypermethylated with age. However, this gene undergoes persistent hypomethylation in the adult prostate of animals exposed neonatally to estradiol or BPA. This epigenetic reprogramming results in overexpression of *Pde4D4* and is correlated with increased susceptibility to develop precancerous lesions in the prostate (Ho et al. 2006).

In some cases, the impact of developmental reprogramming of the epigenome may remain "dormant" until engaged in response to later-life adult events (Tang et al. 2008) such as the presence of ovarian steroid hormones during puberty. Thus, while some epigenetic changes associated with developmental reprogramming can be observed immediately after exposure, both aberrant epigenetic programs (i.e., altered CpG methylation) and altered disease susceptibility may manifest only later in life, long after the environmental exposure occurred. The adult uterus of animals exposed in utero to DES exhibits alterations in methylation of the *Ltf* gene after puberty (Li et al. 1997). If ovariectomized, the aberrant DNA methylation observed in intact animals is not observed in the castrate female uterus. Similarly, early life exposure to DES or the phytoestrogen genistein can reprogram *Nsbp1* gene methylation, causing the promoter of this gene to become hypomethylated in the adult uterus, thus increasing gene expression. However, if genistein-exposed animals are ovariectomized, aberrant hypomethylation of the *Nsbp1* promoter does not occur (Tang et al. 2008). Finally, in Eker rats, ovariectomy before puberty completely eliminates the impact of reprogramming on gene expression and uterine tumor development. As mentioned above, exposure of the developing uterus to environmental estrogens reprograms many estrogen-responsive genes including *Calbindin D9k*, *Gria2, Gdf10,* and *Mmp3*, causing them to become hyperresponsive to hormone (Cook et al. 2005; Greathouse et al. 2008). This increased gene expression is entirely dependent on the presence of ovarian steroid hormones; ovariectomy before puberty completely eliminates the impact of reprogramming on gene expression and uterine tumor development, suggesting an interdependency between early life reprogramming and later-life events.

The mechanisms responsible for such "dormancy" remain to be elucidated. However, one possibility is that the initial reprogramming of the developing epigenome induces changes in histone methylation, which subsequently direct later-life changes in DNA methylation at CpG sites, for example, in response to hormone exposure during puberty. In such a situation, altered patterns of DNA methylation (directed by the reprogrammed aberrant histone methylation) would not be manifested until after puberty when changes in CpG methylation would normally occur.

12.1.5 Linking Environmental Exposures to Reprogramming of the Epigenome

Recently, we identified a direct mechanism by which environmental estrogens can disrupt the epigenetic machinery of a cell during developmental reprogramming (Bredfeldt et al. 2010) (Fig. 12.1). Environmental estrogens can bind to membrane-associated estrogen receptor (ER) to activate rapid, non-genomic ER signaling. Among the targets for this non-genomic signaling are epigenomic writers such as the HMT enhancer of zeste homologue 2 (EZH2), a member of the polycomb repressive complex, PRC2 (Simon and Lange 2008). This pre-genomic signaling activates PI3K signaling and the kinase AKT. Phosphorylation of serine 21 of EZH2 by AKT inactivates EZH2 and reduces the levels of the repressive H3K27me3 methyl mark in the developing uterus. This rapid non-genomic ER signaling provides a mechanism by which environmental estrogens can inappropriately activate kinases such as AKT (and probably others such as ERK in the MAPK pathway) to modulate the activity of HMTs and disrupt the epigenetic machinery during developmental reprogramming.

12.1.6 Windows of Susceptibility for Developmental Reprogramming

The timing of exposure during development also appears to be an important determinant for developmental reprogramming by environmental agents. For some tissues, such as the breast, which continues to develop well into adulthood, this window of susceptibility may be quite large, stretching from in utero to the first full-term pregnancy. In the uterus, development is completed in the first trimester in humans and in the first few weeks of neonatal life in rodents. Although it is not known when the window of susceptibility to developmental reprogramming first opens, we have found that in the Eker rat, the window of susceptibility in the uterus closes around postnatal day 17 (Cook et al. 2007). Rats exposed to xenoestrogens prior to day 17 exhibit developmental reprogramming of estrogen-responsive genes

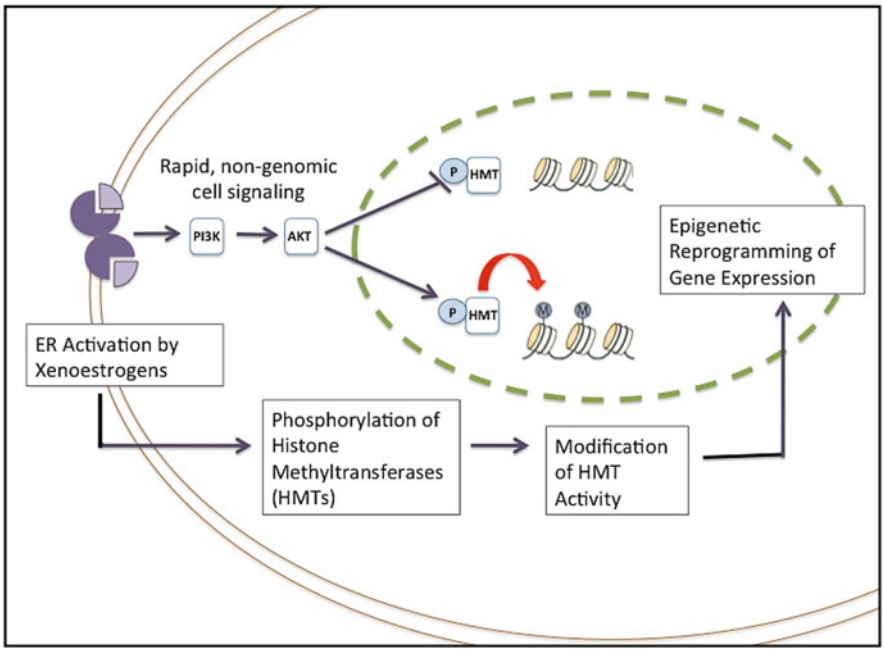

Fig. 12.1 Mechanism of action of xenoestrogen-induced developmental reprogramming. Exposure to xenoestrogens activates non-genomic (or more accurately "pre-genomic") cell signaling pathways such as PI3K/AKT kinase signaling, which in turn phosphorylates HMTs to modify their methyltransferase activity leading to reprogramming of the epigenome

and increased tumor suppressor gene penetrance, whereas neonates exposed after this time do not become developmentally reprogrammed nor exhibit any increase in tumor suppressor gene penetrance.

It remains an open question as to what defines the window of susceptibility for developmental reprogramming, which will likely exhibit both species specificity and tissue specificity. In the female reproductive tract, development occurs in an estrogen-naïve environment; high levels of steroid hormone-binding proteins such as alpha fetoprotein (AFP) protect the developing uterus from maternal hormones of pregnancy, and during neonatal life from estrogen produced by organs such as the adrenal gland. Many environmental estrogens are not recognized by these steroid hormone-binding proteins and thus evade this defense system. In the Eker rat model, timing for window "closure" coincides with the period when the liver ceases production of AFP and steroid hormone-binding proteins are cleared from the neonate; after this time, cells and tissues are exposed to low levels of circulating endogenous estrogens. In this setting, the window of susceptibility for developmental reprogramming may be defined as that period when the uterus is developing in an estrogen-naive environment and that after day 17, the uterus is "ready" to see

estrogens, and therefore neither endogenous nor environmental estrogen exposure disrupts epigenetic programming.

12.2 Conclusions

There is an emerging consensus that development is a time of increased susceptibility to the effects of adverse environmental exposures and that reprogramming of the epigenome by environmental exposures early in life can determine the risk of many adult diseases decades before disease onset. Accordingly, in exposed individuals, the impact of reprogramming events induced by environmental exposures during susceptible windows of development may not be apparent for years or even decades. However, both the long latency period for manifestation of developmental reprogramming and the reversible nature of epigenetic alterations present opportunities for interventions to reverse the adverse effects of developmental reprogramming. Increasing our knowledge about the targets for developmental reprogramming holds the promise for identifying biomarkers of exposure and increased risk, as well as developing epigenetic therapies that can reverse the effects of developmental reprogramming to decrease cancer risk.

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Chapter 13
Human Cancer Epigenetics

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Abstract Research reveals that epigenetic processes are emerging as a central regulatory layer in cell homeostasis and is recognized as a common hallmark in cancer and other diseases. Disrupted DNA methylation profiles, noncoding RNA expression, and histone modification patterns occur at all steps of tumor evolution triggering the malignant phenotype. Evolving cutting-edge technologies are providing new insights into cell physiology and allowing researchers to perform more comprehensive epigenomic studies. In cancer, reliable panels of biomarkers are arising, informative of cancer cell behavior and functional alterations – especially involving changes in DNA methylation – and are being identified as targets for potential therapeutic intervention. It is the aim of this chapter to summarize recent literature on the latest epigenetic advances in the understanding of cancer development and progression, with a special focus on DNA methylation alterations and their value in clinical oncology for cancer management.

Keywords Epigenetics • DNA methylation • Cancer • Biomarkers • Epigenetic drugs

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Abbreviations

ac	Acetylation
CGI	CpG Island
CIMP	CpG island methylator phenotype
CIN	Chromosomal instability
CRC	Colorectal cancer
DNMT	DNA methyltransferases
ES	Embryonic stem (cells)
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HDACi	Histone deacetylase inhibitor
HDM	Histone demethylases
HMT	Histone methyltransferases
K	Lysine
lincRNAs	long intergenic non-coding RNAs
me	Methylation
miRNA	MicroRNA
MLH1	MutL homologue 1
MSI	Microsatellite instability
PcG	Polycomb group proteins
piRNA	piwi RNA
SAHA	Suberoylanilide hydroxamic acid
snoRNA	Small nucleolar RNA
Trx	Trithorax-group protein
TSS	Transcription start site

13.1 Introduction

Epigenetics is devoted to the study of inherited chemical modifications that influence gene expression patterns without altering the DNA code. The term was first introduced by C. Waddington in the early 1940s and has evolved in recent years to emerge as an area of intense research. It encompasses a wide range of processes involved in all steps of ontogeny and influences all cell types in the organism. In fact, every cell type is characterized by a unique epigenome that controls the transcription of the genome and ultimately determines its phenotype (Esteller 2009).

In general terms, epigenetic processes are involved in the tuning of transcriptional programs: DNA methylation at 5-methyl-cytosines, a wide and expanding array of histone modifications, microRNAs and other noncoding transcripts, and

nucleosomal positioning and other processes associated with protein complexes that dynamically remodel chromatin and adjust transcription levels, all of them tightly regulated and working coordinately (Esteller 2009; Portela and Esteller 2010).

13.1.1 DNA Methylation

DNA methylation is the best characterized epigenetic process. It takes place at 5′ position of cytosines which are followed by a guanine – the so-called CpG sites, which tend to cluster in particular regions across the genome. In mammalian and plant genomes, 5-methylcytosine accounts for ~1–6 % of the nucleotides. The overrepresentation of CG dinucleotides constitutes the CpG islands (CGIs), which are, by consensus, regions of 1,000 bases on average with elevated CG presence and frequent absence of methylation, prevalently mapping to the transcriptional initiation sites of approximately 70 % of annotated genes (Deaton and Bird 2011). Nevertheless, there also exist CGIs located at intergenic regions that mark the presence of alternative promoters, transcription start sites (TSS) of noncoding transcripts, and enhancers of transcription, and a connection with gene splicing has recently been suggested. While CGIs mapping gene promoters are prevalently unmethylated, these scattered CGIs display heavy methylation likely due to cell- or tissue-specific expression programs (Flanagan and Wild 2007; Lister et al. 2009; Illingworth et al. 2010; Maunakea et al. 2010).

The enzymes mediating methylation of DNA are the DNA methyltransferases (DNMTs) *DNMT1*, *DNMT3A*, and *DNMT3B*. The first of these is required for the maintenance of DNA methylation patterns during semiconservative DNA replication (Robert et al. 2002), whereas *DNAMT3A* and *DNMT3B* catalyze de novo addition of methyl groups, essentially during development. Interestingly, a recent microarray-based study revealed the existence of new regulatory sequences termed CpG shores, which were defined as regions adjacent to the canonical CGIs – 200 to 2,000 bases away – with an intermediate content of CG. Genes associated with these CpG shores showed a clear inverse correlation between transcription and methylation levels of these newly identified regions. Interestingly, hypermethylation acquired at these regions and expanding to canonical CGIs alters the methylation patterns displayed by the tissue of origin – colon, in this case – whereas hypomethylation occurring further away, 2 to 3 kilobases from the CGIs, recapitulates profiles exhibited by other normal tissues (Irizarry et al. 2009). This agrees with the concept of loss of cellular identity following transformation, since transcriptional programs are profoundly altered following aberrant DNA methylation changes across the genome (Esteller 2009).

13.1.2 Histone Modifications

Another epigenetic regulatory layer is constituted by the posttranslational modifications taking place at N-terminal end of the histone proteins. Histone octamers are globular protein complexes around which DNA is coiled forming the nucleosome and provide a platform for a plethora of covalent modifications at many different residues. The possible combinations have not yet been fully depicted, and the complexity of their actions is only just beginning to be understood. Importantly, these modifications exert a critical role in regulating chromatin structure by affecting inter-nucleosomal interactions and recruiting chromatin-remodeling enzymes that determine chromatin conformation, which consequently affects access of transcription machinery. The list is ever growing, but the best characterized modifications are histone acetylation and methylation. In addition, histone phosphorylation, ADP-ribosylation, ubiquitylation, sumoylation, adenylation, crotonylation, isomerization, and deimination are other types of modifications under investigation (Bannister and Kouzarides 2011; Tan et al. 2011). Histone acetylation levels are maintained by the opposed action of histone acetyltransferases (HATs) and deacetylases (HDACs). The former are responsible for lysine (K) acetylation (ac), thereby neutralizing positive charges and loosening the interaction between DNA and histones and favoring an open conformation of the chromatin which facilitates active transcription. Most modifications (H3K9, H3K14, H3K18, H3K27, H3K56, H4K5, H4K8, H4K12, and H4K16) take place on histone tails; however, some histone modifications take place inside of globular core domains, such as H3K56ac, mediated by *CBP*/*p300* acetyltransferases (Das et al. 2009). Three families of HATs – *GNAT*, *MYST*, and *CBP*/*p300* – carry out the acetylation at multiple sites, and substrate specificity is determined by their association with large multiprotein complexes (Hodawadekar and Marmorstein 2007). Methylation (me) of histones occurs at arginines and lysines as a result of the action of histone methyltransferases (HMTs) and demethylases (HDMs). Further complexity of this modification comes from the fact that arginines can be mono- and symmetrically or asymmetrically dimethylated, whereas lysines can also be mono-, di-, or trimethylated (Ng et al. 2009). Besides, addition of methyl groups does not alter the electric charge, and the effect on chromatin depends on which amino acid is modified and on the degree of methylation. For instance, H3K9 monomethylation (H3K9me1) and H4K20me1 are associated with active transcription, whereas H3K9me3 and H4K20me3 are specific of transcriptional repression (Barski et al. 2007; Wang et al. 2009). Also, H3K4me3 and H3K4me1 represent specific marks enriched at TSS and enhancers of active genes, respectively (Huebert and Bernstein 2005). Both HMTs and HDMs exhibit high specificity, not only recognizing specific residues, but also exerting action dependent on the degree of methylation.

There is an active cross talk between histone modifications that results in diverse effects on the chromatin and gene transcription that has been designated as the histone code (Jenuwein and Allis 2001). Interactions become apparent because of the competitive antagonism between marks targeting the same amino acid (Melcher

et al. 2000), synergistic effects when the addition of a particular mark depends on the previous presence of another (Nakayama et al. 2001), cooperation between different marks in recruiting specific factors (Vermeulen et al. 2010), and on the contrary, exclusive effect that a modification exerts on the presence of another (Rea et al. 2000).

