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Epigenetics in Context

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Epigenetics in Context

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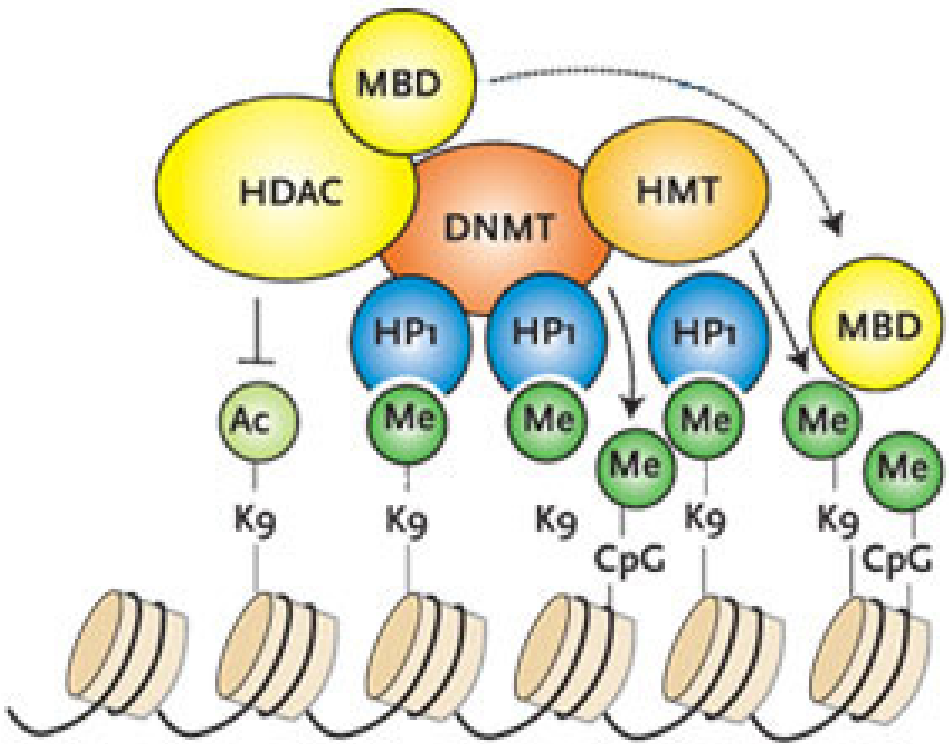

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Epigenetics in context

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Figure on cover

Chromatin machinery: covalent histone modifications such as the acetylation and methylation of histone H3 lysine 9 (K9) create a secondary, self-reinforcing complex that regulates gene expression through transcription. Histone deacetylases (HDAC) remove acetyl groups from the lysine residues making way for methylation. Heterochromatin protein 1 (HP1) binds to the methylated K9 and associates with histone methyltransferases (HMT), DNA methyl-transferases (DNMT) and methyl-C binding proteins (MBD) to aid in spreading the silencing complex (Figure from Greener, 2005).

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Preface

In current biological research, epigenetics has without doubt entered the mainstream. The field now shares the research spotlight with genomics and its entire 'omics' offspring. This report, commissioned by the Netherlands Commission on Genetic Modification (COGEM), aims to present an up-to-date overview of the major topics and trends in epigenetic research in terms of mechanisms, examples and potential applications. This way, it is hoped that the report offers the scientific background to contribute to informed discussions for decision and policy making concerning epigenetics and its applications in the future.

The report is not meant to summarize and describe in considerable detail all data on all epigenetic phenomena and mechanisms published in the literature. The report is based primarily on numerous scientific reviews that were published in the latest years. Such reviews allow the interested reader to gain access to the primary research literature. Given that the 'omics' angle to (epi)genetic research is currently accelerating the discovery and explanation of epigenetic phenomena tremendously, the potential half-life of this report in terms of detailed explanations and models could be -and should be- considered fairly limited.

Jan-Peter Nap
May 2006
Wageningen/Groningen

Samenvatting

De epigenetica bestudeert overerfbare veranderingen in de functie van genen die niet terug te voeren zijn op veranderingen in de onderliggende DNA sequentie. Epigenetisch onderzoek is steeds belangrijker aan het worden in het hedendaagse biologische onderzoek. Het blijkt belangrijk voor het begrijpen van celdifferentiatie en genregulatie tijdens ziekte en gezondheid, groei en ontwikkeling van zowel plant als dier. Epigenetica speelt ook een belangrijke rol in de manier waarop een organisme reageert op zijn omgeving. De code voor epigenetische overerving is even of misschien wel meer complex als de genetische code in DNA. Het omvat allerlei mechanismen die binnen en tussen individuen en generaties kunnen optreden. De moleculaire mechanismen die de epigenetische code vormgeven zijn vooral DNA methylering, histon modificatie, zoals acetylering, RNA interferentie en mechanismen gebaseerd op chromatine (veranderingen).

Epigenetische informatie kan mitotisch (tussen cellen) en meiotisch (tussen generaties) overerfbaar zijn. Mitotische epigenetische overerving beschrijft de overdracht van karakteristieken tussen cellen in een organisme. Het is onderdeel van de normale ontwikkeling van dat organisme en komt tot uiting in fenomenen zoals imprinting, X-chromosoom inactivatie en diverse andere fenomenen. Vooral in het kankeronderzoek is het epigenetische gedachtegoed aan invloed aan het winnen. Meiotische epigenetische overerving is informatie die over verschillende generaties wordt doorgegeven. Het aantal voorbeelden van een dergelijke overerving bij dieren is groeiende en dit zou kunnen betekenen dat het vaker voorkomt dan aanvankelijk werd gedacht. Bij planten komt het in vergelijking vaak voor, waarschijnlijk omdat in planten de epigenetische code vooral op DNA methylering is gebaseerd. Het onderzoek is ook aan het ontdekken dat het verwijderen van epigenetische modificaties minstens zo belangrijk is voor ontwikkeling en regulatie als het aanbrengen van dergelijke modificaties.

De huidige toepassingen van epigenetica richten zich op de diagnose, de preventie en/of het bevorderen van gewenste eigenschappen vooral in relatie tot ziekte en gezondheid, groei en ontwikkeling. Het spectrum van gewenste veranderingen is niet anders dan de wensen in relatie tot genetische modificatie, maar bevindt zich nog in de onderzoeksfase. Diverse epigenetische medicijnen beogen genen te reactiveren die nodig zijn voor normaal functioneren, of genen te deactiveren die betrokken zijn bij ziekte door in te grijpen op DNA methylering, histon acetylering of andere epigenetische modificaties. Hoewel er diverse voorbeelden bestaan van klinisch mogelijk bruikbare toepassingen, blijft pleiotropie een groot probleem. De technologie van RNA interferentie is mogelijk specifieker en geeft mogelijkheden voor onderzoek die op termijn vertaald zouden kunnen worden naar praktische toepassingen, bij voorbeeld in de kliniek of in het veld. De problemen die optreden bij het op een juiste wijze herprogrammeren van zoogdiercellen laat zien hoe complex de rol van epigenetica in groei en ontwikkeling is. Wellicht kan in de toekomst de epigenetica bijdragen aan de verbeterde/verhoogde productie van medicijnen in plantaardige of dierlijke cellen.

Op langere termijn is het voorstelbaar dat alle epigenetische overerving begrepen wordt in termen van samenwerkende eiwitten en niet-eiwitcomponenten. Op dat moment wordt alle epigenetica een vorm van epistasis, samenwerking tussen genproducten met verschillende wijzen van interactie, mogelijk gecombineerd met een aantal stochastische (in de zin van willekeurige) beslismomenten gedurende de ontwikkeling. Waneer alle epigenetische fenomenen uiteindelijk zijn gebaseerd op samenwerkende en interacterende stukken DNA (coderend voor eiwit of niet), dan is het onwaarschijnlijk dat toekomstige epigenetische modificatie, die op het epigenetische niveau beoogt celdifferentiatie en genregulatie te modificeren, zal resulteren in andere veiligheidsoverwegingen dan de veiligheidsoverwegingen die inmiddels bij genetische modificatie als belangrijk zijn geïdentificeerd. Beleidsmakers wordt daarom geadviseerd de ontwikkelingen in het veld van de epigenetica te volgen om te kunnen beslissen of additionele wet- en regelgeving noodzakelijk is, of dat de huidige regelgeving voldoet.

Summary

Epigenetics is the study of changes in gene function that are mitotically and/or meiotically heritable and do not entail a change in DNA sequence. Epigenetics is having an increasingly important role in mainstream biology. It is key to understanding cell differentiation and gene regulation in health and disease, as well as in the interaction between an organism and its environment. The code of epigenetic inheritance is as complex as the genetic code, if not more complex, comprising a variety of mechanisms and events that are now known to occur within and between generations. The molecular mechanisms shaping the epigenome comprise DNA methylation, histone modification, RNA interference and chromatin-based phenomena.

Epigenetic information can be mitotically (within an organism) or meiotically (between generations) heritable. Mitotic epigenetic inheritance describes the transfer of characteristics from cell to cell within an organism. It is part of normal development and is present in phenomena as genomic imprinting, X-chromosome inactivation and others. Notably in cancer research, epigenetic thinking is gaining influence. Meiotic epigenetic inheritance, also referred to as transgenerational epigenetic inheritance, implies transmission of epigenetic marks through the germline. Several examples are now well described and this phenomenon may be more common than was previously thought. In recent years, research is also beginning to appreciate that the removal of epigenetic tags may be as important for regulation as the placing of such modifications.

Applications of epigenetics currently focus on diagnostics, prevention or promotion of traits. In mammalian systems, the applications focus on mitotic epigenetic inheritance. Various epigenetic drugs attempt to reactivate genes required for normal functioning or deactivate genes related to disease development by interference with DNA methylation, histone acetylation or other players in the epigenetics of disease. Although several examples of potentially useful clinical use were demonstrated, pleiotropy remains a major issue. The technology of RNA interference (RNAi) is more specific and may yield possibilities for research that could develop into clinically relevant approaches. The problems in achieving cellular reprogramming show the complexity of epigenetic regulation layers in development. Future applications may involve improved production of pharmaceuticals in cells.

In the longer run, it is feasible that all epigenetic inheritance will be understood in terms of collaborating proteins and non-protein components. Then, all epigenetics becomes epistasis, the result of collaborating gene products. Such epistasis may show different levels of interacting partners and is possibly combined with (few) stochastic (*i.e.* random) decision points along the way. If all epigenetics is based on collaborating and interacting DNA-derived components (either protein or non-protein), it will be unlikely that future targeting the epigenetic layers of cell and gene regulation ("epigenetic engineering") will generate safety issues that are different from the safety issues already encountered in current genetic engineering. Regulators and policy makers in (epi)genetic engineering would therefore be well advised to follow closely the developments in the field of epigenetics to face the challenge of deciding whether additional measures are necessary or existing regulations are sufficient.

Extended Summary

Epigenetics, here defined as the study of changes in gene function that are mitotically and/or meiotically heritable and do not entail a change in DNA sequence, is having an increasingly important role in mainstream biology. Epigenetics is key to understanding cell differentiation and gene regulation, health and disease, as well as the interaction between an organism and its environment. Away from the Lamarckian connotation of 'the inheritance of acquired characteristics', evidence is accumulating that 'on top' of the genetic code of DNA the code of epigenetics is influencing gene expression in a subtly inheritable way. The code of epigenetic inheritance is as complex as the genetic code, if not more complex, comprising a variety of mechanisms and events that are now know to occur within and between generations.

Mitotic or somaclonal epigenetic inheritance describes the transfer of characteristics from parental cell to daughter cell within an organism. It is the mechanism for cell differentiation, allowing organisms to differentiate between cells, tissues and organs that all stem from a single cell. All mitotic epigenetic marks are thought to be erased upon meiosis. Meiotic epigenetic inheritance, also referred to as transgenerational epigenetic inheritance, implies transmission of epigenetic marks through the germline. This phenomenon has been controversial for a long time. It was considered as the witness of errors in erasure of epigenetic marks upon meiosis. Recent evidence from a variety of species, however, suggests transgenerational epigenetic inheritance may be relatively common, notably in plants. It is also clear that the environment has a distinct role in this type of inheritance. In current research, the epigenetic state of whole genomes is studied under the name epigenomics.

In recent years there has been considerable progress in the understanding of the molecular events underlying epigenetic inheritance. Both mitotic and transgenerational epigenetic inheritance are supposed to be based on the same molecular mechanisms. The molecular mechanisms shaping the epigenome of an organism comprise DNA methylation, histone modification, RNA interference and chromatin-based phenomena. In human and plants (but not in fruit fly or *C. elegans*) DNA methylation is the main type of epigenetic modification. DNA methylation is considered the consequence rather than the cause of silencing. Its machinery is supposed to recognize silent genes and result in the irreversible inactivation of such genes. Maintenance methylation, which replicates methylation patterns, should be distinguished from de novo methylation, which changes methylation patterns. Current thinking is shifting towards a balance between methylation and demethylation, but less is known about the biochemistry of DNA demethylation that is thought to be direct or indirect. Indirect DNA demethylation is linked to DNA repair processes.

In addition to DNA methylation, various types of protein modification play a role, most of which target the histone proteins. At least three types of histone modification play important roles: chemical modification, nucleosome remodeling and histone variant exchange. Of these, chemical modifications are best studied and considered most important. A variety of chemical modifications of histones have been described, the most important of which are methylation and acetylation. In general, hypoacetylation and hypermethylation are characteristic for repression of transcription, but it is also known that some methylation events confer transcriptional activation. Research is only beginning to appreciate that the removal of epigenetic tags may be as important for regulation as the placing of such modifications. In addition, the placing of nucleosomes, as well as the use of slightly different types of histone proteins are all described as part of the regulatory repertoire allowing -or denying- DNA binding proteins access to the DNA. In addition to histones, also other proteins, such as the Polycomb and Trithorax-related proteins are involved in maintaining or changing chromatin states. In recent years, the important role of small RNAs (either microRNA or siRNA) in the regulation of chromatin structures is becoming elucidated, whereas future research is likely to demonstrate in more detail that also higher-order chromatin mechanisms, such as chromosome territories and the particular place in the nucleus, are influencing gene activity.

Examples of mitotic epigenetic inheritance are the normal development in mammals and plants. Also genomic imprinting and X-chromosome inactivation are prominent examples of epigenetic gene regulation. Other examples of mitotic epigenetic inheritance include gene bookmarking and heterochromatin replication. Notably in cancer

research, a multitude of epigenetic phenomena is elucidated and the study of epigenetics has changed the way cancer (and cancer treatments) are viewed.

Transgenerational epigenetic inheritance has been convincingly demonstrated in different higher organisms, such as human, mice, yeast and plants. Recent data indicate that fetal programming, defined as the environmental effects on a newborn that have consequences for later life, can be passed on to next generations. In plants, several well established cases are available, that include paramutation, allele methylation and possibly genome rearrangements. The impact of epigenetics on gene expression and gene regulation is driving research into applications that focus on diagnostics, prevention of undesired phenomena, such as disease, and the promotion of desired traits, such as health or yield. In mammalian systems, current applications focus on mitotic epigenetic inheritance, whereas in plants the approach is largely genetic. Most of these possible applications are still in the research phase. True applications of transgenerational epigenetic inheritance are not known, either in mammals or in plants. Epigenetic drugs interfere with DNA methylation, histone acetylation or other players in the epigenetics of disease. Pleiotropy is a major problem. RNAi is yielding possibilities for research that could develop into clinically relevant approaches. Epigenetic modification may help turning animal or cells in more efficient factories for desired proteins, or make genetic engineering more efficient or reliable.

A remarkable issue in epigenetic inheritance and regulation is that so many proteins (and non-protein) components are collaborating. In this sense, understanding the epigenome in its full complexity will require true system's biology. As far as we now know, most -if not all- partners are encoded as gene in the DNA and are subject to regulation. Therefore, when epigenetics is understood to the full, it may be possible to conclude that it is based on epistasis, consisting of collaborations between numerous gene products in combination with different levels of interaction and possibly some chaotic (or stochastic, *i.e.* random) decision points along the way. When epigenetics becomes synonymous with epistasis, the field of epigenetics and genetics will merge. If all epigenetics is based on collaborating and interacting 'genic' DNA (either protein or non-protein), it will be unlikely that targeting the epigenetic layers of cell and gene regulation in future epigenetic engineering will generate safety issues that are different from the safety issues already encountered in genetic engineering. Regulators and policy makers in (epi)genetic engineering would be well advised to follow closely the developments in the field of epigenetics to face the challenge of deciding whether additional measures are necessary or existing regulations are sufficient.

List of abbreviations

5mC	5-methyl-cytosine
Ac	acetylation
DNA	deoxyribonucleic acid
dsRDB	double stranded RNA binding domain
dsRNA	double stranded RNA
DNMT	DNA methyltransferase
HAT	histone acetyltransferase
HDAC	histone deacetylase
HEP	human epigenome project
H2BK20Me3	histone 2B, lysine 20, three methyl groups (example)
HMG	high mobility group
HMT	histone methyl transferase
HP1	heterochromatin protein 1
ICR	imprinting control region
LCR	locus control region
MBD	methyl-CpG binding domain
Me	methylation
miRNA	microRNA
miRNP	micro ribonucleoprotein particle
Pc	Polycomb
PcG	Polycomb group
RDR	RNA-dependent RNA polymerase
PRC	Polycomb repressive complex
PRE	Polycomb responsive element
PTGS	post-transcriptional gene silencing
RdDM	RNA-dependent DNA methylation
RISC	RNA-induced silencing complex
RITS	RNA-induced initiation of transcriptional gene silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
RNA pol II	RNA polymerase II
rRNA/rDNA	ribosomal RNA/DNA
siRNA	small interfering RNA; repeat-associated = rasi; transacting = tasi
TGS	transcriptional gene silencing
TrxG	Trithorax group
TFIID	transcription factor IID
TRE	Trithorax responsive element

1. Introduction

In the biological literature in the 1990s and beyond, the use of the term 'epigenetic' or 'epigenetics' has exploded (Figure 1), firmly establishing epigenetics as a widely recognized subdivision of mainstream biological research. Yet, still in 2001, there was so little consensus over what the term epigenetics actually meant, that it was suggested to abandon the term (Lederberg, 2001).

In this report, a short overview of the largely philosophical discussions over epigenetics and epigenetic inheritance is given. The body of the report is based on what is currently seen by most life scientists as the proper molecular definition of epigenetics: 'the study of changes in gene function that are mitotically and/or meiotically heritable and do not entail a change in DNA sequence' (Wu and Morris, 2001). The regulation of gene expression is complex (Lemon and Tjian, 2000) and a large amount of that regulation is obviously written in the genetic code itself.

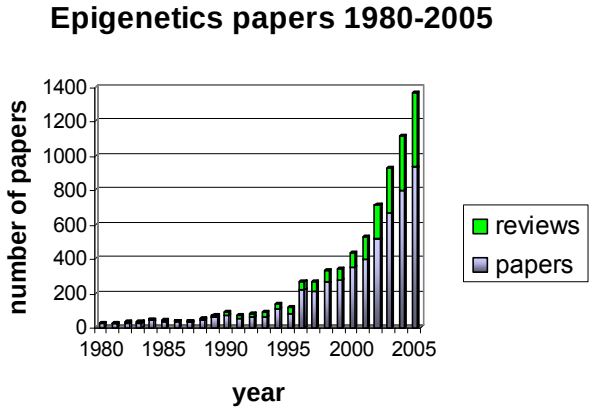

Figure 1. Growth of the number of publications using the term 'epigenetics' and associated terms (epigen*; from Scopus database).

Intermezzo I
Key terms and concepts used

Epigenetics: the study of changes in gene function that are mitotically and/or meiotically heritable and do not entail a change in DNA sequence
Epigenetic inheritance: synonymous with epigenetics
Epigenome: the epigenetic status of the genome per individual cell
Epiallele: gene (primary sequence) plus all its epigenetic information

Promoter sequences with transcription factor binding sites and enhancer sequences are clearly required to give the expression of a gene. Yet, epigenetic inheritance has now been convincingly demonstrated in several different eukaryotic organisms as part of their development (Morgan et al., 2005) and across generations (Chong and Whitelaw, 2004b). The idea that an epigenetic state that is established in the parent, either stochastically or in response to the environment, can then be inherited by the offspring has some Lamarckian flavor and continues to meet with resistance (Chong and Whitelaw, 2004b). The emerging evidence hints that the code of epigenetic inheritance is as complex as the genetic code, if not considerably more complex.

