Epigenetic differences after prenatal adversity : the Dutch hunger winter

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Introduction

Genes, environment and something else

The central dogma of biology states that heritable information is passed residue-by-residue from DNA to RNA and finally protein. Moreover it states that such information cannot be transferred back to protein or nucleic acid[1]. However, the concept of the gene as the sole container of biological information has been challenged by several observations. For instance, the phenomenon that a gene's activity is determined by its place in the genome[2] is explained by the fact that information is also contained in the positional context of genes in the genome. Another observation is that genetic mutations and environmental perturbations during development can give rise to the same phenotype[3]. More recently an enigmatic 'third component' has been found that causes phenotypic variation during development that persists into adulthood in inbred or even monogenetic populations under a constant environment[4,5]. Indeed, it has proven very hard to produce an identical copy of an organism. When the first cat, called carbon copy (a.k.a. copycat), was cloned it had a completely different coat coloring than the mother[6]. All these observations lead to the question what molecular marks could mediate these positional effects, the influence of the early environment or the seemingly stochastic variations in genetically identical organisms.

It is believed that so-called epigenetic marks which envelop DNA may be the mediator. The study of 'epigenetics' has exploded since the year 2000[7], but different researchers have different definitions, which is due to the fact that the term epigenetics arose multiple times during the previous century (**Box 1**)[8]. In this thesis we adhere to the definition by Jaenisch & Bird, namely that of epigenetics as the study of the molecular mechanisms by which heritable changes in gene expression potential occur that are not caused by changes in DNA sequence[9]. This definition calls for a cell-autonomous nature of epigenetic information that is passed during mitosis and possibly meiosis and excludes sustained expression changes mediated by extracellular signals or by morphology[10]. These epigenetic marks may contain stable genomic information potentially providing the molecular basis to explain part of the phenotypic variation in humans[11]. Research on epigenetic marks in human

populations is focusing in particular on those diseases occurring in adulthood that are linked with disturbances of the early environment[12]. In this thesis we lay the first basis to ultimately elucidate the role of epigenetic marks in early development and disease in humans.

Box1: the origins of epigenetics

The term epigenetics was originally coined by Waddington[72], who in 1939[73] started his attempts to conceptually merge embryology, evolution and genetics. He hypothesized that a cell, tissue or organism is formed through dynamic interconnected networks of genes that interact with the environment during development. He put forward the term 'epigenotype' as the whole of these 'organizing relations' standing between the genotype and the phenotype and 'epigenetics' as the discipline studying the epigenotype. His groundbreaking network view of genetics and biology did originally not envision mechanisms outside the gene and the environment[74].

The second major usage of the term epigenetics was introduced by Nanney[75] and refined by Harris as the study of mechanisms that regulate the expression of the genetic information[76]. This concept of epigenetic control was soon adapted to describe the mechanisms underlying cellular inheritance other than that mediated by DNA[77], and to denote mechanisms underlying cellular differentation[78]. These usages denoted mechanism independent or complementary to genes. This is in contrast to Waddington's original usage, although Waddington noted that both concepts 'do not bite' each other.

It has become clear that the molecular mechanisms regulating the potential of a genomic region to become transcribed may be effectuated by genes[79] and shaped by genetic variation[14,30] and environmental conditions during early development[38]. These new insights are very much in the spirit of Waddington's concept of the epigenotype. This has led to a renewed enthusiasm for Waddington's network view on development and biology, which has proven highly influential for recent refinements of evolutionary theory[80,81]. Moreover, (molecular) geneticists coming from the tradition of the second emergence of the term epigenetics recently proposed a merger of Waddington's original theoretical framework with the expanding body of knowledge on 'epigenetic' gene expression regulation during differentation[82]. Development is back in the heart of Biology, Genetics and Evolution by partly re-inventing the concept of the epigenotype and its study: epigenetics.