The distribution of histone marks across the genome is not random (Jenuwein and Allis 2001; Barski et al. 2007; Wen et al. 2008). Specific modifications are overrepresented in euchromatic or heterochromatic regions. For instance, the inactive X chromosome is enriched in H3K27me3 (Brinkman et al. 2006), and centromeres and telomeres are characterized by high levels of H3K9me3 (Regha et al. 2007). By contrast, euchromatin is a less tangled genomic environment. Modifications in this chromatin domain tend to cluster at transcription-regulatory elements or sites of active transcription. Actively transcribed genes are enriched in H3K4me3 at TSS that prevents the recruitment of DNMTs; H3K36me3 expands along transcribed regions (Hon et al. 2009), being interpreted by the splicing machinery as an "exon inclusion mark"; and H3K4me1 indicates the presence of active enhancers (Heintzman et al. 2007).

Interestingly, several studies in embryonic stem (ES) cells have identified similarities between ES cell biology and neoplasia. Proteins common to stem cell biology and neoplastic processes include the polycomb (PcG) and trithorax (Trx) groups, which maintain a subset of genes under a semi-dormant state during differentiation, by marking them with activating H3K4me3 and repressing H3K27me3 around TSS. In this way, genes bearing this bivalent state can be turned on or permanently off in response to requirements of cell commitment. This group of genes does not involve promoter hypermethylation in embryonic or committed cells; nonetheless, 50 % of the genes aberrantly methylated in cancer overlapped these PcG-targeted genes, suggesting that in response to external cues, cancer-initiating stem cells would permanently silence this subset of genes generating a cell population with selective advantages that would foster further tumor formation and progression (Ohm et al. 2007; Widschwendter et al. 2007; Schlesinger et al. 2007).

13.1.3 Nucleosomal Positioning

The dynamic positioning of nucleosomes by chromatin-remodeling factors in an ATP-dependent manner has a profound effect on chromatin conformation and regulates the accessibility of transcription machinery to TSS. Location of nucleosomes at TSS is generally associated with gene repression, whereas their displacement correlates with gene activation (Schones et al. 2008; Cairns 2009). Nucleosome positioning does not only represent a physical barrier to the engagement of transcription factors but also influence DNA methylation patterning at genomic scale (Chodavarapu et al. 2010). Base-resolution DNA methylation maps have revealed that DNA coiled around histone octamers is predominantly

methylated and specially enriched at exons compared with intronic DNA, in agreement with previous observations indicating that DNMTs preferentially target nucleosomal-bound DNA. However, *cis*-acting elements, e.g., *Sp1* elements present in the proximity of TSS of genes constitutively transcribed, enriched in H3K4me3, protect them from de novo methylation during development by means of perpetuating these loci, many of which correspond to tumor-suppressor genes (Brandeis et al. 1994; Schones et al. 2008; Chen et al. 2011).

In addition, specific histone marks have been shown to recruit specific splicing mediators following DNA transcription, influencing splice-site choice (Kornblihtt et al. 2009; Luco et al. 2011). Furthermore, nucleosome positioning is overrepresented around exons, specifically in 50 nucleotides intronic regions preceding and following exons, and also in alternatively spliced exons, suggesting that nucleosome distribution might play also a role both in exon definition (Schones et al. 2008; Tilgner et al. 2009; Chodavarapu et al. 2010). Far from being a static structure, nucleosomes also play a dynamic role, in conjunction with the rest of epigenetic regulatory processes, in shaping chromatin and influencing genome activity.

13.1.4 Noncoding RNAs

Recently, another level of epigenetic regulation is being vigorously investigated: regulation by noncoding RNAs (ncRNAs). Among the ncRNAs, microRNAs (miRNAs) have been the most extensively studied; however, there are multiple classes of ncRNAs, including small nucleolar RNAs (snoRNAs), long intergenic noncoding RNAs (lincRNAs), piwi RNAs (piRNAs), and many others with functions that are currently under investigation.

MicroRNAs were initially identified in *C. elegans* and later documented to be widely expressed in a multitude of living organisms. Observations from many studies indicated miRNAs are essential for modulating expression programs during development and the immune response, as well as many other processes. MicroRNAs modulate gene expression by silencing chromatin, degrading complementary mRNA, or by blocking translation at ribosomes, affecting more than 60 % of protein-coding genes, and thus are involved in virtually all cellular processes.

The first evidence that attributed a tumor-suppressive function to some miRNAs came from a leukemia study where deletions of *mir-15a* and *mir-16-1* were described (Calin et al. 2002). Additional studies have ascribed tumor-suppressive roles as well as oncogenic properties to these noncoding transcripts (Chen 2005). In addition to miRNAs, studies have shown ultra-conserved RNAs' (UCRs) activity is also altered in cancer as a consequence of epigenetic silencing of their TSS (Saito and Jones 2007; Lujambio et al. 2008, 2010). However, the functions of this "dark side of the genome" (noncoding transcripts account for 90 % of the human transcriptome) are just being unraveled and constitute a vast research field that

will exponentially expand with the application of the new high-throughput technologies.

13.2 Aberrant Epigenomic Profiles Correlate with Tumor Progression

Altered epigenomic landscapes are a common feature of human cancer and have been extensively studied in a wide variety of tumor entities. Among these, special attention has been paid to aberrant DNA methylation affecting promoter regions of coding genes. As a result, DNA methylation has been recognized as an alternative mechanism for gene inactivation other than genetic mutation and has also proven to be valuable as biomarker for cancer management.

13.2.1 Field Effect

Alterations in DNA methylation patterns arise very early in the neoplastic sequence. In the 1950s, the concept of field cancerization was proposed to explain the multifocality of neoplasms in patients with no evident familial predisposition to those tumors and the observation that cells in the proximity of tumors bear some of the molecular features exhibited by full-blown cancers (Slaughter et al. 1953). Many studies have reported panels of methylation markers associated with tumor formation and further progression emerging in histologically normal-appearing tissues. On one hand, this provides a potential opportunity to identify informative molecular markers predictive of tumor development and, on the other – from a clinical standpoint – raises an important question since, once the main tumor has been surgically removed, the remaining "field" from which it developed stays and could potentially originate a new neoplasm. Genetic and epigenetic alterations linked to field cancerization have been documented in colorectal, breast, lung, bladder, prostate, and head and neck cancers (Papadimitrakopoulou et al. 1996; Franklin et al. 1997; Shen et al. 2005; Yan et al. 2006; Ramírez et al. 2008; Trujillo et al. 2011). An extended hypothesis explains that these early molecular alterations may arise in organ-specific stem cells that would in turn give rise to abnormal daughter cells and eventually create a field that could finally evolve to form a tumor (Fearon and Vogelstein 1990). Studies carried out in colon cancer have identified genetic abnormalities early in the carcinogenic sequence, as well as epigenetic disruption of genes in normal-appearing colorectal mucosa adjacent to the tumor, including *p16* for control of cell cycle, *MGMT* involved in DNA repair, SFRP family of genes as regulators of Wnt pathway and *GATA-4* and *GATA-5* transcription factors, and E-cadherin, many of which are also found in the neighboring tumor (Ramírez et al. 2008). Additionally, hypermethylation of *DAPK1* has also been

found in precancerous tissues and tumor-adjacent mucosas, being an early event in the development of oral, esophageal, lung, and colon cancers. Interestingly, the detection of hypermethylation in various combinations of biomarkers is also an efficient strategy in detecting early neoplasia yielding high specificity and sensitivity in early diagnosis (Belshaw et al. 2008). The identification of such markers in early-stage neoplastic processes implicates both risk assessment and cancer prevention at a clinical level.

Whether or not field effect cancerization is influenced by environmental factors remains an issue to be resolved. Epigenetics is frequently referred as the mediator between the environment and the genome. In fact, influence of the environment, understood as external cues, on epigenetic drift has been demonstrated comparing methylation histone modification profiles of monozygotic twins in early and advanced ages (Fraga et al. 2005). A critical window of "epigenetic vulnerability" is the embryogenesis period, during which the embryo genome is sculptured after having undergone global demethylation following fertilization. Epigenetic reprogramming is especially active during gestation and also during neonatal development (Szyf 2009). Epigenetic signatures acquired during these stages may have lifelong effects and repercussions on gene expression programs. Diet, alcohol and tobacco consumption, toxic exposures, stress, etc., have a wide impact on the dynamic epigenomic landscapes of cells and may unleash pathological mechanisms. Nevertheless, factors underlying field cancerization are still unknown and further efforts are needed to depict the cues driving tumor initiation and identify informative biomarkers for early diagnosis.

13.2.2 Epigenetics in Cancer: The Paradigm of Colorectal Cancer

Colorectal cancer (CRC) has become an archetype in cancer epigenetics research (Carmona and Esteller 2010). The first report came in the 1980s reporting a global loss of DNA methylation in colon cancer cells (Feinberg and Vogelstein 1983), opening a new dimension in cancer research that has yielded seminal contributions to the field.

Colorectal cancer results from an accumulation of genetic and epigenetic alterations that trigger the cancerous phenotype. Several heterogeneous molecular features classifying CRC define diverse pathways involved in colorectal carcinogenesis. To begin with, chromosomal instability (CIN), observed in 70–80 % or sporadic CRC, encompasses a wide range of genomic alterations such as translocations frequently in 5q, 17p, and 18q; deletions; loss of heterozygosis; and imbalances in chromosome number affecting many key genes, such as *APC*, *KRAS*, or *SMAD4*, whose inactivation promote subsequent tumor formation and progression through the adenoma-carcinoma sequence (Pino and Chung 2010). These alterations occur early and likely result from defects in DNA repair

mechanisms, telomere instability, and impaired chromosome segregation (Stewénius et al. 2005; Dalton and Yang 2007), although the whole picture is poorly understood. Another type of genomic instability is microsatellite instability (MSI), defined as changes on the reading frame due to insertion or deletions, affecting repeat units in microsatellites that occur as a result of defective DNA repair following replication. The major causal feature of MSI in sporadic CRC is the epigenetic inactivation of *MutL* homologue 1 (*MLH1*) mismatch repair gene (Fleisher et al. 1999), which occurs on 15 % of sporadic CRC and the almost all cases of Lynch syndrome, a nonpolyposis form of hereditary CRC. MSI tumors have distinctive pathological and anatomical features, poor differentiation status, and prevalent genetic alterations compared to CIN tumors (Park et al. 2003). A distinct pathway in colon carcinogenesis, the CpG island methylator phenotype (CIMP) characterized by increased "epigenetic instability," was proposed to explain non-CIN colorectal tumors and will be further discussed.

In the adenoma-carcinoma sequence, numerous candidate-gene approach as well as high-throughput studies have reported a valuable list of aberrant DNA methylation events that are associated with specific steps. Early stages – adenomatous and hyperplasic polyps – in CRC formation are characterized by hypermethylation of genes *RASSF1A*, *CDH13*, *MGMT*, *RUNX3*, *p14ARF*, and *SFRP2*, being in some cases the first molecular alteration detectable, suggesting that aberrant DNA hypermethylation may be involved in CRC initiation (Ahlquist et al. 2008; Jones and Baylin 2002). Moreover, global DNA hypomethylation is also a common causal trait in tumor initiation, although it exerts opposing roles depending on the tumor type (Yamada et al. 2005) (Fig. 13.1).

Further on, the increasing number of loci targeted by aberrant DNA methylation and the growing frequency of tumors displaying altered profiles indicate that some inactivating events influence key steps in the carcinogenic cascade. Adenomas frequently exhibit heavy promoter hypermethylation of *p16ARF*, *ITGA4*, *MGMT*, *RASSF2A*, and *HPP1* (Petko et al. 2005; Ausch et al. 2009; Wynter et al. 2006; Akino et al. 2005), among others, and the list increases when compared to invasive carcinomas with prevalent methylation of *hMLH1*, *COX2*, *CDH1*, *CDH13*, (Herman et al. 1998; Toyota et al. 2000; Esteller et al. 2001b; Toyooka et al. 2002) and others (extended in Table 13.1). Regarding their function, genes inactivated along the carcinogenic cascade involve transformation steps as well as tumor aggressiveness and dissemination.

Aberrant DNA methylation has been studied in virtually all tumor types, and specific profiles have been assigned (Esteller et al. 2001a). From a DNA methylation standpoint, aberrant CpG island hypermethylation profiles follow tissue-type (cancer-type) specific patterns, which implies an advantage in the depiction of comprehensive panels of biomarkers for tumor diagnosis using luminal fluids, e.g., saliva, urine, and stool, to unequivocally assess tumor identity or to track back the tumor of origin for those metastases of unknown origin (Fernandez et al. 2011).

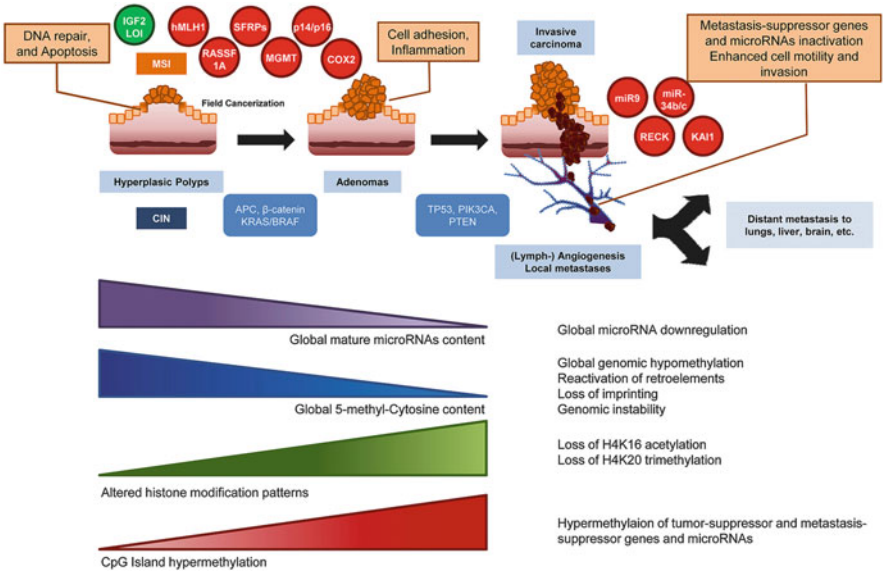

Fig. 13.1 Epigenetic disruption of cancer cells. During CRC progression, the interplay between genetic (*blue squares*) and epigenetic (*circles*) alterations contributes to an overall deregulation of gene expression patterns. The promoter hypermethylation-associated gene silencing affects all major pathways, including DNA repair (*hMLH1*), cell cycle (*p14*/*p16*), and control of apoptosis (*SFRPs*), allowing transformed cells to escape from control checkpoints and fostering the acquisition of malignant features. At later stages of tumor progression, aberrant promoter hypermethylation further promotes the invasive phenotype by inactivating metastasis-suppressor elements. At a global scale, several processes have been identified to become deregulated during the carcinogenic cascade, contributing to the abnormal gene expression patterns observed in cancer cells

13.2.3 Epigenetic Alterations in Metastatic Progression

Despite most epigenetic alterations having been assessed during primary tumor formation, DNA hypermethylation inactivating events occurring during metastatic progression triggers cancer cells dissemination to lymph and bloodstream favoring the colonization and growth of distant metastases (Rodenhiser et al. 2008; Carmona and Esteller 2011b). Knowing the nature of these alterations would permit the design of new therapies that could potentially target this critical step which is responsible for 90 % of cancer-related deaths and identify clinical markers for a more accurate and early diagnosis.