1.1 Definition and scope of epigenetics

In molecular biology today, the definition of epigenetics most familiar to life scientists is 'the study of changes in gene function that are mitotically and/or meiotically heritable and do not entail a change in DNA sequence' (Wu and Morris, 2001; Haig, 2004). Equivalent formulations exist. In this definition, epigenetics is synonymous with 'epigenetic inheritance'. It refers, by definition, to non-Mendelian inheritance. There is some tendency to make epigenetics into a container concept to cover any example of non-Mendelian (or supposedly non-Mendelian) inheritance (Griesemer, 2002). Some authors seem to want to move away from the discussion about inheritance and define epigenetics as a change in gene expression that depends not on a change in DNA sequence, but on covalent modifications of DNA or chromatin proteins such as histones (Comai, 2005). Others see as interesting consequence of recent advances in epigenetics that now phenomena as RNA interference and chromatin-based inheritance (see below) can be studied without the constant need to (re)define epigenetics (Zilberman and Henikoff, 2005).

The need for an epigenetic regulation of gene function stems from the apparent paradox in multi-cellular organisms that every cell in the body arises from a single-cell precursor, the oocyte, yet the adult body is composed of different cells. The differences between these cells are not related to their genetic heritage, so the information in the DNA should be modulated by additional regulatory mechanisms. Such mechanisms are not directly in the DNA code itself, but, literally, 'upon' the genes (Griesemer, 2002). In the genomics era today, it is becoming more and more obvious that biological complexity indeed depends less on gene number or genome size, but on the way those genes are regulated and expressed (used). Defining inheritance (genetically) as the transfer of characteristics from parent to offspring, the definition of epigenetics given above implies that information can flow from a cell to daughter cells (mitotically heritable; within an organism) or from an individual to its descendants (meiotically heritable; between organisms and generations) (Chong and Whitelaw, 2004b). Mitotic epigenetic inheritance, also referred to as somaclonal epigenetic inheritance (Van de Vijver et al., 2002), is an essential mechanism in shaping the body plan and further development of multi-cellular organisms. The inheritance of the epigenetic state through mitotic rounds of cell division is considered to progress relatively faithful and predictable (Chong and Whitelaw, 2004b). It is also becoming clear that the establishment of epigenetic marks during development can be influenced by environmental factors. In other cases, the establishment of epigenetic modifications appears stochastic, but once established, the epigenetic state is maintained throughout the life of the individual (Rakyan and Whitelaw, 2003). In such cases, identical alleles can give variable expression within a population without genetic or environmental heterogeneity. Mitotic epigenetic information is retained in, or rebuilt after, mitosis. Various diseases, notably various forms of cancer, are now associated with defects based on mitotic epigenetic flaws (Egger et al., 2004; Maio, 2005).

At certain times in development (*i.e.* meiosis; either in embryogenesis or in gametogenesis) the epigenetic state is reset; that is, (fully) erased and re-established (Chong and Whitelaw, 2004b). Clearing of the epigenetic state between generations is considered necessary to provide a 'clean state' on which the process of differentiation could occur. This would correlate with the totipotency of the zygote. Meiotic epigenetic inheritance, also and more aptly known as 'transgenerational epigenetic inheritance' (Rakyan and Whitelaw, 2003), that is the transmission of the epigenetic state through the germline, has been controversial for a long time. It is still considered a relatively rare phenomenon, although the evidence that it exists in plants is well documented (Takeda and Paszkowski, 2006). Epigenetic transmission of traits maintained through the production of germ cells from one generation to the next was first observed in maize and is known as paramutation (Chandler and Stam, 2004; Stam and Mittelsten Scheid, 2005). In mammals, various epidemiological studies have provided support for transgenerational epigenetic inheritance, but recent literature indicates that it may be more common than assumed some years ago (Chong and Whitelaw, 2004a). Some parts of the genome are apparently not cleared to completion. This can be due (in part) to environ-mental factors. For example, the epigenetic state of a locus influencing the coat color of mice can be manipulated by altering the diet of the pregnant female (Whitelaw, 2006).

Obviously, all mitotically generated information that is not removed prior to or in meiosis, either as step in development or as an error, becomes transgenerationally inherited. The increased knowledge of epigenetic reprogramming supports the idea that epigenetic marks are not always completely cleared between generations (Tchurikov, 2005). Incomplete erasure at genes associated with a measurable phenotype can result in unusual patterns of inheritance from one generation to the next. Alternatively, epigenetic information may be specifically retained in, altered or put into place during meiosis (Ivanovska et al., 2005). Both types of epigenetic inheritance are thought to be largely based on similar or related molecular mechanisms.

Epigenetics is now studied in various organisms that represent all kingdoms of life, i.e. eubacteria, archaea, fungi, plants and animals. Popular research model organisms next to man are *Saccharomyces cerevisiae* (yeast), *Arabidopsis thaliana* (thale cress, a plant), *Caenorhabditis elegans* (nematode worm), *Drosophila melanogaster* (fruit fly), *Danio rerio* (zebrafish) and *Mus musculus* (mouse). Analyses of human diseases help to advance an understanding of epigenetic mechanisms and the underlying cause of disease (Egger et al., 2004). Concomitant with the rise of the perceived importance of epigenetic inheritance, plants and animals used in agricultural applications are also investigated for epigenetic phenomena (Bisoni et al., 2005; Davis, 2005; Grant-Downton and Dickinson, 2005, 2006). The better understanding of epigenetic regulation in all these organisms may have applications in human and veterinary medicine as well as in agriculture, potentially involving cloning, cell reprogramming, epigenetic engineering, epigenetic medication and/or epigenetic epidemiology.

1.2 Short historical account

Over time, the term epigenetics has had various meanings, in part because the prefix epi- (Greek: $\varepsilon\pi\iota$) has different meanings in English, but also because the term was used in various theories of development and inheritance (Jablonka and Lamb, 2002; Haig, 2004). One could speak of a semantic morass (Lederberg, 2001). The adjective 'epigenetic' has a much longer history than the noun epigenetics (Haig, 2004) and originally it referred to the somewhat different concept of epigenesis. In 1942, Waddington defined epigenetics as 'the branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being' (Jablonka and Lamb, 2002). This definition is clearly different from the meaning as evolved in molecular biology. In nowadays terms, the Waddingtonian definition is more close to the field of developmental biology. Indeed, Huxley (1957) used the term epigenetics 'to denote the analytic study of individual development (ontogeny) with its central problem of differentiation'. This definition of epigenetics is sometimes referred to as 'developmental epigenetics' (Jablonka and Lamb, 2002).

The currently prevailing 'molecular' definition originates from biologists that maintained an important role for extra-nuclear, or cytoplasmic, factors in heredity. Both definitions have coexisted in science for quite some time. The 1987 Holliday paper (Holliday, 1987), suggesting that epigenetic changes were responsible for cancer, may well have triggered the explosion in use of 'epigenetic' in current day biological research (Haig, 2004). The DNA code is long considered the major (only?) focus of understanding the morphology of phenotypes, life histories and physiology. Yet, although epigenetics is defined in terms of DNA and genes, its message is that greater attention should be paid to things that are non-DNA (Wu and Morris, 2001).

To contrast genetics with epigenetics, it should be emphasized that genetics deals with the transmission and processing of information in DNA, whereas epigenetics focuses on the interpretation and integration with information from other sources (Jablonka and Lamb, 2002). In this context, the growing interest in epigenetics may also be related to the current call for a less reductionist, more holistic approach to biology, often referred to as 'systems biology' (Ideker et al., 2001; Ge et al., 2003; Gorski and Misteli, 2005). From a traditional as well as a historical perspective, there is a kinship between genetics and epigenetics. According to some investigators, epigenetics should be considered the causal, logical and consequential successor of genetics.

1.3 Associated and potentially confusing terms and concepts

Concomitant with the rise of epigenetics in biological research, various terms have been modified with the prefix 'epi'. The 'epigenotype' (Holliday, 2005) or 'epigenome' (Murrell et al., 2005) is the overall epigenetic state of a cell. Every cell of an organism is likely to have its own characteristic epigenome. A single nuclear (DNA) genome may therefore give rise to as many epigenomes as there are (different) cells in the organism. The study of the epigenome is now referred to as 'epigenomics' (Beck et al., 1999; Grange et al., 2005). The epigenetic state of a single gene is frequently referred to as epigenetic allele or 'epiallele' (Jacobsen and Meyerowitz, 1997; Kakutani, 2002).

The term epiallele in this context could be considered a new definition of the basic concept of a gene, including both sequence information and possible epigenetic instructions. Alleles that (appear to) receive their epigenetic modifications stochastically are referred to as 'metastable epialleles' (Rakyan and Whitelaw, 2003). A change in epigenetic instructions is called an epimutation. The phenotype as result of epigenetics is called the epigenotype. Related terms to cover epigenetics that occur sporadically in the literature, but have not gained wide acceptance, are paragenetic (Haig, 2004) and epinucleic (Lederberg, 2001).

Potentially confusing terms and concepts are epistasis and eugenics. Epistasis, or epistatic interactions, is a concept from Mendelian genetics, in which the action of one gene is modified by others that segregate independently. Most of the time, it refers (or is thought to refer to) the interaction of two (or more) proteins, where one protein masks the action of another one (Griffiths et al., 1993; Cordell, 2002; Carlborg and Haley, 2004). However, epistasis can also occur at the DNA level, where a gene could encode a protein preventing (or promoting) the

transcription of the other gene (see also http://en.wikipedia.org/wiki/Epistasis). A recent example is the demonstration of epistasis in the Bardet-Biedel syndrome, an oligogenic disease with complex inheritance (Badano et al., 2006). A locus was identified that does not itself cause the disease, but increases the severity. A mutation (C to T) in the locus results in less mRNA and protein, identifying the mutant allele as an epistatic modifier of the syndrome that was confirmed in studies in zebrafish (Badano et al., 2006). In population biology, epistasis often reflects the statistical properties of genetic interactions, such as loss of additivity or the occurrence of modified segregation ratios (Cheverud and Routman, 1995; Wagner et al., 1998). Epistasis can be synergistic or antagonistic. Fitness epistasis is considered to be the cause of linkage disequilibrium. Complex epistatic interactions may be seen as non-Mendelian inheritance and be interpreted as epigenetic. The future may see epigenetic inheritance phenomena be explained in terms of epistatic interactions, or *vice versa*. For example, the role of microRNAs in gene regulation and development (see below) could be considered an example of epigenetics turning epistasis.

Eugenics has nothing to do whatsoever with epigenetics. It is a social philosophy for the supposed improvement of (human) hereditary qualities (Schwartz, 1992). The term means 'well born' or 'good breeding'.

Also other fields than molecular and developmental biology are using the term ‘epigenetic’. In geology, the term epigenetic is used in a completely different connotation and refers to the timing of mineral depositions relative to the age of the surrounding rock material (Sims et al., 2002; Yudovich and Ketris, 2005). In psychology, the term is used for a theory of human development, which stresses psychosocial crises during development. Although development is largely determined by genetics, the manner in which the crises are resolved is not. By analogy with the epigenetic theory of cell differentiation, this manner is called epigenetic (Wallerstein, 1998). The term is also used in combination with other (human) behavior (Harper, 2005).

2. Molecular mechanisms of epigenetic phenomena

In recent years, there has been considerable progress in the identification and detailed understanding of the molecular mechanisms underlying epigenetic inheritance. These mechanisms are all interrelated, but will first be discussed separately below. In the framework of this paper, we distinguish four different levels of epigenetic mechanisms (Tchurikov, 2005):

- DNA methylation (and demethylation)
- Protein (notably histone) modification
- RNA-based mechanisms
- Higher order chromatin-based mechanisms

In order to understand how these mechanisms work and affect gene expression, it is necessary to know how DNA is packed and used in the nucleus of the cell (see Intermezzo II: DNA compaction in the nucleus).

Intermezzo II: **DNA compaction in the nucleus**

(Griffiths et al., 1993; Brown, 2002; Grant-Downton and Dickinson, 2005)

A DNA molecule is a linear chain of nucleotides that in the nucleus is tightly folded around proteins. The combination of protein and DNA is called chromatin. The major protein complex involved is the nucleosome. Nucleosomes can be seen in an electron microscope as bead-like structures along the DNA. The nucleosome consists of two of each of four different 'core' histone proteins, H2A, H2B, H3 and H4. These histones make up the central core particle of the nucleosome and act as spools around which DNA winds.

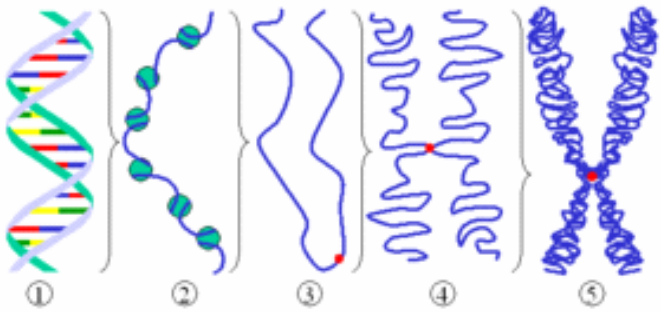

Figure 2. Different levels of DNA condensation. (1) DNA double-strand helix. (2) Chromatin strand (DNA with histones). (3) Condensed chromatin during interphase with the centromere. (4) Condensed chromatin during prophase, in which copies of the DNA molecule are present. (5) Chromosome during metaphase (Figure from Wikipedia; en.wikipedia.org/wiki/Chromatin).

Another, larger histone molecule, H1, sometimes called H5 or the linker histone, binds to DNA molecules which cross over each other and is thought to act as a clamp and have a stabilizing function. The nucleosome together with histone H1 is called a chromatosome. The DNA is wrapped around this protein complex in about 2 turns, comprising about 145 base pairs. Together with the DNA linking two nucleosomes, there are about 200 base pairs of DNA per nucleosome.

The nucleosome core is formed of two H2A-H2B dimers and two H3-H4 dimers, forming two nearly symmetrical halves. These histones are relatively similar in structure. They are highly conserved through evolution, all featuring a 'helix-turn-helix-turn-helix' motif which allows easy dimerisation. They share the feature of long 'tails' on one end of the amino acid structure. Histones allow for different types of physical-chemical interactions with DNA. Their highly basic nature contributes to the water solubility of histones. Histones are found in the nuclei of all eukaryotic cells, but bacteria do not have histones, except in certain Archaea. Sperm cell chromatin is an exception to the above. This chromatin is remodeled into a more tightly packaged, compact, almost crystal-like structure and its histones are largely replaced by protamines, small, arginine-rich proteins.

In the nucleosome, various other proteins are present, such as enzymes and scaffold proteins. The high mobility group (HMG) proteins, such as HMG14 and HMG1, help in conjunction with the nucleosomes to form higher order chromosome structures (Figure 2). Repeating nucleosomes with intervening linker DNA form the 10-nm-fiber. A chain of nucleosomes can be arranged in a 30-nm-fiber, a compacted structure thought to be a zigzag ribbon structure or have no regular structure. Beyond the 30-nm-fiber the structure of chromatin is poorly understood, but it is suggested that the 30-nm fiber is arranged into loops along a central protein scaffold to form transcriptionally active euchromatin. Further compaction leads to transcriptionally inactive heterochromatin (Figure 3).

The extruding N termini of the H3 and H4 histones are positively charged. In chromatin consisting only of DNA and nucleosomes, the positive histone N-termini would interact with the negative phosphate groups of the DNA backbone such that the chromatin is highly compacted ('closed chromatin' or heterochromatin). There are high levels of H1 linker histones in this chromatin.

In a closed chromatin environment, genes cannot be transcribed as the transcription factors are sterically hindered to trigger mRNA synthesis: the genes are silent or silenced. Various modifications (see below) open the chromatin to allow transcription. In general, genes that are active have less bound histones and associated proteins, while inactive genes are highly associated with histones. The tight association presents a fundamental challenge to DNA template processes, such as transcription, replication and repair, which must occur in the context of chromatin. For example, transcription by RNA polymerase II involves a complex wading through nucleosome complexes.

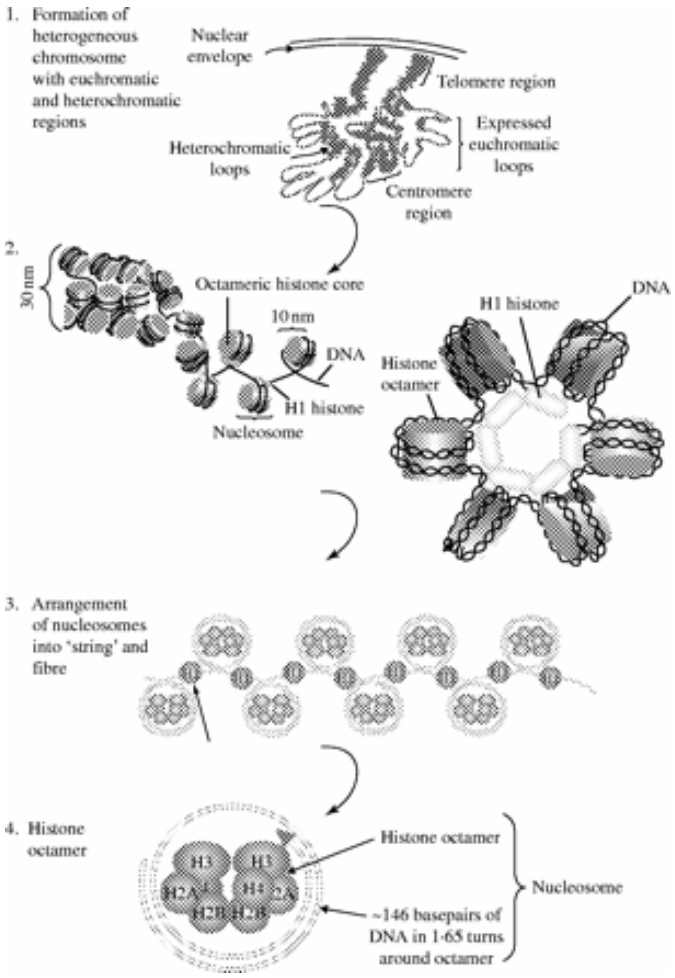

Figure 3. The structure of chromatin (Grant-Downton and Dickinson, 2005).

2.1 DNA methylation and demethylation

DNA methylation is the major modification found throughout genomes (Wade, 2005). DNA methylation is by far the most studied epigenetic modification of DNA. The methylation is a chemical modification which involves the addition of a methyl group to carbon-5 of the cytosine pyrimidine ring (5mC), brought about by enzymes known as DNA methyltransferases (Chen and Li, 2004). Usually cytosines of CpG dinucleotides are methylated. DNA methylation is probably universal in eukaryotes. Methylation is an effective mechanism to turn a gene off. It is seen as a mechanism of gene silencing against the inappropriate expression of for example potentially detrimental transposons (Bender, 2004; Zilberman and Henikoff, 2004, 2005). The role of DNA methylation in gene silencing was recognized before that of histone modification (see below), even though it is less well conserved. The link between DNA methylation and histone methylation is well established in fungi (apart from yeast), animals and plants (Martienssen and Colot, 2001; Geiman and Robertson, 2002; Tariq and Paszkowski, 2004). DNA and histone methylation may have a common origin.

In humans, approximately 1% of the DNA bases undergo DNA methylation. In adult somatic tissues, DNA methylation typically occurs in a CpG dinucleotide context; non-CpG methylation is prevalent in embryonic stem cells (Robertson, 2005; Saxonov et al., 2006). However, some organisms, for example *C. elegans*, have (virtually) no 5mC (Hodgkin, 1994), whereas *Drosophila* has very little 5mC and more often in CpT dinucleotides than in CpG (Field et al., 2004). Whatever epigenetic regulatory mechanism is carried by DNA methylation, the absence of it in such organisms may indicate that other regulatory mechanisms can take over. In this context, the regulation brought about by DNA methylation may be redundant in methylating organisms. In plants and fungi, the system of DNA methylation is more complex (Takeda and Paszkowski, 2006). In addition to CpG, also the cytosines in CpNpG sites can be extensively methylated. In plants, cytosines can be methylated also asymmetrically (CpNpNp), where N can be any nucleotide (Takeda and Paszkowski, 2006).

Two different DNA methylation activities are present in eukaryote nuclei. Maintenance methylation is adding methyl groups to the appropriate positions on newly synthesized DNA during replication. This ensures that the methylation pattern of the parent DNA is maintained in daughter cells (Freitag and Selker, 2005). The second DNA methylation activity is *de novo* methylation, which changes the methylation pattern of DNA. In human, Dnmt1 reproduces the methylation pattern during replication, with an estimated error rate of about 5% (Tchurikov, 2005). The enzymes Dnmt3a and Dmnt3b are responsible for *de novo* methylation. The triggers for such *de novo* methylation are still being investigated. It is thought to depend on the accessibility and/or unusual (repeat?) structures of DNA regions (Bird, 2002).