Epigenetic marks: wrapping DNA

Epigenetic marks are intimately linked to development, for the same genome expresses different parts of its information depending on the cell type. During development different sections of the genome are activated and deactivated and loop together into foci of relative high or low activity[13]. In all organisms in nature the compaction and relaxation of DNA is mediated by the histone proteins forming so-called nucleosome complexes with DNA (**Figure 1**). Multiple of these DNA-protein structures together from a quaternary structure called chromatin and the modifications on the 'tails' of the histones in the

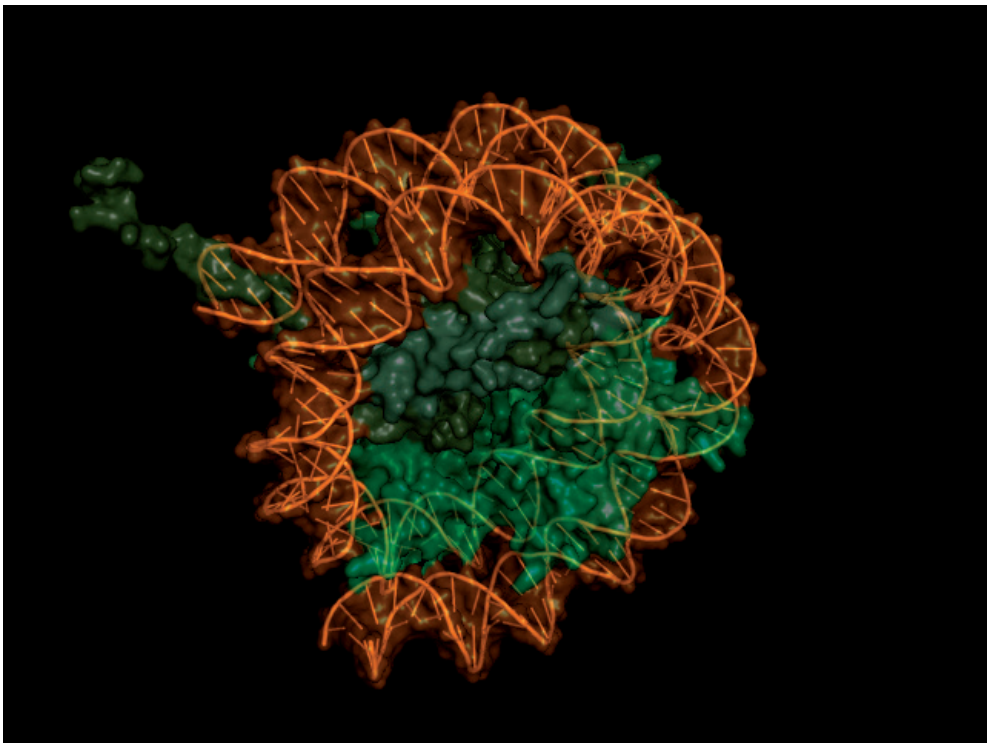

Figure 1. DNA wrapped along a nucleosome core
DNA is wrapped around nucleosomes, complexes of several histone proteins, in the cell core. Visible are the histones in different shades of green, together forming a nucleosome. On several locations the histone proteins protrude over the DNA helix (orange), locking it into place. The strength of this interaction is influenced by modifications placed on these histone tails.

complex help to determine its compactness. DNA positioned within regions with nucleosomes in a more open, euchromatin conformation is more readily transcribed into RNA. DNA positioned within regions where nucleosomes are more densely compressed, in a so-called heterochromatin conformation, is less readily transcribed. The DNA sequence itself influences nucleosome positioning and chromatin formation[14]. However, the correct chromatin state was shown to be faithfully transmitted to daughter cells in yeast despite the deletion of the relevant genetic signals[15] and also the prenatal environment can persistently change chromatin modifications[16,17]. Other, more recently discovered mechanisms may also influence the chromatin landscape. Various forms of non-protein coding RNA is transcribed from the genome and influence chromatin conformations within and between cells[18] and have even been found to influence the epigenetic make-up of the germ line, thereby potentially influencing a following generation[19].