Interestingly, DNA methylation signatures have been described in relation to metastatic spreading (Lujambio et al. 2008). Inactivation of metastasis-suppressor factors facilitates the scattering of tumor cells. Hypermethylation-associated inactivation of miR-148a, miR-34b/c, and miR-9 was reported in primary melanomas, head and neck, and colon cancers in association with lymph node metastasis as a mechanism through which cells acquired a more invasive phenotype by

Table 13.1 Frequent targets of aberrant DNA hypermethylation in human cancer

Cancer type	Gene ID	Cellular component/process	Methylation frequency	Genomic location
Colon cancer				
hMLH1	MutL homolog 1, colon cancer, nonpolyposis type 2	DNA repair	77 % MSI-H CRC	3p21.3
RASSF1A	Ras association (RalGDS/AF-6) domain family member 1	Cytosolic proteins/cell cycle	45 % CRC	3p21.3
DAPK	Death-associated protein kinase	Cytosolic proteins/apoptosis	20–25 % AD, 83 % CRC	9q34.1
TMEFF2/ HPP1	Transmembrane protein with EGF-like and two follistatin-like domains 2	Membrane proteins	63 % HP, 66 % AD, 84 % CRC	2q32.3
SFRP-2	Secreted frizzled-related protein 2	Secreted proteins/Wnt signaling	83–90 % CRC	4q31.3
DKK-1	Dickkopf homolog 1	Secreted proteins/Wnt signaling	13 % CRC	10q11.2
GATA-4/ GATA-5	GATA binding protein 4/GATA binding protein 5	Transcription factors	70 % and 79 % CRC	8p23.1-p2/ 20q13.33
p14	Cyclin-dependent kinase inhibitor 2A	Nuclear proteins/cell cycle	38 % CRC	9p21
p16	Cyclin-dependent kinase inhibitor 2A	Nuclear proteins/cell cycle	32 % CRC	9p21
COX-2	Prostaglandin-endoperoxide synthase 2	Inflammation	14 % AD	1q25.2
Breast cancer				
CDH1	E-cadherin	Cell membrane/cell adhesion	72 % of ductal, lobular, and apocrine carcinomas	16q22.1
CDH13	H-cadherin	Cell membrane/cell adhesion	33 % primary carcinomas	16q23.3
RARβ-2	Retinoic acid receptor, beta	Nuclear receptor/signal transduction	31 % primary carcinomas	3p24
ESR1	Estrogen receptor 1	Nuclear receptor/cell proliferation	46 % primary carcinomas	6q25.1
BRCA1	Breast cancer 1, early onset	Nuclear protein/DNA repair	18 % primary carcinomas	17q21
HIC1	Hypermethylated in cancer 1	Transcription factors	48 % primary carcinomas	17p13.3
SFRP2	Secreted frizzled-related protein 2	Secreted proteins/Wnt signaling	83 % primary carcinomas	4q31.3
Prostate cancer				
GSTP1	Glutathione S-transferase	Cytosolic proteins/apoptosis	80–95 % AD	20q13.2
Laminin-5	Laminin, alpha 5	Nuclear protein	18–45 % primary carcinomas	

(continued)

Table 13.1 (continued)

Cancer type	Gene ID	Cellular component/process	Methylation frequency	Genomic location
RASSF1A	Ras association (RalGDS/AF-6) domain family member 1	Cytosolic proteins	>70 % primary carcinomas	3p21.3
uPA	Urokinase plasminogen activator	Cytosolic proteins/cell proliferation, migration, and ECM remodeling	96 % hypomethylation in primary carcinomas	10q24
Lung cancer				
SEMA3B	Semaphorin 3B	Secreted protein	41 % NSCLC	3p21.3
IGFBP3	Insulin-like growth factor-binding protein 3	Nuclear protein/cell survival	60 % NSCLC	7p14
PAX5	Paired box homeotic gene 5	Transcription factor/differentiation	40–50 % AD and SCC	9p13
p16	Cyclin-dependent kinase inhibitor 2A	Nuclear proteins/cell cycle	25–30 % NSCLC	9p21
RARβ-2	Retinoic acid receptor, beta	Nuclear receptor/signal transduction	40 % NSCLC	3p24

AD adenocarcinoma, *HP* hyperplastic polyps, *NSCLC* non-small cell lung cancer, *SCC* squamous cell carcinoma

upregulation of their oncogenic targets (Lujambio et al. 2008). In many epithelial tumor types, E-cadherin (*CDH1*) is inactivated through promoter hypermethylation in the primary lesion (occurring in 90 % of breast cancer patients) as part of the cadherin switch, by which mesenchymal cadherins (typically N-cadherin) are upregulated and enhance the metastatic phenotype as part of the epithelial-to-mesenchymal transition program (Yoshiura et al. 1995; Graff et al. 1995).

In general, our understanding of the epigenetic involvement in human cancer metastasis is limited and has been predominantly restricted to candidate-gene approaches investigating DNA methylation changes in definite tumor types. This nevertheless has provided valuable insights into the molecular mechanisms driving metastatic dissemination. With the advent of new high-throughput technologies, large-scale unbiased studies have started to come out. Recently, a DNA methylation-based genome-wide approach addressing breast cancer metastasis was published. In the study, a breast cancer methylator phenotype (B-CIMP) was documented by the authors, comprising a subset of hormone-positive tumors, with lower tendency to form metastasis and therefore a better clinical outcome (Fang et al. 2011). The association of CIMP phenotype with good clinical prognosis has been also proposed by some groups for other tumor types but also discussed by others (Yamashita et al. 2003; Anacleto et al. 2005). Interestingly, B-CIMP phenotype seems to initially stimulate cell transformation but restrains later stages of tumor progression due to epigenetic inactivation of key genes, many of which overlap with PcG targets.

Altered patterns of histone modifications have been identified as a frequent trait in human cancer (Fraga et al. 2005) and have proved to be of prognostic relevance (Seligson et al. 2005). Specifically, cancer cells display a loss of monoacetylated and trimethylated forms of histone H4 affecting acetylated Lys16 and trimethylated Lys20 residues of histone H4, which were associated with the hypomethylation of DNA repetitive sequences at different stages of tumor progression. In the field of microRNA regulation, truncating mutations in the microRNA-processing machinery affecting TAR RNA-binding protein 2 (*TARBP2*), encoding an integral component of a *DICER1*-containing complex, and Exportin-5 (*XPO5*), mediator of pre-miRNA nuclear export, have been described in sporadic and hereditary carcinomas with microsatellite instability (Melo et al. 2009, 2010). This provides an explanation for the overall decrease of microRNA functions observed in human cancers (Visone et al. 2007; Ambs et al. 2008) and a therapeutic window since it was experimentally shown that enhancing the microRNA-processing machinery has tumor-suppressive effects in cancer cells (Melo et al. 2011).

13.2.4 Methylator Phenotype: Blind Alley or Right Path?

Over 10 years ago, Toyota and coworkers identified a series of methylation markers whose status defined the CpG island methylator phenotype (CIMP) (Toyota et al. 1999). This proposal emerged as a new pathway for colorectal tumorigenesis, in

addition to the classic mutator or chromosomal instable (CIN) and microsatellite instable (MSI) categories, standing for a subset of sporadic colorectal tumors bearing excessive cancer-specific promoter hypermethylation (Toyota et al. 1999) where epigenetic instability would be the driving force. This molecular subgroup of tumors claimed to group up to 75 % of sporadic CRC with MSI and was initially characterized for exhibiting concordant tumor-specific promoter hypermethylation in a series of markers (*CDKN2A*, *hMLH1*, *THBS1*, *MINT1*, *MINT2*, and *MINT31*). Subsequently, the methylation markers increased, and an association with *KRAS* and *BRAF* mutations and an inverse correlation with *p53* deficiency were reported (Ogino et al. 2007; Lee et al. 2008). From a histological point of view, CIMP has been linked to the development of serrated adenomas (Park et al. 2003), and defenders of this still controversial trend support that it also extends to other tumor types (Noushmehr et al. 2010; Fang et al. 2011) and entails therapeutic implications (Teodoridis et al. 2008; Iacopetta et al. 2008). In addition, little is known about the etiology of CIMP, and some reports even challenge the existence of such a driving mechanism (Yamashita et al. 2003; Anacleto et al. 2005). In summary, the CIMP phenotype remains a controversial issue due to the lack of agreement on panel of markers used to classify CIMP tumors, the increasing subdivision in numerous groups attending to diverse molecular features, and the publication of contradictory results in detecting CIMP or regarding the clinical outcome of this category of tumors. The disparate body of evidence surrounding this concept has not yet dispelled the doubts concerning CIMP, and further evidences will be required to support its validity.

13.2.5 Hypomethylation Defects in Cancer

As previously mentioned, global DNA hypomethylation was the first epigenetic alteration reported in relation to cancer. It is an early event affecting CG sites located outside CpG islands and in 5′ end of non-CpG island genes which typically exhibit restricted tissue-specific expression. Interestingly, some reports document gene re-expression upon hypomethylation of promoters lacking CGIs – low CG density but also influencing gene transcription (Fernandez et al. 2011). The reactivation of these genes that would be normally repressed as part of tissue-specific programs results in an enhanced transformed phenotype and loss of cellular identity, characteristic of the transformed phenotype. DNA hypomethylation also affects transposable elements and repetitive sequences which are found densely methylated in normal conditions. As a consequence, reactivations of these sequences produce genomic instability resulting in chromosomal translocations and insertions that alters gene transcription. The use of a *Dnmt1* hypomorphic mouse model in several studies has ascribed a disparate role to global DNA hypomethylation highly dependent of cell type. Reduced methylation levels were associated with *Apc* loss of heterozygosity, suggesting that DNA hypomethylation could have an initiating role, as was proposed by other studies, possibly by promoting genomic instability. However, while higher incidence of intestinal

initiating lesions was reported, further progression was strongly inhibited, linked to reduced cell proliferation. In other tumor types though, DNA hypomethylation triggers development of full-blown tumors as explored in T-cell lymphoma and hepatocellular carcinoma, highlighting the differing effects depending on cell type and tumor stage (Gaudet et al. 2003; Yamada et al. 2005). Other investigations have focused on the relevance of DNA hypomethylating events as biomarkers for early cancer detection, and *LINE-1* hypomethylation has emerged as a widespread biomarker for colorectal, ovarian, lung, and hepatocellular carcinoma prognosis. In all cancer types, increased levels of *LINE-1* hypomethylation was correlated with poor prognosis, shorter survival, and advanced tumor stages, underscoring the importance that this alteration may have, not only at early stages but also at later stages (Tangkijvanich et al. 2007; Ogino et al. 2008; Pattamadilok et al. 2008; Baba et al. 2010; Saito et al. 2010).

13.3 Translating Epigenetic Research to Clinical Solutions

Specific DNA methylation profiles have been associated to particular cancers, and within a tumor type, methylation biomarkers can distinguish between histological subtypes, prognosis and response to therapy. Therefore, DNA methylation offers opportunities for an improved management of cancer by means of earlier detection at better cost, monitoring prognosis and risk of relapse, and evaluating response to therapy.

13.3.1 Epigenetic Biomarkers for Clinical Management of Cancer

The availability of biomarkers informative of cancer cell behavior is essential in the management of cancer, to better anticipate and eradicate this fatal disease. The advantage of DNA methylation biomarkers rely, firstly, on the stability of methylation levels upon subtle changes in microenvironment, whereas transcription can vary a lot yielding false positive and negative results; secondly, there is a wide range of techniques to detect DNA methylation of a selected gene or in a genome-wide manner at cost-efficient prices, with high sensitivity and requiring little amounts of DNA; thirdly, sources of DNA are available in luminal fluids – sputum, urine/semen, stool, nipple aspirates, etc. – and for many cases, changes in DNA methylation for a given region are qualitative, meaning that results are unambiguous in contrast with expression levels for which varying thresholds have to be established prior to interpretation (Carmona and Esteller 2011a).

Nevertheless, comprehensive panels of biomarkers still remain elusive due to different techniques used, and sample cohorts examined and are frequently influenced by geographic variability (Laird 2003; Mulero-Navarro and Esteller 2008). Until recently, available biomarkers resulted from candidate-gene approach based studies yielded limited information. With the advent of epigenomics, better combinations of markers

are coming out with the promise of becoming clinically available in short term. At the moment, clinically relevant markers for cancer detection are limited. The best examples are O^6-methylguanine-DNA methyltransferase (*MGMT*), whose methylation status is indicator of response to alkylating therapy, such as carmustine, in glioblastoma patients (Esteller et al. 2000), and glutathione S-transferase P (*GSTP1*) that has been almost exclusively found to undergo promoter hypermethylation in prostate cancer patients (Lee et al. 1994; Esteller et al. 1998). Several studies have addressed the identification of reliable methylation biomarkers (Caldeira et al. 2006), (Chang et al. 2002), (Dottori et al. 1999), (Hellebrekers et al. 2009), (Koul et al. 2002), (Kuroki et al. 2003), (Lewis et al. 2005), (Lind et al. 2004), (Liu et al. 2003), (Mittag et al. 2006), (Palmisano et al. 2003), (Parrella et al. 2004), (Pulukuri et al. 2007), (Rawson et al. 2011), (Sathyanarayana et al. 2003), (Simpkins et al. 1998), (Suzuki et al. 2004), (Toyooka et al. 2001), (Wagner et al. 2002), (Wang et al. 2009), (Young et al. 2001), (Zöchbauer-Müller et al. 2001) in many cancers and in association with risk factors such as smoking and diet (Table 13.1). There are ongoing clinical trials testing the power of additional markers in diverse cancer types. A recent report integrating genomic and epigenomic data leads to the identification of a prognostic signature in stage II CRC patients, affecting genes of the extracellular matrix pathway, and is reliable for patient stratification (Yi et al. 2011; Carmona and Esteller 2011c). The presence of aberrantly methylated *SEPT9* in CRC was first reported in 2008 and later confirmed as a powerful screening marker for CRC in blood tests (Grützmann et al. 2008; Lofton-Day et al. 2008; deVos et al. 2009). It is capable of detecting 68 % of cancers with high specificity (89 %) and is currently being evaluated in a large prospective clinical study (www.presept.net). Regarding stool-screening tests, promising results are being developed for the detection of cancers of the gastrointestinal tract, such as pancreatic, esophageal, and gallbladder carcinomas (Ahlquist 2009). Particularly, a study reported extensive hypermethylation at the promoter region of the tumor-suppressor gene *NDRG4*, which occurred in 70–86 % of CRC tissues and was negligible in normal mucosa. In stool DNA, *NDRG4* promoter methylation analysis had a sensitivity of 61 % and 53 % and a specificity of 93 % and 100 % in the training and test sets of CRC patients and control subjects, respectively (Melotte et al. 2009). Additionally, *SYNE1* and *FOXE1* were found to be hypermethylated in high frequency in all stages of CRC, and large prospective trials are in progress to confirm their value as reliable indicators of CRC in plasma tests.

Hopefully, the validation of published results in large prospective studies and the consensus in detection techniques and selected markers will provide helpful tools for the identification of cancer onset and progression, in order to be more effective in cancer prevention and treatment.

13.3.2 Pharmacoepigenomics

Unlike genetic changes, epigenetic modifications have the potential to be reverted, and therefore the alterations leading to expression defects in human disease can be restored to normal levels. Epigenetic alterations can essentially be targeted through chemical agents affecting DNA methylation and histone acetylation at global scale.

In the early 1970s, the first DNA demethylating agents were developed and, soon after, used to reverse aberrant epigenomic profiles arising in cancer cells. Researchers pioneering this field realize that epigenomic plasticity offered a way to turn back on genes that had undergone inactivation through promoter hypermethylation, by using compounds interfering with the DNMTs. These agents, 5-azacytidine (Vidaza) and 5-aza-2′-deoxycytidine (Dacogen), are incorporated into the DNA following replication rounds and act by trapping DNMTs, therefore disrupting the maintenance of aberrant DNA methylation patterns, and theoretically reverting to their normal levels. Their way of action is not so much by actively demethylating but by blocking DNMT activity since it was demonstrated that – especially for DNMT1 methyltransferases – activity maintains aberrant silencing in cancer cells (Robert et al. 2002; Beaulieu et al. 2002). These compounds have been evaluated in clinical trials and were approved by the US Food and Drug Administration (FDA) for the treatment of myelodysplastic syndrome, achieving extended survival and even some remissions in treated patients. Clinical trials are now also being extended to solid tumors as lung, colon, and breast cancers. Besides, inhibitors of histone deacetylation (HDACi) such as suberoylanilide hydroxamic acid (SAHA) have proved synergistic effects in reactivating tumor suppressors when combined with demethylating drugs and are currently being used for the treatment of cutaneous T-cell lymphomas (Kirschbaum et al. 2011; Stathis et al. 2011). Despite the mechanism of action of epigenetic drugs has not been fully understood, the benefits demonstrated in treating certain cancer types provide the basis to extend trials for treatment of other tumor types.