In mammals, between 60-70% of all CpGs are methylated (Saxonov et al., 2006). Unmethylated CpGs are grouped in clusters called 'CpG islands' (Fazzari and Greally, 2004) that are present in the 5' regulatory regions of many genes. Inappropriate methylation is associated with various diseases (Robertson, 2005). For example, in many disease processes, such promoter CpG islands acquire abnormal hypermethylation (Esteller, 2005), which results in heritable transcriptional silencing. Reinforcement of the transcriptionally silent state is mediated by proteins that can bind methylated CpGs. These proteins, which are called methyl-CpG binding proteins, recruit various other chromatin remodeling proteins that can modify histones, thereby forming compact, inactive heterochromatin (Bernstein and Allis, 2005).

There is a prominent link between DNA methylation and chromatin structure. For example, loss of Methyl-CpG-binding Protein 2 (MeCP2) has been implicated in Rett syndrome and the Methyl-CpG binding domain protein 2 (MBD2) mediates the transcriptional silencing of hypermethylated genes in different cancers (Perini and Tupler, 2006). Many tumor suppressor genes are silenced by DNA methylation during carcinogenesis (Das and Singal, 2004; Esteller, 2005). There have been attempts to re-express these genes with epigenetic drugs (Lyko and Brown, 2005), such as by inhibiting *de novo* methyl transfer with the help of 5-aza-2'-deoxycytidine (decitabine). This nucleoside analog inhibits methyl transfer by preventing the ß-elimination step of catalysis and results in degradation of methyl transferase enzymes. However, decitabine is toxic to bone marrow, which limits its therapeutic window considerably (de Vos and van Overveld, 2005). Therefore, attention is also focused on antisense RNA therapies that target the DNA methyltransferases (DMTs) by degrading their mRNAs to prevent their translation (see below).

In plants, methylation is thought to be the main epigenetic mark carried over during meiosis (Takeda and Paszkowski, 2006). This could be explained by either stable transmission of the epigenetic marks from one generation to the next or by a short phase during meiosis where the marks get lost, followed by rapid and reliable reestablishment. Evidence for both scenarios exist (Grant-Downton and Dickinson, 2006). In *Arabidopsis*, the principal DNA methyltransferases, Met1, Cmt3, and Drm2, are similar at a sequence level to the mammalian methyltransferases. Drm2 is thought to participate in *de novo* DNA methylation as well as in the maintenance of DNA methylation. Cmt3 and Met1 act principally in the maintenance of DNA methylation. Met1 has an essential role in keeping epigenetic order through gametophytic development (Grant-Downton and Dickinson, 2006). The specificity of DNA methyltransferases is thought to be RNA-directed. RNA transcripts are produced from a genomic DNA template. These RNA transcripts may form double-stranded RNA molecules and direct DNA methyltransferases to specific targets in the genome (Wassenegger, 2000; Bayne and Allshire, 2005).

Although the understanding of DNA methylation and transcriptional control is growing rapidly, it is still far from complete. Poorly understood are the mechanisms by which (*de novo*) methylation patterns are generated. The primary function of *de novo* methylation may be to memorize patterns of embryogenic cell activity (Morgan et al., 2005). In addition, how DNA methylation represses transcription is not yet completely understood. It can be direct, by either a high density of 5mC's or 5mC's at specific positions. It can also be indirect, by recruiting addition proteins, such as the methyl binding proteins, that help repress and attract yet other proteins, such as histone methyltransferases or deacetylases, to prevent transcription (Fuks, 2005). DNA methylation, histone acetylation and possibly other modifications (see below) are closely intertwined (Figure 4).

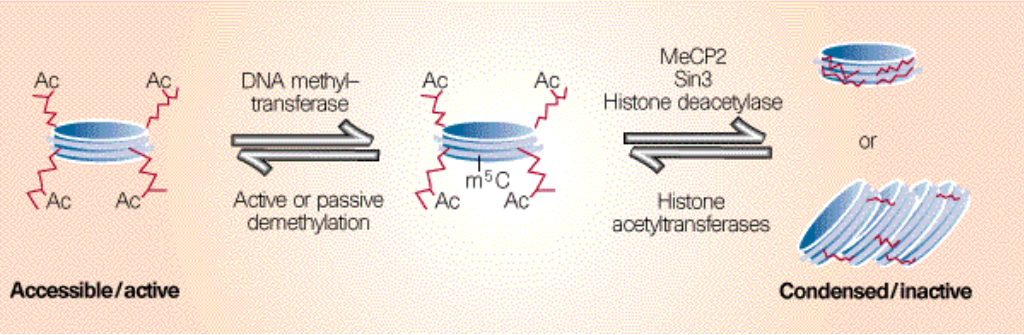

Figure 4. DNA methylation meets histone acetylation (Bestor, 1998).

DNA methylation is not considered the cause of silencing, rather its consequence: methylation does not intervene to silence genes that are actively transcribed, but only affects genes that have already been shut down by other means (Tchurikov, 2005). The methylation machinery is supposed to recognize silent genes and is required for the irreversible inactivation of such genes in somatic cells. In *Xenopus*, it was indeed observed by the transplantation of somatic cell nuclei that in such somatic cells an epigenetic memory is established for active gene transcription (Ng and Gurdon, 2005).

The Human Epigenome Project (HEP) aims to map the full complement of methylation marks in the human genome (Bradbury, 2003). In a pilot study, the methylation sites of active genes in the major histocompatibility complex in seven tissues showed major differences between loci and tissues. The full project will map all DNA methylation sites in all human genes in around 200 different samples with the help of bisulfite sequencing (Bradbury, 2003).

The transfer of a methyl group to DNA can be considered an example of a more general phenomenon of DNA alkylation. Although ethyl and butyl groups have been implied in DNA research, it is supposed that eukaryote genomes do not have the enzymes for DNA acetylation, ethylation, butylation and so on (Mishina et al., 2006).

DNA demethylation

In contrast to the large amount of information that has accumulated on DNA methylation, relatively little is known about DNA demethylation (Kapoor et al., 2005; Morgan et al., 2005). The demethylation of DNA can be either passive or active, or a combination of both. Passive DNA demethylation occurs by inhibition or lack of maintenance DNA methyltransferases throughout cycles of replication, whereas active DNA demethylation is thought to require specific enzymatic reactions. The loss of DNA methylation of the paternal genome in a zygote is likely an enzyme-catalyzed, active demethylation. An oocyte can actively demethylate a transferred somatic nucleus, indicating that the activity responsible is likely to be found in the oocyte rather than the sperm. In mice, global demethylation of the zygotic genome after fertilization appears to occur by an active mechanism, which is then followed by passive demethylation during cleavage stages (Morgan et al., 2005). DNA demethylation has also been shown to be necessary for the epigenetic reprogramming of somatic cell nuclei in *Xenopus* oocytes (Simonsson and Gurdon, 2004). Active DNA demethylases are likely to have critical roles in epigenetic reprogramming during somatic cell cloning and in maintaining stem cells in an undifferentiated state, and in causing the DNA hypomethylation seen in most cancers (Morgan et al., 2005).

A number of candidate biochemical pathways have been suggested (Morgan et al., 2005) that either remove the methyl group in the C5 position of the cytidine ring directly (*bona fide* or direct demethylation) or the entire cytidine base (or nucleoside or nucleotide; indirect demethylation). Direct demethylation is difficult because the carbon–carbon bond of the methyl group is very stable. Dioxygenases can remove methyl groups from the C3 position of cytidine, but no enzymes are known that that can catalyze the oxidative removal of the methyl group from 5mC (Morgan et al., 2005)

The indirect pathways to demethylation all involve DNA repair. DNA glycosylases (such as thymine DNA glycosylase) normally repair T:G mismatches thought to result from spontaneous deamination of 5mC, but have also weak activity on 5mC:G base pairs. This can result in an excision repair where C replaces 5mC. Both RNAs and an RNA helicase are part of the enzyme complex and are involved in the demethylation activity. However, the activities of such enzymes towards 5-methylcytosine DNA substrates are very weak, compared to their activities towards mismatch DNA substrates (Morgan et al., 2005). It is possible that in mammalian systems these enzymes have no strong 5-methylcytosine DNA glycosylase activity *in vivo* (Kapoor et al., 2005). In plants, such activity is well established. The discovery of ROS1 in *Arabidopsis* by a genetic screen and its role in repression of TGS provides strong evidence for the existence of a class of excision repair-related DNA demethylases in plants and their importance in keeping active genes from being silenced. Mutations in the bifunctional DNA glycosylase/lyase ROS1 cause DNA hypermethylation and transcriptional silencing of specific genes. Recombinant ROS1 protein has DNA glycosylase/lyase activity on methylated but not unmethylated DNA substrates (Kapoor et al., 2005). Another example of a so-called helix-hairpin-helix DNA glycosylase in plants is DEMETER, that is known to control the Arabidopsis Polycomb group gene MEDEA (Gehring et al., 2006). No clear functional homologs of ROS1 or DEMETER have yet been identified in mammals.

2.2 Protein modification

In the epigenetic regulation of gene expression, at least three different types of histone modification play important roles. These types of modification are chemical modification, nucleosome (chromatin) remodeling and variant histone exchange (Henikoff, 2005a; Tchurikov, 2005). In addition to histone proteins, other proteins are involved as well to establish and maintain chromatin structures. The Polycomb group (PcG) proteins maintain repressed transcription states, whereas the Trithorax group (TrxG) proteins do the opposite and maintain active transcription states of genes through cell division (Cernilogar and Orlando, 2005; Schubert et al., 2005). Histone methylation serves as a specific mark for PcG and TrxG complexes and others (Daniel et al., 2005).

2.2.1 Chemical modification of histones

Histones can undergo various types of chemical modifications which alter their interaction with DNA and nuclear proteins. These modifications occur on specific amino acid residues, notably lysine and arginine. The H3 and H4 histones have long tails protruding from the nucleosome which can be covalently modified at several places, but also the core octamer histones H2A and H3 can be modified (Cosgrove et al., 2004). The nomenclature of any histone modification takes the name of the histone (*e.g.* H2B), adds the single letter amino acid abbreviation (*e.g.* K for lysine), the amino acid position in the protein from the N-terminal end and specifies the type of modification, such as Me for methylation and, if appropriate, the number of modifications. For example, H2BK20Me3 denotes the presence of three methyl groups in H2B on the 20th lysine from the N-terminal end of the protein. The most important modifications are methylation (Me) and acetylation (Ac). Methylation and acetylation of lysine residues of histones has an important role in chromatin packaging and gene expression. Overall, histone hypoacetylation and hypermethylation are characteristic of DNA sequences that are transcriptionally repressed (LaVoie, 2005).

Histone methylation

The histone methyltransferases (HMT), histone-lysine N-methyltransferase and histone-arginine N-methyltransferase, catalyze the transfer of one to three methyl groups from the S-adenosylmethionine to the lysine and/or arginine residues of the histone proteins (Peters and Schubeler, 2005). In general, methylated histones bind DNA more tightly, which contributes to the repression of transcription. However, detailed elucidation of sites of histone methylation has revealed that some methylation events confer transcriptional activation, while others confer silencing. For example, H3L4Me and H3L79Me are activating and restricted to active chromatin. It was shown that the 5' end of over 300 human genes (about 30% of all genes on chromosome 21 and 22) are highly enriched for H3L4Me, showing variegated histone methylation patterns (Bernstein et al., 2005). Genes that show histone trimethylation at their 5' ends are more active than genes that do not show such modification and the trimethylation could be a good predictor of the start of transcription. It is thought that the Lys4 (tri)methylation facilitates interaction with a particular RNA polymerase isoform, elongation as well as mRNA processing, possibly via recruitment of nucleosome remodeling complexes. In contrast, H3L9Me and H3L27Me are associated with repression of gene expression. The molecular basis for this difference is not yet clear (Bernstein et al., 2005).

A growing group of proteins is shown to have affinity for methylated lysine (Daniel et al., 2005), among which the chromodomain-containing Heterochromatin Protein 1 (HP1) and Polycomb (Pc). Their chromodomain modules translate the methyl-lysine signal into epigenetic gene silencing. Establishment and maintenance of heterochromatin involves HP1-mediated recognition of H3L9Me. Several protein motifs have affinity for methylated histones. For example, WDR5 is (also) an H3L4Me binding protein. It is required for binding of methyltransferase complexes to the histone tails, propagation of H3L4 methylation on chromatin and correct vertebrate development (Wysocka et al., 2005). Various proteins may distinguish the methylation state of methylated histones, further detailing the histone code. For example, a domain known as MBT has affinity for mono- and di-methyl-lysine, but not for tri-methyl-lysine. Histone methylation patterns at orthologous loci are conserved between human and mouse, even when the underlying DNA sequence is not appreciably conserved above background (Bernstein et al., 2005). It is supposed that the relevant regulatory elements may dictate higher-order chromatin structures (Bernstein and Allis, 2005)

Histone acetylation

The histone acetyltransferases (HATs) acetylate lysine residues by transferring an acetyl group (CH3COO-) from acetyl CoA to form ε-N-acetyl lysine (Brown, 2002). This acetylation is associated with the promotion of gene expression. The condensed chromatin is relaxed by this covalently linked acetyl groups. It brings in a negative charge that neutralizes the positive charge normally present. This reduces affinity between histone and (negatively charged) DNA, which renders the DNA better accessible for transcription. Each lysine residue can be a marker for a different signal. The lysine acetylation provides a site of interaction for bromodomain proteins. Recruitment and stabilization of bromodomain-containing complexes at promoter chromatin is important for transcriptional activation. Patterns of histone acetylation have been found to tether HATs and chromatin remodeling complexes to defined chromosomal locations (Yang, 2004). Bromodomain proteins display selective recognition for particular modifications. For example, the Brd2 transcriptional activator associates with acetylated H4 that persists during mitosis, supposedly conveying cellular transcriptional memory across cell division (Yang, 2004).

Other histone modifications

Many other modifications have been described, including phosphorylation, ubiquitination, (iso)prenylation, glycosylation, sumoylation, citrullination and poly(ADP)ribosylation (Jason et al., 2002; Cosgrove et al., 2004). The information on these alternative modifications is limited, but they too can influence chromatin structure and cellular activity. For example, ubiquitination of histone H2B is part of the general role that ubiquitin plays in control of the cell cycle (Robzyk et al., 2000; Jason et al., 2002). Biochemical evidence indicates that there is likely to be a hierarchy of such modifications and of mutually exclusive modifications on particular histones. Such modifications are thought to reduce the strength of the histone-DNA interactions, allowing the chromatin to 'breathe', thus facilitating the various processes involved (Kamakaka and Biggins, 2005). Different modifications of the nucleosome surface may affect histone-DNA interactions either directly or indirectly. How the various modifying enzymes gain access to their target amino acids is being investigated (Cosgrove et al., 2004).

Removal of epigenetic tags: deacetylation and demethylation

Research in chromatin modifications is only recently beginning to appreciate that the removal of epigenetic tags may be as important for regulation as the placing of such modifications (Dokmanovic and Marks, 2005). The pattern of histone acetylation is determined by the action of both HATs and histone deacetylases (HDACs). In the human genome, no less than 18 HDAC genes have been identified, the evolution of which predates that of their substrate histone proteins. One class does not seem to have histones as main substrate. The HDACs are not redundant in their various biological roles (Drummond et al., 2005). HDACs, like HATs, do not interact with DNA directly, but are recruited to multi-protein complexes that associate with DNA. Such complexes differ in composition and their activities are regulated by such composition, as well as by protein modifications that resemble the various histone modifications (Sengupta and Seto, 2004). The removal methylation from histones is not well understood, although it has some mechanistic parallels with DNA demethylation discussed above (Morgan et al., 2005). Direct removal of methyl groups from H3K4Me is catalyzed by a lysine specific demethylase. Histone arginine methylation can also be reversed indirectly by de-imination, the removal of nitrogen at arginine's site of methylation (leaving citrulline, not arginine) or demethylimination, the removal of arginine's monomethylated site (also leaving citrulline), by an enzyme called peptidyl arginine deiminase. The role of this enzyme *in vivo* is not clear yet (Morgan et al., 2005).

Histone code

The various modifications and combinations of modifications are thought to play a critical role in signaling regulatory processes. Studies in many systems have shown that particular histone modifications are enriched at sites of active chromatin, notably histone H3 and H4 hyperacetylation, H3K4 dimethylation and trimethylation, and H3K79 methylation, while others are enriched at sites of silent chromatin, H3K9 and H3K27 methylation (Feinberg et al., 2006). These and other histone modifications can survive mitosis and have been implicated in 'chromatin memory'. The particular combination of these epigenetic tags may represent various types of chromatin and are proposed to constitute a code, the so-called histone code, in analogy to the genetic code (Jenuwein and Allis, 2001). This code, together with DNA methylation, governs the recruitment and assembly of transcription complexes, controls elongation and possibly RNA processing (Perini and Tupler, 2006). Elucidating this code and the mechanism how it is propagated is considered a very -if not the most- important issue in molecular genetics (Feinberg and Tycko, 2004).

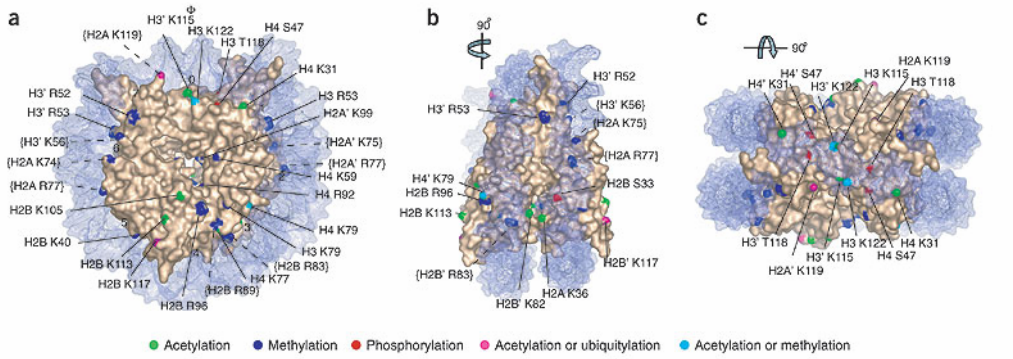

Figure 5. Histone modifications of the nucleosome core particle seen at different angles (Cosgrove et al., 2004)

In recent years, it is becoming increasingly clear that the complexity of this histone code is large (Daniel et al., 2005; Henikoff, 2005a). For example, the yeast Chd1 protein specifically interacts with di- and tri-methylated lysine in histone H3. Another protein (SILK) displays enhanced acetylation activity of H3L4 methylated substrates depending upon methyl binding conferred by Chd1. This indicates that the histone code of transcriptionally active genes may present a binary pattern of acetyl-methyl modifications (Daniel et al., 2005). In such ways, histones are thought to be involved in signaling protein recruitment as well as in mediating enzyme and substrate interactions. Processes as transcription, replication or repair are regulated by a dynamic interplay of histone modifications and downstream recruitment of other chromatin proteins. The various histone modifications (Figure 5) alter chromatin structure either directly by influencing histone-DNA or histone-histone, or indirectly by recruiting non-histone protein complexes. The modifications of the histone tails are considered to serve as a dynamic signaling platform that regulates higher-order chromatin structure in a way that is not understood (Cosgrove et al., 2004).

2.2.2 Nucleosome remodeling

The modification or repositioning of nucleosomes within a (short) region of the genome is a second type of histone modification that allows DNA binding proteins to gain access to the DNA (Brown, 2002). This nucleosomal or chromatin remodeling does not involve chemical alterations to histone proteins, but is an energy dependent process that determines the contact between nucleosome histones and the associated DNA. Nucleosome remodeling involves either a change in the structure of the nucleosome, a physical movement of the nucleosome along DNA or transfer of a nucleosome to another part of the DNA (Brown, 2002).

Chromatin remodeling complexes all have an ATPase subunit to generate the energy for the nucleosome adjustments, but they are diversified and specialized by additional associated proteins (Cairns, 2005). Nucleosome remodeling is likely to occur in tight conjunction with histone acetylation. Remodeling complexes have well-established roles in a wide range of chromosomal processes, including transcriptional regulation and chromatin assembly. Recent work, however, has revealed new functions in which remodeling plays a role, such as histone variant deposition (see below), sister chromatid cohesion as well as RNA transcript elongation and termination. Remodeling complexes are tailored both compositionally and mechanistically to perform particular chromatin functions (Cairns, 2005; Saha et al., 2005).