Epigenetic marks: modifying DNA

Several families of the tree of life have also developed covalent modifications of DNA[20]. Methylation of DNA is limited to the cytosine in CpG dinucleotides in adult humans and several other complex species[21]. DNA methylation results in a more compacted double helix[22]. DNA methylation may also inhibit or promote the binding of certain proteins to DNA. Like chromatin, DNA methylation is highly dynamic during development and functions as a regulator of tissue differentiation[23]. During and directly after fertilization DNA is passively demethylated in the maternal contribution and actively demethylated in the paternal contribution to the zygote's new genome and during this time several intermediates of 5-methyl cytosine are formed through not yet completely elucidated mechanisms[24]. However, the genome is quickly remethylated during the earliest stages of development[25]. For instance, the promoters of genes that regulate pluripotency or lineage commitment are methylated upon differentiation, thereby persistently blocking a return to a more pluripotent cell type[26,27]. Secondly, DNA methylation can stably silence specific alleles of so-called imprinted genes dependent on the parent of

origin[28], a phenomenon that defies classical Mendelian inheritance patterns. DNA methylation is influenced by certain DNA sequences[29] and single nucleotide polymorphisms in cis and trans[30]. However, DNA methylation is also influenced by the before mentioned non-coding RNAs and by environmental factors during development[31]. The latter is the topic of this thesis.

Development, environment and epigenetic change: a hint from animal models

It is suggested by the epidemiological literature that early environmental conditions are linked to later life health and disease[32]. The nature of these associations is and has been hotly debated[33-35]. Experiments modulating animal nutrition and stress levels during development revealed that the precise timing of the environmental exposure and the sex of the exposed fetus is important in determining the phenotypic outcome[36]. Most experiments entailed limiting the amount of protein, calories or folic acid to the developing fetus[37]. From 2003 the wider research community became aware of epigenetic marks as a possible molecular link between nutrition during development and adult phenotypes. In two mouse models, with a repetitive element inserted in front of the gene that influences coat color (*agouti*)[38] and tail shape (*axin* fused)[39] respectively, it was handsomely shown that the amount of methyl donors in the maternal diet could shift the amount of DNA methylation at these elements (**Figure 2**). Such shifts in methylation changed the amount of expression of the neighboring gene and thereby the phenotype. Not long afterwards other examples at 'regular' genes were found[31,40-43]. Several of these experiments found epigenetic changes that influenced genes which have been functionally implicated with health effects that arise as a consequence of prenatal malnutrition. For instance, prenatal protein restriction changed the amount of DNA methylation and expression of the *agtr1b* gene, which is implicated in hypertension[44].