A third class of therapy involving epigenetic modifications is found in miRNA-based therapies. Evidences supporting their efficacy are much more preliminary, restricted to experimental assays developed in the laboratory, but could be considered as a long-term line of action. One example is to take advantage of miRNAs regulatory effect on gene expression in a gene-specific manner. Specifically, it was found that miRNA-29 family was able to reverse altered methylation profiles in a lung cancer cellular model through targeting *DNMT3A* and *DNMT3B*, responsible for aberrant DNA methylation in cancer (Fabbri et al. 2007; Garzon et al. 2009). Another miRNA-based strategy targeting the microRNA-processing machinery also showed striking results in CRC. The administration of a fluoroquinolone, enoxacin, produced a dramatic growth-inhibitory effect in cancer cells without affecting normal counterparts. This molecule enhances the production of miRNAs – which have been shown to be globally diminished in cancer – with tumor-suppressor functions by binding to the miRNA biosynthesis protein TAR RNA-binding protein 2 (*TARBP2*), and restores levels exhibited by normal cells (Melo et al. 2011).

13.4 What Is Next?

In conclusion, it is evident that epigenetic alterations and specifically aberrant DNA methylation in cancer are still a dense and tangled affair that will require huge efforts to unravel. From the molecular point of view, further comprehensive studies

need to be carried out in order to fully understand the role of CGI and non-CGI DNA methylation in stem cell and cancer biology and how the interaction with other epigenetic processes modulates and alters expression programs involved in neoplasia. From the clinical standpoint, protocols for sample storage and processing have to be adopted in order to count with good quality and well-annotated clinical cohorts and shorten the distance between basic research findings and clinical applicability.

Cutting-edge technologies are incorporating the latest discoveries in order to provide researchers with most-up-to-date instruments. The next years will therefore require the incorporation of these methodologies, the design of straightforward protocols for data analysis and interpretation, and validation procedures to identify the most clinically relevant events and the underlying etiology.

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Part V
Epigenetics and the Law

Chapter 14
Legal and Ethical Implications of Epigenetics

Mark A. Rothstein

Abstract Epigenetics raises important legal and ethical issues that have been largely unexplored. Initially, this chapter considers whether legal and ethical issues raised by epigenetics differ from those raised by genetics, about which there already has been much discussion and legislation. Among the key differences are the higher rates of epigenetic marks than genetic mutations from similar environmental exposures and the greater potential for multigenerational harms caused by epigenetics. This chapter discusses the following legal issues: (1) regulation of research, (2) regulation of exposures, (3) discrimination, (4) personal injury litigation, and (5) medical malpractice. The ethical issues discussed are (1) environmental and occupational justice, (2) personal responsibility, (3) privacy and confidentiality, (4) access to healthcare, (5) equality, and (6) intergenerational equity. In general, this chapter discusses how epigenetics raises fundamental issues in a new context, thereby challenging existing legal and ethical doctrines.

Keywords Epigenetics • Ethics • Healthcare • Medical malpractice • Personal injury • Transgenerational

14.1 Introduction

New discoveries in epigenetics have begun elucidating the mechanisms by which environmental factors influence gene expression, thereby holding the promise of novel approaches to disease prevention and treatment. Yet, despite the scientific significance of epigenetics, there has been very little consideration of the ethical, legal, and social issues raised by the research, which contrasts sharply with the

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extensive efforts to study comparable issues surrounding genetic and genomic research resulting from the Human Genome Project.

A foundational issue to consider is whether the legal and ethical issues surrounding epigenetics and epigenomics differ from the legal and ethical issues surrounding genetics and genomics. Unless the social consequences of epigenetics are sufficiently distinct, then the same analyses applicable to genetics would apply. Accordingly, it is essential to compare and contrast the implications of epigenetics with those surrounding genetics. This inquiry into "epigenetic exceptionalism" is analogous to the debate about "genetic exceptionalism," which began in the 1990s and remains ongoing, where the issue is whether genetics and genomics are sufficiently distinct from other areas of biomedical research and clinical application that they warrant special policy treatment (Hellman 2003; Rothstein 2005a).

Although a detailed discussion of the science of epigenetics is beyond the scope of this chapter, the current literature suggests that epigenetics differs from genetics in at least the following ways: (1) There is a higher frequency of epigenetic marks than mutations in DNA sequence from similar environmental exposures. (2) Epigenetic effects are more dose dependent. (3) Epigenetic effects are more responsive to the life cycle stage of exposure. (4) Epigenetic effects are more tissue specific. (5) Epigenetic effects are more species specific. (6) Epigenetic effects are more likely to be reversible. As discussed below, these characteristics have important implications for a wide range of legal and ethical issues.

This chapter considers five of the most significant legal issues raised by epigenetics: (1) regulation of research, (2) regulation of exposures, (3) discrimination, (4) personal injury litigation, and (5) medical malpractice. It then explores six important ethical issues raised by epigenetics: (1) environmental and occupational justice, (2) personal responsibility, (3) privacy and confidentiality, (4) access to healthcare, (5) equality, and (6) intergenerational equity.

14.2 Legal Issues

The following sections are based on the law in the United States, but the principles on which the law is based have widespread applicability. Because no country has yet enacted any laws specifically applicable to epigenetics, the legal analysis initially will focus on whether existing laws are applicable to epigenetics and, if not, whether additional measures are needed.

14.2.1 Regulation of Research

Although some groundbreaking work in epigenetics has involved retrospective studies of human growth and development (Kaati et al. 2002; Pembrey 1996), animal models have been used extensively in experimental epigenetic research

(Dolinoy et al. 2007; Jirtle and Skinner 2007). Many experts believe that new epigenetic research increasingly will focus on humans. Of course, there will not be any experimental designs of the type used with rodents; epigenetic research in humans will involve analyzing tissue samples and health records. In this respect, epigenetics will resemble the large-scale, post-genome research methods used by pharmacogenomics, proteomics, toxicogenomics, and other "omics."

Epigenetics research must comply with regulations applicable to research with human subjects. In the United States, this is known as the Common Rule, because it is a set of rules applicable to most of the federal agencies engaged in sponsoring research with human subjects (Federal Policy for the Protection of Human Subjects 2011). Internationally, the best-known statement of research ethics is the Declaration of Helsinki, developed by the World Medical Association in 1964 and updated periodically (World Medical Association 2000).

Research regulations typically require the prior approval of an independent oversight committee known as an institutional review board, research ethics board, or some other name. Among the key elements of an ethically sound research protocol are informed consent of the subject, equitable selection of subjects, reasonable relation of risks to benefits, and special protections for vulnerable subjects, such as children, prisoners, and mentally disabled persons.

Research oversight bodies also regulate biobanks, which play an increasingly important role in large-scale biomedical research, including epigenetics. Extrapolating from prior estimates, as of 2011, there were over 500 million stored specimens in hundreds of biobanks in the United States, and the number grows by at least 20 million each year (Eiseman and Haga 1999, p. xvii). In addition, large biobanks have been established in numerous countries, including Belgium, Canada, Estonia, Iceland, Japan, Latvia, Singapore, Sweden, and the United Kingdom (Dierickx and Borry 2009; Swede et al. 2007). These biobanks, often in combination with networks of electronic health records, facilitate high-throughput and computer-based research.

An important legal and ethical issue is whether consent is required from individuals whose samples and health records are used in research. As a legal and regulatory matter in the United States and other countries, it depends on whether the samples and records are identifiable or deidentified (Knoppers 2005; Rothstein 2005b). Under the Common Rule, research involving records or specimens is exempt "if the information is recorded by the investigator in such a manner that subjects cannot be identified, directly or through identifiers linked to the subjects" (45 C.F.R. § 46.101(b)(4)). Similarly, under the Health Insurance Portability and Accountability Act (HIPAA) Privacy Rule, "[h]ealth information that does not identify an individual and with respect to which there is no reasonable basis to believe that the information can be used to identify an individual is not individually identifiable information [and therefore not subject to the Privacy Rule]" (45 C.F.R. § 164.514(a)).

The bright-line distinction between identifiable and deidentified samples and records is overly simplistic. It fails to account for the spectrum of deidentification, including anonymization, pseudonymization, deidentification, and various forms of

coding. Furthermore, reidentification of deidentified samples has been demonstrated using publically available databases. In the United States, it has been estimated that between 63 % (Golle 2006) and 87 % (Sweeney 2002) of the population could be uniquely identified by using only gender, postal code (ZIP code), and date of birth.

Reliance on deidentification as a privacy strategy also fails to account for group harms, objectionable uses of samples and information, commercial exploitation, and other concerns associated with research using even deidentified samples and records. In other words, research with deidentified samples and information can still result in a range of serious social harms. Finally, undertaking research using deidentified samples and health records without any notice or consent sharply conflicts with the autonomy interests of individuals to decide how their biological materials and health information will be used (Rothstein 2010a). In short, most members of the public do not follow the regulatory distinction between identifiable and deidentified samples and information (Hull et al. 2008). Evolving regulatory approaches to regulating biobanks will have important implications for genetic and epigenetic research.

14.2.2 Regulation of Exposures

Discoveries in epigenetics are likely to have great significance for laws regulating exposures in the environment, in the workplace, and in pharmaceutical products. Although regulatory agencies are beginning to show some interest in this research, regulatory applications thus far have been quite limited (Rothstein et al. 2009). Because the term "epigenetics" is not used in the enabling legislation, it is not clear whether the statutory grant of regulatory authority extends to regulations designed to prevent the exposure of individuals to substances causing an increased risk of epigenetic marks.

One law illustrating this definitional problem is the United States Occupational Safety and Health Act (29 U.S.C. §§ 651–678). Several questions are raised. First, does the law seek to prevent individuals from suffering epigenetic harms? Section 6(b)(5) of the Act authorizes the Secretary of Labor to establish standards to assure that "no employee will suffer material impairment of health or functional capacity . . ." It is not clear whether this language would include epigenetic harms, although it has been held that the subclinical effects of inorganic lead exposure constitute "material impairment" (United Steelworkers of America v. Marshall, 647 F.2d 1189 (D.C. Cir. 1980)).

Second, at what level is the Secretary of Labor authorized to regulate substances causing epigenetic effects? Again, there is no clear answer, although the Supreme Court has held that the statute was not designed "to require employers to provide absolutely risk-free workplaces whenever it is technologically feasible to do so. . . [but] was intended to require the elimination, as far as possible, of significant risks of harm" (Industrial Union Department, AFL-CIO v. American Petroleum Institute,

448 U.S. 607, 653 (1980)). Thus, it must be determined whether the epigenetic harms are "significant."

Third, are employers permitted to require employees to submit to epigenetic testing as a condition of employment? The Occupational Safety and Health Act is silent on this issue, but another law, the Genetic Information Nondiscrimination Act of 2008 (GINA), contains instructive language. Section 202(b)(5) of GINA permits employers to conduct genetic monitoring of employees to detect the biological effects of toxic substances in the workplace so long as the monitoring is voluntary and the employer receives the results only in aggregate form. This provision does not apply to preexposure genetic testing, and it does not seem to apply at all to epigenetic testing. Thus, without any explicit statutory prohibition, it would appear to be lawful for employers to perform epigenetic screening and monitoring of workers, a position difficult to reconcile with GINA's outright ban on genetic testing.

The context of occupational safety and health illustrates the types of regulatory issues that would be raised under any statute dealing with the human health effects of environmental exposures. Epigenetics and epigenomics have not yet attracted the attention of many elected officials, and when this happens, it will be interesting to see whether any regulatory strategies adopted will follow the same approaches as have been used for genetics and genomics.

14.2.3 Discrimination

Numerous entities, including employers and various types of insurers, have a financial interest in the health status of an individual. Any biomedical information that can be used to predict the future health of an individual, such as preclinical biomarkers or epigenetic marks, might be considered extremely valuable. The use of this type of predictive information to "discriminate" in access to employment, insurance, mortgages, or other important transactions and relationships raises at least three important policy concerns. First, the information might be inaccurate, and some individuals might be denied opportunities when they actually do not have an increased risk of a particular adverse health effect in the future or, even if they do, will never develop the condition. Second, even assuming the information is scientifically accurate, there might be strong policy reasons why it should not be considered, such as promoting the goal of equal opportunity. Third, obtaining, using, and retaining sensitive health information raise important concerns about privacy and confidentiality (Rothstein and Anderlik 2001).

Employment discrimination laws illustrate that it is likely to be difficult to bring epigenetics within the ambit of existing nondiscrimination laws. Many countries have enacted legislation to prohibit discrimination in employment on the basis of physical or mental disabilities (Disability Rights Education and Defense Fund 2011). In the United States, the primary law is the Americans with Disabilities Act of 1990 (ADA) (42 U.S.C. §§ 12101–12213). The United States also has

Table 14.1 Coverage of ADA and GINA

	Asymptomatic	Epigenetic marks, biomarkers, endophenotypes, mild symptoms	"Substantial limitation of major life activity" "Manifestation of disease"
ADA	No	No	Yes
GINA	Yes	No	No

enacted GINA to prohibit discrimination in employment on the basis of genetic information. As depicted in Table 14.1, however, neither the ADA nor GINA would appear to apply to discrimination based on epigenetic information.

To be covered under the ADA as an individual with a disability, an individual must have a current physical or mental impairment that constitutes a substantial limitation of a major life activity (ADA § 12102(2)). To be covered under GINA, the individual's condition must not yet have manifested in disease (GINA § 210). Accordingly, individuals who have any type of "manifestation" or "symptom" (e.g., epigenetic marks) are excluded from coverage under GINA, but the individual is not covered under the ADA unless the condition has become a substantial limitation of a major life activity. Furthermore, under GINA, the definition of a genetic test is limited to "an analysis of human DNA, RNA, chromosomes, proteins, or metabolites, that detects genotypes, mutations or chromosomal changes" (GINA § 201(7)).

There are many other possible bases of epigenetic discrimination, including life insurance, disability insurance, long-term care insurance, mortgages, and commercial transactions. Laws prohibiting discrimination on the basis of genetic information or disability were mostly drafted before recent discoveries in epigenetics and therefore should be reconsidered to ensure that there are no gaps in coverage.

14.2.4 Personal Injury Litigation

Various types of personal injury lawsuits are possible based on epigenetic harms. These are likely to result from hazardous products (e.g., pharmaceuticals) or environmental exposures. The legal bases of the claims probably would be products liability and negligence, although other common law and statutory claims are possible. These legal actions are not likely to be distinct from other comparable types of personal injury cases, except for the issue of whether epigenetic harms in the absence of a fully expressed disease would be actionable.

One possible way in which epigenetics could have a significant effect on toxic tort litigation relates to the burden of proof. It is often quite difficult for an injured plaintiff to prove that his or her injuries were caused by exposure to substances attributable to the defendant. An example will help to illustrate the point. Environmental exposure to benzene causes leukemia (Agency for Toxic Substances and Disease Registry 2011). Not all people exposed to benzene, however, get leukemia.

In addition, not all people who get leukemia have been exposed to benzene. Consequently, how can someone exposed to benzene who develops leukemia prove that exposure to benzene was the legal cause of the leukemia? As a scientific matter, it is impossible to prove, but for legal purposes, many courts have adopted the rule that there must be evidence that exposure at least doubles the risk for an individual; in other words, the relative risk must be equal or greater than 2.0 (Carruth and Goldstein 2001).

It is possible that unique patterns of epigenetic marks could be the molecular signatures of specific environmental exposures and the precursors of disease. If so, it might be possible to dispense with the legal, shorthand presumption that a relative risk of 2.0 or greater establishes causation. Instead, courts could rely on or even require evidence of "specific causation" in the form of epigenetic marks as definitive proof that exposure to a particular substance resulted in harm.