The proteins responsible for remodeling are clustering in large complexes. They are divided into classes on the basis of different protein compositions and functions, and include the SWI/SNF (BAF), imitation switch (ISWI), INO80, sick with rsc/rat (SWR1) and Mi-2/CHD groups (Cairns, 2005). Most are abundant complexes with essential (or important) roles in chromatin biology that are much conserved throughout eukaryotes. For example, SWI/SNF-group remodelers have roles in altering nucleosome positioning at promoters, which can regulate transcription either positively or negatively. Likewise, ISWI-group complexes have established roles in chromatin assembly and in the formation of nucleosome arrays with well-ordered spacing, which might help to promote repression (Cairns, 2005).

A model proposed to explain the working of the histone code is the regulated nucleosome mobility model (Cosgrove et al., 2004): histone tail modifications recruit effector proteins and nucleosome-remodeling activities that ultimately lead to changes in nucleosome mobility, in addition to modifications that function by chemical interference. The latter modifications are located primarily in the nucleosome lateral surface, and include all modifications that alter direct interactions between the histone octamer and DNA (Cosgrove et al., 2004).

2.2.3 Histone variant exchange

A third way to modulate chromatin is via incorporation of histone variants (Kamakaka and Biggins, 2005; Sarma and Reinberg, 2005). Although histones are among the slowest evolving proteins known, there exist variants that can have significant differences in primary sequence. These variants can lead to changes in chromatin structure and dynamics to regulate gene expression and various cellular processes. Some variants have distinct biophysical characteristics that are thought to alter the properties of nucleosomes, while others localize to specific regions of the genome. The variants are usually present as single-copy genes. In general, the ‘standard’ histones are incorporated into the nucleosomes as new DNA is synthesized. Later, some are dynamically exchanged with variant histones as dictated by the conditions in the cell and the transcriptional status of a locus. The mechanisms and consequences of such changes are currently topic of investigations (Henikoff et al., 2004; Kamakaka and Biggins, 2005; Sarma and Reinberg, 2005).

Histone H1 has numerous sequence variants. Most of the sequence differences between the major histone subtypes and the variants occur in the N- and C-terminal tail domains of these proteins. The abundance of these variants fluctuates in different cell types as well as during the cell cycle, differentiation, and development. Which variety is found at a particular site depends on such factors as the type of cell, the cell cycle, and the stage of differentiation (Kamakaka and Biggins, 2005).

Among the core histones, H2A has the largest number of variants (Kamakaka and Biggins, 2005). Some are conserved through evolution, while others are restricted to vertebrates or mammals. The H2A variants are distinguished from the major H2A histones by their C-terminal tails that diverge in both length and sequence, as well as in their genome distribution. H2A may be replaced by its variant H2A.Z at the boundaries between euchromatin and heterochromatin. H2A.Z has been identified in two complexes. One contains the H2A/H2B histone chaperone/assembly protein Nap1, and the other contains a SWI/SNF-like ATPase called SWR1. H2A.Z is also deposited into regions of chromatin that are transcriptionally inactive, but it is not clear whether deposition of this variant is a cause or a consequence of transcription. In contrast, histone H2B is relatively deficient in variants. The few that have been documented completely replace the major H2B subtypes and appear to have very specialized functions in chromatin compaction and transcription repression, particularly during gametogenesis. Additional H2B variants are developmental stage-specific, but their role is unclear. A sperm-specific H2B in sea urchins has a long N-terminal tail that is highly charged. This tail assists in the condensation of chromatin fibers, suggesting that this variant may play a role in packaging the chromatin in the sperm (Kamakaka and Biggins, 2005).

There are two major histone H3 variants called H3.3 and centromeric H3 (CenH3), as well as a mammalian testis tissue-specific histone H3 variant called H3.6. The centromeric H3 variant has many different names, such as CENP-A in mammalian cells. All CenH3 proteins have highly divergent N-terminal tails. H3.3 and H3.6 are the least divergent variants, containing only four amino acid differences compared to H3 in *Drosophila*. There are no known sequence variants of histone H4 (Kamakaka and Biggins, 2005).

Until recently, little was known about the mechanism of variant deposition. Yeast has one H2A variant, Htz1 (similar to metazoan H2A.Z). Htz1 replacement is associated with the action of the SWR1 remodeling complex (see above). A model for remodeling replacement involves the formation of a remodeler-nucleosome complex, where the remodeler deposits DNA into the nucleosome (Cairns, 2005). The SWR1 complex appears quite specialized for this reaction; other remodelers are poor exchangers whereas SWR1 is robust. It is unclear how many other proteins in the SWR1 complex contribute to the targeting, regulation or mechanism of Htz1 replacement. Some are known as histone exchange chaperones. There are also indications that some of these proteins interact with the transcriptional machinery itself, adding to the complexity of how loci are recognized for variant histone exchange. Four members of SWR1 are also members of the H4 histone acetyltransferase (HAT) complex, suggesting further links between histone replacement, histone acetylation and chromatin boundary formation (Kamakaka and Biggins, 2005).

Studies of the yeast *PHO5* (acid phosphatase) promoter suggest that nucleosome loss occurs during *PHO5* activation (Cairns, 2005). This suggests that access to chromatin by activators *in vivo* might be achieved through the active ejection of nucleosomes. The SWI–SNF-related complex RSC, that remodels the structure of chromatin, is capable of nucleosome ejection and/or octamer transfer *in vitro*. Future studies may evaluate the possibility that histone ejection is in equilibrium with replication-independent chromatin assembly, enabling histone variants such as H3.3 to enter active chromatin. Many questions still remain with respect to the role and regulation of variant histones. The future promises to answer many of these questions, but is sure to raise new ones as well (Henikoff et al., 2004; Kamakaka and Biggins, 2005; Sarma and Reinberg, 2005).

2.2.4 Other proteins

Silenced chromatin is generally maintained over most of the lifespan of an organism. This is accomplished by the action of other proteins. A protein mediating such a task is Polycomb. The Polycomb group of proteins (PcG) as important factors in heritable gene silencing was first identified in *Drosophila* (Ringrose and Paro, 2004). Two large multiprotein Polycomb repressive complexes (PRCs) have been identified in *Drosophila* and mammals: the PRC2 complex, also known as the Esc–E(z) complex, which is thought to be involved in the initial maintenance of repressed states; and the PRC1 complex, which is thought to act subsequently to, and synergistically with, PRC2. These complexes have a dynamic composition depending on cell type and developmental stage, both at the level of transcription and of chromatin remodeling. In terms of histone-modifying activities, the evidence points to the PRC1 component Ezh2 being the histone methyltransferase responsible for the tri-methylation of H3K27. In contrast to the Polycomb group of proteins, the Trithorax group of proteins (TrxG) can activate transcription (Ringrose and Paro,

2004). Both types of complexes bind to specific DNA sequences, so-called response elements. A protein known as GAGA factor is part of TrxG and binds directly to the Trithorax response element (TRE). This binding was shown to displace nucleosomes to promote transcription by RNA polymerase II ((Tchurikov, 2005). Although PcG and Trithorax-group (TrxG) complexes have long been thought to have antagonistic effects on target genes, recent results suggest a more complex interplay. PcG response elements (PREs) can overlap with TrxG response elements (TREs) and binding of PcG and TrxG complexes is not mutually exclusive. TrxG proteins have been found at PREs associated with repressed genes and PcG proteins have been identified at core promoters of transcriptionally active genes, suggesting that PcG and TrxG proteins may function in a concerted fashion (Lund and van Lohuizen, 2004).

Other proteins known as insulator and/or enhancer-blocking proteins may also affect gene function. The CCCTC-binding proteins are known to bind to DNA and prevent promoter enhancer interactions. Insulator proteins, such as USF, may function as barrier to block the spread of silencing or activation at sites where they are bound. Such proteins can also facilitate or block the looping of local chromatin and mediate indirectly the interactions of regulatory elements. CTCF is an insulator protein that establishes chromatin boundaries and the binding of which is blocked by DNA methylation (Feinberg and Tycko, 2004). A paralog of CTCF, termed BORIS, is a cancer/testis gene and its hypomethylation might be linked to hypermethylation at other sites, equivalent to the mode of action of EZH2, the human ortholog of the Drosophila chromatin-repressor protein 'enhancer of zeste' (Feinberg and Tycko, 2004).

2.3 RNA-mediated mechanisms

2.3.1 RNA interference

RNAi is a mechanism of epigenetic gene regulation triggered by the formation of double-stranded RNA (dsRNA) in the cell. It is an evolutionarily conserved gene control mechanism, that uses double-stranded RNA (dsRNA) and non-coding small RNAs as sequence-specific regulators (Almeida and Allshire, 2005; Sato, 2005; Tomari and Zamore, 2005). RNAi has been found to play a central role in heterochromatic gene silencing (Wassenegger, 2005), despite the classical view that 'silent' heterochromatin is not transcribed into RNA. For example, the analysis of the fission yeast genome into heterochromatin and euchromatin indicated an important role for RNAi in maintaining genome integrity (Cam et al., 2005). As it is becoming clear that many small RNAs are ‘normal’, non-coding RNA genes in a genome, comparable to tRNAs and rRNAs, or are present in the introns of regular genes (Weber, 2005), it becomes questionable whether they -and/or their mode of action- should still be considered ‘epigenetic’ in the definition of the concept as described in this report. They may represent a case of epigenetics turning into epistasis. However, the field considers RNAi and the existence and mode of action of small RNAs as an integral part of epigenetics, therefore current knowledge will be summarized here.

RNA silencing was first observed in plants (post-transcriptional gene silencing; PTGS) and fungi (‘quelling’), where attempts to overexpress endogenous genes by introducing transgenic copies of the endogenous gene instead blocked expression of both (Matzke and Matzke, 2004). In animals, RNA silencing was first reported when antisense RNA used to block mRNA expression in *C. elegans* showed that the targeted mRNA was also repressed. More experiments showed that double-stranded RNA (dsRNA) was the trigger of gene silencing. Gene expression can also be suppressed through an RNAi-mediated transcription-repressing pathway (transcriptional gene silencing; TGS). RNAi is now but one aspect of a much larger web of sequence-specific, cellular responses to RNA, collectively known as RNA silencing. Systemic silencing, in which a short RNA (or dsRNA) moves from cell to cell to induce silencing at distant sites, was detected in plants and worms, but has not (yet?) been described in *Drosophila* or mammals. Viral proteins that disarm the antiviral defense mechanisms by suppressing silencing are found in plants as well as in animals (Matzke and Matzke, 2004).

In the RNA silencing pathways now known, the dsRNA formed in cells by DNA- or RNA-dependent synthesis of complementary strands, or introduced into cells by viral infection or by artificial expression, is processed into 21-27-nt-long ‘small RNAs’ (Herr, 2005; Kim, 2005; Zamore and Haley, 2005). This term now encompasses small interfering RNAs (siRNAs), repeat-associated small interfering RNAs (rasiRNAs), trans-acting siRNAs (tasiRNAs) and

microRNAs (miRNAs). The latter class will be described separately below. The various silencing pathways share a common set of proteins that produce or amplify small RNAs and couple small RNAs to specific regulatory outcomes (Kim, 2005; Zamore and Haley, 2005). Together, these RNA silencing-related proteins are called the 'RNAi machinery'. Small RNAs guide the cytoplasmatic RNA-Induced Silencing Complex (RISC) or the nuclear RNA-induced Initiation of Transcriptional Gene Silencing (RITS) complex. RISC mediates the degradation of mRNAs complementary to the small RNAs (posttranscriptional silencing; PTGS), whereas nuclear small RNAs incorporated into the RITS complex guide chromatin modification (transcriptional gene silencing; TGS). Members of the Argonaute family of proteins have a central place in these two complexes. In RISC, Argonaute 2 is associated with the identification and subsequent silencing of the target sequences. How the small RNAs within a complex interact with their target sequence(s) remains to be determined (Kim, 2005; Zamore and Haley, 2005). RISC may serve additional functions in cellular regulation, as it was recently associated with the build up of memory in flies (White-Grindley and Si, 2006).

The RNAi machinery includes the multidomain ribonuclease III enzyme Dicer, which produces the small RNAs, and the RNA-dependent RNA polymerase (RDR), which amplifies the silencing triggers (Wassenegger and Krczal, 2006). Both enzymes occur in various isoforms. Ample research is currently devoted to understanding the detailed mechanism of RNAi and the interaction with chromatin as an important parameter of that mechanism (Bernstein and Allis, 2005; Morris, 2005). In *Tetrahymena*, the RNAi machinery is used to delete DNA during nuclear maturation, but there are no indications for small RNA-guided DNA deletions in other organisms (Herr, 2005)

Posttranscriptional small RNA-mediated gene silencing

RNA silencing pathways can be divided into those that require RDRs and those that may not (Wassenegger and Krczal, 2006). In *C. elegans* and fungi like *Neurospora crassa* and fission yeast, RDRs are required for silencing. In plants, RDRs are required for silencing initiated by single-stranded RNA triggers, but perhaps not for silencing triggered by dsRNA. In *C. elegans*, dsRNA is thought to be diced first into rare primary small RNAs and then amplified by RDR enzymes to create more abundant secondary small RNAs. The RDRs might use primary small RNAs to prime the synthesis of dsRNA using the target mRNA itself as a template. Primed synthesis of dsRNA by RDRs can lead to transitive silencing also known as 'spreading', which is the production of small RNAs encoded by the target gene but not by the trigger RNA. Such spreading along the target should only occur 5' to the dsRNA trigger, as is observed in worms. In plants, however, siRNAs can spread both 5' and 3' along the target, relative to the trigger RNA. This suggests the presence of an unprimed RDR pathway, in which single-stranded RNA fragments are copied without small RNA primers. Spreading is linked to systemic silencing, a phenomenon observed in worms and plants in which locally initiated silencing can be inherited or can spread systemically to distant parts of the organism. Recent evidence suggests that protein-bound small RNAs that flow through the vasculature are able to cause systemic silencing in plants. Thus, small RNAs can function as both intracellular and extracellular signaling molecules (Wassenegger and Krczal, 2006). Natural antisense gene pairs (in *cis*) may generate small RNAs that constitute a feedback loop of gene regulation, as in the regulation of salt tolerance in *Arabidopsis* (Borsani et al., 2005).

RDRs do not seem to play any role in the *Drosophila* and mammalian RNAi pathways, where amplification and spreading have not been detected *in vivo*, perhaps because no RDR homologs are present in the genomes of flies or mammals. In this regard, the *Drosophila* and mammalian RNAi pathways resemble the miRNA pathway in plants, which does not involve RDRs or spreading. The absence of an RDR-dependent amplification step may explain the relatively short duration of RNAi in dividing cells in these organisms (Wassenegger and Krczal, 2006).

Transcriptional small RNA-mediated gene silencing

RNAi can suppress gene expression through blocking transcription. Transcriptional gene silencing (TGS) was also first observed in plants (Matzke and Birchler, 2005) and is due to RNA-dependent DNA methylation (RdDM). This pointed to an RNA-guided genome alteration pathway that is now known to exist widely (Bayne and Allshire, 2005). RdDM requires a dsRNA that targets DNA and that is subsequently processed to yield short RNAs. If these short dsRNAs include sequences that are identical to genomic promoter regions, they are capable of inducing methylation of the homologous promoter, resulting in subsequent TGS. In general, transcriptional gene silencing in plants is carried out by a somewhat larger size class of small RNAs, generally 24~26 nucleotides in length. RNAi-mediated

TGS in *S. pombe* has been implicated in regulating heterochromatic silencing through H3K9Me. This interrelates small RNA-specific targeting of histone modifications to specific genomic sequences (Verdel and Moazed, 2005). These subsequently recruit or interact with additional proteins and result in establishing the silenced, heterochromatic state. Dicer-processed dsRNAs interact with several proteins to form the RITS complex that associates with various chromatin-binding factors to silence targeted genomic regions. Among these factors is RNA polymerase II (RNA Pol-II). This suggests that transcription of the homologous target is required to initiate TGS, possibly to open up the targeted promoter to allow promoter-directed small RNAs access to the target (Matzke and Birchler, 2005). There could also be an RNA Pol-II-expressed transcript with sufficient similarity to the target gene to allow the RNAi machinery to direct the chromatin modification of the corresponding genomic region, resulting in TGS. This siRNA/RNA model is thought to define a local 'address' to allow access to the targeted gene (Morris, 2005). The small RNA-containing complexes are indirectly recruiting the HP1 protein component of heterochromatin to bind to nucleosomal histones (Tchurikov, 2005). In this way, RNAi provides a mechanism for programmed, sequence-specific silencing by means of heterochromatic modifications. Although RNAi is believed to have evolved as a defense mechanisms against invasion by parasitic DNA elements, cells are apparently using the ability of the RNAi machinery to serve as key sites for the assembly of chromatin structures (Cam et al., 2005). RNAi-dependent heterochromatin has also been found in Drosophila and is likely to represent a general strategy for creating condensed chromatin (Matzke and Birchler, 2005). Many details of the RNAi-mediated heterochromatic silencing mechanism are still unclear, particularly how specific genomic sequences are targeted. Yet, the existence of RNAi-dependent gene silencing enhances the biological relevance of heterochromatin considerably (Lippman and Martienssen, 2004).

2.3.2 microRNAs

The miRNAs are the cousins of siRNAs: they are endogenous small RNA molecules that repress the expression of target genes (Kim, 2005; Zamore and Haley, 2005). The miRNAs differ from siRNAs in their biogenesis, not in their functions. Like siRNAs, plant and animal miRNAs can direct cleavage of their mRNA targets when the two are extensively complementary, but repress mRNA translation when they are not. The sequence of a miRNA may determine how effectively it directs cleavage or translational repression (Kim, 2005).

The miRNAs are processed from endogenous precursor molecules, which fold into dsRNA-like hairpins. With some exceptions, animal miRNAs regulate gene expression by base pairing imperfectly to the 3′-untranslated region of target mRNAs, inhibiting protein synthesis. In contrast, plant miRNAs generally show nearly precise complementarity to target mRNAs and trigger mRNA degradation (Kim, 2005). The miRNAs form part of RISC-like ribonucleoprotein particles, miRNPs or miRISCs. There is partial overlap in the protein composition of RISCs and miRNPs (e.g. both complexes contain proteins of the Argonaute family), consistent with the ability of miRNAs to act, under some circumstances, as siRNAs and *vice versa*. The number of Argonaute paralogs in different organisms ranges from one in *S. pombe* to twenty-seven in *C. elegans*. The proteins can be divided into two subfamilies, referred to as Ago and Piwi. Humans, as with other mammals, contain four Ago proteins, expressed ubiquitously, and four Piwi family members of ill-defined function, expressed only in testis and hematopoetic stem cells. Five Argonaute proteins are present in *Drosophila*, two of which are best characterized in the context of RNAi and miRNA pathways (Croce and Calin, 2005; Kim, 2005; Tomari and Zamore, 2005; Zamore and Haley, 2005).

Maturation of miRNAs occurs in two steps, each catalyzed by enzymes of the RNase III family, Drosha and Dicer. Droshas are 130–160 kDa nuclear proteins containing two RNase III catalytic domains and a dsRNA-binding domain (dsRBD) in the C-terminal half of the protein, and additional domains of unknown function in the N-terminal half. Drosha does not work in isolation, but exists in a complex with a dsRBD protein called Pasha (in *Drosophila*) or DGCR8 (in mammals). Like Drosha, Pasha/DGCR8 is essential for the processing of primary miRNA transcripts, pri-miRNAs, to ~70-nt hairpins referred to as precursor miRNAs (pre-miRNAs). The large size of the Drosha–DGCR8 complex may be due to dimerization of its components or the presence of additional proteins. In human cells, Drosha also appears to form part of a much larger multiprotein complex, but whether this complex functions in the biogenesis of miRNAs or other RNAs is unclear. Plant genomes do not seem to encode Drosha homologs and, in *Arabidopsis*, all miRNA biogenesis steps may be carried out by one of the four Dicer-like proteins in the nucleus (Kim, 2005; Tomari and Zamore, 2005; Zamore and Haley, 2005).

The miRNA precursor molecules correspond either to transcripts of independent miRNA genes or to fragments, most frequently originating from introns, of protein-coding RNA polymerase II transcripts. The developmental role of miRNAs is still expanding. The miRNA/RISC complex is, for example, shown to be associated with memory in flies (White-Grindley and Si, 2006). As indicated above, the more it is demonstrated that miRNA genes are 'normal', non-coding RNA genes in a genome, comparable to tRNAs and rRNAs, or are present in the introns of regular genes (Weber, 2005), the more it becomes questionable whether they -and/or their mode of action- should still be considered 'epigenetic' in the definition of the concept as described in this report. They may represent a case of epigenetics turning into epistasis.