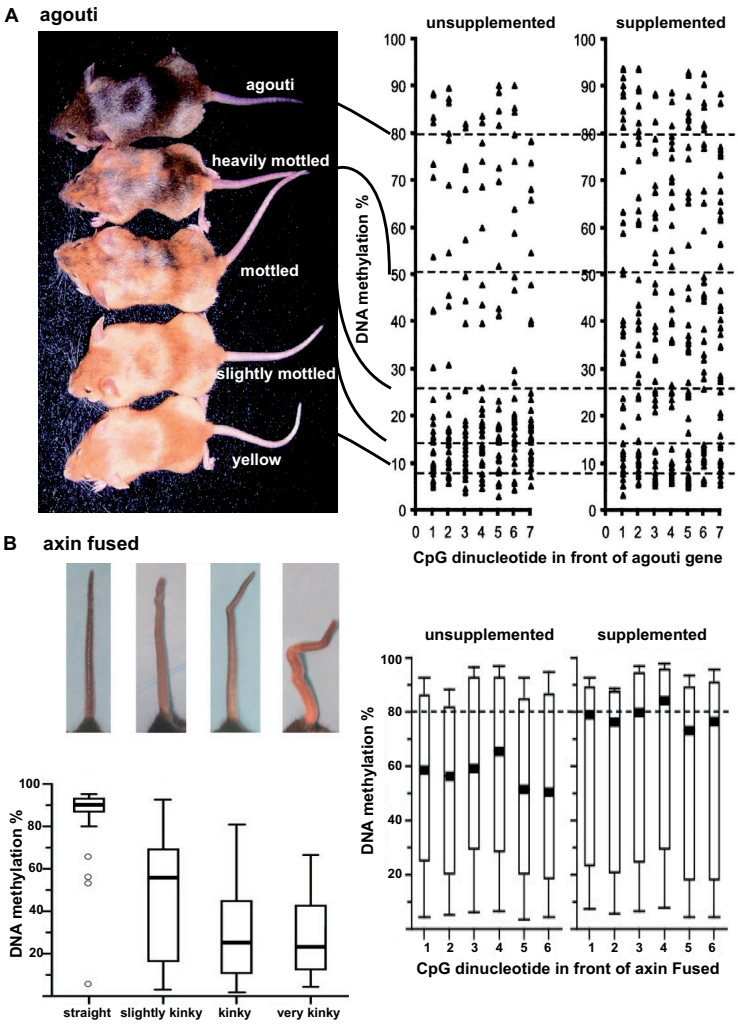

Figure 2. The effect of Supplementation on DNA methylation and phenotype in the *agouti* and *axin* Fused mouse models

A. In a normal litter there are a variety of colors from yellow to agouti (left panel). Upon supplementation of the maternal diet during pregnancy with methyl donors, like folic acid, more offspring is born with an agouti color. This is due to a general increase in the average methylation of 7 CpG dinucleotides at a retrotransposon situated in front of the *agouti* gene (right panel). Copyright© 2003, American Society for Microbiology, *all rights reserved*

B. In a cross resulting in heterozygous *axin Fused* litters, a great incidence of kinked tails is found. Tail form is influenced by DNA methylation in front of the *axin* locus (left panel). Upon supplementation of the maternal diet during pregnancy with folic acid this incidence is greatly decreased (by over 50%). It was found that folic acid supplementation increased the amount of DNA methylation at 6 CpG dinucleotides at this locus (right panel), resulting in litters with more mice with straighter tails. Copyright© 2006, John Wiley and Sons, *all rights reserved*

Development and disease

These animal models were intended to clarify and give body to the hotly debated epidemiological observations in relation to the associations between a low birth weight and uterine growth restriction and an unfavorable body mass index (BMI), type 2 diabetes (T2D) and hypertension[32]. Prenatal growth restriction and a low birth weight are seen in this context as a proxy for malnutrition *in utero*. Recently this view was challenged by observations in large twin cohort studies that have shown that the association between birth weight and BMI or T2D is confounded by genetic factors[45-47] and a large study in parent-offspring trios showing that the association with BMI is not only confounded by genetic but also familial factors[48].

Studies on the consequences of prenatal exposure to famine, which can be analyzed as natural experiments of sorts, show that obesity and diabetes may still arise by a poor prenatal environment and nutrition. An association between prenatal famine exposure and obesity has been found following the Dutch and the Great Leap Forward famines[49-52], where the associations in later life were most prominent in exposed women. The occurrence of diabetes was also found to be increased in two Dutch cohorts[53,54] and in a large population based study in the Ukraine[55]. Another well replicated finding is the association of periconceptional famine exposure with schizophrenia[56]. These replicated findings are either independent of the gestational timing of the famine exposure or specific to early exposure (**Figure 3)**, which unlike mid and late gestational famine exposure is not associated with a reduced birth weight[57]. The famine and twin studies raise questions on the suitability of birth weight and in utero growth restriction as a proxy for prenatal malnutrition, at least in Western cohorts. More defined maternal and environmental characteristics, like maternal BMI, hypertension and gestational diabetes are also associated with the same later life phenotypes associated with a low birth weight[58-60].