14.2.5 Medical Malpractice

Over the next several years, new discoveries in epigenetics are likely to have substantial clinical significance in preventing, diagnosing, and treating a range of illnesses. As with other medical advances, each new discovery in epigenetics places new demands on physicians to understand and, where appropriate, incorporate the developments into their practice. In the United States and in other countries, the new technological imperatives will coincide with shorter times allocated to physicians for clinical encounters. Furthermore, in their role as health counselor, physicians will be responsible for explaining complicated concepts to patients who may have low levels of health literacy or who may suffer from communication or cognitive impairments.

Undoubtedly, some medical malpractice claims might arise from epigenetics, although it is not clear that the overall rate of malpractice claims will be rising (Rothstein 2010b). At least the following types of medical malpractice claims are possible: (1) negligent failure to order an epigenetic test, (2) negligent interpretation of test results, (3) negligent diagnosis, (4) negligent treatment, and (5) negligent prescribing. Physicians will be challenged to expand their knowledge of new developments in epigenetics through continuing medical education and other methods of lifetime learning.

14.3 Ethical Issues

Research on and applications of epigenetics will continue to raise a range of important ethical issues. Unlike the previous sections, where the focus was mostly on laws in effect in the United States, the ethical analysis has a more universal applicability. Furthermore, instead of evoking unique issues, the likely effect of

epigenetics will be to shed new light on existing ethical and societal issues, highlighting new implications, and perhaps pointing the way to their resolution. The following sections consider several of the most important ethical issues.

14.3.1 Environmental and Occupational Justice

Substances that cause epigenetic effects, such as toxic chemicals, airborne pollutants, pesticides, diesel exhaust, and tobacco smoke, are not distributed randomly throughout society. The exposures are frequently linked with poverty, discriminatory land use, substandard living conditions, and hazardous occupational exposures. Populations exposed to these environmental insults also are more likely to have preexisting health problems and comorbidities, often with poor clinical management. If environmental epigenetics adversely affects the most vulnerable populations, should there be a heightened moral duty to remediate the risks and prevent harmful exposures?

Although a Rawlsian argument could be made that there is a moral duty to afford additional protection to those in the most need (Rawls 1971), epigenetics does not help to settle the debate. Indeed, the same moral argument has been made with little success over many decades, involving numerous substances, modes of exposures, and endpoints of adverse health effects. If any aspect of epigenetics could potentially influence this issue, it might be the documented transgenerational effects of epigenetics. A strong case could be made that young or unborn children did not in any way contribute to their exposures and that society has an obligation to remediate the environmental risks to ensure that vulnerable children will have a reasonable opportunity for a healthful life and an open future.

14.3.2 Personal Responsibility

As discussed above, individuals and entities creating epigenetic risks, including manufacturers, polluters, and employers, could face legal liability for their actions. An unresolved question is whether individuals ought to bear some personal responsibility for their own harms. This question assumes individuals have knowledge of the risks and the ability to avoid them. It could be argued that individuals who knowingly and voluntarily expose themselves to the epigenetic and other risks of, for example, tobacco smoke bear some responsibility for the harms caused by the exposure. The issues of personal responsibility become more difficult to assess in the context of other, more diffuse, environmental and occupational exposures.

A common issue of epigenetic ethics is whether the risk of transgenerational harms affects the analysis. It is possible that some responsibility will shift to women for the prenatal exposures of their later-born children. The degree of moral responsibility, however, likely depends on the type of exposure, differing significantly, for

example, between voluntary exposure to tobacco smoke and less voluntary environmental exposure to diesel exhaust fumes. It is questionable whether extensive "victim blaming" promotes any discernible public health policies. It will be interesting to follow whether "personal responsibility" will be invoked as a means for shifting responsibility away from primary polluters to those harmed by exposures.

Another question of personal responsibility involves whether individuals have an obligation to undergo testing for susceptibility to epigenetic marks or indications of prior exposures. In the future, personal responsibility could extend to an obligation to undergo safe, effective treatment to reverse epigenetic harms. Finally, there is the issue of whether individuals have a duty to permit their biological specimens and health records to be used for research, including making the information publically available (Melo-Martin 2008). The latter questions have arisen with greater frequency recently based on the need for more biological specimens and health records in research as well as a sense among some observers that the pendulum has swung too far in the direction of individual rights in research. These issues, however, are not unique to epigenetics.

14.3.3 Privacy and Confidentiality

Epigenetics could create a wealth of new, sensitive information about an individual's likelihood of developing health problems in the future as well as the risk of vertical transmission to his or her later-born children. The increase in sensitive information comes at a time when vast networks of electronic health records are being created in developed countries that make widespread access to and disclosure of health information easy.

An important ethical and policy concern is whether sensitive information will be disclosed to individuals without a legitimate need to know, including some physicians in the clinical setting treating unrelated conditions, other healthcare providers in the clinical setting without a need to know, and non-healthcare providers (e.g., employers, life insurers) pursuant to a "compelled" authorization (Rothstein 2010c).

The ethical imperative to limit unnecessary disclosures has been difficult to translate into practice because of a variety of technical, legal, and policy considerations. Epigenetics is not driving this debate, but it will no doubt be affected by the resolution of it.

14.3.4 Access to Healthcare

Greater understanding of the link between environmental exposures and epigenetic effects increases the importance of prevention, monitoring, and treatment. Many of the individuals most at risk, such as those living and working with hazardous

exposures, are among the least likely to have regular, timely, and comprehensive healthcare. Even for people with access to regular healthcare, it is not yet clear whether expensive epigenetic testing and treatment will be available or whether other demands on scarce healthcare resources will be given a higher priority. The severity of the condition, the availability and efficacy of the intervention, the cost, and other factors all will help determine the priority afforded to epigenetic-related health services.

14.3.5 Equality

Equality of all persons is a fundamental political, social, and ethical concept in Western society. Since the end of World War II, laws have been enacted in many countries to prohibit discrimination in employment, housing, education, and public services on the basis of race, color, religion, sex, national origin, age, disability, sexual orientation, political affiliation, marital status, genotype, and other factors. In many countries, including the United States, virtually all of these laws have been based on a "civil rights" model of equality. Under this approach, all individuals are considered the same because the proscribed criteria for differentiation are officially deemed irrelevant.

As a political ideal, the civil rights model of equality is indisputably appropriate. As a matter of biology or practicality, however, it may be inappropriate. Already, in some situations, public policy has recognized that it is not enough merely to assume that everyone is the same; a good example is the need to provide reasonable accommodations (e.g., ramps, curb cuts) for individuals with disabilities. Treating everyone exactly the same may be inadequate when meaningful differentiation is essential.

Epigenetics establishes an additional way in which individuals differ on a biological level. It remains to be seen how epigenetic variation will be incorporated into prevailing conceptions of equality. It is arguable that society needs to develop a new model of equality that recognizes, respects, and values biological diversity while simultaneously improving individual and population health, ensuring access to healthcare and other social goods, and guaranteeing civil rights (Rothstein 2007).

14.3.6 Intergenerational Equity

Intergenerational equity refers to the relationship each generation has to other generations, past and future, with regard to the natural and cultural resources of the earth. Intergenerational equity has been applied to such vexing issues as the disposal of nuclear waste, extinction of species of plants and animals, climate change, overpopulation, pollution, and destruction of natural resources. In each of these cases, serious harms will persist for many years or they may be irreparable.

If humanity has a responsibility to future generations to refrain from activities that cause harm to the planet, does the same principle extend to harming the genomes and epigenomes of future generations (Rothstein et al. 2009)? If so, when would the obligation arise and how would it be met? Furthermore, is it possible to protect the genetic and epigenetic legacy of future generations without slipping into eugenics? These are indeed weighty issues.

14.4 Conclusion

Epigenetics is emerging as an important area of scientific inquiry. Although the course of discovery remains unclear, epigenetics already has begun to raise a wide range of significant legal and ethical issues. As an initial matter, it is necessary to determine whether and, if so, in what ways, epigenetics raises unique issues not previously addressed in analyses of genetic research and its applications. Some of the legal issues implicated by epigenetics include the regulation of research, regulation of exposures, discrimination, personal injury litigation, and medical malpractice. Ethical issues include environmental and occupational justice, personal responsibility, privacy and confidentiality, access to healthcare, equality, and intergenerational equity. The legal issues are often complex; the ethical issues are often profound. Those who work in the field of epigenetics and those affected by it will have much to consider and discuss in the years ahead.

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Biography

Randy L. Jirtle, Ph.D., directed the epigenetics and imprinting laboratory at Duke University, Durham, NC, until 2012. He is currently a Visiting Professor at McArdle Laboratory for Cancer Research at the University of Wisconsin-Madison, Madison, WI, where he received his B.S. in nuclear engineering in 1970 and Ph.D. in radiation biology in 1976. Jirtle's research interests include epigenetics, genomic imprinting, and the fetal origins of disease susceptibility, and he has published more than 190 journal articles. He was a featured scientist on the *NOVA* television program on epigenetics entitled *Ghost in Your Genes*. He was invited to speak on epigenetics in 2004 and 2011 at Nobel symposia in Stockholm, Sweden. In 2006, he received the Distinguished Achievement Award from the College of Engineering, University of Wisconsin-Madison. Jirtle was nominated for *Time Magazine's* "Person of the Year" in 2007. He was the inaugural recipient of the Epigenetic Medicine Award in 2008 and received the STARS Lecture Award in Nutrition and Cancer from the National Cancer Institute in 2009. In 2011, Jirtle organized the Keystone meeting *Environmental Epigenomics and Disease Susceptibility*. For more information about Professor Jirtle, visit his website (http://www.geneimprint.com).

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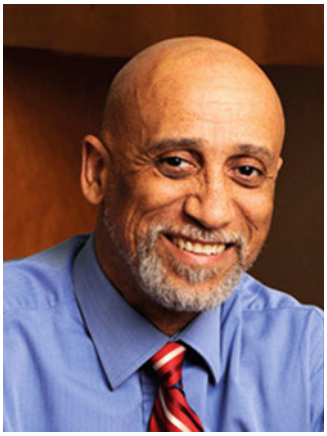

Frederick L. Tyson, Ph.D., is a Scientific Program Director at the NIEHS in the Division of Extramural Research and Training, Cellular, Organs and Systems Pathobiology Branch. Tyson's research portfolio covers a diverse array of topics and has included programs in basic and translational research focusing on mouse genomics, biological mechanisms of health disparities, breast cancer, environmental justice, and community-based participatory research. His portfolio includes projects on marine toxicology and human health risks, transcriptional regulation, chromatin biology, environmental epigenetics, and human epigenomics. Tyson serves as the Program Director for the NIH Roadmap Epigenomics Mapping Consortium (http://www.roadmapepigenomics.org/) and a member of the Executive Committee for IHEC (International Human Epigenomics Consortium (http://www.ihec-epigenomes.org/)). He received his Ph.D. in cell biology and developmental genetics from the Zoology Department, the Graduate School of Rutgers University, and received postdoctoral training in the Laboratory of Developmental Genetics at the Memorial Sloan-Kettering Institute for Cancer Research.

Glossary

2,3,7,8 Tetrachlorodibenzo-p-dioxin (TCDD) A highly toxic polychlorinated dibenzo-p-dioxin compound that is classified as a human carcinogen. It was also a contaminant in Agent Orange, a herbicide used in the Vietnam War.

5-Aza-2-deoxy-cytidine (Decitabine) A cytosine in which the 5 carbon of the cytosine ring has been replaced with nitrogen. Decitabine is exclusively incorporated into DNA, inhibiting mammalian DNA methyltransferases.

5-Azacytidine (AZA) A cytidine RNA analog in which the 5 carbon of the cytosine ring has been replaced with nitrogen. 5-Azacytidine can be incorporated into RNA, and after metabolic activation also into DNA, where it functions as an inhibitor of mammalian DNA methyltransferases.

Acetylation (ac) The enzymatic introduction of an acetyl group to an organic compound, for instance histones.

Acquired immune deficiency syndrome (AIDS) A disease of the human immune system caused by the human immunodeficiency virus (HIV). Presently, there is no cure or vaccine for AIDS; however, antiretroviral treatment can slow the course of the disease, and can lead to a near-normal life expectancy.

Acute myeloid leukemia (AML) A cancer of the myeloid line of white blood cells whose rapid growth interferes with the production of normal blood cells in the bone marrow. AML is the most common acute leukemia affecting adults, and its incidence increases with age.

Acute promyelocytic leukemia (APL) A subtype of AML, a cancer of the blood and bone marrow. Since there is an abnormal accumulation of immature granulocytes called promyelocytes in APL, it is also known as acute promyelocytic leukemia. APL is responsive to all trans retinoic acid therapy.

Adrenocorticotropic hormone (ACTH) A polypeptide tropic hormone secreted by the anterior pituitary gland in response to biological stress.

Agouti gene The murine *Agouti* gene controls fur color through the deposition of yellow pigment in developing hairs. Several variants of this gene exist, and in the Agouti viable yellow (A^{vy}) mouse strain, *Agouti* expression can be heritably modified by epigenetic modifications.

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Alleles Different variants or copies of a gene. For most genes on the chromosomes, there are two copies: one copy inherited from the mother and the other from the father. The DNA sequence of each of these copies may be different because of genetic polymorphisms.

Alpha-thalassemia/mental retardation syndrome X-linked (ATRX) A protein that belongs to the switch/sucrose nonfermentable (SWI/SNF) family of chromatin remodeling molecules that facilitates gene expression by allowing transcription factors to gain access to their targets in chromatin. Mutations in *ATRX* alter DNA methylation, and are associated with an X-linked mental retardation syndrome that is often accompanied by ATRX syndrome.

Alzheimer's Disease (AD) AD is the most common form of dementia. There is presently no cure for this disease, and it worsens as it progresses, eventually leading to death. AD is diagnosed most commonly in people who are over 65.

Androgenote Embryos that develop from two paternal haploid nuclei. These embryos do not develop to term.

Angelman syndrome (AS) A rare pediatric disease caused by chromosomal aberrations or epigenetic inactivation of genes on maternal chromosome 15.

Antioxidant (AO) An antioxidant inhibits the oxidation of other molecules. Oxidation reactions produce free radicals, which can damage DNA and lead to cellular death. Antioxidants, such as glutathione, vitamin C, vitamin A, and vitamin E inhibit these oxidation reactions.

Assisted reproduction technologies (ART) The combination of approaches that are being applied in the fertility clinic, including *in vitro* fertilization (IVF) and intra-cytoplasmic sperm injection (ICSI).

Ataxia telangiectasia mutated (ATM) A serine/threonine protein kinase that is recruited and activated by DNA double-strand breaks. It phosphorylates several key proteins, thereby activating DNA damage checkpoint delay. This results in cell cycle arrest, and subsequent DNA repair or apoptosis.

Autism A neuropsychiatric disorder characterized by impaired social interaction and communication, and by restricted and repetitive behavior.

Autism spectrum disorder (ASD) A range of conditions classified as pervasive developmental disorders that include autism, Asperger syndrome, pervasive developmental disorder not otherwise specified (PDD-NOS), childhood disintegrative disorder, and Rett syndrome. These disorders are typically characterized by social deficits, communication difficulties, stereotyped or repetitive behaviors and interests, and in some cases, cognitive delays.

Avon Longitudinal Study of Parents and Children (ALSPAC) A cohort study of children born in the former county of Avon, England during 1991 and 1992; the initial recruits were 14,000 pregnant women. This study population is used by researchers in health, education, and other social science disciplines. The study is hosted at the University of Bristol, and was initially led by Jean Golding.

Axin fused ($\mathbf{Axin^{Fu}}$) mouse $Axin^{Fu}$ mice have an IAP element inserted into intron 6 of the *Axin* gene, resulting in a number of alternative transcripts. Complete demethylation of the IAP site results in the formation of severely kinked tails,

whereas, the mice have straight tails when the IAP site is fully methylated; partial methylation of this IAP site causes intermediate tail kinking. Supplementation of the mother's diet with methyl donors during pregnancy reduces the incidence of kinked tails in the offspring by increasing DNA methylation at this locus.