2.3.3 Other RNA-based mechanisms

Arabidopsis homozygous for recessive mutant alleles of the organ fusion gene HOTHEAD (HTH) was shown to inherit allele-specific DNA sequence information that was not present in the chromosomal genome of their parents, but was present in previous generations (Lolle et al., 2005b). As this process was found at all DNA sequence polymorphisms examined, it was interpreted as a general mechanism for extra-genomic inheritance of DNA sequence information. It was postulated that these 'genetic restoration' events are the result of a template-directed process that makes use of an ancestral RNA 'sequence cache' (Lolle et al., 2005b). This suggestion has triggered a lot of discussion and alternative explanations (Chaudhury, 2005; Comai and Cartwright, 2005; Henikoff, 2005b; Lolle et al., 2005a; Ray, 2005) and more research seems required to substantiate the claim of the presence of such an RNA cache. No evidence for similar mechanisms in other systems has yet been presented.

2.4 Higher-order chromatin-based mechanisms

Higher-order chromatin configurations are thought to affect gene function in a variety of ways. This is an area of active research, where, however, anecdotal observations tend to outrun explanations in terms of mechanisms. The term "higher order chromatin" is frequently used, or abused, to explain epigenetic effects on gene expression, but what it refers to in molecular terms has not been well defined (Mohd-Sarip and Verrijzer, 2004). Only a small number of studies are beginning to fill the gap between the understanding of the chromatin status of individual genes and loci and more global chromatin structural parameters. When explained, such global views of chromatin should greatly enhance the understanding of the factors that determine how the epigenome is manifested at the level of an individual cell and an organism (Misteli, 2004; Murrell et al., 2005).

Position effects

The influence of chromatin neighborhood on genes and gene expression was first deduced from the large variation in transgene expression among organisms essentially carrying the same (foreign) gene (Mlynarova et al., 1994). The particular place of integration is one of the more prominent reasons for the variation that is observed. This variation is known as 'position effect'. It may be related to position effect variegation and heterochromatin proximity. The surroundings of a gene are supposed to influence the expression of that gene to a considerable extent. Chromatin is now thought to be organized into a series of distinct domains that are functionally independent (Huebert and Bernstein, 2005). Observations that sequences that are supposed to define such domains and interact with the proteins of the nuclear matrix (so-called matrix-associated proteins) can dampen the variation (Mlynarova et al., 1994) confirm the existence of functional domains and the influence of neighboring chromatin. In addition, the actions of enhancers and/or silencers may be based on chromatin interactions (Engel and Bartolomei, 2003).

Looping and local conformations

Chromatin is thought to be organized into a series of distinct domains that are functionally independent (Razin et al., 2003; Wei et al., 2005). Small domains have highly co-expressed genes that may share functional and sequence similarity for co-regulation with nearby regulatory sequences. Genes within large, significantly correlated groups are generally co-regulated at a low level, suggesting the presence of larger chromosomal domains. For example, with

imaging techniques, *HOX* activation during cell differentiation is seen accompanied by chromatin de-condensation and nuclear re-organization, including the looping out of *Hoxb1* from its chromosomal territory. Whereas localization to the nuclear periphery is generally associated with gene repression, localization to the nuclear center is linked to gene-rich, active, chromosomal regions. Furthermore, looping out from core chromosomal structures might be a general feature of active loci. Such large-scale structural reorganizations of chromatin appear to be dynamic and developmentally determined. Further insight into higher-order chromatin organization has emerged from the identifications of long-range regulatory interactions, such as those between the β-globin locus control region (LCR) and specific globin genes. An actively transcribed *β-globin* gene is in close physical proximity to an enhancer element within the LCR located more than 50 kilobases away. The technology of chromosome conformation capture (Dekker, 2006) was used to demonstrate a role for transcription factors in regulating the interaction between these distal loci (Dekker, 2003). Of particular interest are the sequences and mechanisms that define the boundaries between heterochromatin and euchromatin within chromosomes and how these boundaries are established and maintained (Mager and Bartolomei, 2005).

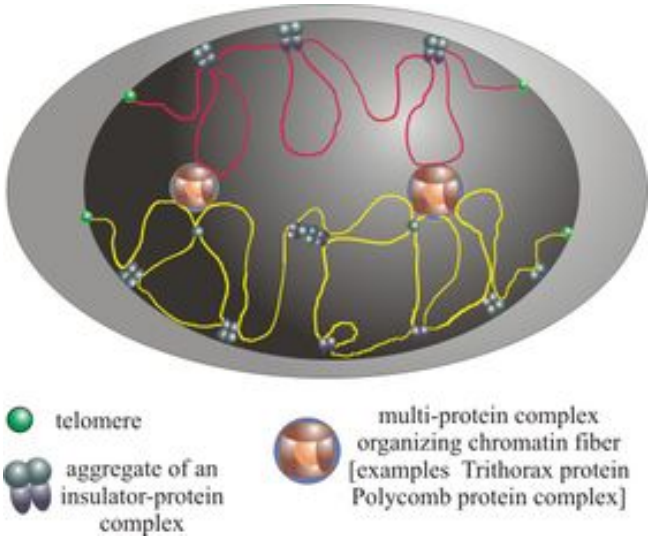

Figure 6. Territorial organization of chromatin in the cell nucleus. The gray oval represents a model of a cell with a nucleus (dark gray oval). Two chromosomes are shown as chromatin fibers (yellow and red lines). Proteins are represented as colored blue rounds. Chromatin components interact with the nuclear membrane. Chromosomes are territorially interlinked by chromatin protein complexes that contain scaffold proteins (Figure from http://en.wikipedia.org/wiki/Chromatin).

Chromosome territories

It is becoming accepted that chromosomes and genes are not randomly positioned within the three-dimensional space of the cell nucleus (Figure 6). Chromosomes occupy their own territory in that space (Speicher and Carter, 2005). Such positioning may affect gene activity and genome stability, and is related to replication and transcription (Chakalova et al., 2005), but the mechanisms and significance of positioning remain to be uncovered. The importance of 3D positioning in the nucleus for gene regulation is beginning to be revealed. It is conceivable that positioning is altered in response to environmental clues. Portions of different chromosomes were shown to interact with each other, suggesting that related genes are brought together to coordinate their expression (Spilianakis et al., 2005). In addition, there may be decisive encounters in early development between different genes or parts of chromosomes in the 3D space of the nucleus that decide on crucial or influential epigenetic marks. High-throughput microscopy methods combined with pattern recognition tools are being developed to understand the role of the three-dimensional nuclear space in chromatin, gene and genome function and epigenetic marking (Speicher and Carter, 2005). This is likely to be a key area of future discovery, notably when it will become possible to study chromosomes in living cells in time with sufficient resolution (Speicher and Carter, 2005).

3. Examples of mitotic epigenetic inheritance

3.1 Normal development in mammals

During normal development, the various cells of multi-cellular organisms carry out different programs of gene expression (Figure 7). This is thought to be substantially regulated by the various epigenetic modifications outlined above. In normal development, some cells subsequently undergo major epigenetic changes, known as 'reprogramming', involving the removal of epigenetic tags and establishment of new tags (Morgan et al., 2005). At fertilization, the parental genomes are in different stages of the cell cycle with very different epigenetic marks and chromatin organization. The paternal genome as delivered by the mature sperm, is single copy (1C), and is packed densely for the most part with protamines rather than histones. The maternal genome is arrested at metaphase II with its 2C genome loaded with histones. Upon fertilization, protamines in sperm chromatin are rapidly replaced with histones, whereas the maternal genome completes meiosis. H3 and H4 in the paternal chromatin are more acetylated than those in the maternal chromatin. It is not clear if this is a passive consequence of the pool of available histones in the cytoplasm being largely acetylated, or due to active incorporation of acetylated histones.

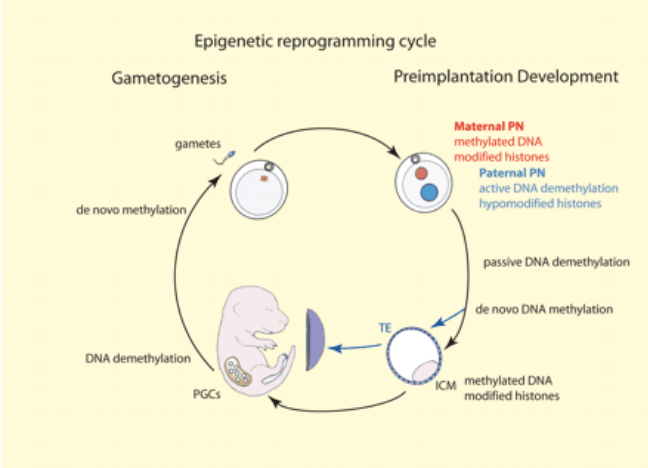

Figure 7. Epigenetic modifications during the life cycle. Primordial germ cells arise from somatic tissue and develop into mature gametes over an extended period of time. These genomes undergo DNA demethylation in the embryo. Upon fertilization, the paternal genome is actively demethylated and its histones initially lack some modifications present in the maternal pronucleus (PN). The embryo's genome is passively DNA demethylated during early cell cycles before blastulation, but imprinted genes maintain their methylation. Histone modifications may also reflect this DNA methylation asymmetry (Morgan et al., 2005).

Closely following the histone acquisition of the paternal genome, a genome-wide loss of paternal demethylation is completed before DNA replication begins in mouse, rat, pig, bovine and human (Morgan et al., 2005). The timing and mechanisms of DNA demethylation in sheep and rabbit appear to differ, but more studies are required to decide what aspects of demethylation are conserved, and what aspects differ, between mammalian species. Paternal demethylation is thought to reprogram paternal germline imprints by the maternally produced oocyte cytoplasm, because the majority of germline methylation imprints is maternal and zygotic demethylation of the paternal genome is only observed in species that have imprinting. In species with a relatively early stage of embryonic genome activation (mouse, pig and human), a more extensive zygotic demethylation is seen than in species where the genome activation occurs later in development (sheep, rabbit). Although demethylation is seen as part of the process to return the gametic genomes to embryonic totipotency, it has yet to be shown that there are, for example, methylated genes in the sperm genome whose demethylation is needed for early transcriptional activity and hence for embryo development (Morgan et al., 2005).

In a study in rats, the methylation status of a glucocorticoid promoter sequence in an adult rat was dependent on experiencing high levels of maternal care (another exponent of 'environment') in early life. In animals that had received high levels of maternal care, the promoter was hypomethylated, whereas lower levels of care are associated with hypermethylation. Infusion of methionine showed that these epigenetic changes are reversible in adulthood, indicating that also mitotic epigenetic tags can be susceptible to plasticity (Weaver et al., 2005; Rowan, 2006). This observation also connects metabolite levels to epigenetic tagging.

3.2 Normal development in plants

In plants, there is no early deposition of the germ line (Takeda and Paszkowski, 2006). Gametes are formed late in development from somatic cells. As a consequence, epigenetic information must be transmitted through many mitotic DNA replications in diploid tissues, through the differentiation of haploid gametophytes. To achieve the double fertilization characteristic of seed plants, the development of male and female gametophytes differs in the number of postmeiotic mitotic divisions. Male gametogenesis is initiated with diploid microspore mother cells. After meiosis, four haploid microspores form a tetrad that releases free microspores. These undergo two asymmetric mitoses to give rise to an immature pollen grain with generative and vegetative cells. The vegetative cell ceases division, whereas the generative cell undergoes an additional mitosis, leading to a mature pollen grain with two sperm nuclei (or 'cells').

The female gametophyte differentiates from a megaspore mother cell, which undergoes meiotic division into four megaspores. In over 70% of flowering plants, including *Arabidopsis* and rice, three of the four megaspores degenerate to result in a single functional haploid megaspore (Takeda and Paszkowski, 2006). In three mitoses, this megaspore produces the eight nuclei of the embryo sack: one of the egg cell, two of the synergid cells, three of the antipodal cells, and two polar nuclei that will undergo a fusion to form the diploid nucleus of the central cell. During fertilization, one haploid sperm nucleus fuses with the egg cell nucleus, and the zygote develops to a diploid embryo. The other sperm nucleus fuses with the diploid nucleus of the central cell to initiate the development of a triploid endosperm. Endosperm nuclei contain two maternal chromosome sets and one paternal chromosome set. This ratio is crucial for the proper development of the endosperm and the epigenetic makeup of the maternal and paternal genomes differs (Takeda and Paszkowski, 2006).

When plant cells and tissues are cultured *in vitro*, regenerated plants can show a high frequency of phenotypic changes that are either stable or unstable (Kaeppler et al., 2000). This is thought to be due to mutations and/or have an epigenetic basis, although mechanisms have not been studied in considerable detail (Grant-Downton and Dickinson, 2005). Most well studied epigenetic effects in plants are (or are supposed to be) transgenerational in nature and will be described in the next chapter of this report.

3.3 Genomic imprinting

Genomic imprinting is the phenomenon in which only one of a pair of genes on homologous chromosomes (in a diploid nucleus) is expressed, the second being silenced (Brown, 2002), as a rule by DNA methylation and/or chemical modification of histone proteins (Wilkins, 2005). Imprinting thus results from a germline mark that causes reduced or absent expression of a specific allele of a gene in somatic cells of the offspring. Imprinting is a feature of all mammals, affecting genes that regulate cell growth, behavior, signaling, cell cycle and transport; moreover, imprinting is necessary for normal development (see above) (Feinberg et al., 2006). Some imprinted genes are expressed from a maternally inherited chromosome and silenced on the paternal chromosome; while other imprinted genes show the opposite expression pattern and are only expressed from a paternally inherited chromosome. Always the same member of a pair of genes is inactive (Brown, 2002).

The imprint is maintained in somatic cells, with the associated patterns of imprinted expression often being both tissue and developmental-stage specific. The epigenetic differences between the two alleles are fully erased through meiosis and then re-established (Rakyan and Whitelaw, 2003). Genomic imprinting reduces expression of a gene from a default state of bi-allelic to mono-allelic expression, but for many genes, the silencing is only partial. The literature typically represents the effects of imprinting with the use of terms such as 'paternally expressed', whereas terms such as 'maternally suppressed', which are being used more frequently in recent literature, are preferable because they reflect the biology of imprinting more precisely (Morison et al., 2005). Imprints can act as a silencer or an activator for imprinted genes. The textbook example of an imprinted gene is *Igf2*, encoding the insulin-like growth factor 2. In mice, only the paternal gene is active, whereas the *Igf2* region of the maternal chromosome is methylated (Brown, 2002; Sakatani et al., 2005).

Most imprinted genes are located in large domains within an Imprinting Control Region (ICR). ICRs acquire DNA methylation marks that result in parent-of-origin-specific gene expression (Delaval and Feil, 2004). Particular mechanisms of imprinting regulation are different for different domains and can involve histone modification and or Polycomb group proteins. In some domains unmethylated ICRs act as insulators, in other domains small RNA interact with the ICR region to attract chromatin- modifying complexes in a mechanism analogous to X chromosome inactivation described below (Tchurikov, 2005).

Several genetic diseases in humans are due to abnormal imprinting (Strachan and Read, 2003). This imprint is then maintained in somatic cells, with the associated patterns of imprinted expression often being both tissue and developmental-stage specific. The pseudo-hemizygosity imposed by imprinting results in genetic vulnerability that contributes to human diseases including Prader–Willi/Angelman syndrome, Beckwith–Wiedemann syndrome and several cancers. For example, the Prader-Willi syndrome is due to two imprinted copies of the chromosome 15 being inherited from the mother. The Angelman syndrome gene is due to two similarly imprinted copies of the chromosome 15 inherited from the father and the locus is similarly imprinted. Each individual who inherits both chromosomes 15 from one parent (so-called uniparental disomy) has either Prader-Willi or Angelman syndrome, depending on the parent-of-origin of the chromosomes (Strachan and Read, 2003).

In sheep, an allele of the 'callipyge' (Greek for 'beautiful buttocks'), or CLPG, gene produces large buttocks of muscle with very little fat. This phenotype occurs when the allele present on chromosome 18 is inherited from a sheep's father and is *not* on the copy of chromosome 18 that is inherited from the mother (Georges et al., 2003).

The phenomenon of genomic imprinting is considered to occur relatively rare in mammals (Brown, 2002). A total of 100–200 imprinted mammalian genes was estimated based on the proportion of mouse loci showing parental effects, and a total gene number 60 000–100 000. An updated prediction based on the current gene count would be ~25 to 84 (Morison et al., 2005). A list of 2100 genes selected based on differential expression between parthenogenetic and androgenetic mouse embryos presents the largest number of predicted imprinted mouse genes to date. There is quite some discussion on this number of genes and confirmatory studies are required (Morison et al., 2005). If parental imprinting is reflected by differential DNA methylation or other chromatin modification without concomitant changes in gene transcription, for example as a mechanism to maintain distinctions between homologous chromosomes for pairing and recombination, the number of parental imprints could be (much) larger than the number of imprinted genes (Morison et al., 2005). A recent survey of imprinted genes in human and mouse lists 83 transcriptional units of which only 29 are shared. There is a high level of discordance of imprinting status between mouse and human, even when cases in which the ortholog is absent from one species are excluded. A high proportion of the imprinted genes are non-coding small RNAs or genes derived by retrotransposition (Morison et al., 2005). All mammalian genes that are known to be imprinted are present in either humans or mice, additional data from other species do not increase the number of known imprinted genes. The presence of small RNA genes within imprinted regions may imply that their expression could contribute to the control of genomic imprinting, either of nearby genes or more globally. Antisense transcripts may be especially common among imprinted genes, but that is not clear from recent studies (Morison et al., 2005).

Imprinted genes with subtle phenotypic effects may remain to be discovered. If they exist, they are likely to be located outside the major imprinted gene clusters now known. In support of the presence of additional imprinted genes are the numerous studies of complex diseases that have shown parent-of-origin effects in linkage studies. Parent-of-origin effects among quantitative trait loci in pigs and other animals also predict that several more imprinted genes may await discovery. Mechanisms implied to regulate allele-specific gene expression involve small RNAs, chromatin modifications and Polycomb proteins (O'Neill et al., 2003; Gehring et al., 2006).

Imprinting is known to cause problems in cloning, resulting in clones having DNA that is not methylated or otherwise correctly marked in the right places (Morgan et al., 2005). There may not be enough time for the reprogramming to be properly achieved. When a nucleus is added to an egg during somatic cell nuclear transfer, the egg starts dividing in minutes, as compared to the days or months it takes for reprogramming during embryonic development. If indeed time is the limiting factor, it may be possible to delay cell division in clones, giving time for proper reprogramming to occur (Morgan et al., 2005).

In plants, parent-of-origin effects on seed development occur after interploidy crosses (Takeda and Paszkowski, 2006), but only a few locus-specific imprinting examples are documented. Imprinting seems to be restricted to the endosperm in the female gametophyte (Arnaud and Feil, 2006), the functional analog of the placenta. Unlike in animals, genes can become imprinted by the removal of methyl groups, not their addition. *MEDEA (MEA)* is an *Arabidopsis* Polycomb group gene that is imprinted in the endosperm. The maternal allele is expressed and the paternal allele is silent. *MEA* is controlled by DEMETER (DME), a DNA glycosylase required to activate expression of *MEA* and *MET1* (encoding a DNA methyltransferase), which maintains CG methylation at the *MEA* locus. DME is responsible for endosperm maternal-allele-specific hypomethylation of *MEA*. However, the paternal-allele silencing is not controlled by DNA methylation. Rather, Polycomb group proteins that are expressed from the maternal genome, including MEA, control paternal *MEA* silencing in what establishes a novel example of self-imprinting. DME establishes *MEA* imprinting by demethylation to activate the maternal allele. *MEA* imprinting is subsequently maintained in the endosperm by maternal MEA, that is silencing the paternal allele (Gehring et al., 2006).

Other examples of genomic imprinting in plants are seen in hybrid systems. Nucleolar dominance is the reproducible silencing of the ribosomal DNA repeat region (which is the core of the nuclear nucleolus) from the genome of one of the parents (Grant-Downton and Dickinson, 2005). In DNA methylation mutants of *Arabidopsis*, a 5S rDNA repeat becomes distinctly marked with H5K27Me3 in leaves in an mono-allelic manner, suggesting the possibility of imprinting in somatic tissue (Mathieu et al., 2005).

The existence of genomic imprinting may imply some selective advantage to mono-allelic expression in spite of the increased vulnerability to mutation. Currently, four hypotheses seem plausible to explain the apparent paradoxical evolution of imprinting from an ancestral state of bi-allelic expression: genetic conflict, the ovarian time bomb, X-linked sex-specific selection and sexually antagonistic selection. Detailed descriptions of each of these hypotheses are available (Morison et al., 2005).