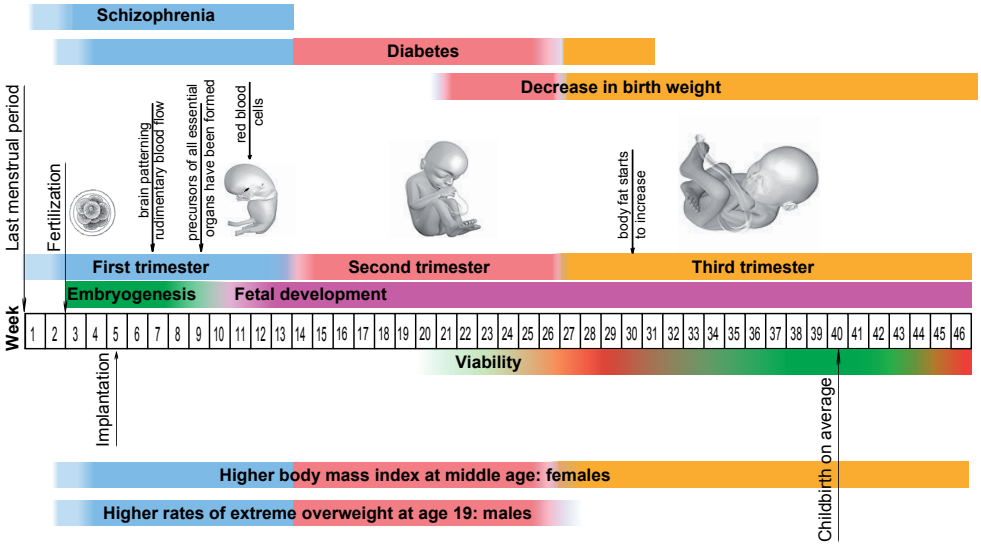

Figure 3. Replicated phenotypic associations with prenatal famine exposure
Human gestation is depicted by week across the 3 trimesters with above the replicated associations found to be independent of gender and below the replicated associations with confirmed or possible gender specificity. *Adaptation from original source on Wikimedia Commons, Mikael Häggström*

Of mice and men: variation and starting material

The data from animal malnutrition studies led to hypothesis that epigenetic processes may underlie the above mentioned link in humans between the prenatal environment and adult health[10,12]. For human studies it was clear that there would be challenges to overcome. First, the animal studies indicated that the differences induced by malnutrition could be small[42] and when the average difference was larger there was often quite some variation, as observed in the *agouti* and *Axin* Fused mouse models[38,39] (**Figure 2A en 2B, right panels**). Secondly, it was shown in animal models that not only the prenatal period but also the postnatal period may be crucial[43,61] and that the postnatal environment may counter effects induced by prenatal conditions[62]. It was also shown in animal experiments that one exposure may counteract the epigenetic effects of another[63,64]. Since human populations are never under controlled homogeneous conditions these latter studies

indicate that confounding will be a serious issue to deal with when studying epigenetic marks in human population studies. Furthermore, both genetic factors[30] as stochastic factors during ageing may influence epigenetic marks in humans[65,66], thereby adding biological noise to studies.

There is also the issue of tissue specificity. Since epigenetic marks are one of the main mechanisms underlying cell differentiation, differences between tissues are bound to be plentiful. In bio banked cohorts often only DNA from whole blood or buccal swaps is available, which are peripheral tissues that may have a different epigenetic pattern to internal tissues[67]. Whole blood is a mixture of highly differentiated cell types which may cause additional non-technical variation, since blood cell populations differ between persons and over time. Both these available sources of DNA are in most cases only suitable for DNA methylation measurements, since the native chromatin structure and non-coding RNA is lost during DNA isolation or storage.

A developmental extreme: The Dutch Hunger Winter Families Study

To reduce variation and increase the chance of success the first genetic studies in humans resorted to family studies, well defined exposures and phenotypic extremes. In this thesis we undertook the same strategy for human epigenetic studies on DNA methylation. In September 1944 the Dutch national railways went on a national strike in support of Allied operation Market Garden, aimed at opening up the main Nazi manufacturing center, the Ruhr region, for a direct assault. This operation stalled after liberating the Southern part of The Netherlands. Little to no food was transported by the Nazi's as military transports were given a higher priority and later on food transports by rail and road were prohibited as a punitive measure, while transportation over water was made difficult by winter conditions and severe fuel shortages. The official government issued rations fell rapidly and went below 1,000 kcals/day by November 1944, reaching as little as 500 kcal/day in April 1945. Before the famine the Dutch population was well fed and the famine rapidly ended after liberation in May by massive Allied relief efforts[68].