Azidothymidine (AZT) A nucleoside analog that functions as a reverse-transcriptase inhibitor. It is used as an antiretroviral drug for the treatment of patients with HIV/AIDS.

Basic helix loop helix (bHLH) The basic helix loop helix motif is characterized by two α-helices connected by a loop. bHLH proteins normally bind to a consensus sequence called an E-box. The canonical E-box, CACGTG, is palindromic; however some bHLH transcription factors bind to related non-palindromic sequences that are similar to the E-box.

Beckwith-Wiedemann syndrome (BWS) An overgrowth disorder usually present at birth that is characterized by an increased risk of childhood cancer and congenital features, such as macroglossia, macrosomia, midline abdominal wall defects, ear creases/ear pits, and neonatal hypoglycemia. More than five distinct errors involving 11p15 have been identified in different BWS patients. Imprinted genes involved in the etiology of this syndrome are *IGF2, CDKN1C, H19*, and *KCNQ1OT1*.

Benzo(a)pyrene (BaP) A polycyclic aromatic hydrocarbon found in coal tar that is classified as a human carcinogen. It was found to be responsible for scrotal cancers in chimney sweeps in the eighteenth century, and skin cancers among the fuel industry workers in the nineteenth century.

Bipolar disorder (BPD) A psychiatric disease defined by the presence of one or more episodes of abnormally elevated energy levels, cognition, and mood with or without one or more depressive episodes.

Bisphenol A (BPA) An organic compound manufactured to make polycarbonate polymers and epoxy resins used in the production of plastics. BPA exhibits hormone-like properties, and studies indicate it increases the incidence of cancer and reproductive problems. Concerns have been raised about its use in consumer products and food containers. Thus, the European Union, Canada, and the United States have banned BPA use in baby bottles.

Bisulfite sequencing (BS) A procedure in which sodium bisulfite is used to deaminate cytosine to uracil in genomic DNA. Conditions are chosen so that 5-methylcytosine is not changed. PCR amplification and subsequent DNA sequencing then reveals the exact position of cytosines that are methylated in genomic DNA.

Bivalent chromatin A chromatin region that is modified by a combination of histone modifications such that it represses gene transcription, but at the same time retains the potential of acquiring gene expression.

Body mass index (BMI) A measure of human body shape defined as the body mass divided by the square of the height (i.e. kg/m^2). The BMI for a healthy weight individual ranges from 18.5 to 25.

Borderline Personality Disorder (BPD) A personality disorder characterized by unusual variability and depth of moods, which may also affect cognition and interpersonal relationships.

Brain-derived neurotrophic factor (BDNF) A protein that acts on neurons of the central and peripheral nervous system, supporting their survival and encouraging the growth and differentiation of new neurons and synapses.

Bromo domain Protein motif found in a variety of nuclear proteins, including transcription factors and HATs involved in transcriptional activation. Bromo domains bind to histone tails carrying acetylated lysine residues.

cAMP response element binding protein (CREB) A transcriptional activator for many immediate early genes.

Cell fate The programmed path of cell differentiation. Although all cells have the same DNA, their cell fate can be different. Some cells develop into the brain, whereas others are the precursors of blood. Cell fate is determined in part by the organization of chromatin – DNA and the histone proteins – in the nucleus.

Cellular memory (epigenetic) Specific active and repressive organizations of chromatin can be maintained from one cell to its daughter cells; this is called epigenetic inheritance. It ensures that specific states of gene expression are inherited over many cell generations.

Cerebellum Region of the brain that plays a role in motor control, as well as language, attention, and some elements of emotion.

Cerebral cortex A sheet of neural tissue that is 2–4 mm thick, and comprised of up to six layers. It covers the cerebrum and cerebellum, and is divided into left and right hemispheres. The cerebral cortex plays a critical role in memory, attention, perceptual awareness, thought, language, and consciousness. It has a grey color in preserved brains, and is also referred to as grey matter.

ChIP-chip After chromatin immunoprecipitation, DNA is purified from the immunoprecipitated chromatin fraction and hybridized on arrays of short DNA fragments representing specific regions of the genome.

ChIP-seq Sequencing of the totality of DNA fragments obtained by ChIP using next-generation sequencing to quantify patterns of enrichment across the genome.

Chromatid In each somatic cell generation, the genomic DNA is replicated in order to make two copies of each individual chromosome. During the M phase of the cell cycle, these copies – called chromatids – are microscopically visible and next to each other before they get distributed to the daughter cells.

Chromatin The nucleo-protein-complex constituting the chromosomes in eukaryotic cells. Structural organization of chromatin is complex and involves different levels of compaction. The lowest level of compaction is represented by an extended array of nucleosomes.

Chromatin conformation capture assay (Hi-C) A high-throughput next generation sequencing technique used to analyze the organization of chromosomes and their interactions. This technique is useful for better understanding gene regulation, nuclear partitioning, and chromatin dynamics.

Chromatin immunoprecipitation (ChIP) This is a method for examining protein–DNA interactions occurring in the cell. DNA-binding proteins are cross-linked to the DNA and enriched using antibodies with specific affinity to particular proteins (e.g. histones) or covalent modifications on proteins. After ChIP, the genomic DNA is purified from the chromatin fragments brought down by the antiserum and analyzed by qPCR, microarray (ChIP-chip), or next-generation sequencing (ChIP-seq).

Chromatin remodeling Locally, the organization and compaction of chromatin can be altered by different enzymatic machineries. This is called chromatin remodeling. Several chromatin remodeling proteins move nucleosomes along the DNA and require ATP for their action.

Chromo domain (chromatin organization modifier domain) A protein–protein interaction motif first identified in *Drosophila melanogaster* HP1 and polycomb group proteins. It is also found in other nuclear proteins involved in transcriptional silencing and heterochromatin formation. Chromo domains consist of approximately 50 amino acids that bind to histone tails methylated at certain lysine residues.

Chromosomal domain It is often observed in higher eukaryotes that chromatin is organized (e.g. by histone methylation) the same way across hundreds to thousands of kilobases of DNA. These "chromosomal domains" can comprise multiple genes that are similarly expressed. Some chromosomal domains are controlled by genomic imprinting.

Chromosomal instability (CIN) An increased rate of chromosome mis-segregation in mitosis that results in a failure to maintain the correct chromosomal complement, thereby causing an increased risk of developing cancer.

Colorectal cancer (CRC) A cancer from uncontrolled cell growth in the colon or rectum. Symptoms of colorectal cancer typically include rectal bleeding and anemia. Screening by colonoscopy is effective at decreasing the chance of dying from colorectal cancer.

Conditioned place preference (CPP) assay This assay is used to evaluate preferences for environmental stimuli that have been associated with a positive or negative reward.

Copy number variation (CNV) Alterations in the DNA of a genome that results in a cell having an increased or decreased number of copies of one or more sections of the DNA. These variations range from kilobases to megabases in size.

CpG dinucleotide A cytosine followed by a guanine in the sequence of bases of the DNA. Cytosine methylation in mammals occurs primarily at CpG dinucleotide positions.

CpG island (CGI) A small stretch of DNA, several hundred bases up to several kilobases in size, that is particularly rich in CpG dinucleotides, and is also relatively enriched in cytosines and guanines. Most CpG islands comprise promoter sequences that drive the expression of genes.

CpG island methylator phenotype (CIMP) Cancers can be classified according to the degree of methylation in their genome. Those with high degrees of methylation are referred to as having a CpG island methylator phenotype, and are characterized by epigenetic instability.

CREB-binding protein (CBP) A protein involved in transcriptional regulation that is often associated with histone acetyltransferases such as p300.

Cytomegalovirus (CMV) A member of the viral genus known as *Herpesviridae*. Herpesviruses can remain latent within the body over long periods. Although they can be found throughout the body, CMV infections are frequently associated with the salivary glands in humans.

Cytosine methylation DNA methylation in mammals occurs at cytosines that are part of CpG dinucleotides. As a consequence of the palindromic nature of the CpG sequence, methylation is symmetrical, and affects both strands of DNA at a methylated target site. When present at promoters, it is usually associated with transcriptional repression.

Deacetylation The removal of acetyl groups from proteins. Deacetylation of histones is often associated with gene repression, and is mediated by histone deacetylases (HDACs).

de novo DNA methylation The addition of methyl groups to a stretch of DNA that is not yet methylated.

Deoxyribonucleic acid (DNA) A molecule encoding the genetic instructions used in the development and function of all known living organisms and many viruses.

Developmental origins of health and disease (DOHaD) A theory that postulates that environmental exposure during early developmental interacts with genotypic variation to change the ability of the organism to cope with its environment in later life, thereby altering the incidence of chronic diseases and neurological disorders in adulthood.

Diabetes Control and Complications Trial (DCCT) A major clinical study conducted from 1983 to 1993. It showed that keeping blood glucose levels as close to normal as possible slows the onset and progression of the eye, kidney, and nerve damage caused by diabetes.

Diethylstilbestrol (DES) A synthetic non-steroidal estrogen first synthesized in 1938; it is also classified as an endocrine disruptor. DES was given to pregnant women between 1940 and 1970 in the mistaken belief it would reduce the risk of pregnancy complications and losses. DES was subsequently shown to cause rare vaginal tumors in the daughters, and developmental malformations in both the daughters and sons who were exposed in utero. Studies are now being conducted to determine if an increased incidence of developmental abnormalities are also present in the grandchildren of the women who were given DES during pregnancy.

Differentially methylated region (DMR) A segment of DNA generally rich in cytosine and guanine nucleotides, with the cytosine nucleotides methylated on only one parental allele. DNA methylation of these regulatory elements is parent-of-origin dependent when they regulate the mono-allelic expression of imprinted genes.

Disomy The occurrence in the cell of two copies of a chromosome, or part of a chromosome, that are identical and of the same parental origin (i.e. uniparental disomy).

Dizygotic twins Fraternal or dizygotic twins develop from two separate eggs that are fertilized by two separate sperm.

DNA demethylation Removal of methyl groups from the DNA. This can occur actively by an enzymatically mediated process, or passively when methylation is not maintained after DNA replication.

DNA methylation A biochemical modification of DNA resulting from the addition of a methyl group to either adenine or cytosine bases. Methylation in mammals is essentially confined to cytosines that are in CpG dinucleotides. Methyl groups can be removed from DNA by DNA demethylation.

DNA methyltransferase The enzyme that adds new (de novo) methylation to the DNA, or maintains existing patterns of DNA methylation.

Dopamine A catecholamine neurotransmitter that has an important role in cognitive function, voluntary movement, reward, motivation, and prolactin production.

Dosage compensation In mammals, the X-chromosome is normally present in two copies in females and only one copy in males. Dosage compensation, by random inactivation of one of the X-chromosomes in females, ensures that in spite of this copy number difference X-linked genes are expressed at the same level in both sexes.

Double strand break (DSB) A break in double-stranded DNA in which both strands are cleaved can result in mutagenic events or cell death if left unrepaired or repaired inappropriately.

Double stranded RNA (dsRNA) RNA with two complementary strands; it is similar to the DNA found in all cells. dsRNA forms the genetic material of double-stranded RNA viruses.

Down syndrome A chromosomal condition caused by the presence of all or part of a third copy of chromosome 21. This syndrome is named after John Langdon Down, the British physician who described it in 1866. Down syndrome is the most common chromosome abnormality in humans. It is typically associated with a delay in cognitive ability and physical growth, and a particular set of facial characteristics.

Dutch famine (Hunger winter) A famine that took place in the German-occupied part of the Netherlands during the winter of 1944–1945 near the end of World War II. A German blockade cut off food and fuel shipments from farm areas to punish the reluctance of the Dutch to aid the Nazi war effort. About 22,000 people died because of the famine. Subsequently, it was determined that the children of pregnant women exposed to the famine were more susceptible to cardiovascular disease, diabetes, obesity, micro-albuminuria, and schizophrenia.

Eight-twenty-one (ETO) This gene derives its name from its association with many cases of acute myelogenous leukemia (AML) in which a reciprocal translocation, t(8;21), brings together a large portion of the *ETO* gene from chromosome eight and part of the *AML1* gene from chromosome 21.

Embryo (EMB) A multicellular diploid eukaryote in its earliest stage of development. In humans, it is called an embryo until about 8 weeks after fertilization, and then it is called a fetus.

Embryonic stem (ES) cells Cultured cells obtained from the inner cell mass of the blastocyst. These cells are totipotent, and can be differentiated into all of the different somatic cell lineages.

Endocrine disruptor A chemical compound that has an antagonistic effect on the action of a hormone to which it is structurally similar. Some pesticides act as endocrine disruptors, and in animal studies are found to have adverse effects on development by altering DNA methylation at specific loci. A well-characterized endocrine disruptor is bisphenol A (BPA), a chemical used for the productions of certain plastics.

Enhancer A small, specialized sequence of DNA which, when recognized by specific regulatory proteins, can enhance the activity of the promoter of a gene(s) located in close proximity.

Enhancer RNA (eRNA) Enhancer regions can produce their own RNA or eRNA that can intensify the ability of cells to produce specific protein coding transcripts.

Epialleles Copies of a DNA sequence or a gene that differ in their epigenetic or expression states without the occurrence of a genetic mutation.

Epigenesis The development of an organism from fertilization through a sequence of steps leading to a gradual increase in complexity through differentiation of cells and formation of organs.

Epigenetic code Patterns of DNA methylation and histone modifications can modify the way genes on the chromosomes are expressed. This led to the idea that combinations of epigenetic modifications constitute a code on top of the genetic code that modulates gene expression, and can be recognized by specific non-histone proteins.

Epigenetic inheritance The somatic inheritance, or inheritance through the germ line, of epigenetic information (i.e. changes that affect gene function without the occurrence of an alteration in the DNA sequence).

Epigenetic marks Regional modifications of DNA and chromatin proteins. This includes DNA methylation and histone methylation that can be maintained from one cell generation to the next, and may affect the way genes are expressed.

Epigenetic reprogramming The resetting of epigenetic marks on the genome so that they become like those of another cell type or of another developmental stage. Epigenetic reprogramming occurs in primordial germ cells brought back to a 'ground state'. Epigenetic reprogramming and dedifferentiation also occur after somatic cell nuclear transfer.

Epigenetics The study of heritable changes in gene function that arise without an apparent change in the genomic DNA sequence. Epigenetic mechanisms are involved in the formation and maintenance of cell lineages during development and in X-inactivation and genomic imprinting; they are frequently perturbed in diseases.

Epigenome The epigenome is the overall epigenetic state of a particular cell. In the developing embryo, each cell type has a different epigenome. Epigenome maps represent the presence of DNA methylation, histone modification, and other chromatin modifications along the chromosomes.

Epigenome-wide association studies (EWAS) The principle of epigenome-wide association studies involves scanning cases and controls to identify epigenetic variations associated with a specific trait or disease.

Epigenotype The totality of epigenetic marks that are found along the DNA sequence of the genome in a particular cell lineage or at a particular developmental stage.

Epimutation A change in the normal epigenetic marking of a gene or regulatory DNA sequence (e.g. DNA methylation) that affects gene expression.

Escape of X-inactivation Regions and genes on the X-chromosomes that are not affected by the dosage compensation/X-inactivation mechanism, and remain active on both X-chromosomes in females.

Euchromatin A type of chromatin that appears lightly stained when observed through the microscope at interphase. Euchromatic chromosomal domains are loosely compacted and relatively rich in genes. The opposite type of chromatin organization is heterochromatin.

Fluorescent in situ hybridization (FISH) A cytogenetic technique that uses fluorescent probes to detect and localize the presence or absence of specific DNA sequences on chromosomes.

Folate A methyl donor obtained primarily from the diet that influences nucleotide synthesis and methylation reactions, including DNA methylation.

