Epigenetics 4:8, 526-531; November 16, 2009; © 2009 Landes Bioscience

The epigenome

Archive of the prenatal environment

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World-wide, research initiatives are in progress to establish the role of the epigenome in human disease. Empirical data are still scarce, but particularly studies investigating how the epigenome links early developmental and adult disease may rapidly change this situation. Recently, several reports showed that prenatal environmental conditions are associated with persistent changes of the human epigenome. The evaluation of candidate loci among individuals prenatally exposed to the Dutch Famine indicates that such changes may be common but individually relatively small and may greatly depend on the timing of the exposure during gestation. These findings suggest that the epigenomic contribution to disease risk may entail the combination of multiple changes especially when adaptive responses are involved to cope with environmental conditions. Well-designed epigenome-wide studies will be crucial in creating a catalog of epigenomic regions that are sensitive to the prenatal environment to evaluate developmental influences on common human disease.

Key words: epigenome, DNA methylation, gestation, development, disease, nutrition

Submitted: 10/05/09

Accepted: 10/06/09

Previously published online: www.landesbioscience.com/journals/epigenetics/article/10265

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Introduction

Studies of the human genome, that started with establishing its sequence[1] and recently witnessed the large-scale discovery of genetic variation affecting common disease,[2] are dramatically increasing our knowledge of human biology and disease. Efforts are being initiated to study the human epigenome in a similar fashion as epitomized by the publication of the first reference methylomes of human embryonic stem cells and fetal fibroblasts.[3] This is a natural next step since the epigenome controls the potential of the genome to become expressed[4] and may have a significant impact on human disease.[5–8] Thoroughly testing this hypothesis, however, will be a substantially greater challenge than it is for genetic variation. In this review, we argue that the best prospects for indentifying epigenomic risk factors for disease may come from studies on the link between early development and disease.[9,10] Our recent studies on genomic consequences of prenatal exposure to famine provided the first evidence that transient environmental conditions in human gestation can be recorded as persistent changes in the epigenome.[11,12] Taking these results as a starting-point, we will discuss the characteristics of the epigenomic changes that can occur as a consequence of adverse prenatal environments, the critical role of gestational timing herein and how these changes can contribute to the risk of disease later in life.

Development

The epigenome refers to the whole of epigenetic marks on the genome that regulate chromatin structure and accessibility of the DNA to the machinery regulating gene expression. The best-characterized epigenetic marks are the methylation of cytosines in cytosine-guanine (CpG) dinucleotides and the modification of histones that package the DNA.[13] These marks are heritable during cell division, particularly mitosis, and are involved in processes that

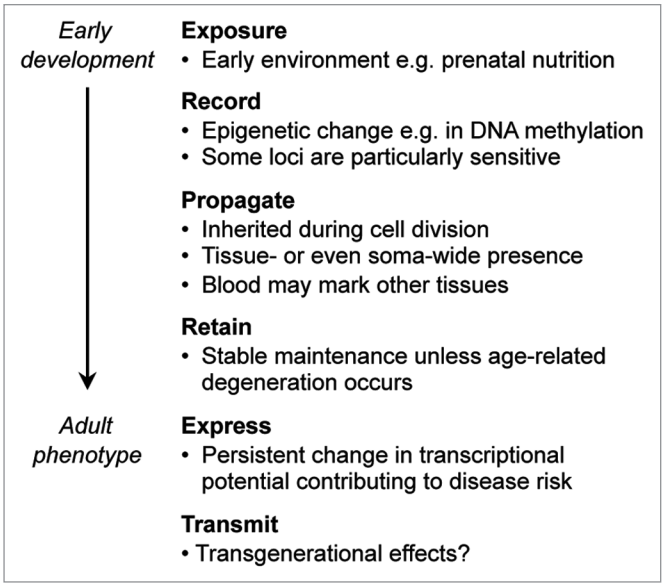

Figure 1. How the epigenome can link early development and adult disease.

require a stable control of gene expression like the selective gene-silencing during cell differentiation,[14] parent-of-origin specific silencing (imprinting)[15] and suppressing the transposition of mobile elements (DNA sequences with the ability to copy themselves throughout the genome).[16] Although generally stable, environmental influences and stochastic events can cause changes in epigenetic marks.[4] In humans, the accumulation of such changes have primarily been studied as a function of age.[17,18] Although potentially relevant for human disease because they can disturb homeostasis,[7] it will be difficult to prove their involvement in human disease if they are highly frequent, largely stochastic,[19] and age,[20] tissue- or even cell-specific.[18] Moreover, they may be the consequence of (subclinical) disease rather than its cause.