3.4 X chromosome inactivation

In mammals, one of the two X chromosomes becomes inactivated in the developing female embryo (Avner and Heard, 2001; Brown, 2002; Heard, 2004). This way, X-encoded gene products are dosage-compensated between males (XY) and females (XX). X-inactivation ensures that females, like males, have one functional copy of the X chromosome in each cell of the body. The X chromosome likely contains between 900 and 1,200 genes (Heard, 2005). Therefore, the process of X-chromosome inactivation may affect about a thousand genes. This makes it one of the most prominent examples of epigenetic gene regulation. It presents a mosaic cellular phenotype (in somatic cells), a mitotic heritability and developmental reversibility of the inactive state. It is characterized by multiple, interconnected layers of epigenetic tags (Heard, 2005). Polycomb group proteins and DNA methylation ensure the inactive state. Gene reactivation on the inactive X chromosome is associated with large regions of promoter demethylation after 5-aza-deoxycytidine treatment, indicating the causal relationship between methylation and gene silencing on the inactive X chromosome (Feinberg and Tycko, 2004). X-inactivation leads to clumped chromatin termed Barr bodies, which are considered inert. However, recent research suggests that the Barr body may be more biologically active than was previously supposed (Carrel and Willard, 2005). Women still express many genes from their inactive X chromosomes, and different women express different genes from the inactive X chromosome. About 15% of the genes on the inactive X chromosome are actually active across all women, and a further 10% of genes are reported to be switched on in some women (Carrel and Willard, 2005).

The key in the differential treatment of the two X chromosomes is the X inactivation centre (*Xic*). This centre ensures that only a single X chromosome remains active in a cell with a diploid autosomal set -a process known as counting (Avner and Heard, 2001)- and it also provides the signal that triggers silencing: the unique, non-coding, Xist transcript (Heard, 2005). This approximately 19 kb-long, untranslated transcript somehow coats the chromosome from which it is expressed *in cis*, and this is rapidly followed by gene silencing across the chromosome during embryonic stem cell differentiation or in early embryos. How Xist RNA coats a chromosome and how this results in transcriptional repression is unclear. It may involve RNAi. Transient homologous chromosome pairing marks the onset of X inactivation (Xu et al., 2006). The Xist RNA may aid in compartmentalizing the X chromosome, supported

by the Scaffold Attachment Factor-A protein, a component of a putative nuclear scaffold that forms part of a stable proteinaceous structure over the inactive X chromosome. Among the earliest chromatin changes that occur during the X-inactivation process are the loss of euchromatin-associated histone modifications (such as H3K9Ac and H3K4Me2 and H3K4Me3) just after Xist RNA coating. Global H4 hypoacetylation occurs shortly afterwards. Following these changes, several new histone modifications appear on the Xist RNA-coated chromosome (Heard, 2005).

An important role in X chromosome inactivation is ascribed to the Polycomb proteins (Heard, 2005). The Polycomb Repressive Complex 2 (PRC2) is involved in X inactivation because one of its components was found to be necessary for the maintenance of the inactive state of the X chromosome in mice. Other components are recruited to the inactive X chromosome, but in the context of the X chromosome, the timing of H3K9 hypoacetylation seems to precede the recruitment of PRC2. The histone methyltransferase responsible for the tri-methylation of H3K27 in general also acts on the inactive X chromosome, whereas also mono-ubiquitination of histone H2A is relevant (Heard, 2005). Two other PRC2 components, Suz12 (mammalian homologue of *Drosophila* suppressor of zeste protein) and Ezh2 (mammalian homolog of *Drosophila* enhancer of zeste protein), are recruited to the inactive X chromosome during embryonic stem cell differentiation. The timing of H3K9 hypoacetylation seems to precede the recruitment of PRC2 to the inactive X chromosome. Members of the PRC1 complex are enriched on the inactive X chromosome in a developmentally regulated fashion. The exact combination of PRC1 components in the complex varies between various studies, probably owing to different cell types and/or antibodies being analyzed, and possibly also to the dynamic constitution of PRC1 complexes. Neither PRC2 nor PRC1 complexes are enriched on the in active X chromosome at later stages of differentiation, suggesting that their association with the inactive X chromosome is linked to early rather than later maintenance events. Genetic and biochemical analyses have also demonstrated that the Ring1 (also known as Ring1a) and Rnf2 (Ring1b) PRC1 proteins appear to be required for the mono-ubiquitination of histone H2A at K119 and that Ring1b is likely to be the E3 ligase responsible (Heard, 2005).

Although Xist RNA is present on the silenced X chromosome, throughout the lifetime of the organism, Xist-induced transcriptional shutdown is thought to occur only during early development (Heard, 2005). This implies either that a silencing partner of Xist is present only transiently or that chromatin is only receptive to Xist-mediated inactivation during early development. It is predicted that new insights will extend beyond the level of chromatin structure, to include changes in chromosome positioning within the nucleus and/or local changes in the nuclear compartment surrounding the X chromosome (Heard, 2005).

Across evolution, different mammals appear to have developed different mechanisms and characteristics related to their inactive X. In humans, the X chromosome that is inactivated appears to be determined by chance. In marsupials, however, the paternal X chromosome is always inactivated. Certain animals have their coloring patterns dictated by X chromosome inactivation. For example, female cats have unique patterns of fur due to certain areas where different X chromosomes are inactivated. The X chromosomes that are activated express different coloring genes and give their mosaic fur color. To date, no *Xist* gene has been identified in marsupials, and these animals do not show DNA methylation on their inactive X chromosome (Heard, 2005).

3.5 Dosage compensation

X chromosome inactivation is an example of dosage compensation, the genetic mechanisms to balancing the relative gene expression between male and female derived genes (Lucchesi et al., 2005). Dosage compensation also occurs in other organisms such as the fruit fly *D. melanogaster* and the roundworm *C. elegans*. These species have different mechanisms of dosage compensation. Drosophila males (XY) double the expression of genes along the X chromosome. In *C. elegans* hermaphrodites (XX), both X chromosomes are partially repressed. Any of these mechanisms balance the relative gene expression between males and females or, in the case of *C. elegans*, hermaphrodites and males. A large variety of chromatin remodeling mechanisms can apparently affect the function of entire chromosomes (Lucchesi et al., 2005). As in X chromosome inactivation, large RNAs can associate with chromatin over the length of whole chromosomes and are crucial for spreading epigenetic changes in chromatin structure. They do not appear to act in a sequence-specific manner but provide scaffolds for co-operative binding of chromatin-associated complexes to enable spreading of chromatin modifications (Wutz, 2003).

In plants, dosage compensation occurs when aberrant meiotic events or mutations result in either aneuploidy or polyploidy (Comai, 2005). Genes on the affected chromosome may be up- or down-regulated to compensate for the change in the normal number of chromosomes present. Studies indicated a balance phenomenon such that changes of individual chromosomal dosage alter the phenotype more dramatically than changes in ploidy. Chromatin remodeling complexes are a major contributor to this balance and this way they become linked to the control of quantitative traits and hybrid vigor (Birchler et al., 2005).

3.6 Gene bookmarking

Gene bookmarking is presented as another mechanism, in addition to the modulation of chromatin structures described above, that is suggested to be crucial for proper gene expression (Sarge and Park-Sarge, 2005). To maintain the phenotype of cell lineages, cells must have a way to remember which genes are active before cell division and to propagate that pattern through mitosis to daughter cells. As in mitosis most sequence-specific DNA-binding proteins are thought to dissociate from the DNA, all active genes should somehow be 'marked' prior to mitosis, so that the daughter cells know what to assemble on the promoters of these genes. The factors or modifications that mark the active genes are called 'molecular bookmarks'. Several types of molecular bookmark and bookmarking mechanism exist and interconnect. Bookmarking can be mediated by selective histone modifications, by the presence of histone variants or by the binding of transcription factor IID (TFIID) to active genes. Other bookmarks are thought to depend on sequence-specific non-histone DNA-binding proteins. Bookmarking mechanisms specific to a gene family could reflect potential regulation, such as in the bookmarking of homeotic genes by PcG or TrxG, or the need to ensure the activity of genes located in chromosomal regions that might be difficult to decompose in mitosis. The observation that TFIID interacts specifically with acetylated Lys9 and Lys14 of histone H3 could explain at the biochemical level how histone modifications characteristic of active genes contribute to maintaining transcriptional memory. Possibly TFIID remains associated with the promoters of these genes during mitosis (Sarge and Park-Sarge, 2005).

3.7 Heterochromatin replication

Heterochromatin is almost exclusively associated with regions of the genome in which genes are not expressed (Wallace and Orr-Weaver, 2005). Heterochromatin is composed of tightly condensed chromatin in which the histones are deacetylated and methylated, and specific non-histone proteins are bound. As discussed above, the DNA within heterochromatin is methylated, in vertebrates and plants. Heterochromatin is a highly organized, compacted chromatin structure, and the details of this structure are being elucidated. However, the occurrence of transcription in heterochromatin and exchange of heterochromatin proteins indicates that the heterochromatic state may be less rigid and compact than originally thought (Martens et al., 2005). Facultative heterochromatin refers to regions that can be transiently condensed and silenced during development of a cell or an organism. These regions can shuttle between heterochromatin and euchromatin. Constitutive heterochromatin, including pericentric and telomeric heterochromatin, refers to the chromatin that remains condensed and silenced throughout development of the organism. Heterochromatin regions play critical roles in chromosome structure and transmission and often consist of highly repetitive satellite DNA and moderately repetitive elements like transposable elements. Although the expression of most genes is repressed by heterochromatin, there are essential genes, such as the *Drosophila light* gene, that is expressed only in a heterochromatic environment (Wallace and Orr-Weaver, 2005).

As the heterochromatic state is stably inherited, replication of heterochromatin requires not only duplication of the DNA, but also reinstalling the appropriate protein and DNA modifications (Wallace and Orr-Weaver, 2005). Analysis of the replication machinery and heterochromatin show that various replication proteins can act both to replicate the DNA and to recruit the heterochromatin binding proteins that epigenetically confer the heterochromatic state. These involve replication proteins, histone modification enzymes, DNA methyltransferase, and chromatin remodeling complexes. New factors required for the maintenance of heterochromatin are the RNAi machinery and the retinoblastoma (Rb) tumor suppressor protein. In fission yeast, *Drosophila*, and mammalian cells, the RNAi machinery is required for heterochromatin protein binding, heterochromatic silencing, and centromere function. The Rb protein family is

required for DNA methylation, hypoacetylation of histone H3, and trimethylation of histone H4, most likely via a direct interaction with the H4K20 trimethyl transferase. It is likely that Rb has a function in reinstating heterochromatin during DNA replication. In addition to the predominant histone proteins, there are histone variants that contribute both to the formation of heterochromatin and protection against the spread of heterochromatin into euchromatic regions (Kamakaka and Biggins, 2005).

Another issue is how all histone modifications and associated chromatin proteins are templated onto the daughter duplex after replication. Given the interdependency of histone modifications, semi-conservative reassembly of the nucleosome could provide a means to re-establish proper histone modifications that could then promote proper protein complex assembly. The evidence to date appears not to favor such an assembly of the nucleosome (Henikoff et al., 2004), but it is not decided yet how the distribution of nucleosomes to daughter strands occurs (Grant-Downton and Dickinson, 2005).

3.8 Cancer epigenetics

There is probably no field of science where the impact of epigenetics has been bigger than in cancer research. An insightful review of the timeline of cancer epigenetics (Feinberg and Tycko, 2004) has been published. After it was observed in 1983 that hypomethylation distinguishes genes of some human cancers from their normal counterparts, considerable advances have been made in characterizing the various genetic and epigenetic alterations that occur in cancers (Feinberg et al., 2006). It has become clear that 'cancer' covers many diseases, ranging from solid tumors to invasive and metastatic tumors, thought to arise from genetic mutations, as well as to leukemias due to chromosomal rearrangements, all resulting in the inappropriate timing of normal cellular functions. It is remarkable to see how in current thinking the ideas on cancer are moving away from the long-assumed primary genetic causes (as mutations and chromosome aberrations) to epigenetic triggers (Baylin and Ohm, 2006; Feinberg et al., 2006).

For a long time, cancer was thought to arise from a single cell through a series of mutations, responsible for clonal selection and tumor cell heterogeneity. This 'clonal genetic model' has been supported by the discovery of dominant oncogenes and recessive tumor-suppressor genes (Feinberg et al., 2006). A mutation leads to the selective overgrowth of tumor cells, and each clinical property of a tumor (invasiveness, metastasis and drug resistance) is related to such a mutation. Epigenetic changes, if accepted at all, are viewed as surrogates for true mutations. This model of cancer has been particularly successful in predicting mutations that seem to be necessary for the earliest stages of tumor growth. Accumulation of genetic changes during tumor progression has been well documented for many tumors. Yet, the model fails in explaining many aspects of cancer initiation and progression (Feinberg et al., 2006).

Epigenetic changes can lead to aberrant activation of growth-promoting genes and aberrant silencing of tumor-suppressor genes. Both DNA hypomethylation and DNA hypermethylation are important in cancer (Feinberg and Tycko, 2004; Baylin and Ohm, 2006; Feinberg et al., 2006). Although hypomethylation was the originally identified epigenetic change in cancer, it was overlooked in preference of hypermethylation for many years. On the basis of hypermethylation of promoter regions, candidate tumor-suppressor genes were identified and more or less random searches to detect changes in methylation and chromatin status is supposed to lead to the identification of important factors (Baylin and Ohm, 2006). Yet, the frequency of hypomethylated sites can in fact be quite high, as indicated by high-throughput genomic-methylation analysis of various tumors, including cancers of the stomach, kidney, colon, pancreas, liver, uterus, lung and cervix. Studies of model organisms support a role of both hypomethylation and hypermethylation in cancer. Depending on the particular cross made, DNA methyltransferase mutants in mice can show an increased frequency of tumors in the lymphoid system or reduced numbers of epithelial tumors. Global DNA hypomethylation generally leads to chromosomal instability and increased tumor frequency (Feinberg and Tycko, 2004).

Epigenetic changes also contribute to chromosomal loss and rearrangements during tumor progression (Feinberg and Tycko, 2004; Baylin and Ohm, 2006; Feinberg et al., 2006). Although individual genes may vary in hypomethylation, in all tumors examined, both benign and malignant, a global reduction of DNA methylation is observed. DNA methylation is changing early and ubiquitously in cancer. Silencing of tumor-suppressor genes is

associated with promoter DNA hypermethylation and chromatin hypoacetylation. Various other epigenetic mechanisms discussed in Chapter 2 are therefore also implied in cancer. There are characteristic histone tags in several types of cancer cell lines and tumors (Greener, 2005). Overproduction of histone methyltransferases that catalyze the methylation of either H3K4 or H3K27, as well as more reductions in mono-acetylated H4-K16 and trimethylated H4-K20 are general features of cancer cells. A histone H3 variant (centromere protein A; CENPA) is overproduced in colorectal cancer and may result in aneuploidy. Other histone variants may play roles in cancer progression (Gregory and Shiekhattar, 2004; Kamakaka and Biggins, 2005)}.

In addition, imprinting is important in cancer. Both gynogenotes (embryos derived only from the maternal genome) and androgenotes (embryos derived only from the paternal genome) form tumors. Various genes show loss of imprinting in cancers. For example, loss of imprinting in the insulin-like growth factor 2 gene accounts for half of all Wilms tumors in children and is a common epigenetic variant in adults associated with increased frequency of colorectal outgrowths. Variations in the stringency of parental imprinting are shown to influence the likelihood of tumor development. Loss of imprinting of the insulin-like growth factor II (IGF2) gene is detected in blood cells of about 10% of the normal human population. This suggests that the stringency of the postnatal maintenance of IGF2 imprinting is (epi)genetically determined. Hybrid mice were generated from females heterozygous for a deleted and differentially methylated region (heterozygosity here means hemizygosity: one of the two copies of the chromosome contained this region, whereas the other did not) and from males with a mutation in a gene that predisposes to precancerous condition (Klein, 2005). All offspring carried the gene mutation, but only half of them inherited the imprinting defect. The frequency of intestinal outgrowths was twice as high in the mice with the imprinting defect as in the controls. It shows that cancer susceptibility is directly influenced by differences in the stringency of epigenetic control (Sakatani et al., 2005).

Tumor tissue appears also to be characterized by over- or misexpression of miRNA genes. Thirteen miRNAs form a signature associated with prognosis and disease progression in leukemia (Calin et al., 2005) and miRNA expression patterns can classify human cancers (Lu et al., 2005). It is becoming clear that miRNAs can function both as tumor suppressors and as oncogenes (Hammond, 2006).

The observations that epigenetic changes occur early in tumor formation, and occur also in normal tissues before tumor formation, has been taken to indicate that early epigenetic changes in so-called progenitor stem cells could provide further insight in the occurrence and development of cancer (Feinberg et al., 2006). Epigenetic disruption of such progenitor cells may be a key determinant not only of cancer risk, but also of tumor progression and heterogeneity late in the course of the tumors that arise from these cells. Epigenetic changes set the stage for genetic alterations and could explain many of the heterogeneous properties commonly associated with tumor cell-growth, such as invasion, metastasis and failing therapy. The epigenetic progenitor model of cancer (Feinberg et al., 2006) proposes that cancer arises in three successive steps: the epigenetic disruption of progenitor cells, an initiating mutation, and genetic and epigenetic plasticity. It should be noted that the second step was long considered to be the first step and has received a lot of research attention over the years (Futreal et al., 2004).

In this epigenetic progenitor model, the epigenetic changes are no longer a surrogate of genetic alterations, but precede and possibly direct the genetic changes (Feinberg et al., 2006). It assumes the existence of tumor-progenitor genes that mediate epigenetic expansion of progenitor cells, and increase their likelihood to promote growth and cancer. For example, enzymatic cytosine deamination would lead to global DNA demethylation as well as the mutations, both general characteristics of cancer. The various arguments for and explanatory power of this model are summarized in detail elsewhere (Feinberg et al., 2006). The model implies that non-cancer (or, more accurately, non-neoplastic) cells should be considered target for cancer risk assessment and therapy. In a way, it is an optimistic model for cure, because epigenetic alterations are or may be easier reversible than genetic changes (Yoo and Jones, 2006).

The single leading risk factor for cancer is probably age. Age itself may disrupt the epigenetic program and increase cancer risk. This relationship could also be true for diseases other than cancer. Epigenetics could explain why most common disorders that involve complex genetics begin later in life (Feinberg and Tycko, 2004).

3.9 Plant epigenetics

Historically, plants have been at the forefront of RNA-mediated silencing research (Matzke and Matzke, 2004). This is due in part to the ability to produce large numbers of transgenic plants, which displayed a rich variety of epigenetic and gene silencing phenomena amenable to analysis. Incentives to find ways to prevent silencing and stabilize transgene expression also boosted research into silencing and its prevention (Matzke and Matzke, 2004). RNA silencing pathways in plants have diversified along with key gene families involved in small RNA biogenesis and regulation. The discovery of an apparently plant specific RNA polymerases (pol IV) linked silencing, histone methylation and heterochromatin formation (Herr, 2005). Although there are many similarities between plant and other systems, it is remarkable that many of the plant proteins involved in chromatin remodeling, histone acetylation or methylation lack the protein-protein recognition modules that are so highly conserved in yeast and mammals, such as chromo- and bromodomains (Brzeski and Jerzmanowski, 2004).

Various examples of mitotic epigenetics plants, such as imprinting and somaclonal variation, have been given above. In addition, there are many instances and possible causes of epigenetic instability in plant polyploids (Adams and Wendel, 2005). Polyploidy is important in plant speciation. Allopolyploidy, or the merging of genomes from separate species, is widespread in plants and produces variable flowering and morphological phenotypes (Comai, 2005). Autoploidy arises through the multiplication of the same chromosome set. Epigenetic resetting in polyploids is likely to be relatively deleterious as it may perturb the regulatory adaptations of the parents. Some, but not all, genes subject to epigenetic regulation in alloploids of Arabidopsis show various alterations in autoploids, although the precise mechanisms will require more study (Comai, 2005).

4. Examples of transgenerational epigenetic inheritance

Current knowledge of epigenetic reprogramming confirms earlier suggestions that epigenetic marks are not always completely cleared between generations (Rakyan and Whitelaw, 2003). Incomplete erasure at genes associated with a measurable phenotype can result in unusual patterns of inheritance from one generation to the next. It is becoming clear that the environment is playing an important role in such phenomena. Although the existence of an epigenetic parental state, established either stochastically or in response to the environment, that is inherited by the offspring, is considered to have a Lamarckian flavor and continues to meet with resistance (Chong and Whitelaw, 2004b), transgenerational epigenetic inheritance has now been convincingly demonstrated in several different eukaryotic organisms. In humans, it is often seen in individuals within single families. The overall biological significance of this particular mode of inheritance is not decided upon.