Fragile X syndrome A genetic syndrome that is the most common single-gene cause of autism and inherited mental retardation among boys. It is associated with the expansion of the CGG trinucleotide repeat affecting the *Fragile X mental retardation 1 (FMR1)* gene on the X chromosome.

γ-Aminobutyric acid (GABA) The chief inhibitory neurotransmitter in the mammalian central nervous system. It helps control neuronal excitability throughout the nervous system, and is also involved in the regulation of muscle tone.

Genome The entirety of an organism's hereditary information that is encoded either in DNA or in RNA for many types of viruses. The genome includes both the genes and the non-coding sequences of the DNA.

Genome-wide association study (GWAS) An examination of all or most of the genes in groups of individuals different for a specific trait or disease in order to identify DNA sequence-based factors that contribute to the origin of such phenotypes.

Genomic imprinting An epigenetic phenomenon that affects a small subset of genes in the genome of Therian mammals, and results in mono-allelic gene expression in a parent-of-origin dependent manner.

Glucocorticoid receptor (GR) A receptor encoded by *NR3C1* that glucocorticoids (e.g. cortisol) bind to it. The GR regulates genes that modulate development, metabolism, immune functions, and stress response.

Glucocorticoids Steroid hormones that bind to the glucocorticoid receptor (GR), and affect development, immunological functions, metabolic processes, and stress response.

Green fluorescent protein (GFP) A protein composed of 238 amino acids, and first isolated from the jellyfish, *Aequorea victoria*. It exhibits bright green fluorescence when exposed to light in the blue to ultraviolet range. GFP is frequently used as a reporter of gene expression.

Gynogenote Embryos that develop from two maternal haploid nuclei; these embryos do not develop to term.

Heterochromatin A type of chromatin that appears dark when observed through the microscope at interphase. Heterochromatic chromosomal domains, found in all cell types, are highly compacted, rich in repeat sequences, and show little or no gene expression. Extended regions of heterochromatin are found close to centromeres and at telomeres.

Hippocampus A region of the brain belonging to the limbic system that plays a role in long-term memory and spatial navigation.

Histone acetylation Posttranslational modification of the ε-amino group of lysine residues in histones that is catalyzed by a family of enzymes called histone acetyltransferases (HATs). Acetylation contributes to the formation of decondensed, transcriptionally permissive chromatin structures, and facilitates interaction with proteins containing bromo domains.

Histone acetyltransferase (HAT) An enzyme that acetylates specific lysine amino acids on histone proteins.

Histone code A theory that specific combinations of histone modifications are recognized by non-histone proteins through specific protein domains, such as bromo and chromo domains, thereby bring about a specific chromatin configuration and expression state (see epigenetic code).

Histone deacetylase (HDAC) An enzyme that removes acetyl groups from histone proteins. This increases the positive charge of histones, and enhances their attraction to the negatively charged phosphate groups in DNA, resulting in chromatin condensation.

Histone deacetylase inhibitor (HDACi) A class of compounds that interferes with the function of histone deacetylases. These compounds are used in psychiatry and neurology as mood stabilizers and anti-epileptics. They are also being investigated as possible treatments for cancer and inflammatory disease.

Histone demethylase (HDM) Proteins catalyzing the active enzymatic removal of methyl groups from either lysine or arginine residues of histones. Prominent examples are LSD1 and Jumonji proteins.

Histone methylation Posttranslational methylation of amino acid residues in histones catalyzed by histone methyltransferases (HMTs). Histone methylation is found at arginine as mono- or dimethylation and lysine as mono-, di-, or trimethylation. Different types of methylation can be found in either open transcriptionally active or closed transcriptionally silent chromatin. Methylated lysine residues are recognized by proteins containing chromo domains.

Histone methyltransferase (HMT) Enzymes catalyzing the transfer of methyl groups from S-adenosyl-methionine (SAM) to lysine or arginine residues in histones.

Histone variants Canonical histones with distinct amino acid changes accumulating at specific chromatin regions associated with the activating or silencing of transcription.

Human chorionic gonadotropin (HCG) A hormone produced during pregnancy that is made by the developing placenta after conception, and later by the placental component syncytiotrophoblast. Measurement of HCG in the blood or urine can be used to test for pregnancy.

Human mammary epithelial cell (HMEC) Mammary epithelial cells line the ducts and lobes of the breast, and they produce milk. Breast cancer most often originates in these epithelial cells.

Hypothalamic-pituitary-adrenal (HPA) axis A complex set of direct influences and feedback interactions between the hypothalamus, the pituitary gland, and the adrenal glands. Interactions between the organs in the HPA axis control the reactions to stress, and regulate body processes, including digestion, the immune system, mood and emotions, sexuality, and energy usage.

Human immunodeficiency virus (HIV) A lentivirus that causes acquired immunodeficiency syndrome (AIDS), an infectious disease in which progressive failure of the human immune system leads to life-threatening opportunistic infections and/or cancer.

Hypothalamus A portion of the brain that links the nervous system to the endocrine system via the pituitary gland and controls body temperature, hunger, thirst, sleep, and circadian cycles.

Immunodeficiency, centromeric region instability, facial anomalies syndrome (ICF) A rare autosomal recessive disease characterized by immunodeficiency and characteristic rearrangements in the vicinity of the centromeres of chromosomes 1 and 16 and sometimes 9. Symptoms of this syndrome include mild facial dysmorphism, growth retardation, failure to thrive, and psychomotor retardation. ICF always involves limited hypomethylation of DNA, and often arises from mutations in one of the DNA methyltransferase genes.

Impaired glucose tolerance (IGT) A pre-diabetic state of hyperglycemia that is associated with insulin resistance and increased risk of cardiovascular disease.

Imprinted brain theory A theory that proposes that autism spectrum disorder (ASD) represents a paternal bias in the expression of imprinted genes, whereas psychotic spectrum disorder (PSD) results from an imbalance in favor of maternal and/or X-chromosome gene expression.

Imprinted genes Genes that show a parent-of-origin specific gene expression pattern controlled by epigenetic marks that originate from the germ line.

Imprinting See genomic imprinting

Imprinting control region (ICR) Region of the DNA that shows germ-line derived, parent-of-origin dependent epigenetic marks that control the parental-specific allelic expression of neighboring imprinted genes.

Imprintome The complete repertoire of differentially methylated imprint regulatory elements in the genome defines the imprintome. Because imprinting is a direct consequence of epigenetic regulation, the imprintome is a subset of the epigenome rather than the genome or transcriptome.

Induced pluripotent stem cells (iPS) Cells derived from differentiated somatic cells by *in vitro* reprogramming. Reprogramming is triggered by the activation of pluripotency factor genes and cultivation in ES cell medium. iPS cells are capable of generating all cell types of an embryo.

Inner cell mass (ICM) In early embryogenesis, the inner cell mass of cells will eventually give rise to the fetus. This structure forms before implantation into the endometrium of the uterus. The ICM lies within the blastocyst cavity, and is entirely surrounded by a single layer of cells called the trophoblast.

Intracisternal A particle (IAP) A family of retrovirus-like elements that encode for virus-like particles found regularly in early rodent embryos. They are also transcribed in a wide variety of neoplasms because of DNA hypomethylation.

Intra-uterine environment The collective conditions affecting a fetus in the uterus.

Intra-uterine growth restriction (IUGR) IUGR refers to poor growth of a baby during pregnancy often resulting from poor maternal nutrition or lack of adequate oxygen supply to the fetus.

In vitro fertilization (IVF) Fertilization of a surgically retrieved oocyte in the laboratory, followed by a short period of *in vitro* cultivation before the embryo is transferred back into the uterus to allow development to term.

Ionizing radiation (IR) Particulate (e.g. electrons and alpha particles) or electromagnetic radiation with enough energy to remove tightly bound electrons from the orbit of an atom, thereby causing the atom to become ionized.

Kinship theory of imprinting An evolutionary theory that attempts to explain the origin and evolution of imprinted genes.

Lamarck Jean-Baptiste Pierre Antoine de Monet, Chevalier de Lamarck (1744–1829) was a French naturalist known best for his theory of inheritance of acquired characteristics or Lamarckism.

Large intervening non-coding RNA (lincRNA) A molecule of RNA 200 to many thousands of nucleotides in length that is transcribed by non-protein coding areas of DNA. These ribonucleotides may play a role in a variety of biological processes, such as cancer formation.

Long interspersed elements (LINE) Highly repeated sequences, 6,000–8,000 base pairs in length, that contain RNA polymerase II promoters. They also have an open reading frame that is related to the reverse transcriptase of retroviruses, but they do not contain LTRs (long terminal repeats). Copies of the LINE1 family form about 15 % of the human genome. LINE elements are usually transcriptionally silent and marked by DNA methylation.

Long non-coding RNA (lncRNA) Non-protein coding transcripts longer than 200 nucleotides. This limit distinguishes long ncRNAs from microRNAs (miRNAs), short interfering RNAs (siRNAs), Piwi-interacting RNAs (piRNAs), and small nucleolar RNAs (snoRNAs).

Long terminal repeat (LTR) Sequences of DNA that repeat hundreds or thousands of times. They are found in retroviral DNA and in retrotransposons that flank functional genes. They are used by viruses to insert their genetic sequences into the host genome.

Major depressive disorder (MDD) A mental disorder characterized by episodes of all-encompassing low mood accompanied by low self-esteem and loss of interest or pleasure in normally enjoyable activities.

Maternal effects Long-term effects on the development of the embryo triggered by factors in the cytoplasm of the oocyte.

Medial prefrontal cortex (mPFC) A part of the prefrontal cortex in the mammalian brain that is implicated in decision making and the processing of risk and fear.

Medial pre-optic area (MPOA) A region in the forebrain rostral to the hypothalamus that is involved in sexual and parenting behaviors.

Messenger RNA (mRNA) A large family of RNA molecules that convey genetic information from DNA to the ribosome, where they specify the amino acid sequence of the protein products of gene expression.

Metatherians Pouched mammals found mainly in Australia and the Americas, such as the opossum.

Methyl-CpG-binding protein 2 (MeCP2) A protein that is essential for the normal function of nerve cells; mutations in this gene cause Rett syndrome.

Methylated DNA immunoprecipitation-microarray (MeDIP-chip) A genome-wide, high-resolution approach to detect DNA methylation in the whole genome or CpG islands. The method utilizes anti-methylcytosine antibody to immunoprecipitate DNA that contains highly methylated CpG sites. The enriched methylated DNA is then interrogated using DNA microarrays.

Methylated DNA immunoprecipitation-sequencing (MeDIP-seq) A genome-wide, high-resolution approach to detect DNA methylation in the whole genome or CpG islands. The method utilizes anti-methylcytosine antibody to immunoprecipitate DNA that contains highly methylated CpG sites. The enriched methylated DNA is then interrogated using massive parallel sequencing techniques.

Methyl-CpG binding domain (MBD) Protein domain in methyl-CpG-binding proteins (MBPs) responsible for recognizing and binding to methylated cytosine residues in DNA. Proteins containing MBDs form a specific family of proteins with various molecular functions.

Methyl-CpG-binding proteins (MBPs) Proteins containing domains (such as MBD) that bind to 5-methyl-cytosine in the context of CpG dinucleotides. MBPs mostly act as mediators for molecular functions such as transcriptional control or DNA repair.

Methyl-DNA binding domain capture-sequencing (MethylCap-seq) A recently developed technique for the genome-wide profiling of DNA methylation. This technique consists of capturing the methylated DNA fragments by their methyl-CpG binding domains (MBDs), and the subsequent deep sequencing of eluted DNA.

Methyl tetrahydrofolate reductase (MTHFR) A key enzyme in the folate S-adenosylmethionine (SAM) pathway.

micro RNA (miRNA) A small non-coding RNA molecule about 22 nucleotides in length found in plants and animals. It functions in transcriptional and post-transcriptional regulation of gene expression.

Microsatellite instability (MSI) A condition manifested by damaged DNA due to defects in DNA repair. Sections of DNA called microsatellites, which consist of a sequence of repeating units of 1–6 base pairs in length, become unstable and can shorten or lengthen during cell division.

Mitogen-activated protein kinase (MAPK) A protein in a cellular signaling pathway that transduces signals from the cell surface to the nucleus, and modifies gene expression by affecting the activities of transcription factors.

Mixed-lineage leukemia (MLL) A type of childhood leukemia in which a piece of chromosome 11 is translocated to another chromosome. Children with this type of leukemia have a particularly poor prognosis. The name comes from the gene expression profiles in this disease being differ than those seen in ALL and AML.

Monozygotic twins Two individuals developing from one zygote that split and formed two embryos, also known as identical twins.

Neural tube closure defect (NTD) One of the most common birth defects, occurring in approximately one in 1,000 live births in the United States. A NTD is an opening in the spinal cord or brain that occurs very early in human development. During gastrulation, specialized cells on the dorsal side of the fetus begin to fuse and form the neural tube. When the neural tube does not close completely, a NTD develops.

Neuronal plasticity The ability of the brain to change as a result of one's experience.

Newborn epigenetics study (NEST) A study initiated at Duke University by Cathrine Hoyo and Susan Murphy in 2004 to prospectively test the influence of in utero environmental exposures on the epigenetic profiles in newborns.

Next-generation sequencing (NGS) A technology similar to capillary electrophoresis-based Sanger sequencing where the bases of a small fragment of DNA are sequentially identified from signals emitted as each fragment is resynthesized from a DNA template strand. NGS extends this process across millions of reactions in a massively parallel fashion, rather than being limited to a single or a few DNA fragments.

N-methyl-D-aspartate (NMDA) receptor An ionotropic glutamate receptor that stimulates intracellular signaling cascades that affect gene transcription, synaptic plasticity, and learning and memory.

Noncoding RNA (ncRNA) RNA transcripts that do not encode for a protein. ncRNA generation frequently involves RNA processing.

Non-Mendelian inheritance The inheritance of traits that do not follow Mendelian rules, and cannot be explained by simple genetic models.

Nucleolus Specific compartments within the nucleus formed by rDNA repeat domains. Nucleoli are marked by specific heterochromatic structures and active gene expression.

Nucleosome Fundamental organizational unit of chromatin consisting of 147 base pairs of DNA wound around a histone octamer.

Nucleosome Free Region (NFR) Regions in the DNA with an increased accessibility to micrococcal nuclease digestion. Thus, NFR refers to a deficiency in experimentally determined nucleosomes, but it does not imply a complete lack of histones. NFRs at the 5′ and 3′ ends of genes are sites of transcription initiation for mRNA and noncoding RNA.

Nucleus accumbens (Nac) A collection of neurons that forms the main part of the ventral striatum. It is thought to play an important role in reward, pleasure, laughter, addiction, aggression, fear, and the placebo effect.

Open reading frame (ORF) An open reading frame is a portion of a DNA molecule that, when translated into amino acids, contains no stop codons.

Paraventricular nucleus (PVN) A neuronal nucleus in the hypothalamus containing neurons that are activated by stressful or physiological changes.

Parthenogenetic (PG) A form of asexual reproduction in which growth and development of embryos occur without fertilization.

Persistent organic pollutants (POPs) Organic compounds that are resistant to environmental degradation through chemical, biological, and photolytic processes.

Phenylketonuria (PKU) An autosomal recessive metabolic genetic disorder characterized by a mutation in the gene for the hepatic enzyme phenylalanine hydroxylase (PAH), rendering it nonfunctional. Untreated PKU can lead to mental retardation, seizures, and other serious medical problems. The primary treatment for PKU is a strict phenylalanine-restricted diet supplemented by a medical formula containing amino acids and other nutrients.

Pituitary An endocrine gland protruding from the hypothalamus that secretes hormones involved in the homeostasis of an organism.

piwi RNA (piRNA) The largest class of small non-coding RNA molecules expressed in animal cells. They form RNA-protein complexes through interactions with piwi proteins. These piRNA complexes are linked to both epigenetic and post-transcriptional gene silencing of retrotransposons and other genetic elements in germ cells.