Epigenetic studies designed within the framework of the developmental origins hypothesis may have better prospects for unravelling the role of the epigenome in human disease. The developmental origins hypothesis is based on numerous epidemiological studies showing a link between characteristics of early development and the occurrence of disease later in life.[21] It states that adverse environmental conditions during specific windows of mammalian development can have lasting effects on metabolic pathways and physiology thereby influencing the susceptibility to disease.[22] Of the mechanisms potentially underlying the hypothesis, environmental influences on the epigenome are receiving increased attention[9,10] since the epigenome can serve as the molecular archive of past environmental conditions.

The first empirical data showing that the epigenome can play this role came from beautiful experiments in an animal model, the agouti viable yellow (A^{vy}) mouse.[23] This agouti strain spontaneously arose through the insertion of a mobile element, namely an intracisternal A particle retrotransposon, upstream of the *agouti* gene. The presence of the mobile element renders the genomic region epigenetically labile. The reason for this is unclear but somehow the epigenetic mechanism that operates to silence the mobile element is not fully effective[16] perhaps because it conflicts with the normal epigenetic programming of the region during development. When agouti mice carrying the mobile element are prenatally exposed to a high level of methyl-donors through feeding their mothers (dams) methyl rich diets during pregnancy, this environmental condition is recorded as a higher level of DNA methylation at the *agouti* locus. This change in DNA methylation can be observed in any tissue suggesting that the epigenetic change is propagated soma-wide due to the transmission of DNA methylation during mitosis. Moreover, the epigenetic change is stably maintained as it persists into adulthood. The epigenetic change is expressed as the silencing of the *agouti* gene that downregulates the synthesis of yellow pigment and leads to a brown coat colour. Thus, early-life environmental conditions can cause epigenetic changes that persist throughout life and result in life-long phenotypic consequences.

The agouti experiments indicate that it may also be feasible to test the association of epigenomic variation with human disease when focussing on prenatal development (**Fig. 1**). In contrast to changes arising with age, the developmental changes in the epigenome may affect many cells or complete tissues increasing the feasibility of studies into their role in human disease. For some loci, particularly those whose epigenetic state is independent of cell differentiation,[15,23] peripheral tissues (e.g., blood) as collected in human biobanks may even be used to estimate DNA methylation in tissues directly involved in pathology.[24,25] Also, epigenetic changes arising during development are not secondary to adult disease. Possibilities to study the contribution of the epigenome to the link between development and disease in humans depend on the occurrence of conditions potentially affecting development. Historical famines are a well-documented example resembling animal experiments of malnutrition during gestation. They may also serve as a model for the study of other prenatal conditions including dietary changes, stress and medical interventions.

The Dutch Famine

In our epigenetic studies we examined individuals who were exposed to the Dutch famine during gestation. This was a severe famine at the end of World War II that affected the western part of The Netherlands from November 1944 to May 1945 (**Fig. 2**). The average daily rations, which the authorities distributed during the famine, were less than 700 kcal (cf. normal daily requirements for women and men are 2,000 kcal and 2,500 kcal, respectively). Prenatal exposure to the famine is associated with various adverse metabolic and mental phenotypes later in life including a higher BMI[26,27] and elevated plasma lipids[28] and increased risks of schizophrenia[29] and possibly cardiovascular disease.[30]

Many of these associations were dependent on the sex of the exposed individual and the timing of the exposure during gestation. The biological mechanisms contributing to these associations are unknown but may involve the epigenome. The first step towards establishing the contribution of the epigenome was to answer the question whether prenatal exposure to famine can lead to persistent changes in the epigenome in humans. To this end we examined the association between prenatal famine and the DNA methylation at candidate loci in our ongoing Hunger Winter Families Study.[31]

The first candidate locus investigated was the well-characterized differentially methylated region of the imprinted insulin-like growth factor 2 (*IGF2*) gene, a key factor in human growth and development.[20,24,32] Individuals who were exposed to famine during early gestation had a lower *IGF2* methylation than controls six decades after the exposure.[11] More recently, we extended this observation by testing a set of 15 additional candidate loci, seven of which were (putatively) imprinted.[12] The loci were implicated in growth, metabolic and cardiovascular phenotypes. Methylation of six of these loci was associated with prenatal exposure to famine (*IL10*, *GNASAS*, *INSIGF*, *LEP*, *ABCA1* and *MEG3*). Of interest, further analysis indicated that some of these associations were sex-specific (*GNASAS*, *INSIGF*, *LEP*), which is in line with previous experiments in sheep[33] but as yet awaits an explanation. The success of the study in detecting these associations may hinge upon its design. We recruited same-sex siblings as controls thus minimizing confounding by sex, early family environment and the influence of genetic variation on DNA methylation.[32] Our studies so far indicate that DNA methylation differences after exposure to prenatal famine may be common and may persist during an individual's life course.