4.1 Human

Epidemiology

Evidence for germline epigenetic inheritance in humans has come almost exclusively from epidemiological studies (Chong and Whitelaw, 2004b). The associations between low birth weight and adult-onset disease are compelling. They have been reviewed several times. They reveal an unexpected plasticity in early mammalian development where environmental cues, such as undernutrition, modify the baby in ways that can have an effect on the health of the adult. This is known as fetal programming. It is proposed that communication about the extra-uterine environment between mother and fetus better prepares the young for survival after birth. Instances of fetal programming in humans, in response to gestational exposure to famine or overfeeding of the paternal grandfather during childhood, have been described. Apparently, the effects of fetal programming can be passed on to the next generation (Chong and Whitelaw, 2004b).

Although most studies of fetal programming only address effects in the first-generation offspring, there are some cases in which programmed phenotypes are maintained for multiple generations (Whitelaw, 2006). It was shown, for example, that the behavior (or the environment) of young boys could influence the phenotype of their sons and grandsons (Pembrey et al., 2006; Whitelaw, 2006). The paternal grandfather's food supply during mid childhood was linked to the mortality risk ratio of grandsons, but not granddaughters. This indicates that in human a one-off event is influencing the phenotype for more than one generation in a sex-specific way. Although epidemiological studies thus provide support for transgenerational epigenetic inheritance in humans, the interpretation of such studies is complicated by the confounding cultural and genetic heterogeneities of the populations investigated (Chong and Whitelaw, 2004b).

Molecular studies

The presence of epimutations, but not mutations, at the *SNURF-SNRPN* locus correlates with loss of imprinting in some patients with the Prader-Willi syndrome (Chong and Whitelaw, 2004b). The epimutation was located on a chromosome with a specific parental and grandparental origin. That is, the paternally derived chromosome carrying an aberrant maternal mark at *SNURF-SNRPN* was inherited from the paternal grandmother. The imprinting defect is thought to be due to incomplete erasure of the grandmaternal mark in the paternal germline. This shows that such epigenetic marks are apparently not always efficiently cleared between generations (Chong and Whitelaw, 2004b).

Another epimutation was found in a DNA mismatch repair gene (*MLH1*) in individuals with a history of multiple cancers. The epimutation was found in tissues derived from all three germ layers (endoderm, mesoderm and ectoderm) and in a low proportion (1%) of sperm, suggested that the epimutation occurred either very early in

development or in the germline of the parents. However, examination of further offspring did not confirm the inheritance of the epimutation. An inherited form of α-thalassemia, a disease normally associated with mutation at the *α*-globin locus, can be caused by a deletion in an adjacent gene (Chong and Whitelaw, 2004b). The deletion yields an antisense (in relation to the *α-globin* gene) transcript and results in the epigenetic silencing and hypermethylation of the *α-globin* CpG island promoter, despite the fact that all *α*-globin *cis* regulatory sequences remain unchanged. Although the epimutation at the *α*-globin locus is observed in an affected mother and son, it does not necessarily imply epigenetic inheritance. It depends on what happens if the DNA sequence in the downstream gene stops to produce the antisense transcript. If the epigenetic change remains stably inherited in the next generation, also in the absence of the initial causative DNA mutation, it would establish yet another confirmed case of transgenerational epigenetic inheritance in human (Chong and Whitelaw, 2004b).

4.2 Other mammalian species

Transgenerational inheritance of epigenetic marks has been observed at several murine transgenes and at a few endogenous loci. The latter include the dominant *agouti viable yellow* (A^{vy}) allele involved in the determination of coat color and the *axin-fused* allele associated with a kinky tail (Rakyan and Whitelaw, 2003; Blewitt et al., 2004). The expression state of these alleles, now referred to as metastable epialleles, varies between animals (variable expressivity) and at A^{vy}, even within an animal (variegation). The differences correlate with cytosine methylation at the locus in genetically identical animals and the phenotypes can be passed on to next generations. The DNA methylation pattern in somatic tissues of the parent is retained in the gametes, inherited by the zygote and then generally, but not always, cleared and re-established some time between fertilization and blastocyst formation (Chong and Whitelaw, 2004b). Therefore, the epigenetic inheritance appears to be the result of a failure in the clearing step. Transcriptional activity of both alleles is under the control of a particular type of retrotransposon that may be resistant to epigenetic reprogramming.

There is a growing body of evidence that the environment can influence the epigenetic state at promoters. Expression at the *agouti viable yellow* allele in mice can be influenced by maternal nutrition. Altering the diet of pregnant female influences the coat color. Progeny of animals that were fed methyl supplements during pregnancy and lactation exhibited substantially increased methylation of the allele in all tissues examined and a shift in coat color (Blewitt et al., 2004; Whitelaw, 2006). It has not yet been reported if such nutrition-induced DNA methylation changes are retained in the gametes, nor whether the phenotype of further offspring continues to be affected.

Work carried out in rats supports the notion that fetal programming can influence subsequent generations also when the environmental inducer is no longer present (Whitelaw, 2006). Transient exposure of pregnant female rats to a fungicide can cause reproductive abnormalities in the male offspring for at least four successive generations (Anway et al., 2004; Anway et al., 2005). As no effect was observed in the female offspring, this rat study could be considered the murine equivalent of the human study discussed above (Pembrey et al., 2006). The mechanism(s) of this type of transgenerational programming is supposed to involve epigenetic gene silencing and/or is related to the Y chromosome.

Female mice were generated that carried a paternally transmitted, modified *Rasgrf1* allele and a maternally derived wild-type *Rasgrf1* allele. The offspring of these females retained the re-activated wild-type *Rasgrf1* allele, in the absence of the modified allele, showing the inheritance of this change in epigenetic state through meiosis (Herman et al., 2003). This phenomenon resembles paramutation-like behavior of alleles as reported in plants (see below).

4.3 Plant species

In plants, newly acquired epigenetic states of transcriptional gene activity are relatively readily transmitted to the progeny (Takeda and Paszkowski, 2006). This is in contrast to mammals. To ensure transgenerational inheritance, the epigenetic information must be maintained during meiosis and various steps afterwards. Germline epigenetic inheritance in plants is thought to rely predominantly on cytosine methylation maintained through meiosis and

postmeiotic mitoses. The maintenance of CpG methylation (5mCpG) in meiosis appears to play a central role, guiding the distribution of other epigenetic signals such as histone H3 methylation and non-CpG DNA methylation. The DNA methyltransferase MET1 is responsible for copying 5mCpG patterns through DNA replication in the gametophyte. The importance of gametophytic MET1 activity is illustrated by the phenotypes of *met1* mutants that are severely compromised in the accuracy of epigenetic inheritance during gametogenesis (Takeda and Paszkowski, 2006). This includes elimination of imprinting at paternally silent loci as *MEDEA* (Gehring et al., 2006).

Paramutation

Paramutation was first used to describe the non-Mendelian inheritance of kernel pigmentation in maize. Paramutation is now known to involve a nonmutation-associated change in gene expression, which is meiotically heritable (Chandler and Stam, 2004; Stam and Mittelsten Scheid, 2005). Changes can occur on a stochastic basis or can be induced by the *trans*-interaction of homologous alleles. Paramutation is affected by environmental temperature. The switch from an active to an inactive state correlates with a change in chromatin structure. Although all genes displaying paramutation in maize are associated with pigmentation, other alleles, with less obvious phenotypes, may behave in similar ways. The two best-characterized endogenous alleles that display paramutation contain tandem repeats, and it has been suggested that this is related to their unusual behavior (Stam and Mittelsten Scheid, 2005).

A similar phenomenon appears to be related to ploidy of a plant (Comai, 2005). Tetraploid (but not diploid) *Arabidopsis* plants (autoploids) carrying a hygromycin phosphotransferase (*HPT*) transgene have variable hygromycin resistance, which correlates with changes in *HPT* transcription and DNA methylation. The hygromycin-sensitive phenotype was stably maintained even after crossing back into the diploid state. The hygromycin-sensitive phenotype was observed even in F_2 seedlings that did not inherit the original silent parental alleles, suggesting an allelic silencing *in trans* that was maintained in the absence of the inactivating allele. This could be interpreted as the occurrence of a paramutation-like event (Comai, 2005).

Stable methylation states

Another example of epigenetic inheritance in *Arabidopsis* is the *FWA* allele (Zilberman and Henikoff, 2005). *FWA* was described fifteen years ago as a dominant mutation that caused a delay in flowering time. *FWA* behaved as a typical Mendelian trait: the phenotype was stable over many generations and segregated with the expected 3:1 ratio. The mutation was mapped to a single gene encoding a putative homeodomain transcription factor. However, no DNA sequence changes could be found in the mutant allele. Introducing a wild type transgenic copy of the gene would occasionally recreate the mutant phenotype. The promoter and transcription start site of FWA are within almost perfect direct repeats. They are methylated in wild type plants, keeping the gene off in all tissues except the endosperm. The *FWA* mutation is caused by loss of DNA methylation in all tissues, resulting in inappropriate expression throughout the plant and the late-flowering phenotype. The methylated *FWA* epiallele is present in numerous locally adopted populations (ecotypes) of *Arabidopsis*, suggesting that it has been stably inherited for thousands of years. The unmethylated form of *FWA* appears to be equally stable. The two states of *FWA* now represent 'true' Mendelian traits, the basis of which is a difference in the methylation of DNA and not in the sequence. In addition to FWA, similar epialleles of several other genes have been described (Zilberman and Henikoff, 2005). The mechanisms for maintaining DNA and histone methylation at the *FWA* locus are now known to involve small RNAs, but many of the details await elucidation.

Another naturally occurring morphological mutant of the common toadflax, *Linaria vulgaris*, in which the fundamental symmetry of the flower is changed from bilateral to radial, is also due to a change in the methylation state of *Lcyc* rather than a change in its primary DNA sequence (Cubas et al., 1999). The epimutation, and hence the peloric phenotype, was transmitted to subsequent generations relatively faithfully (13%). Occasional somatic revertants to the wild type were also observed, concomitant with *Lcyc* demethylation. Sequence analysis of the *Lcyc* locus revealed no differences between mutant and wild-type plants. The switching of *Lcyc* expression, epigenetic state and phenotype among separate branches of the same plant supports the idea that the primary change is epigenetic and not genetic, although it has not yet been established that mutant plants produce more mutant seedlings than plants with the wild-type phenotype (Chong and Whitelaw, 2004b).

Genome changes

In flax (*Linum usitatissimum*), changes in nutrients lead to dramatic phenotypic alterations that are accompanied by gross genomic modifications during vegetative growth, such as consistent reductions in ribosomal gene copy number and widespread insertion events distributed over all chromosomes (Cullis, 2005). An insertion element was identified at the same position in all five independent lines when in the same environment. Amazingly, this particular element was no known transposon, but represented a sequence not present in the original line. The insertion element appears reproducibly at a unique site in a single generation after an environmental stress from a source that cannot be identified as the donor. The sequence of the element suggests it assembled in a series of DNA rearrangement and editing events (Chen et al., 2005). Given that this is only one of many programmed genomic changes that can occur, it seems likely that it is not an isolated example. Programmed genome-wide rearrangements and insertions are seen in various ciliates. Programmed rearrangements and point mutations in the vertebrate adaptive immune system are very well studied biological processes. The existence of such events may establish yet other levels of complexity of the epigenetic regulation of genome function and integrity (Henikoff, 2005b).

4.4 Other organisms

Schizosaccharomyces pombe (fission yeast): the epigenetic modification of a reporter gene placed in the mating-type region of fission yeast could be inherited through mitosis and meiosis. Loci influencing this process were (in)directly involved in the organization of heterochromatin and include histone deacetylases, histone methyltransferases and other structural proteins associated with telomeres and centromeres (Grewal and Elgin, 2002; Chong and Whitelaw, 2004b).

Drosophila melanogaster (fruit fly): the presence of a cis regulatory element upstream of a GAL4-inducible reporter and mini-white gene stably activated reporter gene and mini-white expression in fruit fly upon heat induction. The chromatin-mediated transcriptional activation was inherited through both mitosis and meiosis. The element belongs to the Polycomb response elements (PRE) that bind Polycomb and Trithorax proteins (Ringrose and Paro, 2004). Activity of an endogenous allele of a heat shock protein (Hsp90) produces a heritable array of morphological phenotypes, suggesting that it acts as an epigenetic trigger for phenotypic variation. Drosophila with an abnormal eye phenotype in a fairly complicated genetic setup were fed an Hsp90 inhibitor and showed an increased phenotype that could be maintained for up to 13 generations in the absence of the inhibitor, but only if the most penetrant flies were selected each generation (Rutherford and Henikoff, 2003). This shows that the inducer and the recipient need to be together during only one meiotic generation (Sollars et al., 2003). The phenotype disappeared after 2–3 generations of negative selection. When positively selected progeny were fed histone deacetylase inhibitors, the abnormal eye phenotype was suppressed. This indicates the involvement of chromatin structure, although further molecular mechanisms of the epigenetic modifications are unknown (Ringrose and Paro, 2004). A transposon insertion is possibly related to the epigenetic sensitivity of the eye phenotype. The details of the phenotype establish a relationship between chromatin and the complex inheritance of multigenic traits. It may indicate that also quantitative traits can be epigenetic in origin (Rutherford and Henikoff, 2003).

4.5 Biological role

Epigenetic germline inheritance appears to occur preferentially, possibly exclusively, at transgenes and at genes under the transcriptional control of repetitive elements such as retrotransposons (Chong and Whitelaw, 2004b). Repeat elements are generally concentrated at telomeres and centromeres and play a critical role in chromosome function. The maintenance of the epigenetic state at these structures during meiosis may be necessary for segregation and pairing. The epigenetic inheritance seen at chromosomal positions other than at telomeres and centromeres could be the by-product of a recent genomic rearrangement, such as the insertion of a retrotransposon, which then confers a meiotically heritable form of epigenetic control to this new location. This may provide a special mechanism of rapid adaptive evolution. The metastable phenotypes resulting from an epimutation could give a selective advantage to some individuals within a population (Chong and Whitelaw, 2004b).

5. Applications of epigenetic inheritance

The clear impact of epigenetics on gene expression and gene regulation is an obvious explanation for the current motivation and desire to find applications for the new epigenetic knowledge. The realms of application are generally not very different from applications of gene regulation and genetic engineering that were imagined and/or desired before. These applications focus on diagnostics, the prevention of undesired phenomena like disease and the accomplishment of desired characteristics such as health, yield and/or other (epi)genetic improvements. The (supposed) reversibility of epigenetic changes (Feinberg et al., 2006; Yoo and Jones, 2006), which contrasts markedly with the (experienced) irreversibility of genetic changes, may give epigenetic applications a different scope of success and failure than the genetic approaches taken before. For example, if indeed cancer begins with epigenetic changes in normal cells, it may turn into a disease that is detectable in an early stage and treatable with generic agents (Feinberg et al., 2006). The state of application of the various epigenetic mechanisms described in Chapter 2 also reflects current understanding and mastering of those mechanisms. In mammalian systems, the applications tackle the mitotic epigenetic inheritance, whereas in plants the approach is largely genetic, using transgenic plants. The application of transgenerational epigenetic inheritance is scarcely out of the egg. Currently, applications of epigenetic inheritance may change the epigenomic status of cells beyond the intended effects: a major issue in applications of epigenetic inheritance today is specificity.

5.1 Epigenetic drugs

Given the advance of epigenetics towards cancer, it will come as no surprise that notably in cancer research the use of epigenetic therapies and targets is being evaluated and is reported to show promising progress (Egger et al., 2004; Feinberg et al., 2006; Yoo and Jones, 2006). Epigenetic drugs generally target aberrant heterochromatic regions to reactivate genes that are crucial for the normal functioning of cells. Many compounds are available that alter the methylation or the modification of histones, several of which are currently tested in clinical trials (Egger et al., 2004). Such drugs could be used alone or in combination with chemotherapy, immunotherapy or radiotherapy. Epigenetic drugs could also help to alleviate the resistance to other drugs by reactivating DNA-repair genes. Opportunities from epigenetic alterations in cancer cover the whole chain of early diagnosis, prognostic and/or predictive tests as well as therapy. Hypermethylated genes in serum could be developed as prognostic biomarker (Dueñas-Gonzalez et al., 2005). Epigenetic therapy might also be useful for prevention, especially for those individuals who carry aberrant epigenetic alterations without symptoms. Epimutations, aberrant DNA methylation and histone-modification patterns, could be detected in individuals with no history of malignancy and used as an indicator of the likelihood of developing cancer. Other chromatin changes that are involved could be assayed as well as a molecular marker strategy to aid cancer risk assessment, early detection and prognosis (Baylin and Ohm, 2006). Diagnostic tests are being developed that measure the extent and pattern of DNA methylation to augment conventional tests (Greener, 2005). If such epimutations can be corrected, this can delay or prevent tumor formation. A detailed map of specific epigenetic patterns in each tissue type in their normal and in the various cancerous states would allow early detection of such epimutated situations. Dietary modification of methylation (methionine consumption) could offer additional therapeutic avenues for disorders, for example of the nervous system (Weaver et al., 2005; Rowan, 2006).

Agents that modify the epigenome globally, such as 5-aza-2'-deoxycytidine that inhibits DNA methylation, or SAHA (suberoylanilide hydroxamic acid) that inhibits histone deacetylases, are available (Yoo and Jones, 2006). Most attention on epigenetic drug development is focused on DNA methylation inhibitors and histone deacetylase inhibitors. There are two classes of DNA methylation inhibitors: nucleoside analogues and non-nucleoside analogues. Nucleoside analogues have a modified cytosine ring that is attached to either a ribose or deoxyribose moiety. They are metabolized and incorporated into DNA and/or RNA. DNA methylation is thought to be inhibited when the compounds are incorporated into DNA. Examples are the ribonucleoside analogues 5-azacytidine (5-Aza-CR) and zebularine and the deoxyribonucleoside analogues, 5-aza-2'-deoxycytidine (5-Aza-CdR; decitabine) and 5-fluoro-2'-deoxycytidine (5-F-CdR). 5-Aza-CR and 5-Aza-CdR are extremely potent in inhibiting DNA methylation at micromolar concentration. Their short half-lives in aqueous solution complicate the delivery of these drugs. 5-Aza-CR failed to

obtain FDA approval as a conventional cytotoxic drug about 25 years ago. The recognition that it causes progressive DNA hypomethylation gave the drug FDA approval in 2004 (Greener, 2005). Dihydro-5-azacytidine (DHAC) is hydrolytically more stable and less cytotoxic than 5-Aza-CR, so it may be more promising in future applications (Yoo and Jones, 2006).

Clinical trials have shown that low-dose exposures lead to greater responses and are associated with less toxicity. The compound zebularine (1-β-D-ribofuranosyl-2(1H)-pyrimidinone) is a recent addition to the list of demethylating agents in the family of nucleoside analogues. Treatment with DNA-methylation inhibitors alone in solid tumors has not been successful to date. The demethylating agents do not target cells for immediate death as do most other chemotherapeutic drugs, and the cells must be allowed to proliferate and reactivate genes that have been methylation-silenced for these drugs to take effect. Their effect is generally transient because the aberrant patterns can return with the removal of the drug, allowing the malignant cell population to reappear. Possibly, genomic instability because of hypomethylation could create an adverse long-term consequence (Yoo and Jones, 2006).

Currently there are a handful of non-nucleoside analogues that are known to inhibit DNA methylation (Yoo and Jones, 2006) and only a few have made it into clinical trials, but more compounds in this class are expected in the near future. As these small-molecule inhibitors inhibit DNA methylation by binding directly to the catalytic region of the enzyme, without incorporation into DNA, they may prove to give less problems than the nucleoside analogs (Yoo and Jones, 2006).

Various inhibitors of histone deacetylases (HDACs) are known and described in considerable detail (Dokmanovic and Marks, 2005; Yoo and Jones, 2006): short-chain fatty acids, hydroxamic acids, cyclic tetrapeptides and benzamides. The histone deacetylase inhibitor trichostatin A is already in use and seems to have promising efficacy against leukemias (Feinberg and Tycko, 2004). All HDAC inhibitors prevent hypomethylation of histones, which leads to chromatin remodeling, transcriptional activity, and restoration of malignant cells to a more normal state. Many pathways might be involved in their mode of action. The exact mechanism through which these drugs mediate anti-tumor activity has not been elucidated, although many suggestions have been made as to which cellular pathways are involved (Yoo and Jones, 2006).