Plant homeodomain (PHD) The PHD finger is a Cys_4-His-Cys_3 zinc-finger-like motif found in nuclear proteins thought to be involved in epigenetics and chromatin-mediated transcriptional regulation.

Polycomb group proteins A family of proteins initially discovered in fruit flies that can remodel chromatin such that epigenetic silencing of genes takes place. Polycomb-group proteins are well known for silencing *Hox* genes through modulation of chromatin structure during embryonic development.

Polycomb response elements (PREs) *cis*-regulatory DNA elements that recruit both the Polycomb group (PcG) and Trithorax group (TrxG) proteins that are required for gene silencing and activation, respectively.

Polycyclic aromatic hydrocarbon (PAH) Potent atmospheric pollutants that consist of fused aromatic rings. PAHs occur in oil, coal, and tar deposits, and are produced as byproducts of fuel burning. PAHs are also found in meat cooked at high temperatures such as in grilling or barbecuing, and in smoked fish. As a pollutant, they are of concern because some compounds have been identified as carcinogenic, mutagenic, and teratogenic.

Position effect variegation (PEV) Cell/tissue specific variability of gene expression controlled by the temporal inheritance of certain epigenetic states. PEV is a consequence of variable formation of heterochromatin across the respective gene. A classical example of PEV is found in certain mutations leading to variegated eye pigmentation in fruit flies.

Positron emission tomography (PET) A nuclear medical imaging technique that produces a three-dimensional image of functional processes in the body. The system detects pairs of gamma rays emitted by a positron-emitting radionuclide that is introduced into the body on a biologically active molecule. Three-dimensional images of the radionuclide concentration within the body are then constructed by computer analysis.

Post-translational modification (PTM) Proteins are created by ribosomes translating mRNA into polypeptide chains that then undergo post-translational modifications such as folding and cutting before becoming mature proteins.

Post-traumatic stress disorder (PTSD) A severe anxiety disorder that can develop after exposure to any event that results in psychological trauma.

Potato spindle tuber viroid (PSTVd) A small, circular RNA molecule that contains a pospiviroid RY motif stem loop within the viroidal RNA. All potatoes and tomatoes are susceptible to PSTVd, and there is no form of natural resistance.

Prader-Willi syndrome (PWS) A rare pediatric developmental disorder caused by chromosomal aberrations or epigenetic misregulation of imprinted genes on paternal chromosome 15. Characteristics of PWS are low muscle tone, short stature, incomplete sexual development, cognitive disabilities, problem behaviors, and a chronic feeling of hunger that can lead to excessive eating and life-threatening obesity.

Predictive adaptive response (PAR) A form of developmental plasticity that evolved as adaptive responses to environmental cues acting early in the life cycle, but where the advantage of the induced phenotype is primarily manifested in a later phase of the life cycle.

Prefrontal cortex (PFC) The anterior part of the frontal lobes of the brain, lying in front of the motor and premotor areas. This brain region is implicated in planning complex cognitive behavior, personality expression, decision making, and moderating social behavior. The basic activity of this brain region is considered to be orchestration of thoughts and actions in accordance with internal goals.

Primordial germ cell (PGC) Diploid germ cell precursors that exist briefly in the developing embryo before differentiating to become germ cells.

Prolactin (PRL) Prolactin is a single-chain protein hormone closely related to growth hormone. It is secreted from the anterior pituitary, but it is also synthesized and secreted by a broad range of cells in the body, most prominently various immune cells, the brain, and the decidua of the pregnant uterus. The mammary gland is a major target organ for prolactin where it stimulates mammary gland development and milk production.

Promyelocytic leukemia (PML) A subtype of acute myelogenous leukemia (AML). It is a cancer of the blood and bone marrow with an abnormal accumulation of immature granulocytes called promyelocytes. The disease is characterized by a chromosomal translocation involving the *retinoic acid receptor alpha (RARA)* gene, and is unique from other forms of AML in its responsiveness to all trans retinoic acid therapy.

Protamines Small, arginine-rich proteins that replace histones late in the haploid phase of spermatogenesis during spermiogenesis. They are thought to be essential for sperm head condensation and DNA stabilization. Protamines are removed from paternal chromosomes in the mammalian zygote after fertilization.

Prototherians Egg-laying mammals (i.e. platypus and echidna) are the most ancestral mammals; they are only found in Australia, Tasmania, and New Guinea.

Psychosis An abnormal condition of the mind, described as involving a loss of contact with reality.

Psychotic spectrum disorder (PSD) A group of psychiatric diagnoses that share several clinical features, typically involving reality distortion.

Quantitative real time polymerase chain reaction (qPCR) A laboratory technique based on PCR that is used to amplify and simultaneously quantify a targeted DNA molecule.

Reelin A protein that regulates neuronal migration in the developing brain, and is also involved in important neuronal cell functions in the adult brain like synaptic plasticity, dendrite development, and adult neurogenesis.

Reduced Representation Bisulfite Sequencing (RRBS) A technique that couples bisulfite conversion and next generation sequencing. It is an innovative method that enriches genomic regions with a high density of potential methylation sites, and allows for the determination of DNA methylation at a single-nucleotide resolution.

Regions of altered methylation (RAMs) Persistent RAMs seen in precancerous tissues are thought to play a critical role in the genesis of cancer.

Retinitis pigmentosa (RP2) An inherited, degenerative eye disease that causes severe vision impairment and often blindness.

Retinoblastoma (RB) A rapidly growing cancer that develops in the retina of the eye. There are two forms of this disease, a heritable form and a non-heritable form. The heritable form involves mutations in *RB1*, an imprinted tumor suppressor gene on chromosome 13 that is expressed preferentially from the maternal allele.

Rett syndrome (RTT) A neurodevelopmental disorder of the grey matter of the brain that almost exclusively affects females. Rett syndrome is caused by mutations in *MECP2* located on the X chromosome, and can arise both sporadically or from germline mutations.

Reverse transcriptase (RT) An enzyme used to generate complementary DNA (cDNA) from an RNA template, a process termed reverse transcription. RT is needed for the replication of retroviruses, and RT inhibitors are widely used as antiretroviral drugs. Reverse transcriptase was discovered independently by Howard Temin at the University of Wisconsin–Madison and David Baltimore at MIT; a discovery for which they shared the 1975 Nobel Prize in Physiology or Medicine.

Ribonucleic acid (RNA) A ubiquitous family of large biological molecules that perform multiple vital roles in the coding, decoding, regulation, and expression of genes. RNA is assembled as a chain of nucleotides, but it is usually single-stranded.

RNA-directed DNA methylation (RdDM) An epigenetic process first elucidated in plants whereby small double-stranded RNA (dsRNA) is processed to guide methylation to complementary DNA loci.

RNA-induced silencing complex (RISC) A multiprotein complex that incorporates one strand of a small interfering RNA (siRNA) or microRNA (miRNA). RISC uses the siRNA or miRNA as a template for recognizing complementary mRNA, which is then cleaved by activating RNase. This process is important in both gene regulation and the defense against viral infections.

RNA interference (RNAi) Posttranscriptional regulatory effects on mRNAs (i.e. control of translation or stability) triggered by processed dsRNA and ssRNA. Effects are propagated by enzymatic complexes such as RISC containing the small RNAs bound by Argonaute proteins.

Rubinstein-Taybi syndrome (RTS) A disorder caused by mutations in the *CREBBP* that is characterized by short stature, moderate to severe learning difficulties, distinctive facial features, and broad thumbs and first toes.

S-Adenosyl methionine (SAM) A cofactor for all DNA methyltransferases (DNMTs) and histone methyltransferases (HMTs), providing the methyl group added to either cytosines (DNA) or histones (arginine or lysine).

S-Adenosylhomocysteine (SAH) Hydrolyzed product formed after the methylation reaction catalyzed by DNA and histone methyltransferases using SAM as a methyl group donor. SAH is a competitive inhibitor of SAM for most methyltransferases.

Schizophrenia A mental disorder characterized by disintegration of thought processes and of emotional responsiveness, involving hallucinations, paranoia, delusions, and disorganized speech and thinking.

Serotonin A neurotransmitter produced in the brain that regulates mood, appetite, sleep, and impulse control. It is also known to influence the functioning of the cardiovascular, renal, immune, and gastrointestinal systems.

SET domain A domain found in virtually all lysine-specific histone methyltransferases (HMTs). A protein–protein interaction domain required for HMT activity and modulation of chromatin structure that is frequently associated with cysteine-rich Pre-SET and Post-SET domains.

Sex-determining region Y (SRY) A sex-determining gene on the Y chromosome in Therian mammals.

Short interspersed nuclear element (SINE) Non-long terminal repeat retrotransposons are highly abundant and heterogeneous; their length is about 300 base pairs. The most abundant SINEs in humans are in the Alu family.

Silver-Russell syndrome (SRS) A growth disorder that is one of 200 types of dwarfism. Evidence indicates that one cause results from hypomethylation of the imprinting control region which controls the monoallelic expression of *H19* and *IGF2*. Like other imprinting disorders, the incidence of Silver–Russell syndrome may be increased with the use of assisted reproductive technologies such as IVF.

Single nucleotide polymorphism (SNP) A DNA sequence variation occurring when a single nucleotide in the genome differs between members of a biological species or paired chromosomes.

Small interfering RNAs (siRNAs) RNAs that range in the size between 21 and 24 nucleotides, and are derived from double-stranded long RNAs cleaved by Dicer. siRNAs are incorporated into the RISC complex to be targeted to complementary RNAs to promote cleavage of these mRNAs.

snoRNAs Small nucleolar RNAs involved in processing of small RNAs such as ribosomal RNAs.

Social environment The people and institutions with whom people interact.

Spermatogonia Immature diploid sperm cells that develop into mature spermatozoa or sperm. Major epigenetic changes occur in spermatogonia cells.

Stable isotope labeling with amino acids in cell culture (SILAC) A popular non-radioactive isotopic labeling technique for quantitative proteomics. It is based on mass spectrometry for detecting differences in protein abundance among samples.

Stem cell Non committed cell that has the capacity to self-renew. Stem cells also have the capacity to differentiate into specialized cells.

Suberoylanilide hydroxamic acid (SAHA) An inhibitor of certain histone deacetylases, leading to enhanced levels of histone acetylation.

Sumoylation Addition of a small ubiquitin-like modifier or SUMO group to histone residues that is associated with transcriptional modification.

Supra-optic nucleus (SON) A nucleus of magnocellular neurosecretory cells in the hypothalamus of the mammalian brain; the cells produce antidiuretic hormone.

Tempero-parietal junction (TPJ) An area of the brain where the temporal and parietal lobes meet at the posterior end of the Sylvian fissure. This area is known to play a crucial role in self-other distinction processes and theory of mind. Damage to this area of the brain is implicated in producing out-of-body experiences.

Tetrahydrofolate (THF) A co-enzyme in many reactions, especially in the metabolism of amino acids and nucleic acids. It is produced from dihydrofolic acid by dihydrofolate reductase. It acts as the donor of a group with one carbon atom. A shortage of THF can cause megaloblastic anemia.

Therians A subclass of mammals that give birth to live young without using a shelled egg, consisting of the eutherians (true placental mammals) and the metatherians (marsupials). The only omitted extant mammalian group is the egg-laying prototherians (monotremes).

Totipotency Capacity of stem cells to produce all cell types required to form a mammalian embryo, i.e., embryonic and extra embryonic cells. Totipotent cells are formed during the first cleavages of the embryo.

Tourette syndrome An inherited neuropsychiatric disorder with onset in childhood that is characterized by physical and vocal tics. The exact cause of Tourette's is unknown, but both genetic and environmental factors are involved.

Transgenerational response (TGR) The transmittance of information from one generation to the next that affects the traits of offspring without altering the primary structure of DNA.

Transcriptional gene silencing (TGS) The stable repression of transcription that mainly affects transposons, chromosomal repeats, and transgenic inserts; however, it can also involve protein encoding genes. It results from epigenetic modifications of DNA and histones that create an environment of heterochromatin around a gene, making it inaccessible to transcriptional machinery.

Transcriptome The set of all RNA molecules, including mRNA, rRNA, tRNA, and other non-coding RNA produced in a cell.

Trichostatin A (TSA) An inhibitor of certain types of histone deacetylases.

Trithorax group proteins (TRX) Proteins containing a trithorax-like bromo domain: They are usually involved in recognizing histone modifications marking transcriptionally active regions and contributing to the maintenance of activity.

Trithorax response elements (TRE) Chromosomal regions, a few hundred base pairs long, that maintain the active or silent transcriptional state of their associated genes after the initial determining activators and repressors have disappeared.

Trophoblasts (TB) Cells forming the outer layer of a blastocyst that provide nutrients to the embryo; they develop into the placenta.

Tuberous sclerosis A rare multisystem genetic disease that causes non-malignant tumors in the brain and in other organs such as the kidneys, heart, eyes, lungs, and skin.

Turner syndrome A disorder affecting women that is caused by a chromosomal abnormality in which all or part of one of the X-chromosomes is absent.

Type 2 diabetes mellitus (T2DM) A metabolic disorder that is characterized by high blood glucose coupled with insulin resistance and relative insulin deficiency. The development of type 2 diabetes is caused by a combination of lifestyle and genetic factors.

Ultrabithorax (Ubx) A member of the homeobox gene family. In fruit flies, it is expressed in the third thoracic and first abdominal segments where it represses wing formation.

Untranslated region (UTR) The sections on each side of a coding sequence on a strand of mRNA. It is called the 5′ UTR if it is the leader sequence and the 3′ UTR if it is trailer sequence.

Vascular smooth muscle cell (VSMC) The stromal cells of the vascular wall are involved in the physiological functions, and the pathological changes taking place in the vascular wall. VSMCs of resistance vessels participate in the regulation of blood pressure and also in hypertension.

Ventral tegmental area (VTA) A group of neurons located close to the midline on the floor of the midbrain or mesencephalon. The VTA is the origin of the dopaminergic cell bodies of the mesocorticolimbic dopamine system. It is important in cognition, motivation, drug addiction, intense emotions relating to love, and several psychiatric disorders.

Waddington Conrad Hal Waddington (1905–1975) was a developmental biologist, paleontologist, geneticist, embryologist and philosopher who coined the word epigenetics. He used the term epigenetic landscape as a metaphor for how gene regulation modulates development.

Williams syndrome A rare neurodevelopmental disorder caused by a deletion of about 26 genes from the long arm of chromosome 7. It is characterized by a distinctive facial appearance, along with a low nasal bridge, an unusually cheerful demeanor and ease with strangers, developmental delay coupled with strong language skills, and cardiovascular problems, such as supravalvular aortic stenosis and transient hypercalcemia.

Wilms' tumor (WT) A cancer of the kidney that typically occurs in children. Loss of imprinting (LOI) and overexpression of *IGF2* is the most common epigenetic alteration in Wilms' tumor.

X-chromosome inactivation Epigenetically controlled form of dosage compensation in female mammals resulting in transcriptional silencing of genes on the surplus X-chromosome. X-chromosome inactivation is triggered by the noncoding RNA *Xist*, and it is manifested by various epigenetic modifications, including histone methylation, histone deacetylation, and DNA methylation.

X-inactivation center (XIC) Region at which the XIST-mediated inactivation starts. Allelic differences in the XIC may lead to skewed X-chromosome inactivation.

X-inactive specific transcript (XIST) The mammalian XIST gene encodes for a nonprotein encoding RNA that coats the inactive X-chromosome.

X trisomy A form of chromosomal variation characterized by the presence of an extra X chromosome in each cell of a female. There is usually no distinguishable difference between women with triple X and the rest of the female population.

Yolk sac (YS) A membranous sac attached to the embryo, providing early nourishment in the form of yolk in bony fishes, sharks, reptiles, birds, and primitive mammals. It functions as the developmental circulatory system of the human embryo before internal circulation begins.

Zinc finger (ZNF) A small protein structural motif that is formed by the coordination of one or more zinc ions in order to stabilize the fold. The vast majority of zinc finger proteins function as interaction modules that bind DNA, RNA, proteins, or other small molecules.

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