Figure 2. A typical daily ration distributed by the local authorities in the famine-struck western part of the Netherlands consisted of two slices of bread, two potatoes and piece of sugar beet. The newspaper in the background announces the closure of the soup kitchens.

Timing

DNA methylation may be particularly sensitive to environmental factors during the extensive epigenetic reprogramming early after fertilization.[23,34] Indeed, culturing of preimplantation mouse embryos showed that epigenetic marks are susceptible to nutritional conditions in the very early stages of mammalian development.[35,36] The Famine study can reveal whether this also holds in humans since the timing of the exposure during gestation was recorded.[31] All seven loci for which we found DNA methylation differences were associated with exposure to famine during periconception (i.e., conception occurred during the famine). Methylation of only one locus was also associated with exposure to famine late in gestation (*GNASAS*). The human epigenome may thus be particularly sensitive to the environment during very early development.

Although the interpretation that the DNA methylation differences occurred early in development is compatible with current literature, at least four alternative explanations should be considered. We measured methylation of DNA extracted from leukocytes and adult blood cells stem from the hematopoietic system which is established relatively early in development.[37] Detailed studies of various tissues will be required to test whether the sensitivity of epigenetic marks to the environment is an intrinsic property of early mammalian development or a general feature of newly developing tissues throughout gestation. Another interpretation is that the DNA methylation changes occurred during maturation of the paternal and maternal gametes (note that we observed associations for both paternally and maternally imprinted loci). Also, the differences observed may be a consequence of a compromised placental development. However, individuals who were periconceptionally exposed to the famine did not have a lower birth weight[11] making this explanation less likely. Finally, it cannot be excluded that the DNA methylation differences are related to (sub)clinical disease that may be more prominent among exposed individuals, although preliminary analyses indicated that the DNA methylation differences at individual loci were not associated with risk factors for cardiovascular and metabolic disease. Experiments in animal models that carefully distinguish between exposures prior to conception and during different stages of gestation instead of an exposure throughout pregnancy only and that compare tissues that differentiate during different stages of development will be elemental to solve these issues.

Effect Sizes

Although differences in DNA methylation after famine may be common, the size of the differences observed so far may be regarded as relatively small (absolute differences in DNA methylation between

exposed and controls up to 2.5%). Despite the smaller effects sizes, the associations were statistically robust ($8 \times 10^{-3} < p < 2 \times 10^{-6}$) and remained highly significant after adjustments for multiple testing indicating that the findings are unlikely to be false positives. These smaller effect sizes may be a general phenomenon since they were also observed in human epigenetic studies of child abuse[38] and assisted reproductive technologies.[39]

One of the factors contributing to the small effect sizes is that epigenetic processes have a stochastic component. For example, even in the highly controlled experiments on inbred agouti mice mentioned earlier, maternal methyl donor supplementation was associated with a huge variation in response.[23] It is nevertheless noteworthy that very similar absolute differences in *Ppara* promoter methylation were observed in rats prenatally exposed to a protein-deficient diet (1.6% absolute difference),[40] a much better controlled study than can be achieved in humans with a fraction of the follow-up time. This small difference, however, explained up to 43% of the variance in *Ppara* transcription.[40] Possibly, relative changes in DNA methylation are more relevant than absolute changes. The relative change in the rat experiment was 43% (6.1 vs. 4.5%). In agouti mice, each step in coat colour phenotype from clear yellow to pseudoagouti (brown) via slightly mottled, mottled and heavily mottled is associated with an approximate doubling of the absolute DNA methylation level (7, 13, 26, 54 and 80%).[23] Hence, the greater relative differences in methylation observed in the Famine study, for example the ~10% differences for *LEP* in men and for *IL10*, may be of special interest.

Epigenome-Wide Studies

It is too early to draw definite conclusions as to the magnitude of epigenetic changes in general. Current studies are limited to candidate loci and the complete epigenome awaits to be explored. Fast progress in technology and bioinformatics enables the study of DNA methylation on a genome scale. Various approaches were developed to use micro-arrays for this purpose[14,41,42] but they are rapidly replaced by methods based on high-throughput sequencing. The latter holds the promise of a more complete and unbiased view of the epigenome and of significantly less technical variability because of its digital nature. Also, high-throughput sequencing can be performed on bisulfite treated DNA in which all cytosines are converted to uracil unless methylated.[43] This results in DNA methylation maps at a single base resolution and is considered the gold standard.[44] As yet, it is not feasible to do whole epigenome measurements in an epidemiological setting which requires accurate quantification of DNA methylation. For representative subsets of the human genome, however, this is within reach.[45] It should be noted, that our technological ability to perform genome scale measurements is not matched by a similar ability to interpret the high-throughput data due to a far from incomplete understanding of DNA methylation patterns and immature statistical methodology to sift through the millions of CpG dinucleotides assessed. It will be important to learn from the statistical and bioinformatical methodology under development to analyze complete genome sequences in disease studies for example within the 1,000 genomes project.