Tumor-progenitor genes should present attractive drug targets for therapeutic intervention (Feinberg et al., 2006). Such drugs could prevent the onset of cancer or be useful in preventing relapse after a primary treatment results in remission. Despite the widespread expression of these potential tumor-progenitor genes, specific inhibitors of this class of enzymes might be well tolerated. The key step in identifying such agents will be to focus on epigenetic events in apparently normal tissue that arise long before the recognition of undesired cell activity (Feinberg et al., 2006).

In all epigenetic drugs in development to date, pleiotropy is a major issue (Brueckner and Lyko, 2004; de Vos, 2005). Non-specific epigenetic modification can lead to the activation and silencing of many genes, and it is not yet clear whether regression has an epigenetic basis. Comprehensive knowledge of the epigenome would open up a new avenue for the development of various drugs designed to target a specific region of the genome in which an epimutation has occurred. It will be required to understand the nature of global and gene-specific epigenetic variation in normal cells compared to those with cancer or at risk of developing cancer. The global changes to the epigenome will be made clearer by a systematic examination of the epigenome at the molecular level. DNA-resequencing efforts that are directed at tumors are likely to miss such epigenetic information. The lack of specificity, potential mutagenicity and toxicity are clear disadvantages of current epigenetic drugs, and the development of highly specific drugs targeting a subset of these epigenetic modifiers or a small region of the genomes should be developed. Ultimately, it might be possible to tailor epigenetic therapy to a given epigenetic modification or gene target, for example, by using engineered targeting transcription factors or microRNA (Feinberg et al., 2006; Yoo and Jones, 2006). The lessons from and experiences in the clinic should pave the way to the successful use of epigenetic drugs in practice, alone or in combination (Maio, 2005).

5.2 RNAi-based approaches

RNA interference (RNAi) has quickly become a powerful and indispensable tool in the laboratory to selectively silence essentially any gene in a genome (Robinson, 2004). It also promises potent therapeutic drugs because of the high selectivity: the RNAi sequence seeks out and destroys its target without affecting other genes. The high selectivity of RNAi, combined with its potency, as only a few dsRNAs are needed per cell, make it the tool of choice for functional genomics (determining what a gene product does and with what other products it interacts) and for drug target discovery and validation. By 'knocking down' a gene with RNAi and determining how a cell responds, a researcher can, in the course of only a few days, develop significant insight into the function of the gene and determine whether reducing its expression is likely to be therapeutically useful.

The clinical applications appear endless: any gene whose expression contributes to disease is a potential target, from viral genes to oncogenes to genes responsible for heart disease, Alzheimer's disease, diabetes, and more. Nevertheless, despite all the promise, RNAi therapy is a long way from entering the clinic and is likely to face many future problems. Other RNA-based therapies, antisense and ribozymes, also showed promise at the bench, but have largely stumbled before genuine application in patients (Robinson, 2004), although new developments in chemistry may give antisense approaches a next chance (Juliano et al., 2005).

For RNA-based therapies, the manufacture is not considered a problem, while delivery, stability, and potency may present significant obstacles. Stability and delivery seem major hurdles to any successful RNAi therapy, due to the biochemical nature of RNA itself and defenses against foreign material. To improve delivery, the RNA is complexed with a lipid or its phosphate backbone is modified (Schiffelers et al., 2004). To date, numerous experiments in animal models suggest that RNAi can downregulate a variety of target genes effectively. Whether this will translate into effective therapy is unclear. Its specificity may actually be too high. The more specific it is, the less robust the therapeutic activity is likely to be. In addition, the effect on the normal function of RISC is not known. RNAi may occupy all the available complexes and interfere with normal functioning of the cell after all. The first applications, if any, are likely to be in cancer (targeting out-of-control oncogenes) or viral infection (targeting viral genes). To avoid some of the problems of delivery, initial trials may deliver the RNA by direct injection into the target tissue (for a tumor, for instance) or *ex vivo*, treating white blood cells infected with HIV, but it may take several years to accomplish such applications (Robinson, 2004).

With several recent studies showing the involvement of miRNAs in several diseases, and as miRNAs and siRNAs use the same RNAi machinery to silence genes, the hope is that miRNAs may offer more than one point of therapeutic intervention in the RNAi pathway (Robinson, 2004). Specific miRNAs can be silenced and degraded long-lasting and non-toxic silencing by injecting chemically engineered single-stranded RNA analogues called 'antagomirs' in mice (Krutzfeldt et al., 2005). Apart from knowing what miRNA should be inhibited for what disease, again delivery to the right place at the right time will be a major challenge. Cancer is -again- a focus for investigating miRNA-based therapeutic opportunities. Antiviral challenges are also being looked at. Viral miRNAs have been found in virally infected cells. Human miRNAs have also been identified that can target HIV-1. The number of diseases that can now be attributed to faulty miRNA activity is, however, limited (Petit-Zeman, 2006).

In plants, the RNAi machinery is also extensively used for functional genomics to determine the functions of all the genes in the plant genome, notably in combination with transgenic approaches. RNAi using constructs encoding self-complementary 'hairpin' RNA allow high-throughput plant functional genomics or using virus-induced gene silencing (VIGS) (Waterhouse and Helliwell, 2003; Matthew, 2004). To increase the effectiveness of RNAi, novel RNAi methods (transient RNAi, differential RNAi (dRNAi), comprehensive RNAi with simple construction, quantitatively regulated RNAi, etc.) are available (Sato, 2005). RNAi may also be useful in metabolic engineering. RNAi-based approaches tailored for improved expression in plants showed a 50-fold or higher yield of protein from tobacco leaves using a viral suppresser of gene silencing (Voinnet et al., 2003), opening up an transient expression system that could be of use in future molecular farming. This way, detailed characterization of the mechanism(s) of RNAi could provide the molecular basis for a next generation of gene(tic) engineering.

RNA interference (RNAi) is also applied against various plant parasites (Lindbo and Dougherty, 2005). RNAi-mediated virus resistance can be achieved with the same type of inverted repeat constructs also used for functional genomics (Waterhouse and Helliwell, 2003; Sato, 2005). RNAi occurs in plant parasitic nematodes and possibly other non-viral pests (Bakhetia et al., 2005). Novel resistances could be developed if the plant would generate an effective form of double-stranded RNA in the absence of an endogenous target gene without detriment to itself. If such RNA molecules become available to the nematode, crop resistance could be achieved by introducing a dsRNA in the plant that targets a nematode gene and induces a lethal or highly damaging RNAi effect on the parasite (Bakhetia et al., 2005).

5.3 Cellular reprogramming and cloning

A dramatic way of trying to alter epigenetic marks experimentally is somatic cell nuclear transfer for cloning or stem cell generation in mammalian systems (Morgan et al., 2005) or micropropagation and transformation in plants. The differences between the plant and animal systems are remarkable. In plants, cell reprogramming is generally no problem. Either micropropagation or genetic transformation is routine for many plant species (Veluthambi et al., 2003). It may have to do with the moment in the life cycle of plants that the reproductive tissues are formed and/or it may reflect the presence of epigenetic tags that are relatively easy to remove.

In contrast, reprogramming for cloning in mammals is problematic. Both the erasure of the epigenetic memory of the differentiated donor cells and the establishment of the epigenetic program of the early embryo appear highly defective in most cloned embryos. Cloning requires a differentiated somatic nucleus to become reprogrammed in an enucleated oocyte to a totipotent state without the normal developmental reprogramming. Epigenetic marks in cloned embryos and adults in different mammalian species show abnormalities, and most cloned embryos differ from each other in their precise epigenetic profile. There is great variability in epigenetic marks between individual cloned embryos, and a small proportion of cloned preimplantation embryos have modifications resembling those of natural embryos; these seem to have a better developmental potential than those with highly aberrant epigenetic marks. Epigenetic reprogramming during cloning is apparently a stochastic process, the outcome of which is difficult, if not impossible, to predict (Morgan et al., 2005).

The developmental and the epigenetic abnormalities of cloned embryos tend to be more severe the earlier they are examined, with less abnormal ones surviving to later stages. Epigenetic defects described in cloned offspring include errors in X inactivation, imprinting, DNA methylation in general and of specific gene and repeat sequences, histone acetylation and methylation and widespread alterations in gene expression. Demethylation (active and passive) of the somatic nucleus occurs, but no detailed evaluation of the demethylation is available. Aberrant demethylation of imprinted genes could lead to developmental defects. Histone acetylation and methylation are also only very incompletely reprogrammed and unexpected *de novo* methylation of DNA may occur. Which gene targets are particularly deregulated by abnormal epigenetic marks in cloned embryos is not known (Morgan et al., 2005).

Partly in view of the ethics and scientific feasibility of cloning, research is focusing on alternative technologies for reprogramming. Similar considerations are valid for current stem cell research focused on application (Morgan et al., 2005; Zhang and Rosen, 2006), using either embryonic (Ambrosi and Rasmussen, 2005; Liew et al., 2005) or adult (Mauney et al., 2005) stem cells. Much more knowledge on the mechanisms of epigenetics seems required before mammalian cellular (re)programming will become technically feasible.

5.4 Other epigenetic modifications

Few other epigenetic modifications are being investigated for applications. Production of therapeutic proteins in mammalian cell lines is hampered by low yields and unstable expression. Approaches designed to interfere with epigenetic gene silencing with the aim of enhancing and stabilizing transgene expression include targeting histones, the inclusion of anti-repressor elements to block chromatin-associated repressors as well as targeting sites of high endogenous gene-expression. Employing such epigenetic gene regulation tools, in combination with process optimization, may be a step forward in the production of therapeutic proteins (Kwaks and Otte, 2006).

In plants, considerable variation of transgene expression is often observed within populations of transgenic plants transformed with the same transgene construct. Efforts are being directed toward achieving stable expression of transgenes with an expected level of expression (Butaye et al., 2005). The use of so-called matrix associated regions reduced the variability considerably, supposedly by shielding the transgenes from surrounding chromatin (Mlynarova et al., 1994) and they could also protect against a form of RNA silencing (Mlynarova et al., 2003). Similar results were obtained in different systems (Butaye et al., 2005). The more chromatin remodeling (-like) gene products are implied in plant growth and development, such as during the development of leaves (Yii et al., 2005), the more potential applications will be considered and evaluated in the laboratory. No clear examples are known where transformation of plants changed the epigenomic status of other genes than the incoming DNA, except for the well-documented cases of homology-dependent gene silencing (Matzke and Matzke, 2004) and transformation with genes directly aimed at changing the epigenome.

As application of the occurrence of transgenerational epigenetic inheritance in plants, it would be possible to deliver transiently an epigenetic signal to a plant cell (*e.g.* with a viral vector) and change the epigenetic status of a gene in a stable manner. Examples of this approach have yet to be put forward in the literature. If this approach proves sufficiently stable over generations, it would allow changing a trait in a plant without formal genetic engineering of that plant. This could present a technological alternative to the all-native (or *cis*-genic) DNA transformation strategy (Rommens, 2004). It seems that such an epigenetic strategy, if feasible, would only allow down-regulation of endogenous genes. For transgenerational epigenetic strategies to allow up-regulation of endogenous genes, much more knowledge of the underlying epigenetic mechanism seems required. In the longer run, similar strategies might be developed with respect to histone modifications or chromatin remodeling in plants.

5.5 Prospects and regulation of epigenetic engineering

The more is understood about epigenetic regulation of gene expression, the more feasible epigenetic applications will become, both in the laboratory and outside the laboratory setting. The future development of 'epigenetic engineering', a term here introduced to contrast the approach to the well-established technology of genetic engineering, is therefore inevitable. The realms of desired application are generally not very different from applications imagined or desired before using genetic engineering: health, yield, efficiency, diagnostics, metabolic improvement and/or specialized use. Most experiments now focusing on epigenetics aim at further elucidating the mechanisms and impact of epigenetics, rather than at developing applications. Currently, most applications of epigenetic engineering are in the laboratory stage of development and may never make it to the market or to the field. Many (future) applications of epigenetic engineering will (tried to) be accomplished with the help of genetic engineering to change the characteristics or amount of gene products present.

The first applications leaving the laboratory phase are likely to consist of the use of agents that modify the epigenome globally, such as the inhibition of DNA methylation or histone deacetylation. These may have limited use because of the lack of specificity (pleiotropy). Only when technologies will become available that allow tailoring epigenetic compounds to a given epigenetic modification, preferably at a given genomic position in a desired cell, epigenetic applications are likely to boom and bloom. Further insight in the mechanisms and impact of epigenetic effects may help to understand and master (some of) the variability and pleiotropic effects seen upon genetic engineering.

The problem of pleiotropy will apply equally to all engineering of enzymes that are involved in epigenetic modifications. RNA-based therapies may present solutions to the specificity of interactions, but pose additional problems with respect to stability and delivery. These applications focus on mitotic epigenetic inheritance that is supposed to be erased in the next generation. Commercially and/or ecologically feasible applications of transgenerational epigenetic inheritance would seem to require much more knowledge and mastering of the mechanisms and controls of such types of inheritance.

An important issue along the future development of epigenetic engineering is the issue of biological safety and regulation. Is epigenetic engineering in its concept and potential outcome so different from genetic engineering that it would require an additional or its own regulatory framework? And *vice versa*, is current genetic engineering able to influence epigenetic modifications in such a way that additional precautions should be taken in the safety assessment of genetic engineering? Given the current state of knowledge and level of potential applications, it would seem to be too early to give a definitive solution to this issue, but there is a growing body of evidence that epigenetic engineering may not be essentially different.

For applications of mitotic epigenetic inheritance using compounds that modify the epigenome globally, such as the inhibition of DNA methylation or histone deacetylation, next generations are (or should not be) affected. The individual phenotype aimed for, potential aberrations in that phenotype, as well as the toxicology of the compounds involved would therefore be important criteria. However, even with technologies to establish the epigenomic status of every position in every cell in every condition, it will be difficult, if not impossible, to assess potentially subtle aberrations in the phenotype and/or potential long term effects on the organisms itself or its offspring.
The same will be true for any presumed influence of genetic engineering on the epigenome of the recipient cell/organisms. Effects, if any, are likely to be subtle and pleiotropic, therefore difficult, if not impossible, to measure and even more difficult, if not impossible, to distinguish from the intrinsic variability of biological systems in general. For applications of mitotic epigenetic inheritance where genetic engineering is used to change the genetic make-up of the recipient organism, the genetic alteration may imply that in every subsequent generation a chain of events of modification/removal upon meiosis/modification is triggered that should be evaluated. For transgenerational epigenetic inheritance, once sufficiently mastered to allow applications, a complicating factor will be that the modification may not be obvious in a given generation and show up only in later generations.

In the longer run the increased understanding of epigenetic phenomena may allow the merger with genetics by explaining epigenetic phenomena in the context of epistatic interactions. If so, the regulatory framework for genetic engineering may well prove sufficient for future epigenetic engineering. It would seem sufficiently unlikely that targeting the epigenetic layers of cell and gene regulation in future epigenetic engineering would generate safety issues that are essentially different from the safety issues and other considerations already encountered in genetic engineering (Conner et al., 2003; Nap et al., 2003). This will depend, however, on the outcome of future investigations and the increase in knowledge on epigenetic mechanisms. Regulators and policy makers would therefore be well advised to follow closely the developments in the field of epigenetics to face the challenge of deciding whether additional measures are necessary or existing regulations are sufficient.

6. Concluding remarks

In a relatively short period of time, epigenetics has swept the research community. As a research topic, it is likely to stay at the forefront of mainstream biological research for a long time to come. It offers attractive and novel ways of analyzing and understanding the fundamentals of cell differentiation and gene regulation. Biology is well advised to do away with the supposedly Lamarckian flavor of epigenetics as 'inheritance of acquired characteristics'. Epigenetics is not 'Lamarck's last laugh' (Grant-Downton and Dickinson, 2006), but it represents a further deepening of the understanding of the complexity of biological regulation.

The way the epigenetic way of thought is currently changing the field of cancer research may be taken as pointer to a future to come. The non-gene parts of genomes and organisms deserve more attention in order to understand development and genetics to the full. Without an epigenetic angle, any attempt to develop 'systems biology' (Ge et al., 2003; Mager and Bartolomei, 2005) as a valuable approach is bound to fail. The more is understood about epigenetic regulation, the more feasible epigenetic applications will be, both in the laboratory and outside the laboratory setting. The future development of 'epigenetic engineering' seems therefore inevitable.

Applications of mitotic epigenetic inheritance are reset upon meiosis and are supposed not to be transmitted to future generations. This should be taken into account in discussions about regulation and safety. Transgenerational epigenetic inheritance may turn out to be more widespread in mammals than assumed for a long time (Chong and Whitelaw, 2004b). If behavior and/or environment of an organism are responsible in part for the health and well being of its offspring over multiple generations, as now suggested by the latest results of Prembey *et al.* (2006), such long term effects need to be understood much better. On this level, however, epigenetic regulation is very complex and intrinsically multifactorial in nature. It would be highly advantageous if comparable transgenerational epigenetic inheritance could be demonstrated beyond discussion in a model organism for which large population sizes are easy to establish (such as yeast or fruit fly) and studied without any of the confounding parameters always present in (human) epidemiological studies (Chong and Whitelaw, 2004b). If transgenerational epigenetic inheritance is confirmed, it is likely to have new consequences for ethics and decisions with respect to behavior and environment (Whitelaw, 2006). In plants, epigenetic inheritance over generations is more prominent than in mammals (Takeda and Paszkowski, 2006), possibly because it appears predominantly based on DNA methylation. Yet, future applications seem hampered by the complexity of epigenetic regulation and apparent lack of specificity.

A remarkable feature of all epigenetic regulation as it is now unraveled, is that such regulation involves the collaboration and integration of action of many enzymes, proteins and non-protein components. In view of current knowledge, it seems likely that such interactions are all subject to a, possibly quite subtle, quantitative variation in regulation that current research has yet to learn to appreciate and incorporate. Biological development at its core may also be found to tolerate a given level of stochastic (i.e. seemingly random) decisions to generate variation. The increased knowledge of gene regulation by epigenetics could therefore help the assessment of the safety impact of 'traditional' genetic engineering by opening up the difficult concept of pleiotropic effects.

As knowledge is progressing, it is becoming clear that all collaborating partners in epigenetic regulatory complexes are -and can be- defined in terms of DNA and genes for protein or RNA. In other words, epigenetic players are 'genic' in nature. Such collaboration of genic elements is known in genetics as epistasis. The more the details of the network of epigenetic regulation in terms of DNA and chromatin modification are understood, the more it is likely that epigenetic phenomena can be fully described in terms of protein-protein, protein-RNA, protein-DNA and RNA-DNA interactions, so in terms of epistasis. A clear example of this development is the existence of regulatory microRNAs. These small RNA molecules are an intrinsic part of the epigenetic regulatory machinery, yet it is now clear that they are encoded by and regulated as 'normal' genes. It could be argued that their existence and mode of action should not be considered 'epigenetic' any more, but represents an originally epigenetic phenomenon now explained in terms of DNA and genetics.

In this context, it could be argued that all epigenetics is based on collaborating and interacting 'genic' DNA (either protein-encoding or non-protein). If so, all epigenetics could be considered epistasis, hence 'normal' genetics after all. This way, the fields of genetics and epigenetics could merge. In that case, it would be unlikely that targeting the epigenetic layers of cell and gene regulation in future epigenetic engineering will generate safety issues that are essentially different from the safety issues already encountered in genetic engineering. This will depend, however, on the outcome of future investigations. Therefore, regulators and policy makers in (epi)genetic engineering would be well advised to follow closely the developments in the field of epigenetics to face the challenge of deciding whether additional measures are necessary or existing regulations are sufficient.

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Literature

This report is for the larger part based on (numerous) reviews from the scientific literature. These reviews will allow the readers that want to delve deeper into the broad subject of epigenetics to access and study the primary research literature.

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Website references

In many cases, the Web is an excellent starting point for first information about epigenetic phenomena, people, topics, (abstracts of) research papers, laboratories and companies. The below list is a small selection of sites related to various aspects of epigenetics that could be of potential interest to the readers in relation to the topic of epigenetics. Some sites may require a personal or an institutional subscription.

The reader should please be aware that the web is not always as stable as desired, so sites mentioned here may have disappeared, changed or be temporarily offline. In such cases, targeted inquiries using Google or any other search engine may be just as, or much more, efficient.

http://en.wikipedia.org/wiki/Epigenetic_inheritance
http://en.wikipedia.org/wiki/Epigenetics
http://en.wikipedia.org/wiki/: various other entries
http://geneimprint.com/
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