This discreteness in time, shorter than the nine months of human gestation, and the fact that the health care system remained working, makes that adults can be traced that were exposed during specific periods of their development *in utero*.

For this thesis we make use of The Dutch Hunger Winter Families study[69], which consists of 658 individuals born between 1943 and 1947 in three hospitals in Holland, located in cities exposed to famine in the winter of 1944-'45. For 313 of these individuals a same-sex sibling could be recruited as an unexposed control group. The disaster of the Dutch Hunger Winter, as captured in this cohort, offers a unique quasi-experimental setting for study. We focused our measurements on the individuals that were exposed either early or late in gestation and with a same-sex siblings available for study. This design (partly) matches for genetics and the early familial environment, reducing some of the variation inherent to human studies. Furthermore, the focus on exposure early or late in gestation offers an interesting contrast in relation to the observations relating to a low birth weight and *in utero* growth restriction. Individuals exposed during the last trimester of pregnancy were much lighter at birth, while individuals conceived during the famine and exposed up to 10 weeks into development were not[57] (**Figure 3**). Furthermore, experiments in animals indicate that the period around and just following conception (periconceptional exposure) is a particularly sensitive period of development[31]. Indeed it may be the period during which some of the epigenetic differences arise that were initially discovered following exposure during the entire pregnancy[63]. Differences induced early in development may be mitotically heritable, thus propagated to multiple tissues[70] and we hypothesize that differences induced during this period may be more readily detectible in whole blood, the tissue collected in this cohort, and is more likely to reflect differences in relevant but inaccessible tissues.

Aims and outline of this thesis

In this thesis we aimed to lay the groundwork needed for epigenetic epidemiology in human populations and take the first steps to ascertain if the associations between early development and adult health may in part be mediated by epigenetic mechanisms, as was indicated in animal models. To this end we first studied the variation, stability and tissue specificity of DNA methylation patterns in human blood and buccal cell DNA of candidate loci chosen for their key roles in development, growth and metabolism. Characterizing DNA methylation and investigating its suitability as a marker for molecular epidemiological studies (**Chapter 2**).

Secondly we aimed to discover if we could find associations between DNA methylation and prenatal famine exposure in humans. In particular we hypothesize that most associations should be found in those exposed during periconception. As a proof-of-principle, we studied whether DNA methylation within the key imprinted developmental gene *insulin like growth factor 2* (*IGF2*) is associated with prenatal exposure to the Dutch famine (**Chapter 3**). We then extended this first measurement in the Dutch Hunger Winter Families Study to the fifteen other candidate loci investigated in chapter two. With the aim to investigate if the associations between DNA methylation and prenatal famine mirror the epidemiological findings with timing independent and sex-specific associations (**Chapter 4**).

We then set our observations in the Dutch Famine in the context of prenatal growth restriction and more contemporary prenatal adversities such as maternal hypertension (**Chapter 5**). For this we resorted to the POPS study, which is also a cohort at a developmental extreme. The POPS study is a nation-wide prospective study including 94% of all live born infants born very preterm (<32 weeks) and/or with a very low birth weight (<1500 gram) in 1983[71]. We compared 113 individuals born preterm, but with a normal size for their gestational age, with 38 individuals born preterm and in uterine growth restricted. This to investigate if DNA methylation differences at loci identified in the Dutch Famine are also detectable after early prenatal growth restriction.

Finally, we set out to characterize the DNA methylation differences associated with prenatal famine exposure by extending our measurements to multiple regulatory regions within the *IGF2* locus and contrast the influence of the famine with that of genetic variation at the same locus (**Chapter 6**). After delving deeper, we broadened our inquiries by extending our measurements to a genome-scale using next generation bisulfite sequencing (**Chapter 7**). Investigating what would constitute the normal effect size and the genomic characteristics of regions sensitive to the prenatal environment and extend our analyses away from single genes and loci to entire regions and pathways. The experimental chapters are followed by a summary of the results (**Chapter 8**) and a general discussion (**Chapter 9**). We conclude with a popular summary of the results in Dutch (**Chapter 10**) and the acknowledgements section.

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