Since pure discovery-based strategies are as yet impossible, approaches that combine hypothesis-driven research with genome-wide approaches may be fruitful. Such approaches can study comprehensive sets of loci with a putatively labile epigenetic state which can be scrutinized for methylation differences across the genome for example using padlock probes.[46] These studies will be of particular value if the epigenetic differences observed are interpreted in the context of gene expression data preferably obtained in various tissues to aid the identification of functional epigenetic changes. Imprinted loci were suggested to be good candidates for such studies[47] although our recent findings suggest that these are not necessarily more labile than non-imprinted loci.[12] A more compelling body of literature indicates that methylation marks near active mobile elements in the human genome[48,49] are appealing candidates to be vulnerable to prenatal conditions.[16,47] Furthermore, their relative independence of cell differentiation[23] increases the feasibility of studying them in humans.

Phenotypes

The modest effect sizes observed for DNA methylation in the famine studies justify the question if and how these could contribute to disease later in life. Although larger differences at other loci may have been missed, it is unlikely that the majority of phenotypic consequences arise from single gene effects. This possibility should especially be considered if epigenetic changes arising early in development do not represent undirected damage due to harsh environments but reflect an adaptive response. The combined effects of smaller epigenetic changes may result in an altered tuning of metabolic pathways, allowing an individual to cope with the current environment or, perhaps, prepare for anticipated postnatal environments.[22,50] Of note, prenatal famine was not only associated with lower but also higher levels of DNA methylation in our studies[12] which cannot be attributed to a simple relationship with methyl donor deficiency. Pathway-focused epigenomic studies are warranted to shed light on the involvement of adaptive responses. Irrespective of the nature of the epigenetic changes underlying health outcomes later in life, however, large study samples and replication steps will be required analogous to the state-of-the-art in genome-wide association studies.[51]

We observed transgenerational phenotypic associations in relation to prenatal exposure to famine[52] and it needs to be explored if these could be of epigenetic origin. Evidence for the presence of transgenerational epigenetic effects comes from studies on endocrine disruptors in rats causing male infertility up to the F4 generation[53] and widespread, genetically induced DNA hypomethylation in *Arabidopsis thaliana* significantly contributing to the heritability of flowering time and plant height over eight generations.[54] Finding transgenerational epigenetic effects in humans will be even more challenging than finding phenotypic associations in the exposed generation. From an evolutionary perspective, transgenerational effects may be more likely for

epigenetic damage, like male infertility in rats,[53] than for adaptive responses since they may limit phenotypic plasticity and thus the ability of the subsequent generations to respond to the prenatal environment.

Prospects

Historical famines provide a powerful, quasi-experimental setting in humans for the discovery of epigenetic marks that may be modified by the prenatal environment. Studies focussing on other prenatal exposures can add and confirm such labile epigenetic marks. For example, we recently reported that periconceptional use of folic acid by the mother is associated with higher *IGF2* DMR methylation in the child.[55] Additional prenatal conditions that are being investigated include assisted reproductive technologies,[39] intrauterine growth restriction[56] and maternal depression during pregnancy.[57] Together, these efforts will result in a catalog of loci carrying labile epigenetic marks that are vulnerable to the prenatal environment. This catalog can be used as a monitoring tool for early detection of adverse prenatal conditions. In cohorts that were studied to assess the relationship between prenatal exposure and later health outcomes like the Famine studies, these catalogs can be used to establish associations with disease and its risk factors. Moreover, the catalog may be applied in regular epidemiologic studies without data on development if they focus on diseases with a developmental component like type II diabetes,[58] schizophrenia[29] and cardiovascular disease.[30,59] Studies using biobanks specifically designed for epigenomics research, thus incorporating various tissues or preferably separated cell types, extensive longitudinal sampling, and adequate resources to study the transcriptome, will be most informative.

Despite the challenges that lie ahead, focussing on the early development will be a promising route for the identification of epigenomic risk factors for human adult disease. In particular, studies that aim at the integration of epigenomic and genetic information may eventually reveal genomic risk factors that are more powerful than the current ones solely based on DNA sequence variation.

Acknowledgements

This work was financially supported by grants from the Netherlands Genomics Initiative/NWO (93518027), the US National Institutes of Health (RO1-HL067914), the Netherlands Heart Foundation (2006B083) and the EU funded Network of Excellence LifeSpan (FP6 036894).

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